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ORIGINAL PAPER

Induction of Targeted Deletions in Transgenic Bread Wheat (*Triticum aestivum* L.) Using Customized Meganuclease

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Abstract
Biotechnologies offer breeders good opportunities for breakthrough genetic improvements of bread wheat, one of mankind's main food crops. Since the production of the first transgenic wheat, one of the major concerns has been the removal of selective markers, first because of societal concerns about the antibiotic resistance of some of these genes, and second because removal of a selective marker was the first step toward retransformation using the same selection system. Site-directed nucleases are enzymes that cut genomic DNA in vivo at predefined sites. Among them, meganucleases cut DNA at predefined, long DNA (up to 24 nt) sites, thereby enabling single cuts on large genomes including the bread wheat genome (17 Gbp). In this paper, we describe for the first time the use of a customized meganuclease to cut wheat DNA in vivo. We show that double cuts provoked the deletion of previously inserted DNA cassettes containing the *DsRed* reporter gene, and that in many cases, the meganuclease target site was correctly reconstituted, offering opportunities for subsequent insertion of stacked transgenes to replace the gene of selection. Moreover, perfect deletions were observed not only in the callus after transient expression of the meganucleases, but also in T0 transgenic wheat after stable retransformation with the meganuclease. Future prospects for the removal of selective markers and transgene stacking are discussed.

Keywords Bread wheat · Biolistics · Gene stacking · Meganuclease

Introduction

Over the last 50 years, the increase in the world population has been accompanied by a major increase in food production that has reduced hunger and malnutrition worldwide (World Bank 2008). Data concerning the ongoing century show that the world population will probably increase to between 9.6 and 12.3 billion by 2100 (Gerland et al. 2014). To avoid a drastic increase in hunger, the world will need 70 to 100% more food by 2050 (World Bank 2008). Wheat (*Triticum aestivum* L.) is a major staple food crop worldwide and improving wheat yield will be a major objective for breeders in the coming decades. This improvement will be constrained by global warming that could have deleterious effects on wheat yields (Asseng et al. 2015) and the sustainable intensification of breeding will be required to reduce negative effects. Among many areas of potential improvement (Godfray et al. 2016), biotechnologies, and more precisely genetically modified crops, offer breeders opportunities for breakthrough genetic improvements (Kamthan et al. 2016).

Bread wheat transformation (for a review, see Hardwood 2012) was first achieved by particle bombardment of embryogenic callus (Vasil et al. 1992). Some years later, Cheng et al. (1997) succeeded in *Agrobacterium*-mediated transformation. Despite this success, even after significant improvement in transformation efficiency, *Agrobacterium*-mediated transformation has been limited to specific wheat

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genotypes (Risacher et al. 2009). In contrast, biolistic have succeeded in transforming a wider range of wheat genotypes (Sparks and Jones 2009). Moreover, the combination of linear gene cassettes and small quantities of DNA for microprojectile bombardment allowed the production of a majority of single copy events (Tassy et al. 2014). Although some results were reported on wheat transformation without a selectable marker (Permingeat et al. 2003), most transgenic wheat lines were obtained after selection of the transformed cells using co-transformation with marker genes such as *bar* (Vasil et al. 1992) for phosphinothricin resistance, *nptII* (Cheng et al. 1997) for kanamycin or G418 resistance, or *pmi* (Gadaleta et al. 2006) for mannose availability as a plant nutrient. Despite their interest as biotechnology tools, these selectable markers raised concerns because they remain in the genome of the GM plants, and could, for example, possibly result in herbicide resistance via gene flow to other crops or wild relatives (Chèvre et al. 1997), or the putative transfer of antibiotic resistance genes to soil bacteria. Consequently, the production of GM plants free of selectable markers is of particular interest for field trials and commercial products. To this end, different strategies have been developed to remove marker genes from transgenic plants (for a review, see Yau and Stewart 2013). The Cre/loxP DNA recombination system was successfully used in *Arabidopsis* with a Cre recombinase under the control of an inducible promoter (Zuo et al. 2001) and in *Citrus* (Peng et al. 2015), potato (Orbegozo et al. 2016), and wheat using an elegant induction of the Cre recombinase by cold/vernalization treatment (Mészáros et al. 2015). In the same way, the R/Rs recombination system from *Zygosaccharomyces rouxii* was used in tobacco (Sugita et al. 2000), strawberry (Schaart et al. 2004), apple, and pear (Righetti et al. 2014), and the FLP/FRT system from *Saccharomyces cerevisiae* was developed for rice (Woo et al. 2015) and grapevine (Dalla Costa et al. 2016). Transposon Ac/Ds from maize and *piggyBac* from cabbage looper were also used to induce deletions in rice (Gao et al. 2015; Nishizawa-Yokoi et al. 2015). Overall, recombinases and transposons were clearly shown to be very efficient systems to remove selectable markers from transgenic events, but the two systems did not allow subsequent targeted insertion of a new transgene at the same locus in order to facilitate backcrossing of transgenic traits (Ainley et al. 2013). This feature, named gene stacking (for a review, see Srivastava and Thomson 2016), was of particular interest in research on the insertion of a transgene in a particular target genomic region with good lasting gene expression, and for breeders to facilitate the backcross of multiple transgenes in elite varieties. Deletion of a selectable marker gene cassette and the subsequent generation of a landing pad for future integration of a new transgene can be performed using site-directed nucleases (SDN) like meganucleases, zinc finger nucleases, transcription activator-like effector nucleases (TALEN), or the CRISPR-Cas9 system (for a review, see Zhu et al. 2016). Meganucleases were first used for targeted mutagenesis in human cells (Delacôte et al. 2013), but they were also shown to be efficient for the generation of targeted and inherited short deletions in *Nicotiana alata* (Honig et al. 2015) and to succeed in targeted transgene insertion in cotton (D'Halluin et al. 2013). This type of nuclease was also shown to be efficient in inhibiting virus infections in cultured cells (Grosse et al. 2011). Meganucleases are, thus, versatile tools to cut plant genomic DNA in vivo and to generate deletions of previously defined fragments, typically selected genes, on a transgenic locus. They can also be tested for transformation improvements as already demonstrated in anemone (Renfer and Technau 2017) and zebrafish (Grabher et al. 2004). These applications can be used in the industrial property context.

