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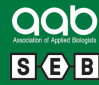

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Transcriptional gene silencing in bread wheat (*Triticum aestivum* L.) and its application to regulate male fertility for hybrid seed production

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Summary

Transcriptional gene silencing (TGS) can offer a straightforward tool for functional analysis of plant genes, particularly in polyploid species such as wheat, where genetic redundancy poses a challenge in applying mutagenesis approaches, including CRISPR gene editing. In this study, we demonstrate efficient TGS in wheat, mediated by constitutive RNA expression of a promoter inverted repeat (pIR). pIR-mediated TGS of two anther-specific genes, *TaMs45* and *TaMs1*, abolished their function resulting in male sterility. Whilst TGS of *TaMs45* required transcriptional silencing of all three homoeologs, a B-genome-specific pIR for *TaMs1* was sufficient to confer male sterility. We further show that the pIRs effect TGS of *TaMs45* gene through DNA methylation of homologous promoter sequence, successfully suppressing transcription of all three homoeologs. Applying pIR-mediated TGS in wheat, we have generated a dominant male fertility system for production of hybrid seed and demonstrated the efficacy of this system under greenhouse and field conditions. This report describes the first successful TGS in wheat, whilst providing a dominant negative approach as alternative to gene knockout strategies for hybrid wheat breeding and seed production.

Introduction

Wheat is one of the most widely grown crops in the world, providing 20% of food calories and protein in human diet. With the predicted growth in world population, a continued improvement in wheat yield is required (Gaffney *et al*., 2016; Tilman *et al*., 2011; West *et al*., 2014). Breeding for hybrids to exploit hybrid vigour in wheat is one way to increase stagnant yields (Longin *et al*., 2012; Mette *et al*., 2015). Commercialization of hybrid wheat varieties will require an efficient hybridization system to economically produce hybrid seed. Ongoing research and limited commercial efforts have focused on utilizing either chemical hybridizing agents (CHAs; McRae, 1985; Whitford *et al*., 2013) or cytoplasmic male sterility (CMS; reviewed in Kempe and Gils, 2011; Whitford *et al*., 2013) to produce hybrid seed in wheat. Each of these systems has limitations, such as induction of male sterility for CHAs and restoration of male fertility for CMS, that make full-scale deployment for commercial hybrid seed production challenging (Adugna *et al*., 2004; Kempe and Gils, 2011; McRae, 1985; Mukai and Tsunewaki, 1979; Wilson and Ross, 1962). Recently, Wu *et al*. (2016) have demonstrated the utility of Nuclear Genetic Male Sterility (NGMS) in maize for hybrid seed production, which could be applied to other crops such as rice, sorghum (Cigan *et al*., 2017) and wheat (Tucker *et al*., 2017). Due to functional redundancy of the three genomes (A, B and D), male sterility system comparable to NGMS described with maize *ms45* gene in wheat requires knockouts in all three homoeologs to generate male sterile lines (Singh *et al*., 2018). Therefore, a dominant, yet reversible, male sterility system based on a single dominant locus will be an ideal system for a polyploid species such as wheat.

Transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) provide alternate approaches to mutagenesis and gene editing to abolish gene function. Whilst PTGS acts through the cleavage or translational arrest of the targeted mRNA (Ali *et al*., 2010; Frizzi and Huang, 2010; Mansoor *et al*., 2006), TGS involves inhibition of transcription of a gene, generally through methylation of its promoter via RNA-dependent DNA methylation (RdDM) pathway (Law and Jacobsen, 2010; Matzke and Mosher, 2014). TGS is triggered by double-stranded RNA processed from a promoter sequence transcribed as a self-complementary RNA (Matzke and Mosher, 2014). TGS plays an important role in plant development as many native genes are regulated through DNA methylation-derived silencing (Brukhin and Albertini, 2021; Yasuda *et al*., 2016). Differential methylation and TGS during polyploidization is an important evolutionary aspect in plants. Combining of the three subgenomes of wheat during polyploidization resulted in chromatin and DNA methylation changes that correlate with altered gene expression (Yuan *et al*., 2020). Leach et al. observed that ~55% of the wheat genes are expressed either from one or two homeoloci through a combination of extensive transcriptional silencing and homoeolocus loss (Leach *et al*., 2014). Similarly, Gardiner et al. observed differential methylation between the three subgenomes of wheat and a correlation between the subgenome-specific promoter methylation and decreased gene expression levels (Gardiner *et al*., 2015). TGS is also involved in plant defence mechanism against transposon and viral DNA by inactivating these through silencing (Wang *et al*., 2012). Silencing of transgenes, also triggered by the plant defence mechanism, is another important aspect of TGS (reviewed in (Rajeev Kumar *et al*., 2015)). Virus-induced gene silencing (VIGS) has

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been manipulated as tool for functional genomics, especially for species recalcitrant to genetic transformation (Dommes *et al*., 2018).

Since its discovery, gene silencing has been extensively used as a tool to suppress gene activity for functional studies. The majority of these applications, however, involve PTGS-mediated RNAi silencing in plants, including monocots (Bae *et al*., 2010; Czarnecki *et al*., 2016; McGinnis *et al*., 2005; Mourrain *et al*., 2000; Zhang *et al*., 2017), whereas application of TGS has not been widely demonstrated in plants. Deng *et al*. (2014) reported silencing of four Arabidopsis endogenous genes either using double-stranded IR or single-stranded anti-sense repeat, although the penetrance of silencing was variable. Okano *et al*. (2008) performed a comprehensive analysis of TGS in rice through analysis of seven genes, of which only one gene was silenced, whilst the other six genes were recalcitrant to silencing. Cigan *et al*. (2005) analysed TGS of five tapetum-specific genes in maize and reported complete silencing of two genes, whilst another two genes were moderately silenced, and one gene was recalcitrant to TGS. In potato, Heilersig *et al*. (2006) reported strong silencing of granule-bound starch synthase I (GBSSI) gene with full-length promoter IRs but not with shorter or allele-specific IRs. Other reports have suggested only moderate silencing for endogenous genes in rice and petunia (Okano *et al*., 2008; Sijen *et al*., 2001).

