Targeted mutagenesis of a conserved anther-expressed P450 gene confers male sterility in monocots

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Targeted mutagenesis using programmable DNA endonucleases has broad applications for studying gene function in planta and developing approaches to improve crop yields. Recently, a genetic method that eliminates the need to emasculate the female inbred during hybrid seed production, referred to as Seed Production Technology, has been described. The foundation of this genetic system relied on classical methods to identify genes critical to anther and pollen development. One of these genes is a P450 gene which is expressed in the tapetum of anthers. Homozygous recessive mutants in this gene render maize and rice plants male sterile. While this P450 in maize corresponds to the male fertility gene Ms26, male fertility mutants have not been isolated in other monocots such as sorghum and wheat. In this report, a custom designed homing endonuclease, Ems26+, was used to generate in planta mutations in the rice, sorghum and wheat orthologs of maize Ms26. Similar to maize, homozygous mutations in this P450 gene in rice and sorghum prevent pollen formation resulting in male sterile plants and fertility was restored in sorghum using a transformed copy of maize Ms26. In contrast, allohexaploid wheat plants that carry similar homozygous nuclear mutations in only one, but not all three, of their single genomes were male fertile. Targeted mutagenesis and subsequent characterization of male fertility genes in sorghum and wheat is an important step for capturing heterosis and improving crop yields through hybrid seed.

Introduction

Crop improvement on a global scale is required to meet growing food demands (Parry and Hawkesford, 2012; Ray et al., 2013). The expansion of yield gains made by developing hybrid crops, rather than varieties, would be an important step to achieve such progress where harnessing the advantages of heterosis is the main objective of hybrid breeding (Duvick, 1999; Melchinger, 1999; Tester and Landry, 2010). Genetic divergence between parent lines has been demonstrated to correlate with the extent to which heterosis of a hybrid increases. Hybrid seed production requires that a male parent inbred pollinates a genetically distinct female inbred parent that is either male sterile or has been emasculated. This is straightforward in monocious plants such as maize that have separate male (tassel) and female (ear) flowers on the same plant, where detasseling can emasculate the female parent during hybrid seed production. While detasseling has been used for hybrid maize seed production, physical emasculation is not a scalable solution for crop species with perfect flowers such as rice, sorghum, barley, millet and wheat. Historically, cytoplasmic male sterility (CMS) has been used in these crops for hybrid seed production; however this method is not effective in all germplasm due to the dependence on nuclear restorer genes specific for a given cytoplasm (Dwivedi et al., 2008; Li and Yuan, 1999; Reddy et al., 2005; Whithford et al., 2013). Recently, a novel hybrid seed production system has been described for maize that is not limited by the genetic background (Wu et al., 2015). This Seed Production Technology (SPT) system involves restoring pollen formation while maintaining a homozygous nuclear recessive mutation in the maize male fertility gene Ms45. Although homozygous ms45 maize plants are male sterile, pollen formation can be restored in plants that contain a transformed version of the Ms45 gene. In the SPT system, only the ms45 allele and not the Ms45 restoration cassette is transmitted through pollen. The resultant ms45 progeny are used as females during hybrid production; thus, emasculation by detasseling is not necessary as these females are genetically male sterile. Although this system originally incorporated the maize Ms45 gene, it is applicable to other crops and to other nuclear male sterile mutations, whether dominant or recessive.

Extending this hybrid seed production system to other monocious crops could afford the ability to maximize yield potentials through heterosis despite important differences such as asynchronous flower development and architecture. This is particularly relevant for rice, sorghum and wheat. However, deployment of a genetically based hybrid seed production system in these crops requires the identification and isolation of male fertility genes. While male fertility genes and mutants have been described and isolated in maize (Cigan et al., 2001; Neuffer et al., 1997) and rice (Guo and Liu, 2012; Yamagata et al., 2007) by classical genetic approaches, there is a paucity of available mutants in crops such as sorghum and wheat. As opposed to the use of chemicals or irradiation to generate random mutations and the associated time-consuming screening of mutagenized plants, recent progress in DNA double-strand break (DSB) technologies holds great promise for the systematic generation of male sterility mutants in these agriculturally important crops. A variety of...
nucleases can be employed for the generation of targeted DSBs; zinc-finger nucleases (ZFN), i-Crel-based homing endonucleases (meganucleases), transcription activator-like effector nucleases (TALEN) and the Cas9-guide RNA system (often referred to as 'CRISPR-Cas') have been used to produce mutations in various plant species (Kumar and Jain, 2015; Osakabe and Osakabe, 2015; Svitashev et al., 2015; Voytas and Gao, 2014). DSBs are repaired through endogenous cellular processes of nonhomologous end-joining (NHEJ) or homology-directed repair; the NHEJ repair pathway can often be inexact resulting in mutations at the site of the DSB and in this way targeted mutagenesis is achieved. Such a directed mutagenesis strategy utilizing a redesigned i-Crel homing endonuclease to target the Ms26 male fertility gene in maize has recently been reported (Djukanovic et al., 2013). As demonstrated in rice, the rice Ms26 ortholog encodes for a cytochrome P450 mono-oxygenase enzyme (OsCYP704B2) and is expressed in the tapetum and microspores in the developing anther (Hobo et al., 2008; Li et al., 2010). The homozygous recessive ms26 mutants in maize and rice are defective in both tapetum and microspore development resulting in male sterility (Li et al., 2010; Loukides et al., 1995). The redesigned endonuclease described by Djukanovic et al. (2013), Ms26+, targets a 22-nt sequence found in the 5th exon of Ms26. DSB generated by Ms26+ within the predetermined chromosomal recognition site and NHEJ repair resulted in mutations in the Ms26 gene and loss of function. Maize plants containing homozygous recessive ms26 mutant alleles were phenotypically normal, producing tassels that did not shed pollen. These mutant ms26 alleles were transmitted to progeny at the expected segregation ratio (Djukanovic et al., 2013). The male sterile phenotype generated by targeted gene disruption using the Ems26+ endonuclease was indistinguishable from ms26 maize mutants generated by transposon-tagging (Loukides et al., 1995; Fox, T., DuPont Pioneer, unpublished observation).

