Editorial

Efficient TALEN-mediated gene editing in wheat

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Plant genome editing is a major advance in the production of novel plant genotypes. There is, however, only a single previous report of applying this technology to hexaploid wheat using TALEN-mediated gene editing to produce heritable modifications (Wang et al., 2014). Here, we describe highly efficient TALEN editing of a uidA transgene and a second endogenous gene, Lr21, in bread wheat with efficiencies exceeding most previous TALEN and CRISPR/Cas9 reports in this species.

A TALEN pair (Figure 1a) targeting the E. coli uidA gene was co-transformed into embryos from a wheat cultivar Fielder line segregating for a Ubi-uidA transgene. Twelve lines were produced containing both TALENs and at least one copy of uidA. T0 genomic DNAs of these plants were restricted with BclI, as this enzyme cleaves the target site, and the target region was then PCR amplified (Figure 1b). Products were amplified from three of the 12 (25%) DNAs (plants P21, P38 and P45), and Sanger sequencing identified deletions of 3, 13 and 4 bp, respectively, that destroyed the BclI site (Figure 1b).

T1 analysis showed that P38 and P45 were hemizygous for uidA and that only edited uidA alleles were transmitted to progeny (nine edited-uidA: six null and 10 edited-uidA: 5 null), consistent with an editing event subsequently dominating the majority of germinal tissue in each plant. In contrast, Mendelian inheritance occurred in P21 T1 progeny (6 wt-uidA; 12 presumed heterozygotes (p-hets): six edited-uidA, $x^2 = 1.0$), indicating the parent plant was uidA/uidA and a single edited allele predominated in germinal tissue. No Gus staining occurred in P38 and P45 T1 plants containing 13- and 4-bp deletion alleles due to loss of uidA function, whereas P21 progeny homozygous for a 3-bp deletion allele showed Gus staining, presumably because the uidA ORF remained in-frame (Figure 1c).

Most gene editing reports have produced targeted gene knockouts. Herein, we attempted to reactivate a pseudogene ($l$r21${}^P$) present in Fielder wheat. The functional Lr21 gene (GenBank AH012974) encodes a nucleotide binding site leucine-rich repeat protein (NLR) that provides race-specific resistance to leaf rust disease caused by Puccinia triticina (Huang et al., 2009). Lr21${}^P$ differs to Lr21 by 3 nonsynonymous SNPS (498 G/D, 854 M/I, 1055 R/S) and a single base deletion that destroys the gene ORF (Huang et al., 2009). Previously, a recombinant allele encoding Fielder Lr21, Lr21${}^P$, regulatory sequences gave functional Lr21 resistance (Huang et al, 2009). Given this functional expression and near sequence identity between Lr21${}^P$ and Lr21, we reasoned that restoring the Lr21${}^P$ ORF by editing the 1-bp deletion site may reconstitute a functional resistance gene.

Forty T0 plants were produced containing a TALEN pair (Figure 1d) targeting the Lr21${}^P$ 1-bp deletion site (Figure 1e). DNAs from each plant were PCR amplified using primers flanking the Lr21${}^P$ target site and amplicons MiSeq sequenced with an average of 2266 ± 800 (standard deviation) reads analysed per sample. Seventy-three different edited alleles were identified amongst T0 amplitcons with 71 encoding deletions (1–11 bp; Figure 1f) and two encoding small indels.

Substantial Lr21${}^P$ editing occurred with 85% of T0 plants (34/40) having between 15% and 100% of amplitcons edited. On average, 55% ± 38% (standard deviation) of amplitcons from each T0 plant were edited. Individual allele frequencies within DNA samples ranged from 0.25% to 98.41% of amplitcons, and most alleles (50/73; 68%) were common to at least two plants. The eight most common modified alleles with their respective frequencies are shown (Figure 1e). Nucleotides in the middle of the target site were more commonly deleted than those adjacent to TALEN binding sites (Figure 1g). Target site analysis of 3 Fielder control DNAs showed a very low error rate in this analysis with 0%, 0% and 0.12% of amplitcons differing to the wild-type Lr21${}^P$ sequence.