In this study, we demonstrate TGS of two wheat genes, *TaMs45* and *TaMs1*, which abolished their function and resulted in a male sterile phenotype, similar to the knockout mutations described by Okada *et al*. (2019) and Singh *et al*. (2018). It is further demonstrated that the *TaMs45* pIR-induced male sterility can be restored through the expression of a maize or rice *Ms45* gene with the maize *Ms45* promoter. Moreover, a male fertility control system is described through silencing and complementation of *Ms45* gene that can be applied for hybrid seed production in wheat. This report describes the first successful TGS of wheat genes and their utility for hybrid wheat breeding. Whilst the recent advancements in gene editing and wheat germplasm independent transformation have been made, dominant gene silencing systems like TGS, offer an attractive means of deploying traits in polyploids that would otherwise require multiple recessive knockout alleles for commercial applications.

Results

TaMs45 and *TaMs1* promoter sequence analysis and pIR design

Previous studies of TGS of endogenous genes in plants have used pIRs ranging from 184 bp to almost 1 kB in length (Cigan *et al*., 2005; Deng *et al*., 2014; Heilersig *et al*., 2006; Okano *et al*., 2008). For TGS of maize *Ms45* gene, Cigan *et al*. (2005) constitutively expressed two pIRs of 184 bp and 390 bp in length using the maize *Ubiquitin* promoter. Both of these pIRs could induce transcriptional silencing which conferred a male sterile phenotype (Cigan *et al*., 2005, 2014). It has also been determined that 184 bp sequence of the maize *Ms45* promoter (−264 to −80) was sufficient to regulate the pollen-specific expression of the maize *MS45* gene (Cigan *et al*., 2001). To design a pIR for the wheat *Ms45* genes, promoter sequences of the three homoeologs were obtained from various wheat sequence databases (Helguera *et al*., 2015; Hernandez *et al*., 2012; Wilkinson *et al*., 2012) through BLAST analysis of the maize (*Zea mays*) *Ms45* sequence (accession NM_001324231.1) (Cigan *et al*., 2001). 500 bp sequence of the *TaMs45-D* homoeolog upstream of ATG start site was analysed through alignment with maize *Ms45* promoter sequence. Overall, 52% sequence identity was observed between *TaMs45-D* and maize *Ms45* promoter (Figure S1). The *TaMs45-D* sequence from nucleotide positions −133 to −70 showed high identity (76%) with the maize *Ms45* promoter (Figure S1), suggesting conservation of regulatory elements in this region, which is important for anther-specific expression in maize (Cigan *et al*., 2001). Alignment of promoter sequences of *TaMs45-A*, *-B and -D* homoeologs showed identity of 94–95% (Figure S2). It has also been previously demonstrated that all three *Tams45* homoeologs are expressed at similar levels and only one functional allele of any homoeolog is sufficient for male fertility, suggesting that promoter of each homoeolog contributes equally towards male fertility (Singh *et al*., 2018). For TGS of *TaMs45*, an inverted repeat sequence corresponding to the 440 bp of *TaMs45-D* promoter, including the −133 to −70 region, was synthesized flanking a *Nopaline Synthase* (*NOS*) promoter spacer sequence and linked to the maize *Ubiquitin* promoter in the TAMS45-PIR construct (Figure S4a). The *TaMs45-D* designed pIR sequence was 95% and 94% homologous to the promoters of *A*- and *B*-homoeologs, respectively.

For TGS of *TaMs1*, a pIR was designed from the promoter sequence of the *TaMs1* gene on chromosome 4 B. It is known that the *B*-genome homoeolog is essential for male fertility and mutations in this homoeolog (*TaMs1*) are sufficient to confer a male sterile phenotype (Tucker *et al*., 2017). The promoter of *TaMs1* was also previously confirmed to regulate *TaMs1* gene for complementation of *ms1d* mutation (Tucker *et al*., 2017). Sequence alignment of promoters from the three homoeologs showed that *TaMs1-B* has an 88% homology with the *A*- and *D*-homoeologs (Figure S3). A 581 bp sequence immediately upstream of the ATG translation initiation site of *TaMs1* was used to design the inverted repeat with the intervening *NOS* promoter spacer sequence and linked to the maize *Ubiquitin* promoter in the TAMS1-PIR construct (Figure S4a). TAMS45-PIR and TAMS1-PIR constructs were introduced into the wheat variety Fielder through *Agrobacterium*-mediated transformation.

TaMs45 and *TaMs1* pIR expressing plants exhibit dominant male sterility

For TAMS45-PIR, a total of 31 independent T_0 plants containing one- and two-copy T-DNA insertions were identified and analysed for vegetative and reproductive development. Although the *TaMS45* pIR is constitutively expressed, vegetative growth and flowering characteristics for all the transformed plants were similar to wild-type non-transformed Fielder plants. TAMS45-PIR plants, however, exhibited anther defects and male sterile phenotype was observed in 77% of T_0 plants (Figure 1a,c; Table 1). The observed male sterility co-segregated with the TAMS45-PIR TDNA confirming the pIR as the causal factor of male sterility. Male sterility was observed in both single- and multi-copy T-DNA insertion events at approximately equal frequency (Table 1, Table S1). Microscopic examination of mature anthers from several independent T_0 plants revealed anthers that lacked pollen as compared to similarly staged anthers from untransformed Fielder plants (Figure 1b,c). Microspores in the anthers of male sterile plants were observed to break down soon after the quartet stage of development (Figure 1c). The stage of

microspore breakdown was similar to that observed in maize and wheat *ms45* mutants (Cigan *et al*., 2001; Singh *et al*., 2018). These data indicated that the pIR designed to the *TaMs45* promoter is able to induce male sterility in wheat plants when present as either single- or multi-copy insertion events.