This report demonstrates that the Ems26+ endonuclease can generate targeted mutations in the orthologs of Ms26 genes of rice (Oryza sativa, Os), sorghum (Sorghum bicolor, Sb) and common wheat (Triticum aestivum, Ta). Orthologous Ms26 mutations in rice and sorghum plants, such as maize (Zea mays L., Zm), confer a recessive male sterile phenotype, and restoration of fertility in these mutant sorghum plants was achieved by a transformed copy of maize Ms26. In addition, using a conditionally regulated Ems26+, mutations in the wheat Ms26 ortholog were recovered in all three genomes of hexaploid spring wheat. These results demonstrate the utility of nuclease-facilitated gene editing to produce male fertility gene mutants for the purpose of unlocking yield potential through exploiting heterosis in these major food crops.

**Results**

The maize Ms26 gene is a member of a P450 protein family whose expression is limited to developing anthers (Cigan et al., 2005). Biochemical characterization indicated putative roles of the rice Ms26 ortholog (OsCYP704B2) catalysing ω-hydroxylated C16 and C18 fatty acids as well as synthesis and transport of sporopollenin precursor components important to pollen wall formation (Li et al., 2010). Maize and rice plants carrying homozygous recessive mutations disrupting the coding-frame of the Ms26 and OsCYP704B2 genes, respectively, resulted in an inability of these plants to generate fertile pollen grains (Djukanovic et al., 2013; Li et al., 2010). Consistent with the importance of this P450 for pollen development, protein sequences with high homology to the maize Ms26 were identified in sorghum and wheat by searching plant sequence databases. The sorghum and wheat Ms26-like sequences along with the rice OsCYP704B2 gene were, therefore, referred to herein as SbMs26, TaMs26 and OsMs26, respectively. Although there is significant sequence identity across these crop species (Figure S1 and Text S1), nothing is known regarding the functional conservation of this P450 in sorghum and wheat. As shown in Figure 1, the 22-nt Ems26+ recognition site is conserved across these monocots and resides in the last exon of each gene, and this exon contains the important haem-binding loop common to haem-thiolate cytochrome P450s (Werczik-Reichhart and Feyereisen, 2000). To determine whether the Ms26 gene is required for male fertility, several directed mutagenesis approaches using the Ems26+ endonuclease were developed and tested in rice and then used to determine whether this gene is required for male fertility in sorghum and wheat.

**Ems26+-generated mutations in rice**

Young rice callus [(Oryza sativa L. ssp. Japonica (cv. Kitaake)] initiated from germinating seed was used as transformation targets for biolistic- or Agrobacterium-mediated delivery of vectors that contained the custom endonuclease, Ems26+. For biolistic transformation, two vectors were constructed: (i) a vector containing a codon-optimized Ems26+ under the transcription regulation of the maize ubiquitin promoter (UBIpro:Ems26+) enabling constitutive expression, and (ii) a vector containing a herbicide resistance selectable marker phosphinotrichcin acetyltransferase (PAT) fused to the red fluorescence protein (RFP) gene and placed under the regulation of the maize END2 promoter. This vector allowed transformed rice cells to grow in the presence of the herbicide glufosinate while monitoring red fluorescence, when codelivered into 2-week-old, seed-derived rice callus. Ems26+ contains a nuclear localization signal at the N-terminus and an intron to eliminate expression in bacterial cells used for vector propagation (Djukanovic et al., 2013). Approximately 6 weeks after bombardment, callus sectors that grew on media containing glufosinate were screened for mutations at the 22-nt Ms26 target site. Due to the presence of a BsWI restriction site (5′-CGTAGC-3′) across the centre of the Ems26+ recognition sequence and at the site of Ems26+-mediated DNA cleavage (Figure 1), PCR amplification and digestion by BsWI was used as an initial screening tool to detect Ms26 mutations. PCR amplicons resistant to BsWI digestion would be indicative of sequence changes at this recognition site due to cutting by Ems26+ and imprecise repair by NHEJ (Djukanovic et al., 2013). Genomic DNA isolated from these glufosinate-resistant callus sectors was amplified using the primer pair UNIMS26 5′ and UNIMS26 3′-1 to generate a 653-bp amplicon that was then subjected to digestion with BsWI. Products of these reactions were electrophoresed on 1% agarose gels and screened; amplicons containing wild-type sequence across the Ems26+ recognition site would produce 376- and 277-bp DNA digestion products (Figure S2, lanes 2, 4). Screening of 292 glufosinate-resistant callus sectors that contained PCR products resistant to BsWI digestion. Subcloning and DNA sequence analysis of these PCR products revealed a variety of mutations across the Ms26 target site, which included single- and multiple-nucleotide insertions and deletions (Figure 2).
To examine the male fertility phenotype of rice plants containing OsMs26 mutations generated by Ems26+, plants were regenerated from glufosinate-resistant callus sectors, analysed for OsMs26 mutations and allowed to set self-pollinated seed (T1 seed). T1 seed from three plants containing nonidentical OsMs26 mutations, but lacking the randomly integrated transformation vectors, were advanced for progeny testing. Using the BsiWI digestion assay, genomic DNA collected from progeny plantlets was amplified with the UNIMS26 primer pair and screened for mutations at the Ms26 target site. Heterozygous OsMs26/Osms26 (Figure S2, lane 4) and homozygous Osms26/Osms26 (Figure S2, lane 6) mutant plants were advanced and scored for their ability to form pollen by analysing seed set upon self-fertilization. Throughout vegetative growth, these plants appeared phenotypically normal and flowered at the same time as wild-type plants with the exception that dehisced anthers were not observed in the homozygous recessive Osms26 plants (Figure 3a). Microscopic examination of anthers from OsMs26/OsMs26 plants staged at late uninucleate microspore development revealed normal appearing microspores, which continued to develop into mature, fertilization-competent pollen. However, examination of anthers from Osms26/Osms26 plants revealed that microspores arrested development immediately after tetrad release, whereupon these plants contained anthers devoid of mature pollen (Figure 3b). These observations were similar to those reported by Loukides et al. (1995) and Li et al. (2010).

