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Brief Communication

Construction of genic male sterility system by CRISPR/Cas9 editing from model legume to alfalfa

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Alfalfa (*Medicago sativa* L.) is an important forage crop with high nutrient values. The molecular breeding improvement of alfalfa has been stunted due to complex heterozygous tetraploid genomes, difficult genetic manipulations, and other specific physiological characteristics. Although alfalfa has heterosis, partial self-incompatibility, and cross-pollination by insects to promote natural hybridization, its own compatible pollens retain a risk of inbreeding, that challenges commercial breeding and seed production. Thus, it is necessary to construct male sterile lines for emasculation. However, random mutation causes male sterility to occur at a low frequency that requires long-term and intensive labours for identification and further utilization.

Gene editing techniques have a great potential in precise crop breeding (Lyzenga *et al.*, 2021). Though there have been examples in alfalfa genome editing, the inefficiency of these methods constraints further practices. Meanwhile, poor understanding of male fertility regulation in alfalfa or its related species limits the choices of candidates. Therefore, identification of novel targets to male sterility along with high-efficient editing tools is the key issue for applications in alfalfa.

Here, we first filtered the candidates of male fertility regulators by the reverse genetic strategy in a diploid model *M. truncatula*. Several studies discovered male sterility in Poaceae caused by single mutations of the genes encoding putative Glucose-Methanol-Choline (GMC) oxidoreductases, such as *OsNP1* (*Oryza sativa No Pollen 1*) and *HTH1* (*HOTHEAD-like 1*) in rice and *IPE1* (*Irregular Pollen Exine 1*) in maize, that were supposed to participate in oxidation of C16/C18 ω-hydroxy fatty acids (Chang *et al.*, 2016; Chen *et al.*, 2017; Xu *et al.*, 2017). But none of those cases in dicots have been reported. Thus, we hypothesized that there might be a member(s) in *M. truncatula* with similar biochemical functions in this multigene family that involves in the male gamete development. We conducted the phylogenetic analysis of GMC oxidoreductases in *M. truncatula*, *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays*, and found that Medtr5g011010 and Medtr5g011020 share closer genetic distances with the branches of OsNP1, HTH1, and IPE1, which could be the candidate orthologs (Figure 1b).

Since stamen-specific expression pattern is essential for most male sterile genes, we searched the transcriptome sequencing data of different tissues in *M. truncatula* (http://medicmine.jcvi.org/). We found a weak expression signal of *Medtr5g011020* in the bud, but none in other tissues. The RT-qPCR assay showed that *Medtr5g011020* was highly expressed in flower buds <1 mm (Figure 1c1). By dissecting stamens and pistils from flower buds <1 mm, we also noticed that *Medtr5g011020* was preferentially expressed in stamens (Figure 1c2). The transgenic reporter line, *Medtr5g011020 pro*::*GUS*, showed distinct staining signals in anthers at Stage 8, especially in the tapetum and microspores, with a diminishing effect at the latter stages (Figure 1d). All the clues above indicated a probable role of *Medtr5g011020* as a male fertility regulator. Hence, it was referred to as the candidate *MtNP1*.

Next, we applied a *Medicago*-optimized CRISPR/Cas9 toolkit (Zhu *et al.*, 2021) to target *MtNP1*(*Medtr5g011020*) with two sites in the wild-type R108 (Figure 1a), and obtained transgenic lines with an efficiency of 94.4% (34/36). Among the transgenic-positive seedlings, editing events occurred in all the Target 1 alleles (100%) (Figure 1e), while only four lines were mutated at Target 2. Thus, we targeted the parallel genomic region with the more effective Target 1 for further genome editing in the tetraploid alfalfa.

For phenotypes of these *Mtnp1* mutants (Figure 1f), we did not observe any vegetative growth defects. During the reproductive stage, the biallelic or homozygous *Mtnp1* mutants with frameshift mutations flowered but did not yield any fruits, while the control plant produced seedpods normally (Figure 1f1). For detailed morphology assay, though outlooks of their flowers were similar (Figure 1f2), the infertile *Mtnp1* mutants did not release pollen grains, while the control had obvious mature pollens (Figure 1f3). The Alexander's staining (Figure 1f4), I_2-KI staining (Figure 1f5), and transverse sections (Figure 1f6) revealed that the infertile *Mtnp1* mutants did not have vigorous pollens. Furthermore, the artificial cross of the *Mtnp1* mutants (♀) with the wild-type R108 (♂) suggested that female fertility was not impaired (Figure 1g). Thus, the results above demonstrated that the infertile *Mtnp1* mutants were genic male sterile (GMS) lines.