In this paper, we used a customized meganuclease (CMN), a derivative of *I-CreI* (for a review, see Arnould et al. 2011) that was engineered to target the human *RAG1* gene (Muñoz et al. 2011), and we describe—to our knowledge—for the first time, the generation of CMN-mediated targeted deletions of a reporter gene on a landing pad inserted in the wheat genome, with subsequent regeneration of the meganuclease site. These deletions were obtained after transient expression of the nuclease in a callus, or by stable expression in a wheat transgenic plant after retransformation with the nuclease. Future prospects for marker gene cassette deletion and gene stacking in wheat are discussed.

Material and Methods

Plant Material

Triticum aestivum cv. Bobwhite S26 was used for all experiments. The donor plants for embryo transformation and transgenic plants were grown in a greenhouse under controlled conditions with 16 h daylight at 18 °C and 8 h of night at 15 °C.

Construction of a NptII-DsRed Cassette (Fig. 1a)

The *NptII-DsRed* cassette was assembled from synthetic DNA fragments (Genscript) in the plant binary vector pSCV1 using standard cloning techniques. The following fragments were assembled to form the plasmid pBIOS03146: (1) *PlexSc4* promoter (Schünmann et al. (2003)) plus *AtFAD2* intron linked to the CMN target sequence (5′ ttgttctcaggtacctcagccaga 3′), the *NptII* selectable marker followed by a *Nos* polyadenylation sequence. (2) An enhanced *35S* promoter plus the maize *Adh1* intron linked to the *DsRed2* gene (Clontech) followed

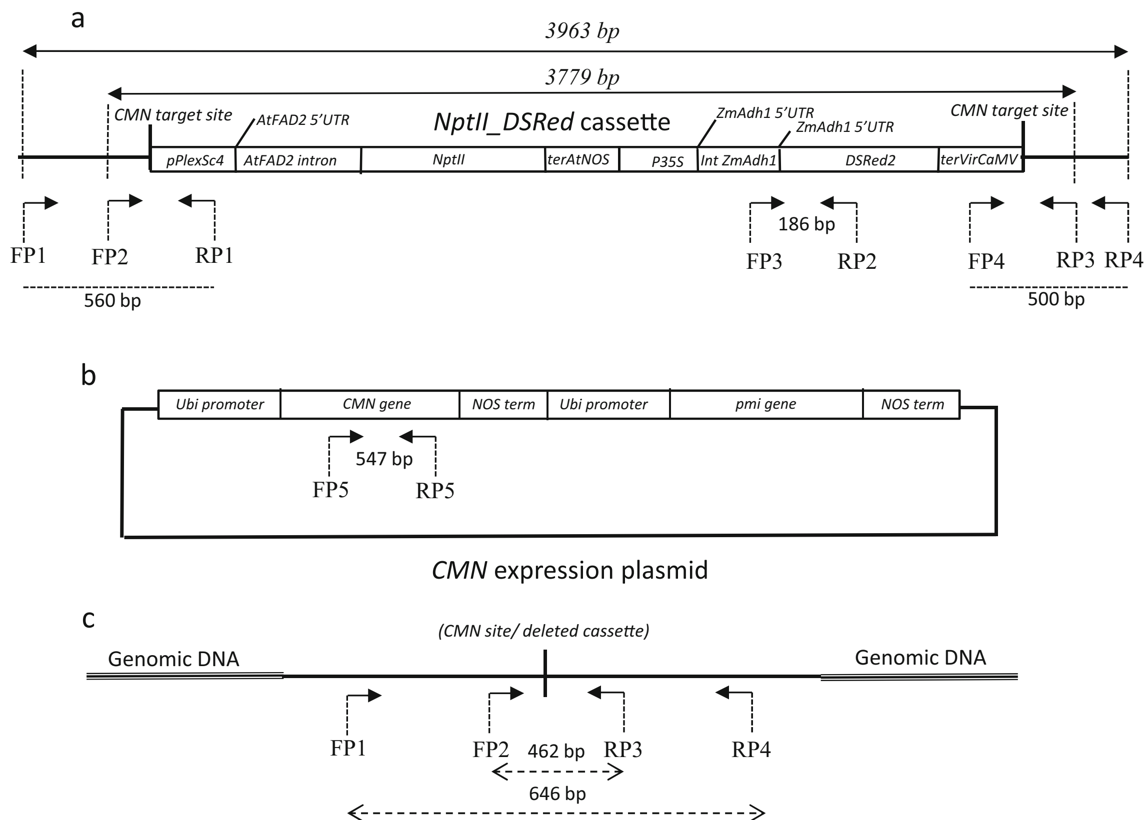

Fig. 1 DsRed cassette and CMN plasmid used for wheat transformation. **a** *NptII* and *DsRed* expression units are flanked by two *CMN* sites that allow deletion of the cassette. The positions of the PCR primers used for PCR-based characterization of the structure of the cassette are indicated. **b** *CMN* gene expression unit. PCR primers used for *CMN* gene detection are indicated. **c** Theoretical structure of a deleted cassette and position of the primers used for PCR/nested PCR amplification

by a *35S* terminator and the CMN target sequence. (3) A 5′ truncated *GFP* gene followed by an *Arabidopsis thaliana Sac66* polyadenylation sequence (Jenkins et al. (1999)).