Table 1 Male fertility assessment of T_0 and T_1 plants with TAMS45-PIR and TAMS1-PIR constructs

pIR copy #	Sterile[†]	Fertile[†]
TAMS45-PIR (T_0)		
1	14	4
2	10	3
Total	24	7
TaMS1-PIR (T_0)		
1	6	0
2	8	0
>3	2	0
TaMS1-PIR (T_1)		
1	4	0
0	0	4

[†]Number of plants.

To analyse the plants with TAMS1-PIR, 16 T_0 plants containing single- and multi-copy T-DNA insertions were selected and grown to flowering stage. All TAMS1-PIR plants showed wild-type like vegetative and floral development but were male sterile due to collapsed pollen produced as a result of defective tapetum in the anthers (Figure 1f; Table 1). Five of the 16 TAMS1-PIR plants were fertilized with pollen from wild-type Fielder plants. Seed set was observed from these crosses indicating normal female reproductive development in these plants (Table S2). Eight T_1 plants were grown from the crossed seed of a T_0 plant with one copy T-DNA insertion and genotyped for T-DNA insertion. Four of these eight plants carried the T-DNA insertion, whilst four were null segregants. Although all T_1 plants showed normal vegetative development, TAMS1-PIR plants were male sterile and did not set seed through self-pollination (Table S2). In comparison, the null segregants were male fertile and set seeds comparable to wild-type plants (Table S2). Transcript accumulation of *TaMs1* gene was determined by qRT-PCR in anthers at early uninucleate stage of pollen development utilizing the PCR primers designed to *TaMs1* sequence (B-homoeolog, (Tucker *et al*., 2017)). It was

Figure 1 Anther and pollen development in TAMS45-PIR, TAMS1-PIR and TAMS45-PIR/ZMMS45PRO:ZMMS45 restored plants. (a) Heads showing anther development and extrusion in a Fielder wild-type plant (left), a TAMS45-PIR male sterile plant (middle) and a TAMS45-PIR/ZMMS45PRO:ZMMS45 restored plant. (b) Anthers at uninucleate microspore stage, wild-type plant. (c) a plant with the TAMS45-PIR construct. (d) Heads showing anther development and extrusion in wild-type plant (left) and a TAMS1-PIR male sterile plant. (e) Anthers at uninucleate microspore stage of a wild-type plant. (f) anthers at uninucleate microspore stage of a TAMS1-PIR plant.

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observed that the expression of *TaMs1* was significantly reduced in the TAMS1-PIR plants as compared to wild-type plants (Figure 2d). Similar to the observations of *TaMs45* pIR, these results indicated that a constitutively expressed pIR designed to the *TaMs1* promoter could induce male sterility in wheat.

Restoration of fertility in TAMS45-PIR plants through expression of *Ms45* gene with a heterologous promoter

Since the TAMS45-PIR plants exhibit dominant male sterility, a complementation strategy was devised to restore male fertility. This strategy involved the expression of *Ms45* coding sequence in the TAMS45-PIR plants, using the maize *Ms45* promoter. The maize *Ms45* promoter is only 52% homologous to the *TaMs45-D* promoter; it is therefore not expected to be silenced by the TAMS45-PIR. A complementation construct ZMMS45PRO: OSMS45 (Figure S4b), wherein the maize *Ms45* promoter was used to express the rice *Ms45* gene, was introduced into Fielder variety through *Agrobacterium*-mediated transformation. T_0 plants containing ZMMS45PRO:OSMS45 were grown to maturity and were similar to wild-type Fielder plants with respect to vegetative development, fertility and seed set. ZMMS45PRO: OSMS45 construct was then combined with the TAMS45-PIR using a traditional crossing strategy (Figure S5). F_2 plants containing TAMS45-PIR with and without ZMMS45PRO:OSMS45 were grown to maturity and male fertility phenotype was recorded. As shown in Table 2, the plants containing only TAMS45-PIR were male sterile and did not set any seed. In contrast, TAMS45-PIR plants containing ZMMS45PRO:OSMS45 T-DNA shed pollen and were capable of self-fertilization like the wild-type plants (Table 2). These results demonstrate that the heterologous *Ms45* promoter from the ZMMS45PRO:OSMS45 construct is not silenced by the TAMS45-PIR and is capable of restoring fertility to plants exhibiting dominant male sterility.

PIRs designed to the *TaMs45* promoter methylate homologous DNA sequence and cause transcriptional gene silencing

Methylation of the cytosine bases of DNA is associated with the sequences targeted for TGS, via the RdDM pathway in plants (Cigan *et al*., 2005; Deng *et al*., 2014; Okano *et al*., 2008; Sidorenko *et al*., 2009). To investigate the basis of TAMS45-PIR-induced male sterility, methylation of *TaMs45* promoter and its consequence on expression of *TaMs45* was investigated. Eight plants homozygous for the TAMS45-PIR were selected for further analysis. Since the pIRs in these plants are constitutively expressed, pIRs transcript was examined in the developing leaves through analysis of the *NOS* promoter spacer sequence. The spacer sequence forms the hairpin loop and is not a part of the

