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Genome editing in wheat microspores and haploid embryos mediated by delivery of ZFN proteins and cell penetrating peptide complexes

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Introduction

Bread wheat (*Triticum aestivum* L.) is a source of ~20% of the world's food and is considered one of the most important staple crops. Wheat belongs to the cool season crops; therefore, increase in the global temperature can significantly hamper its cultivation in the world.

Conventional breeding practice of the small grain cereals relies on efficient production of fully-homozygous plants with the fixed beneficial traits. The ability to work with haploid cells/tissues is desirable for biotechnology applications since it significantly reduces time and cost required for generation of complete homozygous lines with the altered genome. Microspores are predecessors of male gametes that under certain conditions can undergo embryogenesis *in vitro* to produce green plants. Being single cell and haploid in nature, the cells provide an opportunity to produce homozygous plants with edited or disrupted locus without germline-chimerism in a single generation.

Recent advances in genome editing (GE) technology resulted in wide adoption of the method for rapid and efficient creation of crops with novel traits. There is still some uncertainty of how genome edited crops will be regulated depending on geographies. Some of the concerns are stemming from the type of molecules used to deliver DE (e.g., DNA, RNA or protein) inside the plant cells. If the enzymes (ZFN, TALEN or Cas9/gRNA) are delivered in the form of DNA there is a risk of unintentional extraneous integration of the complete or fragmented plasmid in the genome, therefore triggering regulation statutes pertaining to transgenic plants.

We provide evidence for the first time of genome editing through direct delivery of purified ZFN proteins into unmodified microspores with intact walls. We used purified ZFN monomers from bacterial culture complexed with cell penetrating peptides (CPP) and internalized into the plant cells. As a target we chose the *INOSITOL PENTAKISPHOSPHATE KINASE 1* (*IPK1*) gene, the product of which is involved in catalyzing the final steps of phytic acid production, which is an anti-nutritional component of feed grain. We recovered edits at the target site for both – microspores and haploid embryo-like structures (ELS). Curiously, although ZFN-IPK1 was designed to target conservative region in both subgenomes A and B, we observed clear dominance of indels, as a result of ZFN activity, only at the subgenome A.

Material and methods

Cloning of IPK1 homolog from wheat cultivar AC Andrew

Wheat IPK1 homolog for subgenome A was was mapped to chromosome 2AL, 2BL and 2DL. All three copies were amplified from AC Andrew gDNA using subgenome specific primers, cloned and sequenced to confirm sequence identity with the published database of IWGSC. ZFN constructs were designed to target specifically *IPK1*-sgA and *IPK1*-sgB copies.

Cloning of recombinant plasmids

Several ZFNs targeting endogenous *TaIPK1* gene were designed by Sangamo Biotherapeutics and tested in a yeast proxy assay (Ainley, Sastry-Dent et al. 2013). ZFN monomers were cloned into protein expression vector to generate the plasmids pET45b(+)::ZFN-L and pET45b(+)::ZFN-R. To assess ZFN cleavage activity *in vitro*, an *IPK1* fragment containing ZFN recognition site was amplified form the subgenome B and cloned into pUC18 plasmid.

Overexpression and purification of ZFN monomers from bacterial culture

Purification of ZFN proteins from bacterial culture was done according to protocols described in (Tovkach, Zeevi et al. 2011, Gaj, Guo et al. 2012).

In vitro ZFN cleavage activity

Activity of purified ZFN proteins was assessed using an *in vitro* cleavage assay (Tovkach, Zeevi et al. 2011).

Labeling of ZFN monomers with the fluorophore, *in vitro* conjugation to CPPs and transfection of microspores

To assess CPP-mediated delivery of ZFN proteins, ZFN-R monomer was labeled with Alexa Fluor 647 (AlF-647) protein labeling kit (ThermoFisher Scientific, cat.no. A20173) according to the manufacture's protocol. Conjugation reaction was done with 7 µg of ZFN-R-AlF647 (1.59 µM in His-Tag buffer B, pH 7.5) by adding different amounts of cys(Npys)-(D-R)9 (Anaspec) and incubating the mixture for 2 hours at room temperature. 100,000 cells in CIMC7 wash medium (Bilichak, Luu et al. 2015, Bilichak, Luu et al. 2018) were directly mixed with (D-R)9-ZFN-R-AlF647 conjugate and the volume was adjusted to 100 µl. Transfection was done for 2 consecutive days followed by washing of the microspores for 3 times with CIMC7 medium. The visual examination of transfected microspores was done using confocal laser scanning microscope Olympus, FV1000 with 594/633 laser line.