Allele inheritance was investigated in five T1 families from plants L14, L16, L20, L24 and L27 using target site PCR amplification and Sanger sequencing. In two T1 families, L14-T1 and L16-T1, single edited alleles (Figure 1h) showed Mendelian inheritance (8 wt: 25 p-hets: 12 ed; $x^2 = 0.53$ and 7 wt: 25 p-hets: 9 ed; $x^2 = 0.34$, respectively). The L14-T1 allele was the most frequent (49.54%) edited sequence amplified from the L14 T0 parent, while the L16-T1 allele represented only 8.51% of plant L16 amplicons. Progeny of L20 and L24 inherited two deletion alleles in each family (Figure 1h) although with non-Mendelian ratios (P20, 3:1; P24, 0 wt: 40 p-hets: 6 ed with 4-bp deletion: 2 ed with 6-bp deletion). In both families, these alleles were the most abundant amplitcons from the T0 parent (L20, 48.27% and 47.21% of amplicons; L24, 49.49% and 47.91%). In contrast, no modified Lr21${}^P$ alleles were inherited by progeny of L27 consistent with few amplitcons from the parent also being edited (0.46%).

Two edited alleles inherited in T1, progeny encoded restored Lr21${}^P$ ORFs (Figure 1i). One, present both L14 and L20 T1 families, encoded a 5-bp deletion (Figure 1h) which restored the gene ORF, albeit with the loss of two amino acids (Figure 1i). The second allele, also inherited by L20 progeny, had an 11-bp

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Figure 1  TALEN editing in wheat. (a) Constructs used for TALEN editing of a uidA transgene present in Fielder wheat. Each TALEN, located on a separate binary vector, was under the regulatory control of maize polyubiquitin promoter (Ubi) and Agrobacterium nopaline synthase 3' transcription termination sequences (nos). (b) The uidA target site sequence with TALEN binding sites shown in blue font. A Bch restriction enzyme site is indicated in red font. Edited allele sequences from P21, P38 and P45 are shown. uidA sequence corresponds to nucleotides 7799–7852 of GenBank AB489142.1. Note the actual right-hand TALEN binding site sequence is the reverse complement as only a single DNA strand is shown for convenience. (c) GUS staining of tissue from T1 plants for uidA alleles inherited in T1 progeny of plants L14, L16, L20 and L24. (i) Predicted peptides encoded by in-frame edited alleles from P21 (panel 2), P38 (panel 3) and P45 (panel 4). Panel 1 shows wild-type uidA staining of unedited P21 sib tissue. (d) TALEN constructs used for editing of lr21Ψ. Each TALEN gene was again regulated by maize ubiquitin promoter and Agrobacterium nos terminator sequences. (e) The eight most common edited alleles present in 217 T0 DNAs with their frequency amongst amplicons from all 40 T0 DNAs shown on the right. Sequence shown is equivalent to nucleotide coordinates 1264–1314 of the Fielder lr21Ψ sequence (GenBank F1876295). TALEN binding sites are highlighted in blue. A single nucleotide deletion (A) pre-exists in the Fielder lr21Ψ target site amongst 40 T0 DNAs. Boxed sequences on the X-axis are TALEN binding sites with the reverse complement of the right-hand TALEN binding site shown. (f) Edited lr21Ψ alleles inherited in T1 progeny of plants L14, L16, L20 and L24. (i) Predicted peptides encoded by in-frame edited alleles shown in (h). Eight most common edited amplicon sequences recovered from protoplasts transformed with lr21Ψ TALENs. Asterisks highlight alleles also present in T0 DNAs (panel e). (k) Screening of TALEN-edited plants for new leaf rust resistance. The left image shows functional Lr21 resistance present in wheat accession CS1DS406 when compared with the susceptible cultivar Fielder (lr21Ψ lr21Ψ). TALEN-edited plants L14 and L20 contained restored lr21Ψ ORFs as shown in panels (h) and (i). T1 progeny of these plants were grown and genotyped for zygosity of alleles with restored ORFs (wt = wild lr21Ψ, p-het = presumed heterozygous, ed = homozygous for the edited allele), and these plants then challenged with P. triticina. Heterozygotes are described as presumed as plants producing PCR products with mixed sequencing traces could also be biallelic or chimeric. All seedlings were fully susceptible and indistinguishable to the Fielder control shown on the left. (l) Potential lr21Ψ TALEN off-target sites (OTS) amplified from the Fielder wheat genome. OTS1 and OTS2 correspond to annotated wheat genes TraesCS1B02G0052400 and TraesCS1A02G0067000, respectively. Other off-target sites are present in the Chinese Spring genome sequence that could not be amplified from Fielder presumably due to sequence polymorphism existing between these two cultivars. TALEN binding sites are highlighted in blue on the lr21Ψ and OTS sequences. Mismatched nucleotides at the TALEN binding sites of OTS sequences are highlighted in red. Immediately beneath each OTS are variant sequences identified amongst 40 T0 DNAs with frequencies indicated.

