Journal of Genetics and Genomics 44 (2017) 465–468

Contents lists available at ScienceDirect

Journal of Genetics and Genomics

Journal homepage: www.journals.elsevier.com/journal-of-genetics-and-genomics/

Letter to the editor

Generation of thermosensitive male-sterile maize by targeted knockout of the *ZmTMS5* gene

Maize (*Zea mays* L.) is one of the most important cereal crops, with a global production of 1.02 billion tons in 2013 (Baldaufa et al., 2016). Heterosis is widely used to increase the productivity of maize, and the first commercial hybrid maize was introduced in the 1930s (Duvick, 2001). The high-yield maize varieties used today are all hybrids, and the production of hybrid maize seed requires sterilization to prevent self-pollination. Conventional seed production of hybrid maize relies upon detasseling — the removal of pollen-producing flowers using either manual methods or semi-automated processes — to ensure the purity of the hybrid. However, this process is costly and inefficient, and it is difficult to remove the functional pollen grains completely. Lines of hybrid maize that have male sterility do not require sterilization and are therefore ideal for hybrid seed production. Male sterility can be caused by cytoplasmic male sterility (CMS) or genic male sterility (GMS). CMS, which is a maternally transmitted defect in pollen production, was the technique first used to generate hybrid maize. In the 1970s, however, the outbreak of southern corn leaf blight almost destroyed maize CMS-T (CMS-Texas) lines in the USA and, as a consequence, CMS lines were no longer used to generate hybrid maize seeds (Levings, 1993). Thus, GMS lines, including photoperiod-sensitive GMS (PGMS) lines and thermosensitive GMS (TGMS) lines, might be used for hybrid maize seed production in the future.

Genome editing technologies that use sequence-specific nucleases (SSNs), including zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) (Boch et al., 2009) and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated nuclease protein 9 (Cas9) system (Cong et al., 2013; Mali et al., 2013), provide opportunities for crop improvements. SSNs create targeted DNA double-strand breaks, which are mainly repaired by either error-prone non-homologous end joining (NHEJ) or by high-fidelity homologous recombination (Symington and Gautier, 2011). The homologous recombination is a less frequent process, in which precise gene modification can be created using a homologous donor (Puchta et al., 1996). The NHEJ pathway, in which the broken DNA ends simply rejoin (Gorbunova and Levy, 1997), creates small insertions and/or deletions (indels) at the break point, and has been exploited to create targeted gene knockouts in various organisms including plants (Weeks et al., 2016). ZFN and TALEN techniques have enabled genetic engineering in multiple biological systems by using the protein-based recognition of DNA, but these techniques are limited by their variable efficiency and cumbersome assembly. The CRISPR/Cas9 system consists of a Cas9 endonuclease derived from *Streptococcus pyogenes* and a chimeric single guide RNA (sgRNA) (Jinek et al., 2012). Because of its simplicity, high efficiency and low cost, the CRISPR/Cas9 system is becoming the most promising genome editing tool, and has been exploited in staple crops, such as rice, maize, wheat and soybean (Shan et al., 2013; Liang et al., 2014; Wang et al., 2014; Li et al., 2015).

With increasing global demand for food production, the development of more efficient seed-production strategies is important for sustainable agriculture. In a recent study, researchers found that a C-to-A transition at position 71 of the gene *thermosensitive genic male-sterile 5* (*TMS5*) in rice causes the TGMS trait in most GMS lines used in hybrid rice seed production (Zhou et al., 2014). In the current study, we used the CRISPR/Cas9 system to generate TGMS maize by targeted knockout of the *ZmTMS5* gene. If successful, this technique could simplify hybrid maize seed production.