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Surprisingly, as shown in Figure 3b, the in-frame deletion of nine nucleotides in Osms26-Δ9.2 conferred a male sterile phenotype. In summary, all OsMs26/Osms26 plants were male fertile, and all Osms26/Osms26 plants were male sterile (Table 1). In addition, homozygous Osms26/Osms26 plants in this study were female fertile as demonstrated by their ability to set seed when fertilized with wild-type rice pollen.

Inducible Ems26+ for producing double-strand breaks

Given the ability to successfully direct mutations to the rice Ms26 ortholog that confer a male sterile phenotype, the requirement of Ms26 for pollen development in sorghum and wheat was investigated. Relative to maize and rice, transformation of sorghum and wheat is challenging (Casas et al., 1998). Moreover, gene editing in wheat is complicated due to the size and polyploid nature of the genome. Thus, as an alternative to biolistic delivery of Ems26+, an in planta method to stimulate targeted mutagenesis in sorghum and wheat was devised. This strategy depended on the generation of plants containing a stably integrated, temperature-regulated Ems26+ expression cassette delivered on a T-DNA vector by Agrobacterium-mediated transformation. Ems26+ was placed under the transcriptional control of a maize temperature-regulated promoter, MDH (ZmMDHpro: Ems26+) (Svitashev et al., 2015), which allowed for temperature-inducible expression of the nuclease in plant tissues. In this strategy, immature embryos from plants containing conditionally expressed Ems26+ would be heated at 37 °C for 24 h to generate mutations. This T-DNA also contained a blue fluorescence gene (Cyan) regulated by an embryopreferrered EN2 promoter (ZmEnd2pro:Cyan). This reporter served as a visible marker for selection of transformed callus, fertilized immature embryos and mature seed. A disrupted copy of the DsRED gene transcriptionally regulated by a maize Histone 2B promoter was also present in this T-DNA to monitor endonuclease activity. This nonfunctional colour marker consisted of duplicated DsRED gene fragments (RF-FP) with a 369-bp overlap separated by a 136-bp spacer containing the Ems26+ recognition site (Text S2). DSBs in RF-FP would either be repaired by NHEJ or intramolecular recombination within the repeated RF-FP fragments (Figure 4a). Duplicated gene coding regions interrupted by a DSB target site(s) have been demonstrated to be useful visual reporters to detect nuclease activity (Puchta and Hohn, 2012). As such, one outcome of a DSB at the target site would be to stimulate intramolecular recombination within the repeated RF-FP restoring DsRED gene function as revealed by the appearance of red-fluorescing callus cells against a background of blue fluorescence. Thus in contrast to control callus where no red fluorescence would be expected, the appearance of red-fluorescing cells in treated samples would demonstrate activity of Ems26+ endonuclease and suggest potential cutting and, therefore, mutagenesis within the endogenous Ms26 gene.

