Brief Communication

Site-directed mutagenesis in bread and durum wheat via pollination by cas9/guide RNA-transgenic maize used as haploidy inducer

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The present approach relies on the expression of cas9 and wheat gene-specific gRNA in maize sperm cells. Therefore, transgenic maize carrying a ubiquitously expressed GFP was analysed, and conspicuous fluorescence was found in sperm cells (Figure 1a). Two Cas9/gRNA target motifs for TaBRI1 and one for TaSD1 were selected. These proved to be conserved across all two (AABB) or three (AABBDD) homeologues of the target genes in durum and bread wheat, respectively. Corresponding gRNAs were cloned into generic vectors used to transform maize (Budhagatapalli et al., 2016). Two hundred maize T0 plants carrying wheat target-specific cas9/gRNA-encoding T-DNAs were pre-screened by qRT-PCR analysis. Per target motif, five maize transgenics with high cas9 and gRNA expression were selected for pollination of wheat (Figure 1b). Upon these intergeneric crosses, embryos were rescued in vitro. Regenerated wheat plants were then subjected to PCR-based mutation analysis by Sanger sequencing. For BRI1 target motif 1, three, two and one mutants were obtained out of 83, 44 and 10 plants in genotypes BW, W5 and D6, respectively. Two plants out of 4 and 3 carried mutations for BRI1 target motif 2 in genotypes W5 and D7, respectively. In addition,-seven mutants for the SD1 target motif 1 were obtained from 17, 5 and 8 plants in genotypes BW, K15 and S96, respectively (Figure 1c). Subcloning and Sanger sequencing of target motif-derived amplicons of M1 plants indicated that all bread wheat mutants for BRI1 and SD1 were invariably homozygous, whereas those in durum genotypes D6 and D7 were chimeric (Figure 1c). This may be due to differences in cas9 and gRNA expression, the time point of male genome activation and the activity of DNA repair.

In the present approach, mutations can be induced at various phases before and after the zygote undergoes mitosis (Figure 1d). Mutations induced during G1 and early S phase are more likely to occur owing to Cas9 and gRNA molecules pre-produced in the sperm rather than to zygotic de novo transgene expression. Resultant embryos are expectedly non-chimeric with regard to the induced mutations (Figure 1d-i). Alternatively, after chromatid duplication, Cas9 may trigger mutations in one chromatid or independently in either of the sister chromatids (Figure 1d-ii). In this scenario, the daughter cell that has received a mutated wheat chromatid during the first embryonic mitosis itself undergoes S phase, by which the mutated allele becomes genetically fixed across the two sister chromatids, while the other daughter cell has received a non-mutated or differently mutated chromatid and thus gives rise to a genetically distinct sector. Consequently, embryos formed via mutagenesis during G2 phase are expectedly chimeric (Figure 1d-ii). In the course of initial embryonic cell divisions upon wheat x maize crosses, maize chromosomes are

Received 24 January 2020; revised 1 May 2020; accepted 12 May 2020.
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Keywords: BRI1, Cas endonuclease, CRISPR, doubled haploid, SD1, targeted mutagenesis.
eliminated due to asynchronous processing in terms of DNA replication, condensation and centromere formation (Laurie and Bennett, 1988).

In total, 15 independent target gene-specific mutants were identified out of 174 wheat plants from which good-quality Sanger sequences of target motifs had been retrieved. Mutants were obtained in six wheat backgrounds, including the three spring-type bread wheats BW, W5 and K15, the winter-type bread wheat S96, and the two durum wheats D6 and D7 (Figure 1c). Mutations were found in all three target motifs addressed (Figure 1c). None of the 15 mutants carried any transgene. Across the genotypes, the efficiency in mutant plant formation ranged from 3.6% to 50% (Figure 1c).

The BRI1 and SD1 genes are known to play an important role in plant height. Therefore, loss-of-function mutants may entirely fail to develop. In addition, knockouts of BRI1 and SD1 in Arabidopsis lead to male sterility, as they regulate key genes of anther and pollen development (Plackett et al., 2012; Ye et al., 2010). The haploid plants obtained in the present work were subjected to colchicine treatment. As a result, 7 out of 15 mutants were fertile (Figure 1c). In M2, progenies of doubled-haploid mutants SD1-TM1-DH04-AABBdd mutant (n = 10), K15-wt (n = 10) and SD1-TM1-DH04-AABBdd mutant (n = 10). Significant differences between mutants and wt counterparts are indicated by asterisks, with **** representing a P-value < 0.0001 according to unpaired t-test; n is the number of plants analysed, and error bars represent the standard deviation.

In conclusion, the principle of haploid induction coupled with site-directed mutagenesis was exemplified in wheat using the two

Figure 1 Site-directed mutagenesis in bread and durum wheat via pollination by cas9/gRNA-transgenic maize. (a) GFP accumulation in maize sperm cells (arrows); bright field (left) and GFP (right) filter images with 200x magnification. (b) Expression of cas9 and gRNAs. Graphs represent the mean values of cas9 and gRNA, and error bars represent the standard deviation derived from three replications. (c) TaBRI1 and TaSD1 target motifs are highlighted in red with the protospacer-adjacent motifs (PAM) being underlined. Mutations are indicated by green font, and the numbers given to the right represent the concerned nucleobases. (d) Mutations induced in hybrid zygotes at various phases before and after mitosis. (e) Tiller height of representative doubled-haploid SD1 M2 plants at anthesis stage. The graph represents the mean height values of the first three tillers from the BW-wt (n = 10), SD1-TM1-DH07-AABBdd mutant (n = 17), K15-wt (n = 10) and SD1-TM1-DH04-AABBdd mutant (n = 10). Significant differences between mutants and wt counterparts are indicated by asterisks, with **** representing a P-value < 0.0001 according to unpaired t-test; n is the number of plants analysed, and error bars represent the standard deviation.
target genes BRI1 and SD1 which control the agronomically important trait plant height. Major advances achieved in this work include reduced genotype dependence of site-directed mutagenesis in wheat, the opportunity of creating a whole variety of mutations using just one cas9/gRNA-transgenic (pollinator) plant as well as the production of T-DNA-free and frequently homozygous M1 plants. There is still scope for increasing the efficiency of this approach, for example by stronger transgene expression at the relevant time point or by the development of improved protocols for in planta production of doubled haploids.

Acknowledgements

We thank Andrea Müller, Petra Hoffmeister, Josef Bergstein, Margit Lang, Anke Halbach and her team, Janett Paper, Jenny Osterburg and Dr. Katja Kempe for their excellent support. We also thank Dr. Andreas Jacobi and Dr. Edgar Müller for selecting and providing appropriate wheat genotypes. The study was financially supported by the Federal Ministry of Food and Agriculture (FKZ 2814603113).

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

J.K. conceived the project concept, and N.B., T.H., H.B. and A.E.M. designed and performed the experiments. N.B. and J.K. wrote the manuscript, and T.H., S.H. and A.E.M. reviewed and edited the manuscript. All authors read and approved the manuscript.

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