Construction of a CMN Cassette (Fig. 1b)

The [*Ubi-CMN-NosTer*] cassette contained the customized meganuclease (CMN) gene under the control of the maize *Ubiquitin* promoter followed by the *nopaline synthase* terminator. CMN is a single chain derivative of I-CreI containing the scV3(K7E-K96E)V2(E8K-E61R) modifications described by Muñoz et al. (2011, GenBank accession 3MX9A) as well as a N-terminal SV40 nuclear localization signal followed by a 6His tag (see an annotated sequence in Supplementary data 1). The DNA fragment was obtained by DNA synthesis with optimization of maize codon usage (Integrated DNA Technologies). The cassette was constructed using Gateway MultiSite technology (Life technologies) according to the manufacturer's instructions, and using the following plasmids: Entry No. 1/Maize *Ubiquitin* promoter in pDonr p4-p1R (construction previously realized in our lab), Entry No. 2/*CMN* gene in pDonr221, Entry No. 3/*Nos* terminator + selection cassette (*Ubi* promoter/*pmi* gene/*nos* ter) in pDonr P2R-P3 (construction previously carried out in our lab).

Bread Wheat Transformation

Immature seeds were harvested 12–14 days post anthesis, and were sterilized for 15 min in a 20% sodium hypochlorite solution containing 1 drop per 200 mL of Tween 20. The immature embryos were isolated microscopically in a sterile environment and the embryo axis was removed to prevent precocious germination. The embryos were transferred on plasmolysis medium and transformed by particle bombardment using a PDS 1000 He device (BioRad) with 900 psi rupture discs at a target distance of 7 cm. Gold microprojectiles (1 mg), diameter 0.6 μM, were sonicated for 1 min and suspended in 10 μL (300 ng) of DNA solution. Next, 20 μL of 0.1 M spermidine and 50 μL of 2.5 M CaCl2 were mixed with particles coated with DNA and incubated for 15 min at room temperature under gentle agitation and then centrifuged for 1 min. The supernatant was removed, and after being washed in 99.8%

ethanol, the gold bullets were re-suspended in 100 μL of ethanol; 10 μL of suspension was used for each shot. The transformation experiments and wheat regeneration were performed according to the protocol described by Pellegrineschi et al. (2002). *NptII* selection was done with G418 (geneticin) at a concentration of 50 mg/l, according to Ye et al. (2002). The selection of *pmi* transgenic events was performed as described by Wright et al. (2001).

PCR Analysis of the DsRed Gene, CMN 5′ and 3′ Sites, and CMN Gene Detection

Wheat genomic DNA was isolated from young leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Tassy et al. 2006). Cassette components were detected by PCR amplification using 50 ng of total genomic DNA as template and *Taq* DNA polymerase (Q-BIO gene) following the manufacturer's instructions, using the primers FP3 and RP2 for the *DsRed* assay, FP1 and RP1 for the *CMN* 5′ site, FP4 and RP4 for the *CMN* 3′ site (Fig. 1a, Table 1 and Supplementary data 2 for sequences), and FP5 and RP5 for the *CMN* gene (Fig. 1b, Table 1 and Supplementary data 2). Microsatellite *gwm*257 (Röder et al. 1998) was used as internal positive control for the PCR amplifications.

The PCR reaction mixture (50 μL) contained 1× Taq polymerase buffer (Qiagen) containing 1.5 mM $MgCl_2$, 150 μM of each dNTP, 20 pmol of each primer, and 1 unit of Taq polymerase (Qiagen). The PCR was performed using a MJ research PCR thermocycler with one cycle at 94 °C for 5 min, 34 cycles at 94 °C for 45 s, at 62 °C for 45 s, at 72 °C for 4 min, and a final extension step at 72 °C for 4 min for the *DsRed* and *CMN* gene assay, and with one cycle at 95 °C for 4 min, 34 cycles at 95 °C for 30 s, at 60 °C for 30 s, at 72 °C for 1 min, and a final extension step at 72 °C for 2 min for the *CMN* 5′ and 3′ site assays. The 186-, 547-, 560-, and 500-bp PCR products for *DsRed* and *CMN* genes, and CMN 5′ and 3'sites, respectively, were detected by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

DsRed Fluorescent Protein Detection

DsRed protein detection was performed by direct observation of the transformed growing callus using a Leica MZFL III fluorescence stereomicroscope equipped with a ET DsRed filter.