Using homology alignment, we found a sequence for *ZmTMS5*, which contains six exons and five introns in the maize genome and encodes 302 amino acids (Fig. 1A). Therefore, we designed one sgRNA (T1) to target the first exon of *ZmTMS5* and two sgRNAs (T2 and T3) to target the second exon of *ZmTMS5* (Fig. 1A). All targets contained restriction enzyme sites at the Cas9 cleavage site, 3 bp upstream of the protospacer-adjacent motif (PAM). The activities of the resulting sgRNAs were first checked in maize protoplasts by polymerase chain reaction (PCR)/restriction enzyme (RE) assays (Fig. 1B), and then indels at the target sites were revealed by sequencing (Fig. 1B). Off-target effect is a major concern in the application of the CRISPR/Cas9 system. Thus, we next predicted potential off-target sites for the T1, T2 and T3 sgRNAs in the maize genome using Cas-OFFinder (Bae et al., 2014). The potential off-target sites to T1 have at least one nucleotide mismatch (Table S1). Using the PCR/RE assay, no off-target mutations were detected at any of the four most likely off-target sites for T1 in the maize protoplasts, suggesting that T1 sgRNA is specific for its recognition site. However, more than one T2 or T3 sgRNA target sites existed in the maize genome (Table S2). Because the maize genome is large and complex, it is necessary to use genome-wide sequence analysis to check for the uniqueness of the sgRNAs. The design of the sgRNA sequences must also take into consideration the fact that Cas9 generally tolerates no more than three mismatches in the sgRNA–DNA paired region, and the presence of mismatches near the PAM may greatly reduce the affinity of Cas9 for the target site (Cong et al., 2013; Bortesi and Fischer, 2015).

Next, we transformed the targeting vector pBUN411–T1 into immature Hi-II embryos by particle bombardment (Frame et al., 2000). Seven putative transgenic bialaphos-resistant lines (named as T_0-1 ~ T_0-7) were obtained, and four of them (T_0-1, T_0-4, T_0-5 and T_0-7) were identified as biallelic mutants by the PCR/RE assay (Fig. 1C). Sanger sequencing results confirmed that these four mutant lines had indels at the targeted T1 site (Fig. 1C). Lines T_0-

http://dx.doi.org/10.1016/j.jgg.2017.02.002

1 and T_0-4 with a 1-bp insertion and T_0-5 with a 1-bp deletion were biallelic homozygous mutants, and these 1-bp indels will cause frame shifting during translation. Line T_0-7 was a biallelic heterozygous mutant (a 1-bp deletion in one chromosome and a 27-bp deletion in the other chromosome). Furthermore, no off-target mutations were detected in the four *tms5* mutant lines (Table S1).

To investigate whether the indels generated by the CRISPR/Cas9 system could be transmitted to the next generation, the four mutant plants grown at a low temperature were self-pollinated and their T_1 progenies were analyzed using the PCR/RE assay and sequencing. Among the 18 T_1 seedlings of T_0-7, four were biallelic homozygous mutants with 1-bp deletion, five were biallelic homozygous mutants with 27-bp deletion, and the remaining nine seedlings were biallelic heterozygous as their parent. The rest T_1 plants