For practical utilization in cultivated alfalfa, we designed two target sites (fused to sgRNA cassettes driven by *MtU6-6p* and *MtU6-5p*) in the alfalfa reference genome according to the previous editing events in the diploid model (Figure 1h). Stable transformation in the genetic background of the Chinese landrace 'Baoding' resulted in 19 regenerated seedlings, among which 16/19 (84.2%) were transgenic-positive. All the transgenic-positive lines were successfully modified at *MsNP1* (16/16), among which 14/16 were edited at all the four alleles, while 2/16 (L11 and L14) retained a wild-type copy (Figure 1i). Therefore, the editing efficiency in this case was 96.9% (62/64).

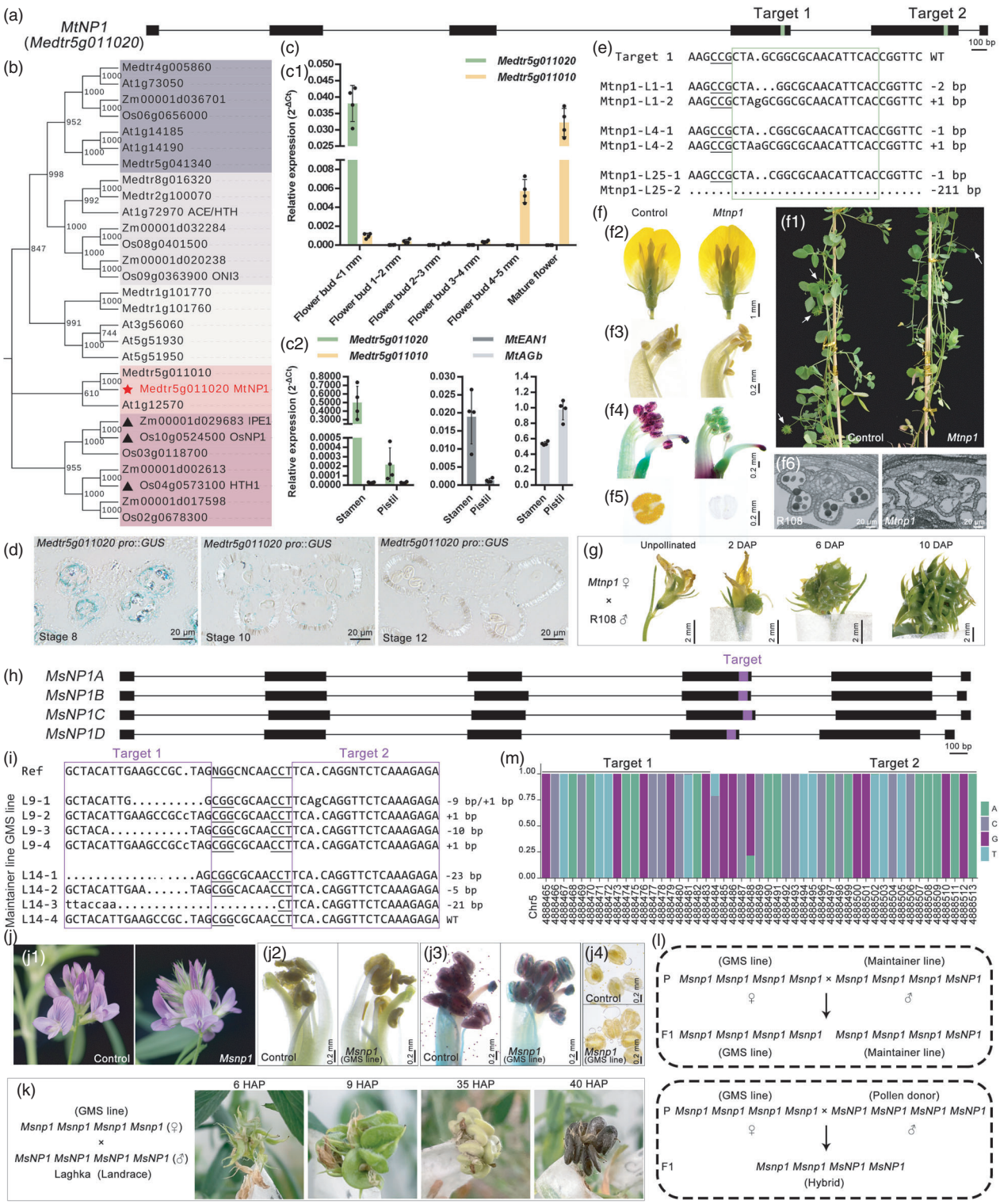

Figure 1 The CRISPR/Cas9 mutagenesis of *MtNP1* or *MsNP1* led to male sterility in *M. truncatula* or *M. sativa*. (a) Gene structure of *MtNP1* (*Medtr5g011020*). (b) Phylogenetic analysis of the putative GMC oxidoreductases. (c) RT-qPCR assay of *Medtr5g011010* and *Medtr5g011020* in R108. (c1) Flowers divided by lengths. (c2) Dissected tissues confirmed by anther-specific *MtEAN1* and pistil-specific *MtAGb*. Error bars, ±SD of four replicates. *MtGAPDH* was adopted for normalization. (d) GUS staining assay of the *Medtr5g011020 pro*::*GUS* transgenic line. (e) Representative mutations of *MtNP1* at Target 1. (f) Phenotypic analysis of the *Mtnp1* mutant by the whole plant (f1), flower (f2), stamens and pistil (f3), Alexander's staining (f4), and I_2-KI staining (f5). (g) Artificial cross between the *Mtnp1* mutant and the wild-type. (h) Gene structures of *MsNP1A/B/C/D* in the alfalfa reference genome. (i) Representative mutations of the *Msnp1* mutants at the target sites. (j) Phenotypic analysis of the *Msnp1* mutant by inflorescence (j1), stamens and pistil (j2), Alexander's staining (j3), and I_2-KI staining (j4). (k) Artificial cross between the *Msnp1* mutant and the wildtype. (l) Utilization of the GMS system in cultivated alfalfa. (m) Single nucleotide variations in the *MsNP1* target sites among alfalfa global core germplasms.