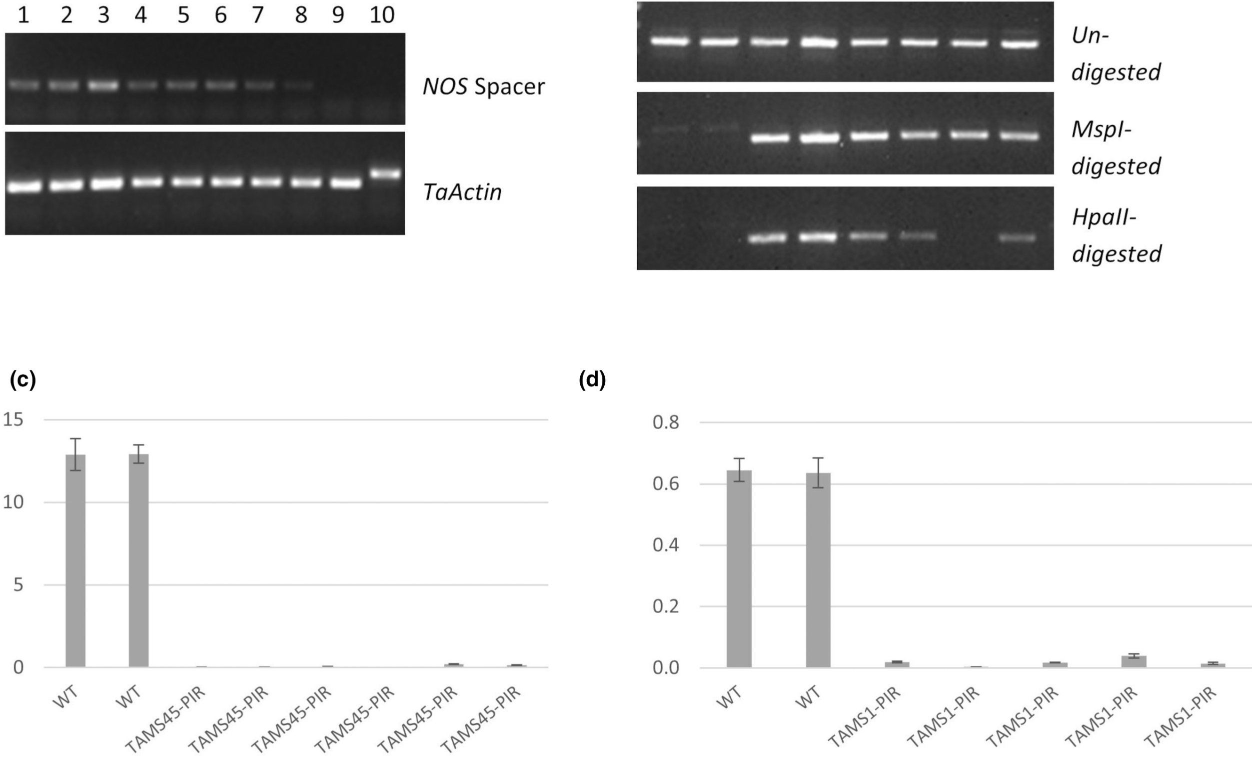

Figure 2 *TaMs45* promoter DNA methylation and transcript abundance analysis in TAMS45-PIR plants. (a) Analysis of transcription of *TaMs45* pIR. Semi-quantitative RT-PCR of *NOS* spacer transcript in the leaves of TAMS45-PIR plants was taken as a measure of the transcription of pIR. 1–8, homozygous TAMS45-PIR samples; 9, wild-type Fielder control; 10, genomic DNA control. Upper panel, *NOS* spacer transcript amplification; Lower panel, *TaActin* transcript amplification. (b) DNA methylation of *TaMs45*-A promoter in WT or TAMS45-PIR plants determined by *HpaII/MspI* restriction enzyme analysis. Leaf genomic DNA from WT or TAMS45-PIR plants was digested with *MspI* or *HpaII* restriction enzymes and amplified with *TaMs45-A* specific primers encompassing the restriction site. Undigested genomic DNA was utilized for control amplifications. (c) *TaMs45* transcript accumulation in the anthers of wild-type and TAMS45-PIR silenced plants. (d) *TaMs1* transcript accumulation in the anthers of wild-type plants and TAMS1-PIR silenced plants. *TaMs45*and *TaMs1* profiles were determined relative to *TaActin* expression. The *y* axis shows arbitrary units. Error bars indicate SE.

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Table 2 Male fertility assessment through complementation of TAMS45-PIR plants with restorer construct ZMMS45PRO:OSMS45 or maintainer construct ZMMS45PRO:ZMMS45

# of plants	pIR copy #	Complementation TDNA	Phenotype
ZMMS45PRO:OSMS45			
7	2	1	Fertile
5	2	NULL	Sterile
ZMMS45PRO:ZMMS45			
9	2	1	Fertile
12	2	NULL	Sterile
2	WT	WT	Fertile

repeat sequence, it is therefore expected to be transcribed but not processed into small RNAs. It was observed that the *NOS* promoter spacer sequence was transcribed in the leaves of the all eight TAMS45-PIR plants (Figure 2a).

Further, to determine if the transcription of the pIRs resulted in methylation of the *TaMs45* promoter sequence, DNA methylation was analysed. A *MspI/HpaII* site is present in the promoter of *A*-genome homoeolog of *TaMs45* gene and to investigate this site, *A*-genome-specific PCR primers encompassing this restriction site were designed (Figure S7; Table S4). The isoschizomer restriction enzyme pair, *MspI* and *HpaII*, is sensitive to CNG and CG methylation, respectively. Genomic DNA from leaf tissue of six TAMS45-PIR plants and two wild-type Fielder plants was digested with *MspI* or *HpaII* and PCR was performed with *A*-genome specific primers. The wild-type DNA was completely digested with both the restriction enzymes and no amplification products were obtained, whereas amplification products were observed in all the TAMS45-PIR plant samples for both enzymes, except for one sample digested with *HpaII* (Figure 2b). DNA from the five TAMS45-PIR samples was resistant to digestion with both *MspI* and *HpaII* indicating methylation in both CG and CNG contexts. These results indicated that the promoter of *TaMs45-A* is hypermethylated in TAMS45-PIR expressing plants as compared to wild-type plants, consistent with the hypothesis of TGS through RdDM pathway.

To determine if the methylation of *TaMs45* promoters causes transcriptional silencing of *TaMs45* genes, transcript abundance of *TaMs45* homoeologs was determined by qRT-PCR in anthers at early uninucleate stage of pollen development. The PCR primers were designed to conserved sequence in all three homoeologs and have been shown to amplify transcripts of all three homoeologs with equal efficiency (Singh *et al*., 2018). It was observed that the expression of all homoeologs was significantly reduced in the TAMS45-PIR plants as compared to wild-type plants (Figure 2c; Figure S8). Observations on promoter methylation and *TaMs45* transcript abundance showed that plants with TAMS45-PIR exhibit transcriptional gene silencing due to methylation of the *TaMs45* promoter induced by the inverted repeats likely through the RdDM pathway as evident in maize (Cigan *et al*., 2005; Sidorenko *et al*., 2009).