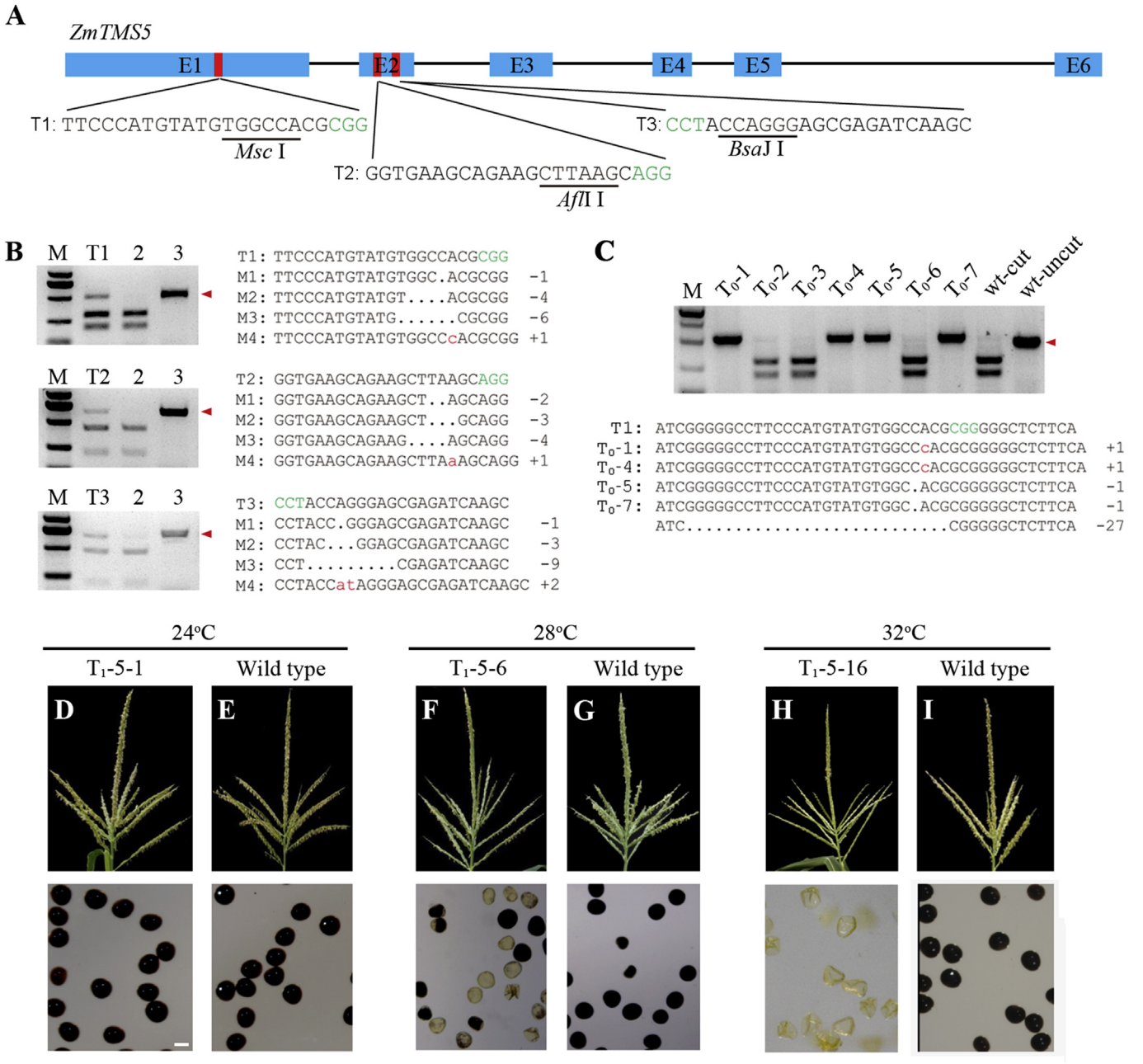

Fig. 1. sgRNA-induced targeted mutations in *ZmTMS5* and fertility phenotypes of *tms5* mutants at different temperatures. **A**: sgRNAs targeting exon 1 and exon 2 of *ZmTMS5*. The sgRNA target sequences (T1, T2 and T3) are indicated in red boxes and the PAM is shown in green. The restriction enzyme sites (*Msc* I, *Afl*I I and *Bsa*J I) used for the PCR/RE assays are underlined. **B**: PCR/RE assays for detecting sgRNA-induced mutations in *ZmTMS5* in maize protoplasts. Lanes T1, T2 and T3 are from digested sgRNA-transformed protoplasts; lanes 2 and 3 are from digested and undigested wild-type controls, respectively. Red arrowheads indicate bands with mutations. The sequences of sgRNA-induced mutations are shown on the right-hand side. Deletions and insertions are indicated by dots and red letters, respectively, and the numbers indicate how many nucleotides are involved (+, insertion; −, deletion). **C**: PCR/RE assays for detecting T1 sgRNA-induced mutations in T_0 plants. In lanes T_0-1 to T_0-7, PCR fragments were amplified from the transgenic maize plants and digested by *Msc* I. Lanes wt-cut and wt-uncut showed PCR fragments amplified from a wild-type control plant with and without digestion, respectively. The sequences of sgRNA-induced mutations in the T1 target site in T_0 plants are shown beneath. **D** and **E**: Tassel (upper panels) and pollen (lower panels) in *tms5* mutant (T_1-5-1) and wild-type plants at the low temperature (24°C). The pollen (stained with I_2–KI solution) was fertile in both *tms5* mutant and wild-type plants. **F** and **G**: Tassel (upper panels) and pollen (lower panels) in *tms5* mutant (T_1-5-6) and wild-type plants at 28°C. Pollen displayed a fertility transition in the *tms5* mutant plant, but fertile in the wild-type plant. **H** and **I**: Tassel (upper panels) and pollen (lower panels) in *tms5* mutant (T_1-5-16) and wild-type plants at the high temperature (32°C). Pollen was sterile in the *tms5* mutant plant but fertile in the wild-type plant. In **D**–**I**, bars = 100 μm.