Callus DNA Extraction

After particle bombardment, the embryos were placed on MSE3 medium (Pellegrineschi et al. (2002) for 15 days in order to produce callus structures. Calli were collected on Deepwell plates (1.2 mL, 96 wells, and 6 calls per well). Two stainless steel beads (3 mm in diameter) and 350 μL of extraction buffer (0.1 M TrisHCl, 0.05 M EDTA, and 0.5 M NaCl) were added to each well. Grinding was performed for 30 s at 1200 rpm on FastPrep_96™ homogenizer (Mp biomedicals). After grinding, a fresh volume of 350 μL of extraction buffer was added to each well. Fresh buffer and the callus extract were mixed and placed in a water bath for 1 h at 94 °C. Next, 10 μL of RNAse buffer (10 mg/mL) were added to each well, and after mixing, the solution was incubated in a water bath for 20 min at 37 °C. The plates were then centrifuged for 10 min at 6000 g. The supernatant (400 μL) was recovered and mixed in a new DeepWell plate with isopropanol (600 μL) and ammonium acetate (75 μL, 7.5 M). The plates were mixed and allowed to cool at − 20 °C for 2 h. DNA was pelleted by centrifugation for 10 min at 4 °C (6000 g). The supernatant was discarded and replaced by 400 μL of 70% EtOH. After another centrifugation for 5 min at 6000 g, the supernatant was discarded and the DNA pellets were allowed to dry overnight. Each DNA pellet was dissolved in 100 μL of ultrapure water by horizontal shaking at 120 rpm for 2 h then stored at 4 °C. DNA quality was checked by electrophoresis on 1.5% agarose gel.

PCR and Nested PCR Analysis for Cassette Deletion

Deletion of [*CMN-NptII-DsRed-CMN*] cassette was detected by direct PCR amplification using primers FP1 and RP4 (Fig. 1, Table 1, and Supplementary data 2), or by nested PCR using

Table 1 Primer combinations used for PCR amplifications and theoretical size of the resulting products

Forward primer	Reverse primer	Target	PCR product size (bp)
FP1	RP4	Cassette	3963
		Deleted cassette	646
FP2	RP3	Nested cassette	3779
		Deleted nested cassette	462
FP1	RP1	5′ *CMN* site	560
FP4	RP4	3′ *CMN* site	500
FP3	RP2	*DsRed*	186
FP5	RP5	*CMN*	547

a second amplification of the previous PCR product using the two internal primers FP2 and RP3 (Fig. 1, Table 1, and Supplementary data 2). The first reaction was carried out as follows: The PCR reaction mixture (25 μL) contained 12.5 μL of Taq Gold polymerase mix (Qiagen), 1 μL of GC enhancer (Qiagen), 7.5 pmol of each primer, 5 μL of genomic DNA (10 ng/μL), and 5 μL of ultrapure water. PCR was performed using a MJ research PCR thermocycler with one cycle at 95 °C for 4 min, 44 cycles at 95 °C for 30 s, at 60 °C for 30 s, at 72 °C for 45 s, and a final extension step at 72 °C for 1 min. PCR products were subjected to agarose gel electrophoresis (2% in 1× TAE buffer). Nested PCR was performed under the same conditions as the first reaction except that 5 μL of the first PCR product was used instead of genomic DNA.

PCR Product Sequencing and Sequence Analysis

Sequencing reactions were performed by Eurofins Genomics, and sequences were analyzed using Serial Cloner (Serialbasics) software.

Results and Discussion

Generation of Wheat Transgenic Plants for the Cassette [5′ CMNsite-NptII-DsRed-3′ CMNsite]

The [*5′ CMNsite-NptII-DsRed-3′ CMNsite*]/[*CNRC*] cassette (Fig. 1) was prepared and purified as described in Tassy et al. (2014). Two thousand six hundred and forty-four immature embryos were shot with this cassette. In vitro culture was performed under geneticin selection pressure and regenerated plantlets were subjected to *DsRed* PCR detection. Finally, 27 independent transgenic plants were identified as *NptII* positives (selection) and *DsRed* positives (PCR). These plants were tagged DS1 to DS27 according to the nomenclature described in Table 2.

Transformation efficiency with the *NptII*/geneticin system was 1%, which is much lower than the 205% efficiency currently observed in our conditions with the *pmi*/mannose system (Tassy et al. (2014)), and also lower than the efficiency reported by Richardson et al. (2014; 5% for Bobwhite accession), but is in the range of wheat transformation efficiencies reported by Nehra et al. (1994; 0.5–2.5%). PCR amplification of the two flanking *CMN* sites (Fig. 1) was performed for a more precise check of the integration of the entire [*CNRC*] cassette (Supplementary data 3). Among the 27 transgenic events tested, 24 showed positive amplification for the three PCR tests, indicating that at least one complete cassette was likely present. These results were consistent with previous results showing that simple, low complexity events could be obtained using linear cassette DNA without vector DNA or dephosphorylated cassettes (Tassy et al. (2014); Mészáros et al. (2015)).

DsRed protein expression was checked at all steps of the plant transformation process. Fluorescent red spots were detected on calli 2 weeks after immature embryo transformation (Supplementary data 4), indicating that the *DsRed* expression cassette was functional and that stable transformation had occurred. DsRed fluorescence was more difficult to detect on regenerated plantlets, except in the non-photosynthetic, white parts of the plantlets, and was unfortunately not detectable on leaves because of the high level of auto-fluorescence detected on control plants. In the first description of DsRed protein in plants, Jach et al. (2001) showed that transgenic tobacco leaves harbored stronger red fluorescence than untransformed control plants. Wenck et al. (2003) detected red spots on leaves of *DsRed* transgenic wheats. In our study, the fluorescent pattern in wheat plants transformed with the [*CNRC*] cassette (Supplementary data 4) did not differ substantially from the pattern detected in control plants. For this reason, in subsequent experiments, we decided to track the presence of the *DsRed* cassette using PCR amplification.