TaMs45 pIR-based male sterility system for hybrid seed production in wheat

For hybrid seed production in wheat, it would be advantageous to generate a maintainer for increasing the pIR male sterile inbred line. As shown above, it is possible to restore the *TaMs45* pIR-induced male sterility through expression of a heterologous *Ms45* promoter-gene combination. This ability to restore pIR-induced male sterility was utilized to devise a system in wheat similar to a seed production technology (SPT) for maize described by (SPT; Wu *et al*., 2016). To accomplish this, a complementation construct, ZMMS45PRO:ZMMS45 (Figure S4c), was utilized, wherein the maize *Ms45* promoter was used to express the maize *Ms45* gene. In addition to the maize *Ms45* genomic sequence (including 250 bp of the promoter sequence and 400 bp of the 3′ UTR), this construct also included DSRED, as a fluorescent selection marker; and maize alpha-amylase gene to prevent the transmission of T-DNA through pollen (Figure S4c). This construct was introduced into Fielder variety by *Agrobacterium*-mediated transformation and combined with TAMS45-PIR plants using a traditional crossing strategy as outlined in Figure S6. Plants homozygous for TAMS45-PIR and hemizygous for ZMMS45PRO:ZMMS45 were identified from the F_2 generation for further analysis. These plants were male fertile due to expression of the *ZmMs45* gene (Table 2; Figure S8) and represented the maintainer plants to self-pollinate and maintain TAMS45-PIR dominant male sterility.

Self-pollinated seeds from a maintainer plant segregated 1:1 for red-fluorescence and no-fluorescence (Figure 3a). Red-fluorescing seeds were homozygous for TAMS45-PIR and hemizygous for ZMMS45PRO:ZMMS45, whilst non-fluorescing seeds are homozygous for TAMS45-PIR and null for ZMMS45PRO:ZMMS45 (Figure 3a). Progeny from the non-fluorescing seed represents the male sterile female inbred line for hybrid seed production. The red-fluorescing seed will produce male fertile maintainer plants to provide a supply of male sterile inbred line (Figure 3a). To test the functioning of this system, 100 red-fluorescing and 100 non-fluorescing seeds from two maintainer plants, each with an independent event for TAMS45-PIR, were planted in the greenhouse. Ninety-three and 92 plants obtained from red-fluorescing and non-fluorescing seeds, respectively, were grown to maturity and analysed for male fertility. All plants from non-fluorescent seeds were male sterile, whilst all the seeds from red-fluorescing seeds were male fertile (Figure 3b,c, Table S3). The seed set on the plants from red-fluorescing seeds was comparable to wild-type wheat plants (Table S3). Further, to assess the efficacy of pollen-expressed alpha-amylase gene in preventing the transmission of ZMMS45PRO:ZMMS45 T-DNA through pollen, the TAMS45-PIR male sterile plants were crossed as females to male fertile plants containing ZMMS45PRO:ZMMS45 T-DNA. In total, 1953 crossed seeds were obtained, which were screened for DSRED fluorescence. No red fluorescing seeds were observed from the crossed seed. These results demonstrated the high efficacy of pollen-expressed alpha-amylase gene in preventing transmission of ZMMS45PRO:ZMMS45 T-DNA through pollen in wheat.

Seeds from the greenhouse-grown maintainer plants were also planted in the field to test various components of the *TaMs45*-pIR hybridization system. TAMS45-PIR plants were analysed for male sterility, whilst TAMS45-PIR/ZMMS45PRO:ZMMS45 were analysed for male fertility and thus the maintainer function. In addition, TAMS45-PIR/ZMMS45PRO:OSMS45 F_1 hybrid plants, produced from a cross of homozygous TAMS45-PIR plants with homozygous ZMMS45PRO:OSMS45 plants, were also tested for fertility restoration. Six rows of 120 non-fluorescing seeds homozygous for TAMS45-PIR were grown to maturity. Complete male sterility was observed in these plants with no seed set obtained through self-pollination (Table 3). The rows of maintainer and F_1 plants were completely male fertile (Table 3). These observations on male sterility, maintainer and restoration of