For phenotyping, all the *Msnp1* mutants showed no defects at the vegetative stage and had ordinary inflorescences during the flowering phase (Figure 1j1); but there were no visible pollen grains in the all-allelic *Msnp1* mutants. In the control plant, however, pollens were dispersed and surrounded the anthers and stigma (Figure 1j2). The Alexander's staining (Figure 1j3) and I_2-KI staining (Figure 1j4) barely showed any vibrant pollen grains. By crossing with another landrace, Laghka, the all-allelic *Msnp1* mutant could develop normal seedpods, which confirmed female fertility (Figure 1k). Thus, the all-allelic *Msnp1* mutants could be a promising original breeding material for alfalfa GMS lines.

The heterozygous triple-allelic *Msnp1* mutants (L11 and L14) were confirmed male fertile, that could serve as the maintainer lines. By crossing with the GMS lines, the genotypes of the progeny were segregated as *Msnp1/Msnp1/Msnp1/Msnp1* (GMS lines) and *Msnp1/Msnp1/Msnp1/MsNP1* (maintainer lines) distinguished by specific molecular markers at the *MsNP1* locus, which reserved the parent materials sustainably by sexual reproduction. As the male sterile lines and the maintainer lines were obtained in a single round of transformation procedure, and specific restorer lines were not required in the GMS system, the hybrid breeding programme was much simplified (Figure 1l). Moreover, the target region located at *MsNP1* was quite conserved among the alfalfa global core germplasms by resequencing (Figure 1m). This protocol therefore could be extended to different genetic backgrounds in alfalfa as long as feasible transformation procedures are established.

In summary, the reverse genetic strategy by targeting a male fertility regulator, *MsNP1*, was effective in creating the GMS system in alfalfa with a much-shortened breeding cycle, greatly promoting hybrid vigour utilization in the global alfalfa industry.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Q.Y., J.D., and T.W. designed the experiments and wrote the manuscript. Q.Y., X.M., H.C., J.W., L.Z. C.S., D.G., Y.Z., J.L., and Q.X. performed the experiments.

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