Eight T1 seeds of each of the 24 selected DS plants were sown, and the resulting plantlets were analyzed by PCR to detect the presence of the *DsRed* transgene. For each T0 plant, we observed the presence of a segregating *DsRed* PCR signal in the progeny (data not shown). T1 plants originating from 5 DS T0 plants (i.e., homozygous or heterozygous for [*CNRC*] cassette) were used for transient expression experiments.

Transient Expression of CMN Gene in Calli of DS Plants

T1 plants in which the presence of a cassette was confirmed were used as donor plants for the production of T2 immature embryos. For transient expression experiments, 342 embryos were shot with the plasmid containing the *CMN* cassette and then subjected to in vitro culture for callus production for 2 weeks. The 342 resulting calli were collected 6 by 6 to constitute 57 pools for DNA extraction. DNA quality was checked by agarose gel electrophoresis (Fig. 2a). High molecular weight DNA was obtained from each pool, which was important to avoid amplification of low molecular weight PCR fragments due to partial or total DNA degradation

Table 2 Nomenclature of the different wheat plants

	Nomenclature
[*CNRC*] T0 plants	Dsx
Stable Shooting on T2 embryo DS x, regenerated event y	T2 DSx/ T0-y *CMN*

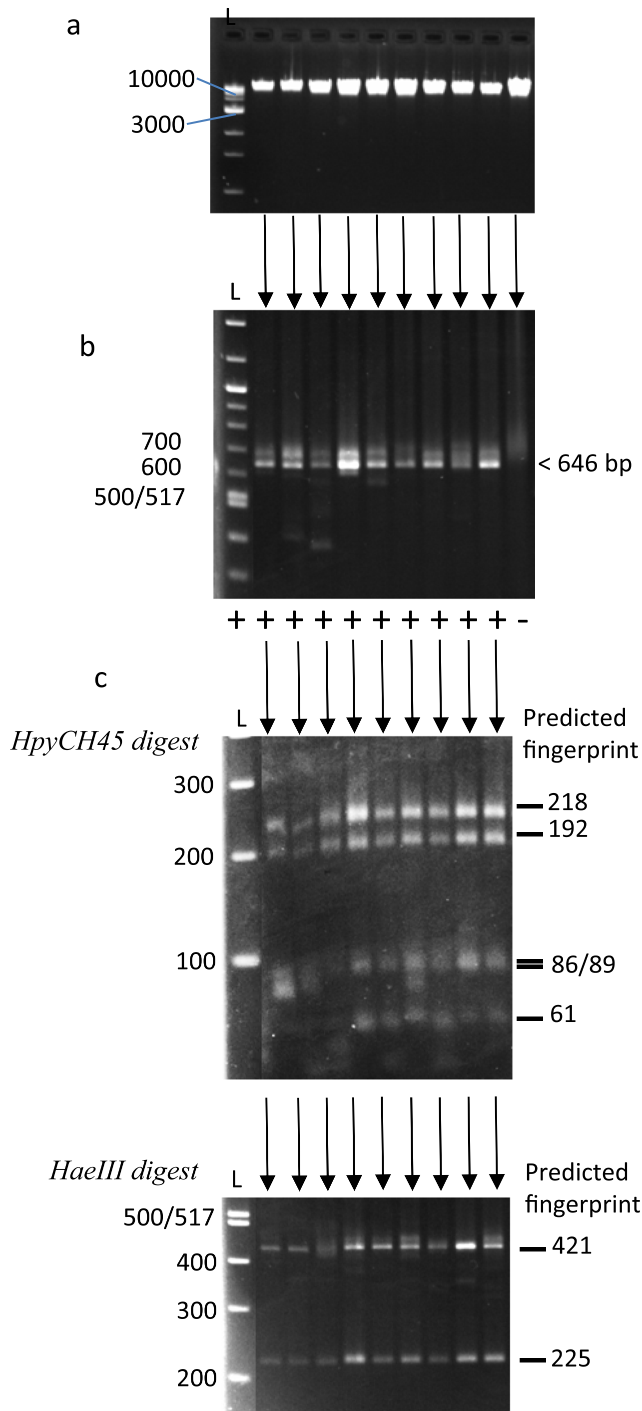

Fig. 2 Detection of targeted deletions on wheat callus after transient expression of CMN. (a) Quality check of DNA samples extracted from pools of calli. Compact bands indicate that intact high molecular weight DNA was extracted from the pools (example for ten callus pools extracted from progeny of DS T0 plant 26) (b) PCR amplification of the 646 bp fragment resulting from deletion of the [5′ CMN site-NptII-*DsRed*-3′ CMN site] cassette of callus pools presented in Fig. 2a. (c) Fingerprints of the PCR amplified fragments using *HpyCH45* and *HaeIII* restriction endonucleases. L: NEB DNA ladder 1 kb (Fig. 2a) and 100 bp (Fig. 2b, c)

