Wheat cell suspensions as tool for CRISPR/Cas9 constructs evaluation

Krzysztof Michalski[1*], Anna M. Linkiewicz[1,2]

[1]GMO Controlling Laboratory, Plant Biotechnology and Cytogenetics Department, Plant Breeding and Acclimatization Institute—National Research Institute, Radzikow, 05-870 Blonie, Poland;

[2]Institute of Biological Sciences, Faculty of Biology and Environmental Sciences, Cardinal Stefan Wyszynski University in Warsaw, Woycickiego 1/3 Street, 01-938 Warsaw, Poland

*krzysztof.michalski@ihar.edu.pl

INTRODUCTION

Utilization of site-specific nucleases (CRISPR/Cas, TALEN, ZNF, etc.) for precise plant genome modifications has become a hot topic within last few years. Despite huge optimization effort the method still shows one major drawback, namely the necessity to test activity of every nuclease construct before using it *in planta.* Several strategies of genetic evaluation were proposed, based mainly on *in vitro* approaches, where transfection of seedling-derived protoplast using PEG-mediated delivery of plasmid became the most popular. However, we have found out that in case of triticale (haxaploid species) the results from PEG-mediated protoplast transfection as a method for CRISPR/Cas9 construct did not corelated with *in planta* findings when we compared transformation results of polyploid cereal species, like triticale. Therefore, we developed a system based on Agrobacterium-mediated transformation of wheat cell suspension cultures which was used for evaluation of CRISPR/Cas9 construct designed for ABA 8'-hydroxylase 1 gene editing. The efficiency was verified by Sanger sequencing and bioinformatic analysis. We discuss advantages and possible future developments of this method as more consistent with in planta transformation techniques.

MATERIALS & METHODS

Non-embryogenic and fast growing friable calli derived from wheat anther cultures cv. Svilena were cultured on solid 190-2 [1] medium and subsequently used for establishment of cell suspension cultures. To initiate the suspension, 1-2g of actively growing callus was transferred to a glass flask containing 30 ml of liquid 190-2 medium and placed on rotary shaker (120 rpm). Every week ¾ of suspension volume was being replaced by fresh medium till stable growth was achieved.

Two gRNA/Cas9 constructs targeting first two exons of *ABA 8'-HYDROXYLASE 1* gene, ABA/1/364 and ABA/2/323 respectively, enhanced with *Three prime repair exonuclease 2* (*TREX2*), were used for stable transformation of wheat cell suspensions using *Agrobacterium tumefaciens* AGL1 strain. Both constructs were previously tested in triticale protoplasts, where they produced desired on-target mutations with the same efficiency on respective sub-genomes [2]. *Agrobacterium* inoculation and co-cultivation was based on modified protocol described by Kumlehn et al. (2006) [3]. 48h after inoculation suspensions were washed with liquid medium supplemented with antibiotics and evaluated under a fluorescent stereo microscope for transient GFP expression. Suspensions with high GFP expression, indicating efficient T-DNA transfer, were placed on a stack of sterile filter papers to remove liquid medium and finally, transferred to petri dishes (90mm) with higromicine-supplemented solid medium. Readily growing calli lines on primary selection medium were transferred to a fresh medium for proliferation.

TRIzol method was used for simultaneous extraction of genomic DNA and total RNA from selected calli. Transgenic character of calli was confirmed by Reverse Transcriptase PCR. Finally, genomic DNA of transgenic calli was amplified using genome-specific primers [2] and Sanger sequenced. Indel frequency was quantified with TIDE software [4].