deletion (Figure 1h) and encoded an in-frame ORF with four codons deleted at the editing site (Figure 1h, i). However, Lr21 resistance to P. triticina was not recovered in seedlings homozygous for either of these alleles or amongst 443 T1 seedlings from 10 other active lr21Ψ TALEN lines which potentially contained additional allelic variants (Figure 1k).

Eight potential lr21Ψ TALEN off-target sites are present in the Chinese Spring wheat genome, and two of these sites, corresponding to genes TraesCS1B02G0052400 and TraesCS1A02G0067000, were successfully amplified from Fielder DNA. MiSeq sequencing of amplicons from all 40 T0 DNAs identified two potential editing events, a 6-bp and a 1-bp deletion, at
germplasm is the only other available source. Programs, which is laborious if wild relatives or unimproved footprints may be tolerated. Potentially, pseudogene reactivation attempt was unsuccessful, others may function was not, possibly due to editing footprints. While this will maximize wheat editing opportunities. Without a PAM sequence requirement, and using both platforms to edit. TALEN editing therefore provides an efficient alternative, Potentially, this is problematic if an unamenable target site is critical edited in wheat, likely due to sgRNA and genomic target site accessibility. Alternatively, we used Agrobacterium transformation rather than biotics which may cause significant TALEN expression differences. It is noteworthy that Wang saw higher editing in protoplasts compared with biolistics.

A variety of stable and transient CRISPR/Cas9 editing approaches have also been used in wheat with Tg editing efficiencies usually around 1%–10%, (Howells et al., 2018) and references therein; Zhang et al., 2019; Kumar et al., 2019; Kelliher et al., 2019; Okada et al., 2019), which is low compared with rice (Zhang et al., 2014). While CRISPR/Cas is technically simpler than TALENs and far more accessible. Alternatively, we used Agrobacterium transformation rather than biotics which may cause significant TALEN expression differences. It is noteworthy that Wang saw higher editing in protoplasts compared with biolistics.

These data show highly efficient wheat TALEN editing of a transgene (25%) and an endogenous gene (85%) in Tg wheat plants and modified alleles having high heritability. Previously, the wheat Mlo gene was TALEN edited with 3.4%–6.0% efficiency in Tg plants although with multiple homoeologous loci simultaneously modified in some plants and co-inherited in Tg progeny (Wang et al., 2014). Both our and Wang’s studies used maize polyubiquitin promoters for TALEN expression, so the large editing efficiency differences between studies suggest variation in TALEN target site accessibility. Alternatively, we used Agrobacterium transformation rather than biotics which may cause significant TALEN expression differences. It is noteworthy that Wang saw higher editing in protoplasts compared with biolistics.

While the ir21Ψ ORF was successfully restored, resistance gene function was not, possibly due to editing footprints. While this pseudogene reactivation attempt was unsuccessful, others may succeed if less constrained proteins are targeted where editing footprints may be tolerated. Potentially, pseudogene reactivation could be beneficial in introducing a functional allele in breeding programs, which is laborious if wild relatives or unimproved germplasm is the only other available source.

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Conflict of interest
The authors declare that there is no conflict of interest.

Author contributions
ML, HL, SC and AR undertook experiments; DB, SL and TR produced wheat transgenics; RM and TL designed and produced TALEN constructs; NU and ML undertook bioinformatic analysis; ML and MA designed experiments and wrote the manuscript.

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