(Alaeddini et al. 2010). Primers FP1 and RP4 (Fig. 1a) were used to perform a standard PCR amplification. Under standard PCR conditions and using standard *Taq* polymerase, it was not possible to amplify the complete cassette because of its large size (3963 bp), but if the complete cassette was deleted due to a *CMN* endonuclease double cut, the size of the amplicon (646 bp) become compatible with standard PCR conditions (Fig. 1c). The deletion was detected in 26 callus pools out of the 57 tested (Fig. 2b) independently of the T0 plant used to provide immature embryos. Although a PCR fragment was indeed of the correct molecular weight, we still had to check if it was really the expected fragment. To do so, each amplified fragment was isolated and successively subjected to fingerprinting using *Hpy*CH4V and *Hae*III restriction endonucleases (Fig. 2c). All the amplified fragments presented the correct fingerprint for the two enzymes, indicating that we had detected at least 26 independent deletion events of the [*CNRC*] cassette. Although meganuclease *DFR-MN* has been shown to be efficient in vivo in *Nicotiana alata* (Honig et al. 2015), *I-SceI* cutting for site-specific transgene integration has been demonstrated in maize (D'Halluin et al. 2008), *I-CreI* has produced targeted mutagenesis in maize (Djukanovic et al. 2013) and has been used for trait stacking in cotton (D'Halluin et al. 2013). To our knowledge, this is the first report of in vivo activity of a custom made meganuclease in wheat. Our result showed that *CMN* was transiently expressed in wheat immature embryos and/or calli, and that the CMN efficiently cut DNA in vivo. We also showed that CMN cutting can cause the deletion of large DNA fragments located between two *CMN* sites.

Sequencing analysis of the first batch of PCR amplicons was not performed because they probably resulted from multiple independent deletion events that had occurred at different points in time in different cells. The presence in some amplicons of a faint band of higher molecular weight than expected (Fig. 2b) possibly reflected a heteroduplex fragment resulting from double strand association of heterogeneous DNA strands possibly indicative of heterogeneity of the PCR product. This heterogeneity made it impossible to perform sequencing reactions. To overcome this problem, we amplified very rare, or unique, deletion events on the 31 negative samples using nested PCR. From these 31 samples, 25 nested PCR amplification signals were obtained, and good quality sequencing results were obtained for 11 PCR fragments (Fig. 3). Sequencing data showed that among the 11 samples, six were a perfect reconstruction of the *CMN* site with no detectable mutation, two presented an indel or a SNP that did not disrupt the *CMN* recognition sequence, and three presented SNP or deletions that disrupted the CMN recognition sequence (Fig. 3 and Supplementary data 5). Interestingly, the A-G point mutation detected in the meganuclease recognition site was chimeric (Fig. 3b T4 and Supplementary data 5), indicating that more than one deletion event occurred in a single pool comprising six calli. On the other hand, the single A deletion (Fig. 3b T2) or the A-G point

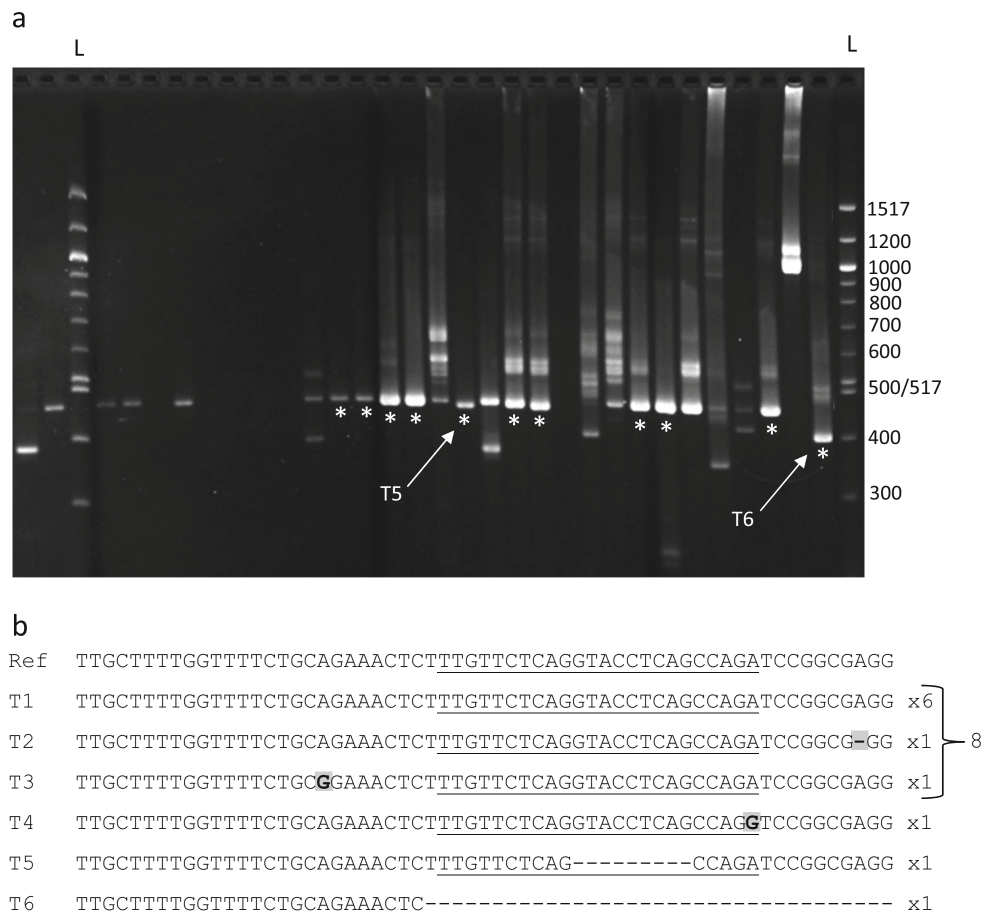

Fig. 3 Detection of targeted deletion of the [5′ CMN site-NptII-*DsRed*-3′ CMN site] cassette by nested PCR on wheat callus after transient expression of CMN. **a** Nested PCR amplification. Fragments subjected to sequencing are indicated by asterisks. **b** Six different sequence types identified. T1 is a perfect reconstitution of the *CMN* site, T2 and T3 showed one? indel and one? SNP mutation (gray), respectively, near the *CMN* site that did not disrupt the recognition sequence, T4, T5 (arrow), and T6 (arrow) showed SNP or deletions that disrupt the recognition site. The complete sequence of T6 is presented in Supplementary Data 5. To the right of the sequences, xN indicates the number of times a sequence was retrieved in independent nested PCR fragments. Eight perfectly reconstructed *CMN* sites (sum of T1 + T2 + T3) were obtained after cassette deletion. L, 100 bp DNA ladder (NEB)

