DOI 10.18699/SBB-2022-353

Development of CRISPR/Cas9-based genome editing constructs for Nevsky and Udacha potato cultivars

Larichev K.[1, 2]*, Sergeeva E.[1, 2], Karetnikov D.[1, 2], Salina E.[1, 2], Kochetov A.[1, 2]

[1] *Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia*

[2] *Kurchatov Genomic Center of the Institute of Cytology and Genetics, SB RAS, Novosibirsk, Russia*

** k.larichev@g.nsu.ru*

Key words: CRISPR/Cas9, guide RNA design, potato, U6 promoter

Motivation and Aim: Potato (*Solanum tuberosum*) is a staple food crop that has a significant impact on the world's food supply, warranting the continuous development of potato's breeding techniques. Apart from being autotetraploid and heterozygous, there is a large degree of heterogeneity between various cultivars of potato. Because of this, utilization of conventional breeding techniques is very challenging, which complicates development of potato cultivars with desirable properties. CRISPR-based methods of genome editing are able to address these issues by allowing breeders to introduce new traits without disrupting the existing genetic background. As a result, currently CRISPR\Cas9 editing is the most promising approach to developing new commercially viable potato varieties. However, high heterogeneity and heterozygosity of potato still complicate the use of CRISPR based methods of genome editing, as they rely on having the exact sequence of target region, and, in case of potato, publicly available reference sequences may differ significantly from the sequence of a particular cultivar.

In this work, we have focused on designing guide RNAs (gRNAs) to induce knock-out mutations in granule-bound starch synthase (GBSS) gene of Russian potato cultivars Nevsky and Udacha. We have also aimed at constructing plasmid vectors for efficient gRNA expression in potatoes by using cultivar-specific U6 snRNA promoters. The GBSS gene was chosen as it is responsible for amylose content in starch, and it's knock-out is known to result in amylose-free starch phenotype [1–3]. This amylose-free starch is better suited for certain industrial applications than the wild-type starch.

Methods and Algorithms: Genomes of cultivars Nevsky and Udacha were sequenced on the Illumina MiSeq platform. Sequences of GBSS gene from Nevsky and Udacha were derived from genome assemblies using BLASTn algorithm and a GenBank GBSS sequence X58453 as a reference. In order to find gRNA target regions in GBSS (13 exons, 2948 bp length), which would be identical for all the alleles in both cultivars, we aligned Nevsky and Udacha GBSS sequences with reference GenBank sequences A23741 and X58453 using UGene (alignment algorithm ClustalΩ). Regions that were identical in all sequences were chosen as query sequences for gRNA design. gRNA design was performed in three programs: CRISPOR, CRISPRdirect and CRISPR-P 2.0. We only selected gRNAs which had the highest specificity according to all three programs. The secondary structure of selected gRNAs was analyzed in RNAfold. Only gRNAs with optimal secondary structure (mostly free 5' end, three characteristic stem loops) were chosen [4]. As a reference, we also used gRNA to first exon designed by Johansen et al [3].The lack of heterogeneity in sites, targeted by designed gRNAs was further confirmed using data from Illumina reads of PCR-amplified GBSS fragments form cultivars Nevsky and Udacha. gRNAs were either synthesized with GeneArt gRNA

Precision Synthesis Kit (Invitrogen), or by *in vitro* transcription from pUC57-sgRNA plasmid [5]. gRNA cleavage efficiency was tested *in vitro* and *in vivo*. For *in vitro* assay we used PCR-amplified fragments of GBSS gene containing the target site. Fragments were incubated with Cas9 protein and one of gRNAs. Cleavage efficiency was determined by gel-electrophoresis in agarose gel. For *in vivo* assay, protoplasts were transformed with either ribonucleoprotein complexes (RNP) of Cas9 and gRNA, or with pHDE-35S-Cas9-mCherry plasmid, encoding Cas9 protein and one of gRNAs. To determine efficiency of transformation, DNA of transformed protoplasts was extracted and used as a template for PCR-amplification of target regions. These PCR products were sequenced with either Sanger sequencing, or on the Illumina MiSeq platform. To drive more efficient gRNA expression from plasmid vectors in potatoes we used potato U6 snRNA promoters. U6 promoter sequences of cultivars Nevsky and Udacha were acquired from genome assemblies of these cultivars using BLASTn algorithm and GenBank potato U6 promoter reference sequences Z17290, Z17292, Z17293, Z17301. Identified promoter variants were generated by PCR-amplification and inserted into pAtU6-sgRNA plasmid instead of *Arabidopsis thaliana* U6 promoter by NEBuilder HiFi DNA Assembly (New England Biolabs), effectively making it into a pStU6-sgRNA plasmid. The entire gRNA expression cassettes (U6 promotor, gRNA, U6 terminator) were then inserted in pHDE-35S-Cas9-mCherry binary vector [6], suitable for both biolistic and *Agrobacterium*-mediated transformations.

Results: We designed five gRNAs with high predicted specificity: three for the first exon, one for the second and one for the tenth. *In vitro* efficiency was the highest for gRNAs 3, 4 and 5. None of our gRNAs performed intended cleavage *in vivo* in PEG-mediated RNP transformation of potato protoplasts. Investigation of plasmid-based transformation efficiency is still ongoing. We identified five distinct U6 promoter variants in both cultivars. Promoter sequences varied between cultivars. Each promoter was used to create new vectors – pStU6-sgRNA, suitable for gRNA expression in plants. Efficiency of identified promoters is currently being tested.

Conclusion: Using assembled genomes of cultivars Nevsky and Udacha we were able to design five gRNAs targeting GBSS gene with high predicted specificity, and to identify five distinct variants of U6 promoter in each cultivar. The latter were used to construct new expression vector – pStU6-sgRNA, which can be used to efficiently express gRNAs with a specific target sequence in potato plants. It is worth noting that currently there are no plasmid vectors containing potato U6 promoter available for purchase, which makes our vector especially valuable.

Acknowledgmens: The study is supported by the Kurchatov Genomic Center of the Institute of Cytology and Genetics, SB RAS (075-15-2019-1662).

References

1. Veillet F. et al. The Solanum tuberosum GBSSI gene: a target for assessing gene and base editing in tetraploid potato. *Plant Cell Rep*. 2019;38(9):1065-1080.
2. Andersson M. et al. Efficient targeted multiallelic mutagenesis in tetraploid potato (Solanum tuberosum) by transient CRISPR-Cas9 expression in protoplasts. *Plant Cell Rep*. 2017;36(1):117-128.
3. Johansen I.E. et al. High efficacy full allelic CRISPR/Cas9 gene editing in tetraploid potato. *Sci Rep*. 2019;9(1):1-7.
4. Liang G. et al. Selection of highly efficient sgRNAs for CRISPR/Cas9-based plant genome editing. *Sci Rep*. 2016;6(1):1–8.
5. Shen B. et al. Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nat Methods*. 2014;11(4):399-402.
6. Gao X. et al. *Plant Physiol*. 2016;171(3):1794-1800.