To test this scheme, rice plants were generated to contain a stable, randomly integrated ZmMDHpro:Ems26+ expression casette as described above in the rice genome. Four independently transformed plants were allowed to self-pollinate, and blue-fluorescing seed (ZmEnd2pro:Cyan) were selected to initiate rice calli (Hervé and Kayano, 2006; Toki, 1997). Calli from these four events were allowed to grow at 26 °C (control) for 30 days, whereupon a portion of callus from each event was then incubated at 37 °C (treated) for 48 h. The temperature-treated calli were returned to 26 °C, upon which control and treated samples were examined for the appearance of red-fluorescing sectors. Over the course of 2 weeks, red sectors were readily visible in three of the four independent callus isolates treated at 37 °C (Figure 4b) and none in the control calli. Detection of red fluorescence indicated DSBs as a result of active Ems26+; therefore, genomic DNA was isolated from several red-sectoring callus events and screened for mutations at the endogenous OsMs26 gene. A DNA fragment spanning a portion of the OsMs26 gene was amplified from control and temperature-treated calli and screened using the BsaWI digestion assay. Analysis of amplified products from the three callus events demonstrated RF-FP repair and revealed BsaWI-resistant fragments present only in genomic DNA derived from these callus sectors incubated at 37 °C. Uncut amplified PCR products from control and temperature-treated callus DNA were sequenced.

Table 1 Male fertility phenotypes of T1 progeny plants with heterozygous or homozygous OsMs26 mutations

| Mutation | Plants | OsMs26/Osms26 | OsMs26/Osms26 |
|----------|-------|---------------|---------------|
|          |       | Male sterile | Male sterile |
| OMs26-Δ9.2 | 18    | 0             | 0             | 8             |
| OMs26-Δ36  | 9     | 0             | 0             | 4             |
| OMs26-Δ65A1| 15    | 0             | 0             | 4             |

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These blue-only-fluorescing embryos were incubated at 26 °C. Four independent T-DNA insertion events were harvested 10 immature embryos (10 plants, 16 embryos per plant, representing several plants containing single-copy ZmMDHpro:Ems26 alleles and the absence of red-fluorescing seed suggested that the Ems26 recognition site (position identified by the black bar above spacer sequence). These observations demonstrated that a stably integrated and conditionally regulated endonuclease could be used in rice to generate site-specific mutations and, thus, it has the potential to perform similarly in other monocots such as sorghum and wheat.

**Mutations in the sorghum Ms26 ortholog confer male sterility**

To determine whether the ortholog of maize Ms26 in *S. bicolor* was required for pollen formation, Ems26+ was used to direct mutations to the conserved recognition site in the 5th exon of this single-copy gene (*SbMs26*) present on Chromosome 1 (Sbo1g045960). Immature embryos from *S. bicolor* [Tx430 (Miller, 1984)] were transformed with the ZmMDHpro:Ems26+ T-DNA using *Agrobacterium*-mediated delivery. Six independent blue-fluorescing callus sectors, indicative of the presence of a stably integrated copy of ZmMDHpro:Ems26+ T-DNA, were used to generate sorghum plants (approximately 2–12 plants per callus sector). Intactness and copy number of the T-DNA insertion were determined in these plants by DNA hybridization analysis (Southern, 1975) while amplification and sequencing of genomic DNA confirmed the presence of only wild-type *SbMs26* alleles in these plants. In addition, no red fluorescence was detected when progeny seed were screened visually. The presence of only wild-type *SbMs26* alleles and the absence of red-fluorescing seed suggested that the Ems26+ was not activated in these T0 plants. Several plants containing single-copy ZmMDHpro:Ems26+ T-DNA insertions were allowed to self-pollinate, and blue-fluorescing immature embryos (10 plants, 16 embryos per plant, representing four independent T-DNA insertions) were harvested 10–15 days postfertilization to be used for *SbMs26* mutational analysis. Visual inspection of these harvested embryos revealed that there were no red-fluorescing sectors or red-fluorescing embryos present. These blue-only-fluorescing embryos were incubated at 26 °C or 37 °C for 48 h and examined for RF-FP repair. In contrast to control embryos maintained at 26 °C, red-fluorescing sectors were readily detected to varying degrees in all embryos incubated at 37 °C indicating the presence of active Ems26+. Calli from embryos treated at 37 °C were grown for 4 weeks and used to regenerate plants that were subsequently screened for mutations at the *SbMs26* target site on Chromosome 1. In total, approximately 20% of the plants (n = 440) regenerated from these four independent T-DNA insertion events revealed heterozygous mutations within the *SbMs26* gene when screened by PCR amplification and digestion with *BsaW1*. Although expected at a low frequency, biallelic mutations were not detected in these sorghum experiments. Similar to the experiments in rice, DNA sequence analysis of these PCR products (n = 50 independent *BsaW1*-resistant PCR fragments) revealed a variety of mutations across the *SbMs26* region that consisted of deletions and insertions. In total, 16 nonidentical mutations were identified in these regenerated sorghum plants (Figure S3 and Text S3).

Sorghum plants containing heterozygous mutations at *SbMs26* were allowed to flower, and seed from self-pollinated plants was advanced to examine male fertility phenotype in progeny plants. T1 progeny containing heterozygous *SbMs26* mutations [36-bp deletion (*Sbms26*-Δ36), 59-bp deletion with 11-bp insertion (*Sbms26*-Δ59 + 11I) and 78-bp deletion (*Sbms26*-Δ78)] were phenotypically identical to wild-type sorghum Tx430 plants and were male fertile (Table 2). As shown in Figure 5a, panicles from *SbMs26*/*sBms26-*A78 plants extruded anthers that contained pollen and had begun to set seed as demonstrated by the appearance of collapsed stigmas after anthesis. Homozygous *SbMs26* plants were also normal in stature and development with the exception of being completely male sterile: *SbMs26*-Δ78 plants extruded small shrivelled anthers, did not shed pollen and did not set seed upon self-pollination (Figure 5b and c). Upon closer examination, pollen was easily detected in anthers from *SbMs26*/*sBms26-*A78 plants (Figure 5d and g), while pollen was not observed in anthers from *SbMs26*-Δ78/*sBms26-*A78 plants (Figure 5e and h). In summary, all *SbMs26*/*sBms26-*A78 plants were male fertile, while all *SbMs26*/*sBms26-*A78 plants were male sterile (Table 2). These male sterile plants were female fertile as demonstrated by their ability to set seed when pollinated with wild-type sorghum pollen.