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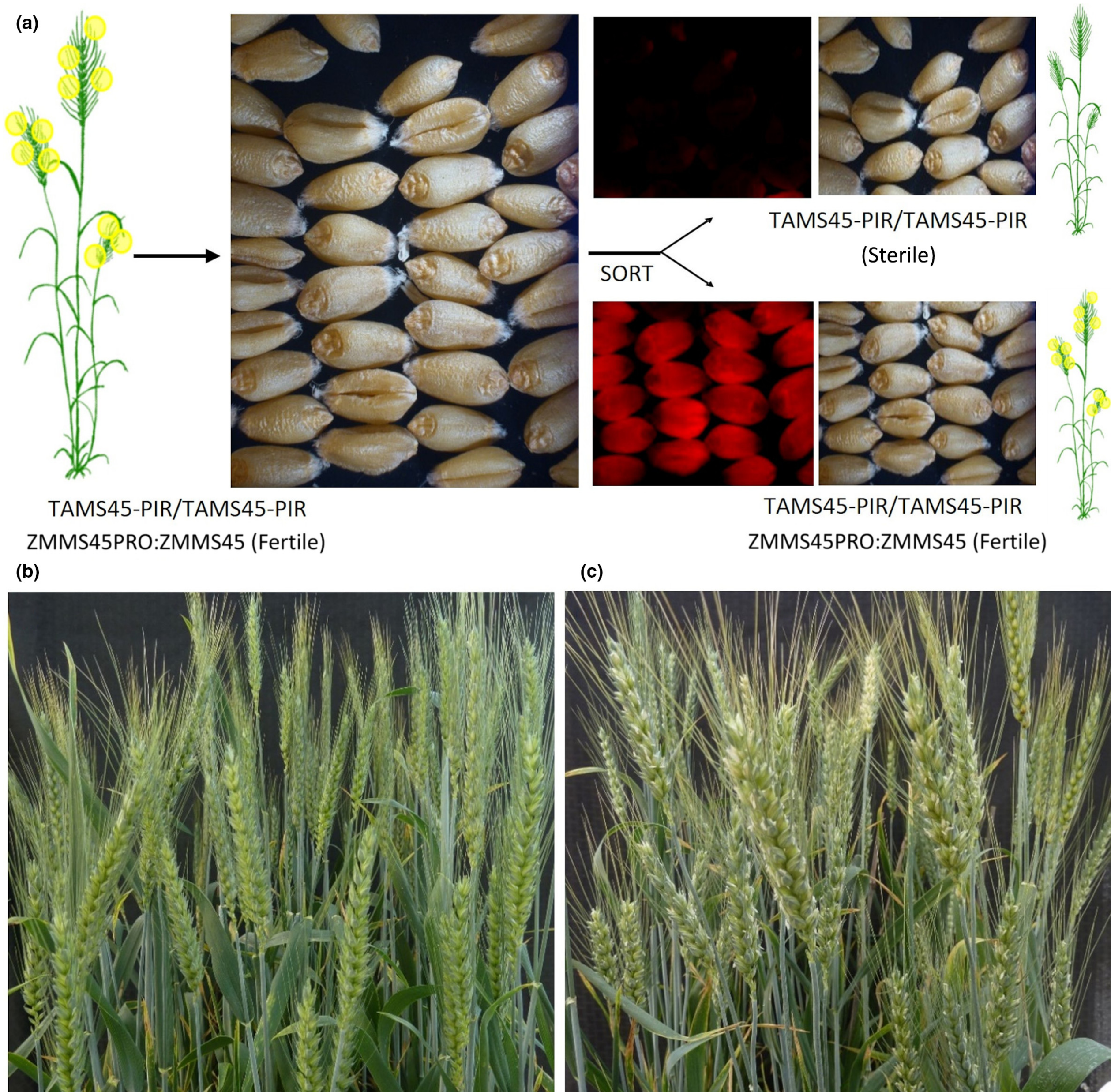

Figure 3 Dominant male sterility system in wheat. (a) Diagrammatic representation of the dominant male sterility system. (b) Segregation for male sterile plants; and (c) male fertile plants grown from non-fluorescing and red-fluorescing seeds, respectively, obtained by self-pollination of a TAMS45-PIR/ZMMS45PRO:ZMMS45 maintainer plant.

fertility in the field demonstrated the stability and utility of *TaMs45*-pIR-based dominant male sterility system for hybrid seed production in wheat.

Discussion

Transcriptional gene silencing in wheat

TGS of endogenous genes has been proposed as a tool to study gene function in plants. However, this technique has not been widely applied, and the number of endogenous genes silenced in plants using TGS has been limited and with variable degrees of success (Cigan *et al*., 2005, 2014; Eamens *et al*., 2008; Okano *et al*., 2008). This study shows the feasibility of TGS in wheat through efficient and complete silencing of two endogenous genes, *TaMs45* and *TaMs1*. In addition to their diverse role in wheat anther development, *TaMs45* and *TaMs1* also represent two diverse examples of functional redundancy in hexaploid wheat. In the case of *TaMs45*, all three homoeologs are functional and should be silenced to confer a male sterile phenotype, whereas for *TaMs1*, silencing of only 4B homoeolog can be expected to achieve the same phenotype. Demonstration of male sterility in T_0 generation for both TAMS1-PIR and TAMS45-PIR showed both single and three homoeolog loci could be silenced utilizing pIRs. However, T_0 fertility data did indicate that *TaMs1* was more efficiently silenced compared to *TaMs45*, as 100% of the TAMS1-PIR plants showed male sterile phenotype compared to 77% for TAMS45-PIR plants. In the T_1 generation, the penetrance of male sterile phenotype was similar for *TaMs45*

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Table 3 Field assessment of male sterility, maintenance and fertility restoration with TAMS45-PIR, TAMS45-PIR/ZMMS45PRO:ZMMS45 and TAMS45-PIR/ZMMS45PRO:OSMS45 constructs, respectively

Row	Category	Plants per row	Phenotype
1	pIR (white seeds)	120	Sterile
2	pIR (white seeds)	120	Sterile
3	pIR (white seeds)	120	Sterile
4	pIR (white seeds)	120	Sterile
5	pIR (white seeds)	120	Sterile
6	pIR (white seeds)	120	Sterile
7	Maintainer (red seeds)	120	Fertile
8	Maintainer (red seeds)	120	Fertile
9	Maintainer (red seeds)	120	Fertile
10	Maintainer (red seeds)	120	Fertile
11	Maintainer (red seeds)	120	Fertile
12	Maintainer (red seeds)	120	Fertile
15	Restored	32	Fertile
17	Restored	32	Fertile
18	Restored	31	Fertile
20	Restored	30	Fertile
21	Restored	31	Fertile
22	Restored	31	Fertile
23	Fielder	36	Fertile
24	Fielder	31	Fertile

and *TaMs1*, suggesting that once the *de novo* DNA methylation is established, it can be maintained as effectively for both single and three homoeolog loci. Whilst these observations suggest higher efficiency of TGS for single gene loci in T_0 generation, variation in expression or stability of pIR transcript cannot be ruled out, that can also affect the efficiency of TGS.

Previous studies have shown that *de novo* methylation of homologous DNA sequence via the RdDM pathway is associated with transcriptional gene silencing induced through pIRs (Cigan *et al*., 2005; Deng *et al*., 2014; Okano *et al*., 2008). The role of RdDM pathway in pIR-mediated TGS in maize has been confirmed by demonstrating that *Mop2*, a component RdDM pathway which encodes the second largest subunit of the plant-specific RNA polymerases, is required for pIR-mediated TGS in maize (Sidorenko *et al*., 2009). Observation of *TaMs45* promoter methylation in TAMS45-PIR plants suggests a role of RdDM pathway for TGS in wheat. DNA methylation was observed in both CG and non-CG contexts in the *TaMs45-A* promoter and was concomitantly accompanied with reduction in accumulation of *TaMs45* transcript. However, the RdDM pathway in wheat is likely to be more complex due to functional redundancy that accompanies hexaploidy. Indeed, it has been shown in wheat that the three homoeologs of the *Argonaute4_9* genes, another central component of the RdDM pathway, are differentially expressed in the developing embryos suggesting subfunctionalization of homoeologs (Singh *et al*., 2013).