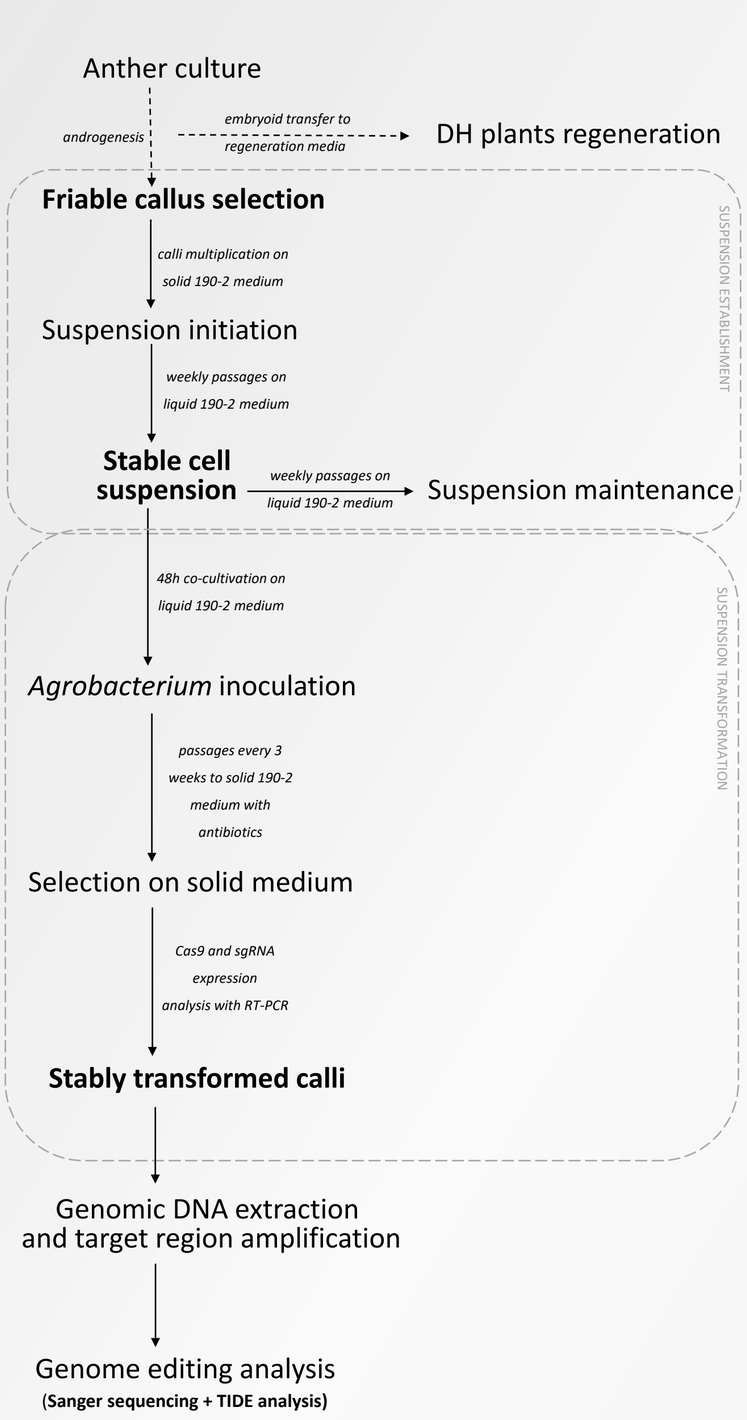

Figure 1. Main steps of the wheat suspension culture establishment and *Agrobacterium* transformation protocol evaluation of gRNA/ Cas9 constructs.