**Figure 4 OsMs26 mutations in rice generated using a stably integrated cassette containing Ems26+ under transcriptional control of a maize temperature-regulated promoter.** (a) *Agrobacterium* vector for stable integration of the ZmMDHpro:Ems26+. The vector also contained a blue fluorescence gene (Cyan) as a visible marker regulated by the maize END2 promoter (END2). The disrupted DsRED gene (H2Bpro:RF-FP) contained duplicated DsRED gene fragments (RF, FP) separated by a 136-bp spacer containing the Ems26+ recognition site (position identified by the black bar above spacer sequence). (b) Blue and red fluorescence overlay image of rice calli containing stably integrated ZmMDHpro:Ems26+ after treatment at 37 °C for 48 h. Images captured 7 days after treatment. (c) Deletions in OsMs26 achieved via targeted mutagenesis. Shown in the reference sequence, the 22-nt Ems26+ recognition sequence is highlighted in grey and yellow. The latter indicates the 4 bp overhang, 5′-GTAC-3′, produced by nuclease cutting and is highlighted throughout for orientation purposes. Nucleotide shown are deleted sequence in seven of eight identified mutations, one larger deletion not shown. ZmMDH, Maize Mannitol dehydrogenase promoter; Ems26+, homing endonuclease targeting Ms26 target site; E, CaMV 35S promoter; H2B, maize Histone 2B promoter.
To further investigate that the male sterile phenotype observed in these sorghum mutant plants was directly due to deletions at *SbMs26*, restoration of fertility was tested using a copy of the maize *Ms26* gene (also referred to as *ZmMs26*) introduced by transformation. Wild-type sorghum plants were generated to contain a single copy of the *ZmMs26* gene introduced on a T-DNA expression cassette and crossed with plants carrying either the *Sbms26-A36* or the *Sbms26-A78* deletion mutation. From these crosses, F1 plants were screened and those that contained both the *Sbms26* mutation and *ZmMs26* were allowed to self-pollinate to generate F2 seeds. F2 plants were grown under greenhouse conditions and genotyped for the *Sbms26* mutation and presence of *ZmMs26*. A set of F2 plants homozygous for the *Sbms26* mutation but segregated for the *ZmMs26* were allowed to self-pollinate and embryos were harvested 15 days postfertilization. Blue-fluorescing embryos (n = 12) isolated from each plant were either held in the dark at 26 °C (control, n = 4) or incubated at 37 °C for 24 h (treated, n = 8), whereupon the treated embryos were returned to 26 °C and allowed to grow in the dark. As observed for the rice and sorghum experiments, approximately 72 h after treatment, wheat embryos incubated at 37 °C began to develop red-fluorescing sectors, consistent with the presence of active *Ems26* endonuclease (Figure S6). No red-fluorescing sectors were detected in control, nontreated wheat embryos. To ascertain whether *Ems26*-generated mutations at endogenous *TaMs26* sequences, total genomic DNA was isolated from temperature-treated and control embryos and amplified using UNIMS26 primers. Deep sequencing analysis of these amplicons (approximately 5 million reads per sample) revealed that 1.6% of the amplicon reads from the temperature-treated embryos contained mutations as compared to <0.007% from control embryos (Table S2 and Figure S7). Callus from embryos treated at 37 °C was used to regenerate 121 plants that were then screened for mutations using the *BsiWI* digestion assay; ten wheat plants containing *BsiWI* digestion-resistant amplicons. DNA sequencing of these resistant PCR products identified seven different mutations within the *Ems26* recognition site. Together these experiments demonstrated that a conditionally expressed *Ems26* was capable of generating targeted mutations at the *TaMs26* gene in *planta* and that single-genome mutants in this P450 are not sufficient to confer a male sterile phenotype in the spring wheat.

### Table 2 Male fertility phenotypes associated with sorghum mutants and complementation of male sterility using maize Ms26 gene

| Mutation | Plants | Male fertile | Male sterile | Male fertile | Male sterile |
|----------|--------|--------------|--------------|--------------|--------------|
| *SbMs26/Sbms26* | | | | | |
| *Sbms26-A36* | 6     | 3            | 0            | 0            | 3            |
| *Sbms26-A78* | 6     | 3            | 0            | 0            | 3            |
| *Sbms26-A59 + 111* | 6 | 3 | 0 | 0 | 3 |

| Complementation data | | | | | |
|----------------------|--------|--------|--------|--------|
| *Sbms26/Sbms26* | + *ZmMs26* | | | |
| Male fertile | Male sterile | Male fertile | Male sterile |
| *Sbms26-A36* | 10³ | 0 | 4 | 6 |
| *Sbms26-A78* | 15¹ | 0 | 10 | 5 |

*SbMs26*, sorghum Ms26 ortholog; *ZmMs26*, maize Ms26 gene.