Fertility control in wheat using TGS

The highly effectual and stable TGS of male fertility genes, *TaMs45* and *TaMs1*, provides an opportunity to regulate male fertility in wheat. Based on TGS of the *TaMs45* gene, we developed a male fertility control system for wheat hybrid seed production. This system offers a simpler control of male fertility compared to other genetic male sterility systems such as cytoplasmic male sterility (CMS). TGS-induced male sterility is dominant and requires only one factor as compared to three mutations in the case of *Tams45*-based recessive male sterility. TGS-induced male sterility may also be beneficial for single homoeolog-based mutants such as with *TaMs1*. Although a single genome mutation is required to achieve male sterility in the case of *TaMs1*, the penetrance of this phenotype is variable (Tucker *et al*., 2017). This leakiness of phenotype may be due to activation of the A- and D-genome homoeologs that are epigenetically silenced in hexaploid wheat (Wang *et al*., 2017). Thus, TGS may create a more stable male sterility through silencing of all three *TaMs1* homoeologs.

From a practical hybrid breeding perspective, a dominant male sterility approach is preferred since contamination due to out-crossing to a wild-type wheat plant can be contained. In a recessive male sterility system, out-crossing to a wild-type plant during seed production will result in contamination with fertile plants. Further, dominant sterility conferred by a single genetic locus would also be simpler to introgress and maintain than a trait system that required triple knockout to confer male sterility. For crop species, especially for polyploids like wheat, introducing a single dominant suppression construct is now achievable given the recent development of gene targeting via CRISPR-Cas and improvements in plant transformation that is germplasm independent. In this approach, a dominant suppression construct could be reliably and repeatably inserted at a chosen location in the plant genome of different female inbred backgrounds and maintained and increased using an SPT-like system. During hybrid seed production, male sterile females could then be fertilized by male lines that contain a promoter version of the fertility gene that is not silenced by TGS dominant silencing. To this end, we demonstrated that a heterologous source of *Ms45* could restore fertility to pIR-induced male sterile plants. In this report, to restore fertility of TAMS45-PIR plants, the promoter expressing *MS45* gene was derived from maize. These results show that promoters even with high homology in short stretch of sequence (76% between nucleotide positions −133 to −70 of *TaMs45-D* and maize *Ms45* promoters) are recalcitrant to TGS-mediated suppression if the overall homology in the pIR region is low (52% between *TaMs45-D* and maize). Promoters from plant species that mimic the temporal and spatial expression pattern of *TaMs45* gene, but are divergent in sequence, could also be used to complement TAMS45-PIR plants (Cigan *et al*., 2005, 2014). Whilst in this report, a transgenic version of *Ms45* was used to complement silencing of *TaMs45* homoeologs, gene-targeted modification of the wheat *Ms45* promoter in the male inbred lines used to make hybrid seed could be sufficiently altered to express *Ms45* and restore fertility in the hybrid plant as they would not be silenced by the dominant pIR introduced by the female inbred. Thus, by combining gene editing, elite plant transformation to introduce effective dominant systems, silencing or otherwise, it is conceivable that traits could be rapidly deployed as modules in specific inbred backgrounds removing the need to conventional backcross breeding.

As demonstrated in this study, TGS can be an attractive tool for analysis of gene function in wheat. Cultivated wheat varieties are either hexaploid or tetraploid in genomic composition with extensive functional redundancy of the resulting homoeologs. Traditional mutagenesis techniques, including modern genome editing technology, can pose a challenge to the functional analysis of genes in polyploid wheat. TGS can be utilized to

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control plant development in wheat in a dominant manner, as demonstrated through male fertility control in wheat. This technology presents an opportunity for hybrid seed production in wheat and for developing other important agronomic traits for wheat improvement.

Methods

Plant material and growth conditions

Plants were grown in the greenhouse and in the field at Corteva Agriscience research facilities in Johnston, Iowa. The greenhouse conditions were set to 22 °C and 20 °C for average day and night temperatures, respectively, with 30% humidity and a 16 h. day length period. Standard plant care practices were followed including fertilizer and pesticide applications.

Promoter DNA sequence analysis

To obtain the promoter sequence for wheat A-, B- and D-homoeolog of *Ms45* gene, the Wheat Genome 50× Survey Sequence and the Wheat Genome 454 reads databases (Helguera *et al*., 2015; Hernandez *et al*., 2012; Wilkinson *et al*., 2012) were searched through (Basic Local Alignment Research Tool (BLAST); Altschul *et al*., 1990) analysis with the *Zea mays Ms45* sequence (Cigan *et al*., 2001). Sequence comparisons and alignments were performed with DNA analysis software Sequencher 5.1 (Genecodes, Ann Arbor, MI, USA) and Vector NTI (Life Technologies Inc., Carlsbad, CA, USA).

T-DNA constructs and plant transformation

Vectors for plant transformation were assembled using standard molecular biology techniques and using components described in (Cigan *et al*., 2001). To construct TAMS45-PIR, a 440 bp sequence of wheat *TaMs45-A* promoter was amplified and cloned in forward and reverse orientation with a NOS promoter spacer fragment between the two inverted fragments. The pIR was operably linked to the maize ubiquitin promoter-intron sequence to make the TAMS45-PIR construct (Figure S4a). The final vector also contained AM-CYAN1 gene under the control of seed-specific *END2* promoter and *CAMV35S* enhancer, which served as a selection marker for wheat transformation and seed selection. To assemble TAMS1-PIR construct, a 581 bp sequence immediately upstream of the ATG translation initiation site was cloned in forward and reverse orientation with a *NOS* promoter spacer between the inverted sequence fragments. The inverted repeat was operably linked to the maize ubiquitin promoter-intron sequence to make the TAMS1-PIR construct (Figure S4a).