mutation (Fig. 3b T4) in 5′ of the *CMN* recognition sequence showed no evidence of chimerism (Supplementary data 5), indicating that a single deletion probably occurred in these two pools of calli. Nucleotide deletions observed inside the CMN recognition site (Fig. 3b T5) or large deletions that included the CMN site (Fig. 3b T6) indicated that the amplification fragments were not generated by artifactual PCR recombination between the two CMN recognition sites. We postulate that the mutations detected, located near or inside the meganuclease recognition site, were generated during the process of DNA repair after cassette deletion. So, our sequencing results confirmed the results we obtained previously using restriction endonucleases, and showed that in a large number of cases, the *CMN* recognition site was perfectly reconstructed after cassette deletion, indicating that the site was available for subsequent site-directed insertion of a new transgene, a targeted insertion that has been demonstrated in cotton using I-CreI based customized designed nuclease (D'Halluin et al. 2013). Our results, which were obtained after transient expression of CMN nuclease in callus, now needed to be confirmed on regenerated plants after stable transformation with *CMN*.

Cassette Deletion after Stable Transformation of CMN Gene

Ten independent T0 DS events were randomly chosen and 8 T1 plants for each T0 were sown to produce immature embryos. 13 T1 plants shown to be positive for the presence of the [*CNRC*] cassette by PCR were retransformed with the [*CMN*] cassette. 61 T2 DS/T0 CMN plants were regenerated (Supplementary data 6), tested by PCR for the presence of *CMN* and *DsRed* transgenes and for the deletion of the [*CNRC*] cassette. Among them, 46 were positive for the *CMN* transgene, 39 were positive for *DsRed*, 31 were positive for both genes (Fig. 4a), and three regenerated plants were positive for *CMN*, *DsRed* and cassette deletion (Fig. 4b).