Generation of NGS library and bioinformatic analysis of indels at the target site

The NGS library was generated by amplification of IPK1 target regions using primers specific for SNPs in subgenomes at both 5' and 3' regions in regards to ZFN cut site. 100 ng of gDNA was used for amplification of the target regions using either Q5 or Phusion DNA polymerase (NEB). Amplification was done for 35 cycles in total volume of 30 µl each. This was followed by gel purification of PCR fragments. Every primer combination had a unique barcode for discrimination among samples during bioinformatic processing of the NGS data. Sequencing of the NGS libraries was conducted using Illumina NextSeq platform with single-end sequencing runs and 150 cycles. Each sample generated from 2.5 to 10 million HQ reads with the overall average of 7 million HQ reads per sample. Reads were trimmed to 80 bp for the analysis and processing of the data was done by Dow AgroSciences LLC bioinformatic pipeline (Elango 2012). Background correction was done by removing sequences with point mutations or less than 2 indels from both sets and activity is determined relative to the control sample. The frequencies of the identified high-quality alignments were represented as percentage of high-quality trimmed reads sequenced. Multiple sequence alignment of the reads showing indels at the ZFN cutting site was done and visualized using MAFFT aligner tool in Unipro UGENE program (Okonechnikov, Golosova et al. 2012).

Results and discussion

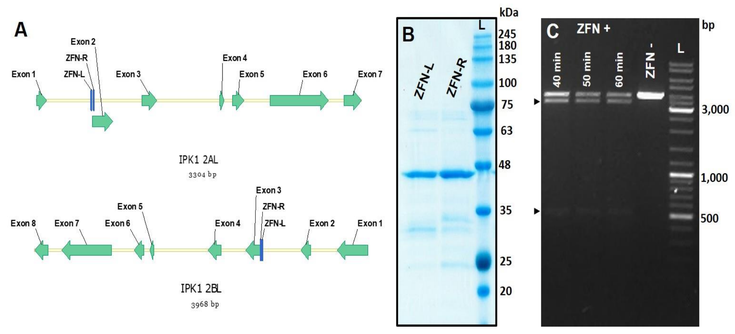

Figure 1. Recognition sites (A), purification (B) and cleavage activity of ZFN-*IPK1* protein *in vitro* (C). A – schematic map of the *IPK1* homoeologs for subgenomes A and B showing binding sites for ZFN-L and ZFN-R. B – left and right ZFN monomers purified from bacterial culture and analyzed on 10% PAGE, showing ZFN-L and ZFN-R proteins (42 and 46 kDa, respectively), C – cleavage activity of purified ZFN-*IPK1 in vitro*

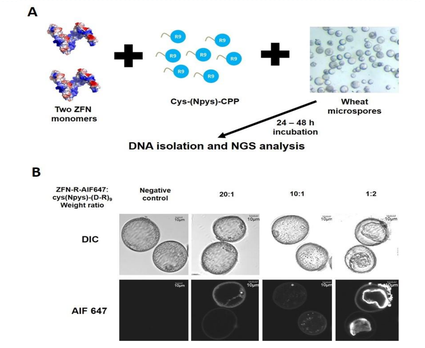

Figure 2. Transfection of wheat microspores with ZFN monomers using CPPs. A – experimental design for transfection of either wheat microspores or haploid embryos with ZFN monomers combined with CPPs. B – transfection of wheat microspores with ZFN-R monomer labeled with Alexa Fluor 647 using cys(Npys)-(D-R)$_9$ CPP and visualization using direct or fluorescence microscopy.

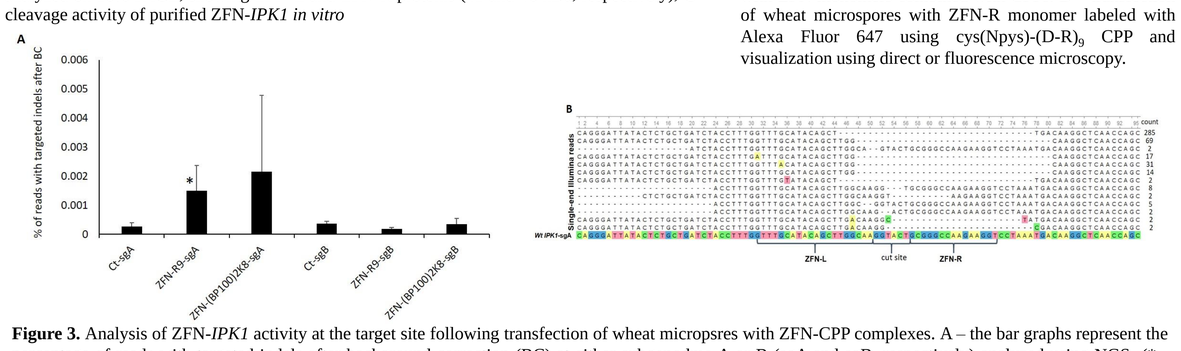

Figure 3. Analysis of ZFN-*IPK1* activity at the target site following transfection of wheat micropsres with ZFN-CPP complexes. A – the bar graphs represent the percentage of reads with targeted indels after background correction (BC) at either subgenodme A or B (sgA and sgB, respectively) analyzed using NGS (* - Student's t-test, p<0.1); B – representative indels at the subgenome A target site for both CPPs used. Ct – no CPP-ZFN transfection control. All treatments were done in two biological replicates.