The assembly of the complementation vector ZMMS45PRO: OSMS45 (Figure S4b) is described elsewhere (PHP37034; Singh *et al*., 2018). In brief, the rice *Ms45* coding region was operably fused to the maize *Ms45* promoter. Assembly of ZMMS45PRO: ZMMS45 vector (Figure S4c) is described elsewhere (Wu *et al*., 2016). In brief, this construct consisted of the maize *Ms45* genomic sequence (including 250 bp of the promoter sequence and 400 bp of the 3′ UTR); DSRED, a selection marker operably fused to endosperm-specific *LTP2* promoter: and a maize alpha-amylase gene operably linked to pollen-specific *PG47* promoter. All the constructs were introduced into wheat variety Fielder through *Agrobacterium*-mediated transformation as previously described (Cigan *et al*., 2017). qPCR analysis was used for AM-CYAN1 and DSRED copy number determination as described in Wu *et al*. (2016) and taken as a measure of pIR and restorer copy number, respectively.

Promoter methylation analysis

Promoter methylation was analysed through use of *HpaII* and *MspI* restriction enzymes, which are sensitive to CNG and CG methylation, respectively. Genomic DNA was extracted from leaves of *TaMs45* pIR and wild-type plants at the seedling stage using CTAB DNA extraction method (Springer, 2010). One µg DNA was digested with *HpaII* or *MspI* for 3 h and heat inactivated following the manufacturer's protocol. About 50 ng of DNA was used for PCR amplification for 35 cycles utilizing the *TaMs45-A* specific primers described in Table S4. Undigested genomic DNA was utilized for control amplifications. PCR products were analysed on a 1% agarose gel.

RT-PCR and qRT-PCR analysis

For *TaMs1* and *TaMs45*, RT-PCR anthers were collected for stages corresponding to early and late uninucleate microspores. For NOS promoter spacer RT-PCR, young leaf tissue was collected from 3–4 leaf seedlings. RNA was extracted using TRIZOL reagent (Invitrogen Inc.) following manufacturer's protocol. To remove DNA contamination, a total of 1 µg RNA was treated with DNase I enzyme (Invitrogen Inc., Waltham, MA, USA) following the manufacturer's recommendations. Reverse transcription was performed with 500 ng of DNase-treated RNA utilizing the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). The transcribed cDNA was diluted 5× in sterile water and used for PCR. Primer pair *TAMS45*-F, *TAMS45*-R, *TAMS1*-F, *TAMS1*-R and *TAACTIN*-F, *TAACTIN*-R (Table S4) were used to amplify *TaMs45*, *TaMs1* and *Actin* transcripts, respectively. qRT-PCR was performed with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a ViiA™7 System or QuantStudio 6 Flex Real-time PCR System (Thermo Fisher Scientific) and analysed with the accompanying software. Relative *TaMs45* gene expression was obtained by normalizing with *Actin* expression. *NOS* promoter spacer transcript was amplified for 25 cycles with *NOSPRO*-F and *NOSPRO*-R primers (Table S4) and resolved on a 1% agarose gel.

RNAseq analysis

Total RNA was isolated from two biological replicates for each sample and used to create Illumina TruSeq cDNA libraries and sequenced on a Illumina HiSeq2500 instrument (Illumina, San Diego, USA). Images from sequencing run were analysed via Illumina analysis pipeline. Resulting sequences were filtered for quality and split on index. High-quality sequences aligned to TAGI_cDNA plus MS45 seqs database using bowtie_V1.0, maximum alignment = 100. Sequences were summed on the transcript level, normalized to Relative Parts per Kilobase per 10 Million (RPKtM) and displayed in Genedata.

Microscopy

For confocal scanning laser microscope (CSLM), flowers were collected and fixed in 2% paraformaldehyde:4% glutaraldehyde in PBS buffer and vacuum infiltrated at 10 psi to increase the penetration of fixative. Samples were rinsed in PBS and then cleared in graded series of 2,2′-Thiodiethanol (TDE) (10%, 25%, 50%, 75% and 97%). Samples were mounted on slides in TDE and imaged using the Leica TCS SPE CSLM (Leica Microsystems, Wetzlar, Germany) with 405 nm, 488 nm and 532 nm laser lines.

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Conflict of interest

AMC and MS are inventors on pending patent applications on this work and are current or former employees of Corteva Agriscience who own the pending patent applications. MK and KEC are current employees of Corteva Agriscience.

Author contributions

Conception and design: A.M.C. and M.S.; Analysis and interpretation of data: M.S, A.M.C., M. K. and K.E.C.; Preparation of manuscript and editing M.S., K.E.C., A.M.C.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Sequence alignment of the maize *Ms45* gene with the promoter of *TaMs45-D* homoeolog.
Figure S2 Promoter sequence alignment *TaMs45* homoeologs and *TaMs45 pIR*.
Figure S3 Promoter sequence alignment *TaMs1* homoeologs and *TaMs45 pIR*.
Figure S4 TDNA used for transcriptional silencing of *TaMs45* and *TaMs1* genes and complementation of *TaMs45* silenced plants.
Figure S5 Crossing strategy to combine TAMS45-PIR and ZMMS45PRO:OSMS45
Figure S6 Crossing strategy to combine TAMS45-PIR and ZMMS45PRO:ZMMS45 to generate a maintainer plant for generating male sterile inbred seed.
Figure S7 Schematic diagram of TaMs45 promoter DNA methylation analysis.
Figure S8 Expression of TaMs45-A, -B, -D and ZmMs45 genes in wild type, TAMS45-PIR and TAMS45-PIR/ZMMS45PRO:ZMMS45 plants based on RNAseq data.
Table S1 Male fertility assessment of T_0 plants with the TAMS45-PIR construct.
Table S2 Male fertility assessment of T_0 and T_1 plants with the TAMS1-PIR construct.
Table S3 Phenotype and fertility of progeny from TAMS45-PIR/ZMMS45PRO:ZMMS45 maintainer plant.
Table S4 List of primers used in the study.