all had biallelic homozygous mutations, as had their respective parents (Table S3). The next generation of T_0-5 plant was selected for further fertility analysis.

Growth chambers were used for growing T_1-5 plants at different average day temperatures (see the Materials and methods section) under fixed photoperiod conditions. At the low temperature of 24°C, *tms5* mutants were male-fertile; the anther split normally (Fig. 1D and E), and the female organ developed normally and could accept the male gamete, similar to the wild-type plants. At the temperature of 28°C, the *tms5* mutants displayed a fertility transition (Fig. 1F and G). At the high temperature of 32°C, however, *tms5* mutants were male-sterile and the anther split abnormally in comparison to the wild-type plants (Fig. 1H and I).

An advantage of the SSN-mediated gene editing approach is the potential to produce progeny that have the targeted gene mutation but are also free of any transgene. To evaluate whether it was possible to achieve *tms5* mutants without the presence of foreign DNA (pBUN411–T1 construct) in the T_1 maize genome, PCR-based assays were performed, employing the following primer pairs: F1/R1, specific for the *OsU3* promoter region and target site; F2/R2, specific for the *Cas9* gene; F3/R3, specific for the *Bar* gene in the plasmid pBUN411–T1 (Fig. S1 and Table S4). PCR assays did not detect *Cas9*, *Bar* or the *OsU3* promoter region in 2 of 42 (4.8%) T_1 plants derived from the T_0-5 line (Fig. S2). The pBUN411–T1 construct was segregated by simple self-fertilization, and the targeted *tms5* mutation was transmitted independently (Fig. S2). These results indicate that Cas9-free plants carrying only the desired *tms5* mutation can be obtained by segregation, and provide a useful germplasm that is potentially used to simplify hybrid maize seed production in the future.

In our work, we targeted knockout of the *ZmTMS5* gene using the CRISPR/Cas9 system. All of the edited T_0 plants showed biallelic modification (Fig. 1C), and the *tms5* mutants were transmitted faithfully to the next generation. Homozygous T_1 *tms5* mutants are male-sterile at 32°C, but male-fertile at 24°C. Notably, there was a fertility transition at 28°C, suggesting that the cultured temperature was an important factor influencing the fertility of *tms5* mutants. In addition, plants with the TGMS trait that are created by altering only a few nucleotides among the billions that comprise the genome of maize might prove to be more acceptable to the public than plants that carry foreign DNA in their genomes. In conclusion, our work on the targeted modification of *ZmTMS5* without foreign DNA integration indicates that the CRISPR/Cas9 system is a successful approach to targeted mutagenesis for creating new agronomic traits in maize which might be used for molecular breeding and basic research.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (Nos. 31501376 and 31570369), the National Key Research and Development Program of China (No. 2016YFD0101804) and the National Transgenic Science and Technology Program (No. 2016ZX08010002).

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2017.02.002.

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9 December 2016
Available online 21 February 2017

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