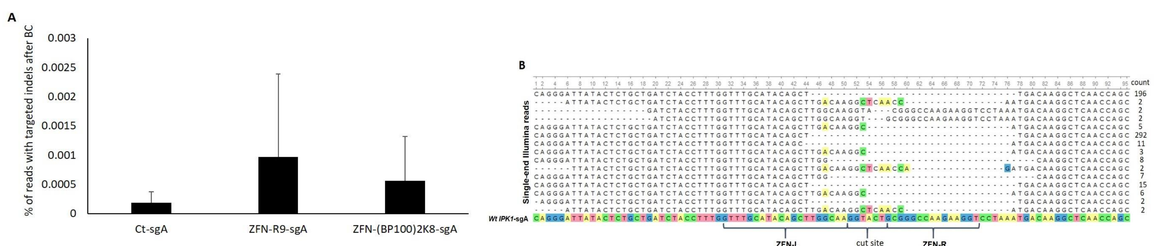

Figure 4. Analysis of edits at ZFN-*IPK1* target site in ELS regenerated from transfected microspores. A – the bar graphs represent the percentage of reads with targeted indels after background correction (BC) at subgenome A (sgA) analyzed using NGS; B – representative indels at the *IPK1*-sgA site in ELS regenerated from microspores transfected with ZFN-cys-cys-CPP. Ct – no CPP-ZFN transfection control. All treatments were done in three biological replicates.

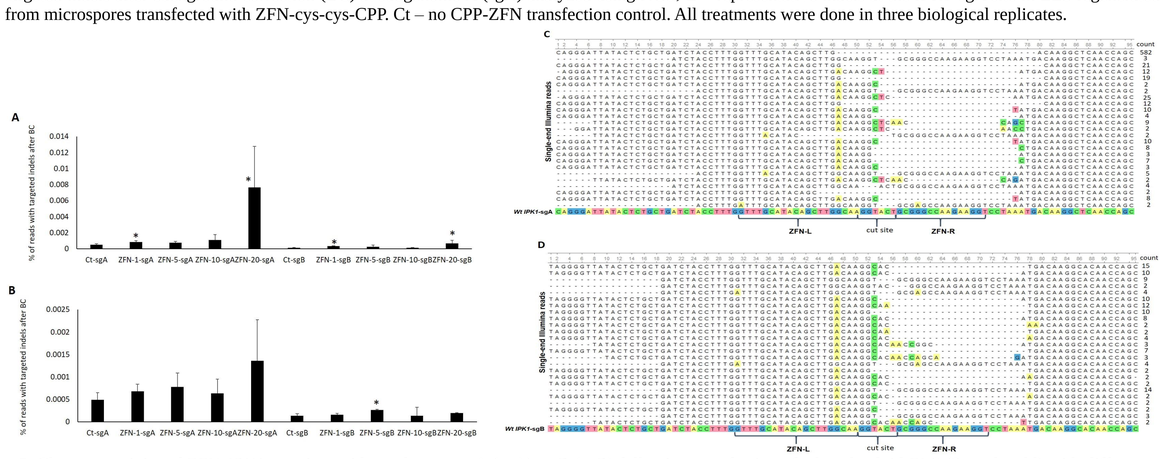

Figure 5. Cleavage activity of ZFN-*IPK1* protein at either subgenome A (sgA) or B (sgB) following transfection of wheat haploid ELS (3 weeks old) with different CPP-ZFN complexes. A and B – the bar graphs represent the percentage of reads with targeted indels after background correction (BC) at subgenomes A and B as analyzed using NGS for either cys(Npys)-(D-R)$_9$ (A) or cys(Npys)-(BP100)$_2$K$_8$ (B) CPP, respectively. Experiment was done in 2 biological and 3 technical reps, the bars marked with asteriks indicate statistically significant difference as compared to corresponding controls (Student's t-test, p<0.1). C and D - representative types of edits detected using NGS at either subgenome A or B for both CPPs, respectively.

Conclusions

- We present an approach of nucleic acid-free genome editing directly in isolated predecessors of plant gametes by delivery of purified ZFN proteins using CPPs.
- NGS analysis of microspores and haploid embryos transfected with CPP-ZFN complex demonstrated enrichment of edits at the target site as compared to non-treated control reads.
- Further optimization of the technology includes testing CPPs with different properties for their ability to deliver DE in the form of proteins into plant cells and including epigenetic drugs (e.g., zebularine) to potentially decrease inhibitory effect of DNA methylation at the endogenous target loci.

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