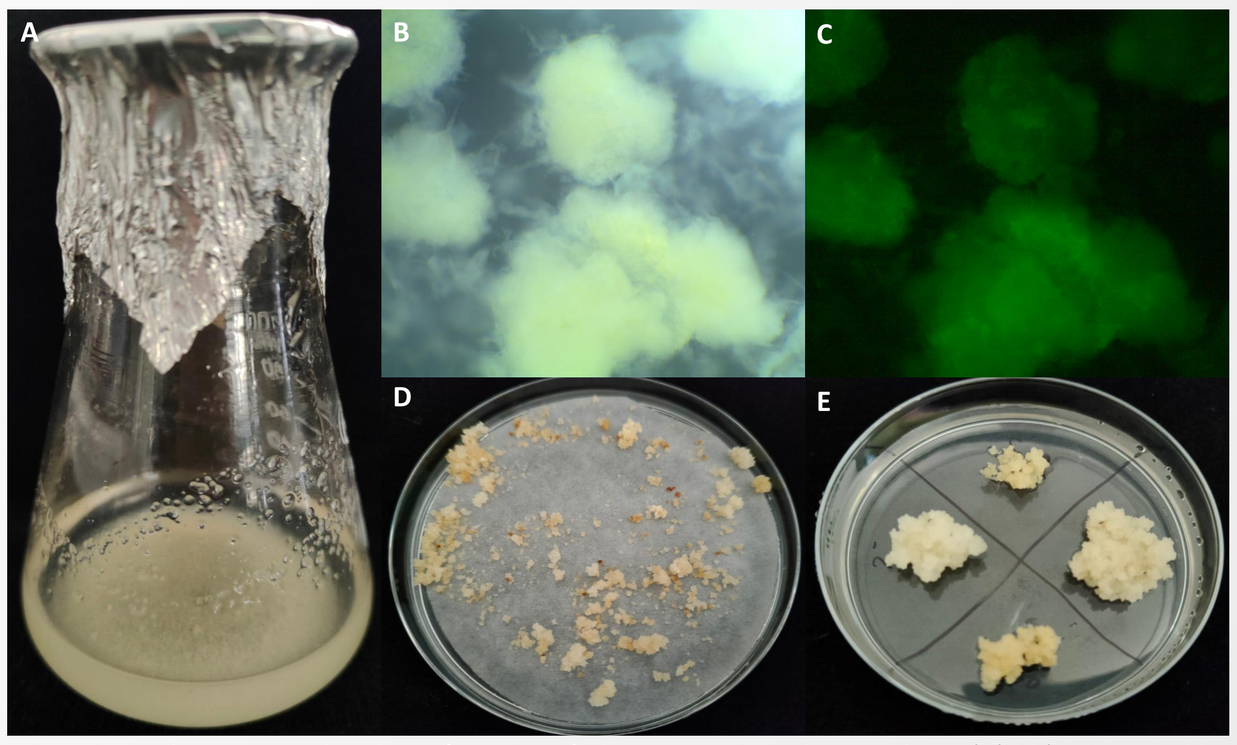

Figure 2. *Agrobacterium*-mediated transformation of wheat suspension cultures with ABA/1/364/Cas9: **A** – stable wheat cell suspension after 3 months of weekly passages in liquid 190-2 medium, **B**, **C** – cell suspension aggregates (magnif. 32x) after 48h co-cultivation with *Agrobacterium* in brightfield and GFP fluorescence, respectively, **D** – suspension-derived calli 6 weeks after transfer onto solid 190-2 medium supplemented with higromicine, **E** – variations in morphology and growth rate among Hpt$^+$ resistant single aggregate-derived lines of calli.

gRNA	line	Genome A	Genome B	Genome D
gRNA-ABA/1/364	2D1	65,7%	61,8%	27,7%
	2D2	85,1%	75,4%	86,8%
	2D3	77,5%	ND	97,7%
gRNA-ABA/2/323	3D1	76,4%	ND	63,5%
	3D2	97,4%	ND	19,4%
	3D6	91,3%	ND	32,7%

Table 1. Two gRNA/Cas9 constructs targeting *ABA 8'-HYDROXYLASE 1* gene were used. Indel induction rates for respective wheat A, B, D sub-genomes in transgenic callus lines shown in percent of modified amplicons, based on Sanger sequencing and TIDE analysis. ND – non detected.

RESULTS & DISCUSSION

A useful and efficient system of gene editing and gRNA/Cas9 constructs evaluation is crucial for gene functional studies as well as genetic engineering. Site directed mutagenesis that proved to be efficient in model plants remains problematic in wheat due to its polyploidy and low *in vitro* transformation-regeneration response. The efficacy of gRNA/Cas9 constructs must be verified to estimate the effectiveness of plant transformation experiment. We show that wheat suspension cultures can be established within 3 months (Fig. 2A). The transformation and selection takes 6-8 weeks and routinely provides 10-30 transgenic aggregates per 1 ml of inoculated suspension (Fig. 2B-E). Cell suspensions remain stable for 3 years. It can be easily multiplied and is always ready for transformation with selected gRNA/Cas9 constructs. We were able to detect mutations in *ABA 8'-HYDROXYLASE 1* gene induced by gRNA-ABA/1/364 and gRNA-ABA/2/323 constructs on each of respective wheat sub-genomes (A, B, D), except in genome B for gRNA-ABA/2/323. Mutation rates ranged from almost 20% to 98%. With gRNA/Cas9 delivery via *Agrobacterium* we observed considerable differences in nuclease efficiency toward distinct sub-genomes. No such differences in indel frequency were detected when these constructs were delivered into triticale protoplast via PEG-mediated transfection [2].

REFERENCES

1. Pauk J, Mihály R, Puolimatka M. 2003. Protocol of wheat (Triticum aestivum L.) anther culture. in: Maluszynski M, Kasha KJ, Forster BP and Szarejko I (eds), Doubled Haploid Production in Crop Plants, a manual, 59-64. Kluwer Academic Publishers, Dordrecht, The Netherlands
2. Michalski K, Hertig C, Mańkowski DR, Kumlehn J, Zimny J, Linkiewicz AM. Functional Validation of cas9/GuideRNA Constructs for Site-Directed Mutagenesis of Triticale ABA8'OH1 loci. International Journal of Molecular Sciences. 2021; 22(13):7038. https://doi.org/10.3390/ijms22137038
3. Kumlehn J, Serazetdinova L, Hensel G, Becker D, Loerz H. Genetic transformation of barley (Hordeum vulgare L.) via infection of androgenetic pollen cultures with Agrobacterium tumefaciens. Plant Biotechnol J. 2006 Mar;4(2):251-61. doi: 10.1111/j.1467-7652.2005.00178.x. PMID: 17177801.
4. Eva K. Brinkman, Tao Chen, Mario Amendola, Bas van Steensel, Easy quantitative assessment of genome editing by sequence trace decomposition, Nucleic Acids Research, Volume 42, Issue 22, 16 December 2014, Page e168, https://doi.org/10.1093/nar/gku936