1Includes events #3 and #4.

2Includes events #1, #2, and #3.

**Ems26**-targeted mutations in the wheat *Ms26* ortholog

As described above, the maize *Ms26* polypeptide sequence was used to search the translated hexaploid wheat genome database (Chinese spring) (Consortium, 2014) for homologous sequences. A 4.2-kb contig derived from Chromosome 4AS was found capable of encoding a 550 amino acid polypeptide having 91% sequence similarity to maize *Ms26* protein (Figure S1 and Text S4). Additional BLAST (NCBI, Bethesda, MD, USA) search yielded wheat 4BL and 4DL contigs that only partially overlapped the region corresponding to the 2nd (4DL) and last exons (4BL and 4DL) of the 4AS sequence. To ascertain whether the *Ems26* recognition site was conserved across all three wheat genomes, UNIMS26 primers were used to amplify genomic DNA from the hexaploid spring wheat cultivar Fielder. DNA sequence analysis of these amplified products, when compared to the contigs described above, suggested that the 22-nt Ems26+ recognition site was conserved. The A-, B- and D-genomic copies of the wheat *Ms26* ortholog (referred to as *TaMs26*) were assigned to the Fielder genome (Figure 5A). Distinguishing features within this amplified region include a six-nucleotide, two-amino acid gap; functional relevance of this sequence difference is not yet known, but interestingly is found in a region that is diverse across maize, sorghum, rice and wheat Mt26 protein alignments (Figure 5B).

To examine whether the *Ems26*+ endonuclease was competent to generate targeted mutations at the *TaMs26* gene orthologs in hexaploid wheat, Fielder plants containing the *ZmMDHpro:Ems26*+ T-DNA were generated by Agrobacterium-mediated transformation. Immature Fielder embryos were used as transformation targets. Blue-fluorescing callus sectors indicative of T-DNA integration were selected to initiate plant regeneration. Young wheat plantlets grown under greenhouse conditions were analysed for *ZmMDHpro:Ems26*+ T-DNA copy number by qPCR. Four independently transformed wheat plants containing single- or low-copy *ZmMDHpro:Ems26*+ T-DNA were allowed to self-pollinate and embryos were harvested 15 days postfertilization. Blue-fluorescing embryos (n = 12) isolated from each plant were either held in the dark at 26 °C (control, n = 4) or incubated at 37 °C for 24 h (treated, n = 8), whereupon the treated embryos were returned to 26 °C and allowed to grow in the dark. As observed for the rice and sorghum experiments, approximately 72 h after treatment, wheat embryos incubated at 37 °C began to develop red-fluorescing sectors, consistent with the presence of active *Ems26*+ endonuclease (Figure S6). No red-fluorescing sectors were detected in control, nontreated wheat embryos. To ascertain whether *Ems26*+ generated mutations at endogenous *TaMs26* sequences, total genomic DNA was isolated from temperature-treated and control embryos and amplified using UNIMS26 primers. Deep sequencing analysis of these amplicons (approximately 5 million reads per sample) revealed that 1.6% of the amplicon reads from the temperature-treated embryos contained mutations as compared to <0.007% from control embryos (Table S2 and Figure S7). Callus from embryos treated at 37 °C and containing *ZmMDHpro:Ems26*+ was used to regenerate 121 plants that were then screened for mutations using the *BsiWI* digestion assay; ten wheat plants contained *BsiWI* digestion-resistant amplicons. DNA sequencing of these resistant PCR products identified seven different mutations within the *Ems26* recognition site consisting of a single-nucleotide addition and deletions of various sizes; a nonoverlapping subset of these mutations was found within each genome copy of *TaMs26* (Figure 6). Plants containing *TaMs26* mutations were allowed to self-pollinate for progeny analysis. Inheritance analysis determined that, with the exception of the single-C-nucleotide-insertion mutant (no seed set), mutations in all three genomes were sexually transmitted to progeny plants. These progeny plants containing single-genome, homozygous mutations in the A-, B- or D-genomic copies of *TaMs26* were phenotypically normal and male fertile (Table S3). Together these experiments demonstrated that a conditionally expressed *Ems26*+ was capable of generating targeted mutations at the *TaMs26* gene in *planta* and that single-genome mutants in this P450 are not sufficient to confer a male sterile phenotype in the spring wheat.
cultivar Fielder. Studies that genetically combine these different A-, B- and D-genome mutations are in progress to determine the requirement of Ms26 for pollen development in wheat.

**Discussion**

The ability to perform targeted mutagenesis in plants is a valuable tool for gene functional analysis and generation of genetic diversity. In this report, targeted mutagenesis of the monocot male fertility gene, Ms26, was accomplished in three different plant species using either a constitutively or conditionally expressed custom designed I-Cre endonuclease, Ems26+. In rice, approximately 7% of the primary plants contained mutations across the highly conserved Ems26+ target site; these mutations ranged from single-nucleotide deletions to large insertions. Similar to mutations identified by classical mutagenesis of maize and rice, targeted mutations to the rice Ms26 ortholog also resulted in complete male sterility by preventing the formation of pollen in homozygous recessive mutant plants. During these studies, it was determined that plants carrying a constitutively expressed Ems26+ endonuclease did not promote mutagenesis in subsequent generations of plants suggesting that mutations occurred early in the plant transformation process and functional endonucleases were not stably transmittable. In contrast, when Ems26+ was introduced into transformed plants and placed under the transcriptional control of a conditional promoter, the endonuclease was functional in progeny sorghum and wheat plants.

