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 foremascula. 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 (Lyzena 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 OstNP1 (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-PCR 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-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 (9) with the wild-type R108 (d) 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 I2-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 I2-KI staining (j4). (k) Artificial cross between the Msnp1 mutant and the wild-type. (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 I2-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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