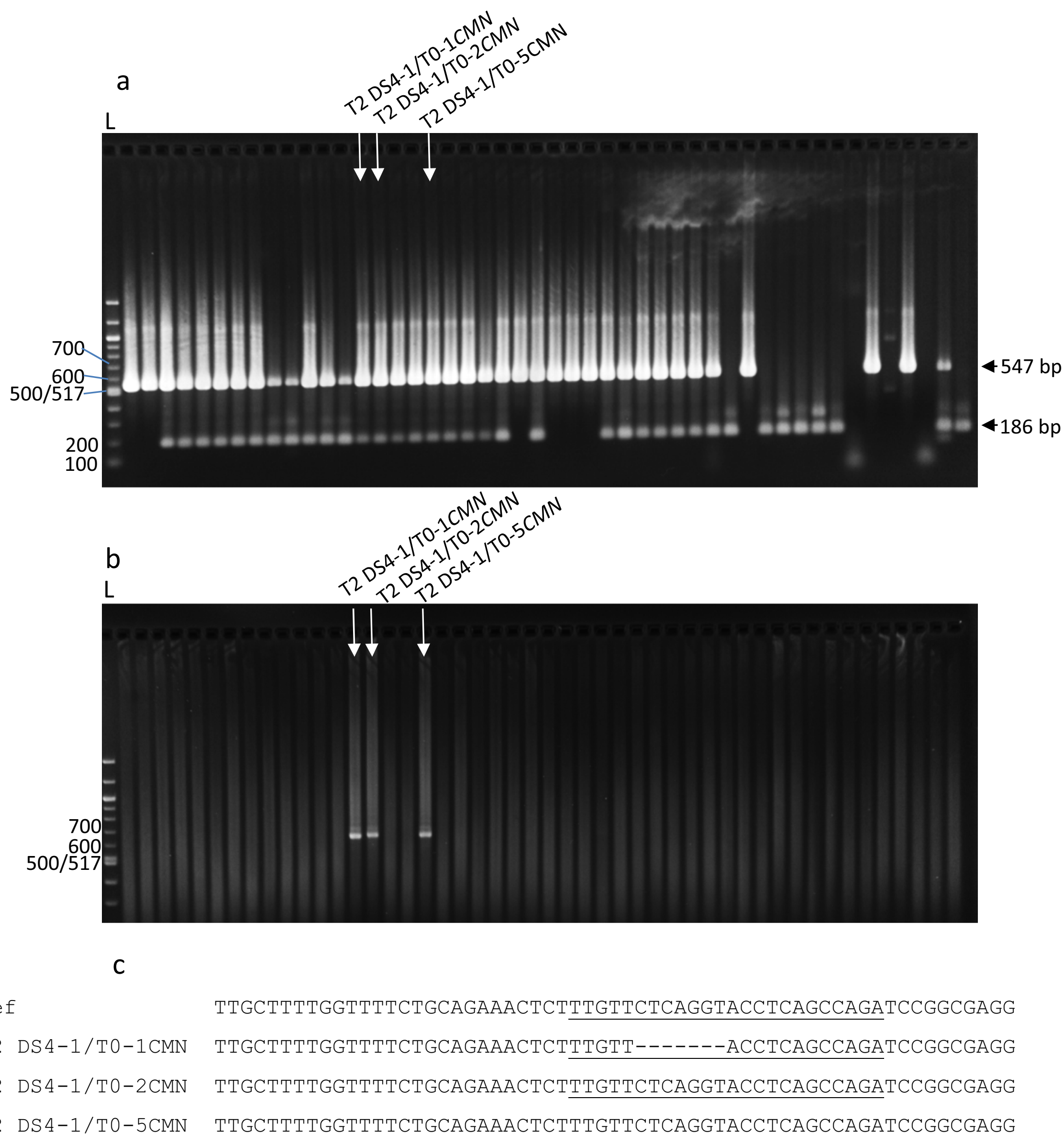

Fig. 4 Detection of targeted deletions in transgenic wheat plants after stable expression of CMN. **a** PCR amplification of *CMN* (547 bp fragment) and *DsRed* (186 bp fragment) on T0/T2 double transgenic plants (only the first batch of 48 regenerated plants out of the 61 tested are presented). **b** PCR amplification of the 646 bp fragment indicating deletion of the [5′ CMN site-NptII-*DsRed*-3′ CMN site] cassette on T0/T2 double transgenic wheat plants. **c** Alignment of the sequences surrounding the *CMN* site of the three fragments showing cassette deletion. Ref: theoretical reference sequence of the deletion. Plant T2 DS4-1/T0-1 CMN showed a 7 bp deletion after reconstitution of the *CMN* site, and plant T2 DS4-1/T0-2 CMN and T2 DS4-1/T0-5 CMN showed perfect reconstruction of the *CMN* site (underlined). L, 100 bp DNA ladder (NEB)

The three amplified fragments corresponding to a deleted cassette were sequenced (Fig. 4c). Plant T2 DS4-1/T0-1*CMN* showed a 7 bp deletion after reconstitution of the CMN site, and plant T2 DS4-1/T0-2*CMN* and T2 DS4-1/T0-5*CMN* showed perfect reconstruction of the CMN site. The 7-bp deletion was probably due to non-homologous end joining (NHEJ) repair, but the two perfect reconstructions of the CMN site could also result from homologous recombination (HR) that could have used intact CMN sites as matrix. The PCR amplification was performed on 50 ng of wheat DNA, corresponding to approximately 1000 wheat cells, and thus, to at least 2000 DNA targets. Artifactual amplification, if it occurred, cannot reproduce a very rare event like the deletion of seven short nucleotides we observed so many times. Considering this result, the hypothesis of an artifactual amplification by PCR recombination can be excluded. We also checked that all 5 T0 and 13 T1 plants used for the production of immature embryos in both transient and stable transformation experiments were negative for deletion fragment amplification (Supplementary data 7). These data showed that a

[*CNRC*] cassette was deleted in three independent events (3/61, 5% efficiency), with a perfect reconstruction of the site in two transgenic plants (2/61, 3% efficiency), opening perspectives for future insertion of a new transgene at the same locus. Cassette deletion was detected in three independent T0 *CMN* events originating from T2 *DsRed* embryos provided by the same T1 *DsRed* plant, indicating that independent *CMN* transformation events can lead to cassette deletion, but also that location of the cassette insertion site on chromosomes could be important for efficient meganuclease activity. The presence of the *DsRed* cassette in deleted events indicated that the cassette was present in more than one copy in the *DsRed* insertion locus, or that the cassette was present at the homozygous stage in T2 embryos of DS T0 plants, and that only one copy was deleted. Five T2/T0 plants were sterile (5/61, 8%). Unfortunately, among these five plants, four originated from the T2 DS4-1 event, including T2 DS4-1/T0-1, 2 and 5*CMN* plants that showed a cassette deletion (Supplementary data 6). It is unlikely that sterility was a consequence of *CMN* expression because it was observed in only five events out of 61, and because four of these five independent events were generated on the same DS4-1 transgenic background. On the other hand, it is unlikely that sterility was due to a DS4 event as we did not observe any sterility in the T0 and T1-1 generation. Consequently, we postulate that sterility resulted from *CMN* activity in this particular event. One possible hypothesis is that the insertion locus of this event was particularly accessible due to its chromosomal context/position, as has been described for customized meganucleases in human 293-H cells (Daboussi et al. 2012). This accessibility could have caused a high rate of cassette deletion, but could have also triggered chromosome instability during meiosis, which resulted in sterility. One way to overcome this problem would be to try to regenerate plants showing cassette deletion induced by transient expression of *CMN*. Evidence of cassette deletions obtained by transient expression in our work is the first step to achieve this goal.

Conclusion

Our results demonstrated the feasibility of meganuclease-mediated targeted deletion of a marker gene in the wheat genome. Meganuclease activity was first demonstrated by transient expression on calli, which paves the way for rapid tests of the usefulness of other types of SDN, and which provide an interesting alternative to the previously described transient tests in protoplasts (Shan et al. 2014). Moreover, the recognition site of the meganuclease was reconstructed after elimination of the DNA fragment in the majority of deletion events, offering the opportunity to use *CMN* for targeted insertion of a new construct at the same genetic locus. For gene stacking through backcross of multiple transgenes in elite accessions, the use of *CMN* could be more suitable than a CRISPR-*Cas9*-based system because of its reduced off-target effects and established intellectual property landscape.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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