In previous reports, the low frequency of stable event recovery has been suggested to be an indicator of cytotoxicity due to off-target recognition for ZFNs in human cell lines and plants (Cornu et al., 2007; Szczechek et al., 2007; Townsend et al., 2009). This suggestion is supported by the observation that in maize and rice experiments, in contrast to control experiments that did not introduce UBipro:Ems26+, event recovery was lower when Ems26+ was delivered by biolistic transformation. Although it cannot be ruled out that reduced transformation frequency in these experiments was simply a consequence of the high concentration of vectors containing Ems26+ delivered by particle bombardment when compared to *Agrobacterium*
Figure 6  TaMs26 gene mutations isolated from Fielder wheat A-, B- and D-genomes via targeted mutagenesis. Ems26+ recognition sequence is highlighted in yellow above the A-genome sequence. Deleted nucleotide sequence is represented by a hyphen ("-"). Inserted nucleotide sequence is in white font and highlighted in black. Sequence differences between the A-, B- and D-genomes are highlighted in grey. White space indicates a gap in sequence.

This observed functional conservation is particularly relevant to transformation, differences in delivery and regulation of Ems26+ were important design considerations for these targeted mutagenesis experiments. The establishment of pre-integrated and conditionally regulated endonuclease was one strategy that mitigated potential toxicity by taking advantage of a promoter capable of induction at higher temperatures and the temperature-dependent biochemical activity of this class of endonucleases (Wang et al., 1997). In our studies, Ems26+ demonstrated increased nuclease activity at 37 °C both in vitro and in planta. Thus, short pulses at elevated temperatures during tissue culture or plant growth resulted in both increased transcription and nucleolytic activity of the Ems26+ gene and protein, respectively. This dual switch was an effective method for controlling DSB activity in these experiments and has been exploited as a means to rapidly generate large collections of allelic variants for gene discovery.

Pollin formation in plants is highly conserved at the biochemical and genetic level (for review see Shi et al., 2015). A number of pollen- and anther-specific genes have been identified whose function is conserved across plants. The maize Ms26 and rice OsCYP704B2 genes encode a cytochrome P450 that plays a critical role in male fertility. In this study, conservation of sequence within these orthologous genes was used to introduce targeted mutations in rice, sorghum and wheat. Importantly, this is the first isolation and functional characterization of a male fertility gene in sorghum, a close relative to maize. As in rice, sorghum plants containing homozygous recessive mutations in the ortholog of the maize Ms26 gene were male sterile. Moreover, a transformed copy of the maize Ms26 gene was able to restore fertility in these sorghum mutant plants. The ability to restore fertility in these sorghum mutants using a maize Ms26 gene further supports biochemical conservation of P450 function. This observed functional conservation is particularly relevant to the foundational experiments described in this report where the orthologs of the maize Ms26 gene were targeted in wheat. To date, there are only two recessive male fertility mutants that have been described in Triticum: Ms1 and Ms5 (Whitford et al., 2013). Given the high sequence similarity across maize, sorghum and rice, it is likely that orthologs of Ms26 in wheat are also required for pollen development. In contrast to these diploid species, wheat is an allohexaploid. In this study, it was observed that single-genome homozygous TaMs26 mutations in hexaploid wheat did not confer a male sterility phenotype. These results suggest that no single TaMs26 genomic copy from the A-, B- or D-genome is essential to fertility in wheat, as the other wild-type TaMs26 copies present in these plants are likely to function to maintain pollen development and a male fertility phenotype. It is interesting to speculate that despite the high amino acid sequence conservation across all three wheat genome copies, functional differences may be revealed and associated with relatively minor sequence changes, in particular within the regions of amino acid diversity (Figure S5). Thus, combining these TaMs26 gene mutations via genetic crosses will allow examination of the effects of various combinations of homozygous TaMs26 mutations in the three genomes of hexaploid wheat (i.e. in one, two or all three single genomes) on male fertility. In this report, targeted mutagenesis of the monocot male fertility gene, Ms26, was made possible by advances of genomics, gene editing and plant transformation that overcome two barriers in crop improvement. First, having the ability to specifically target and modify fertility genes would extend SPT systems to crops having perfect flowers enabling yield gains by heterosis not yet fully realized. Second, gene editing, when combined with advanced plant transformation methods, would allow for precision breeding of elite germplasm reducing the time needed for introgression of induced sequence diversity by backcrossing. Classical methods for introducing genetic diversity into plants include mutagenesis with chemical and physical agents or transposition. These nontargeted methods result in random and
often additional and undesired changes in the genome. These approaches are both time-consuming and resource-intensive requiring multiple generations of backcrossing and selection to convert back to the original state of elite genetics for maximal crop yields. In contrast to these nontargeted approaches that slow discovery and crop improvement, adoption of the precision breeding innovations described above is essential to sustaining agriculture as population increases and as protection of natural resources becomes more challenging (Tian et al., 2016; Tilman et al., 2011; West et al., 2014). Targeted mutagenesis of the Ms26 gene is a good example of how advanced technologies could be deployed to rapidly and precisely generate mutant fertility alleles in elite germplasm of maize, rice, sorghum and potentially wheat. While not exclusively considered a replacement for classical breeding methods, these advances could have a positive impact and change modern agriculture. As with classical mutagenesis methods, should undesired genome modifications occur (i.e. random integration of plasmid DNA or off-target mutations), these changes could be easily bred away by genetic crosses to elite parental lines, which would eliminate unwanted genome alterations yet maintain targeted sequence variation. In addition, high-resolution sequencing is particularly useful in the design and testing of nucleases with unique recognition and cutting specificity, thus further improving and advancing gene editing as an accelerated breeding tool.

**Experimental procedures**

**DNA methods**

Standard DNA techniques as described in Sambrook et al. (1989) were used for vector construction. The Ems26+ was maize codon-optimized and designed to include an amino-terminal nuclear localization signal SV40 (MAKKKRKV, nucleotide position 2837–30) to accommodate translational fusion to a maize proteinase inhibitor II gene (PinII) [nucleotides 2–2016 The Authors. Plant Biotechnology Journal published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., 15, 379–389

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amplifications were then excised and purified with a Qiagen gel extraction spin column. The concentration of the gel-purified amplifications was measured with a Hoechst dye-based fluorometric assay, combined in an equimolar ratio, and single-read 100-nt-length deep sequencing was performed on an Illumina Genome Analyzer Ix (Illumina Biopharmaceuticals, Inc., Hayward, CA, USA) with a 30%–40% (v/v) spike of PhiX control v3 (Illumina, Inc., San Diego, CA, USA) to off-set sequence bias. Only those reads with a ≥1 nucleotide indel arising within a 6-nt window centred over the expected site of cleavage and not found in a similar level in the negative control were classified as NHEJ mutations. The total numbers of NHEJ mutations were then used to calculate the % mutant reads based on the total number of reads of an appropriate length containing a perfect match to the barcode and forward primer.

**Plant methods**

Rice, sorghum and wheat plant transformation and tissue culture details are outlined in Data S1.

**Microscopy**

Sorghum samples were dissected to remove the glumes and expose the anthers, and fixed in 2% paraformaldehyde and 4% glutaraldehyde overnight under vacuum (10 psi). After fixation, the samples were rinsed three times in 1 x PBS (Phosphate Buffer Solution). Samples were then cleared in graded TDE (2, 25%, 50% and 97%), and the 97% TDE was repeated to assure optimal clearing. Samples were mounted in 97% TDE and the coverslip was sealed with nail polish. Confocal images were taken with the Leica (Wetzlar, Germany) TCS SPE using the solid-state 405 nm with the DAPI setting (emission: 430–480); the 488 nm with the GFP setting (emission: 500–600); and the 561 nm with the DRED setting (emission: 650–700) laser lines and merged using the Leica LAS X software. Final image adjustments were accomplished with Adobe Systems Photoshop CS6 (San Jose, CA).

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**Conflict of interest**

AMC and JY are inventors on pending applications on this work and are current or former employees of DuPont Pioneer who owns the pending patent applications. MS, GB, LF, MK, M-JC, NS and SS are current or former employees of DuPont Pioneer.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Ms26 protein sequence is highly conserved across crop species.

Figure S2 BsmI restriction enzyme resistance assay screen for OsMs26 mutations.

Figure S3 BmMs26 gene mutations in sorghum achieved via targeted mutagenesis.

Figure S4 DNA sequences corresponding to the IWGSC contigs containing the wheat 4AS, 4BL and 4DL genomic regions orthologous to maize Ms26 gene and aligned with DNA sequences from Fielder.

Figure S5 Predicted protein translation products of amplicons derived from UNIMs26 primers.

Figure S6 Harvested Fielder wheat embryo containing EmS26+ under transcriptional control of a maize temperature-regulated promoter.

Figure S7 Top 10 amplicon sequences containing mutations from genomic DNA derived from Fielder wheat callus transformed with ZmMDHpro:EmS26+.

Table S1 Restoration of male fertility to homozygous Sbms26/ Sbms26 plants with a transformed copy of maize ZmMs26 was achieved using four nonidentical events in two different Sbms26 mutant backgrounds.

Table S2 Percent (%) mutant reads at the wheat EmS26+ target locus identified by deep sequencing.

Table S3 Male fertility phenotype for wheat TaMs26 mutant lines.

Text S1 4.194-kb region containing wheat Ms26 ortholog IWGSC 4AS.

Text S2 Spacer sequence (136 bp) containing multiple nuclease target sites.

Text S3 Nucleotide sequence of EmS26+ target site (underlined) present in Chromosome 7 in Sorghum bicolor Tx430.

Text S4 Predicted 550 amino acid sequence encoded by wheat Ms26 ortholog IWGSC 4AS coding region.

Text S5 Maize-optimized EmS26+ coding region.

Data S1 Experimental procedures detailing plant transformation and tissue culture methods.