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Targeted genome modification in protoplasts of a highly regenerable Siberian barley cultivar using RNA-guided Cas9 endonuclease

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The modification of crop genomes employing functional components of the microbial CRISPR/Cas immune system is a rapidly developing area of applied research. Site-directed plant genome modification by this technology involves the construction of Cas endonuclease- and guide-RNA-encoding vectors, delivery of the plasmid DNA into plant cells, processing of the chosen genomic target site by the corresponding gene products and regeneration of plants from modified cells. The utilization of this technology in local breeding programs is mainly limited by the typically strong genotype dependence of gene transfer and *in vitro* regeneration procedures, which holds particularly true in cereals. In the present study, an evaluation of *in vitro* regeneration efficiency of immature embryos of ten Siberian barley cultivars revealed that only one of these is on a par with the experimental standard cultivar Golden Promise. This cultivar, namely cv. Aley, was consequently chosen for further experiments on site-directed mutagenesis in leaf mesophyll protoplasts. Two genes controlling hulled *vs* naked (*Nud*) and two-rowed *vs* six-rowed barley (*Vrs1*) were used as targets to be modified via polyethyleneglycol-mediated cellular uptake of guide-RNA/Cas9-encoding plasmid DNA. Deep-sequencing of amplicons obtained from protoplast genomic DNA revealed that 6 to 22 percent of the target sites were mutated. The detected modifications comprised deletions in all three target sites and of various sizes, whereas insertions were observed in only one of the target genes (*Vrs1*) and were confined to the size of 1 nucleotide. This study demonstrates the possibility of site-directed genome modification in Siberian barley. Further steps in technology advancement will require the development of protocols with reduced genotype dependence in terms of both the gene transfer to totipotent cells and the subsequent plant regeneration originating from such cells.

Key words: CRISPR/Cas; *Cas9*; barley; protoplasts; *Nud*; *Vrs1*; regeneration; transfection; site-directed mutagenesis; *in vitro* culture.

Применение РНК-направленной нуклеазы Cas9 для сайт-специфической модификации генома в протопластах сибирского сорта ячменя с высокой способностью к регенерации

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Модификация геномов культурных растений при помощи компонентов бактериальной защитной системы CRISPR/Cas в настоящее время является быстроразвивающейся областью прикладной науки. Методика индукции сайт-специфических изменений в растительных геномах, как правило, включает такие этапы, как конструирование генетических векторов, содержащих гены нуклеазы Cas9 и химерной направляющей РНК, доставку плазмидной ДНК или рибонуклеопротеиновых частиц в клетки растений, что приводит к внесению изменений в выбранный сайт геномной ДНК, и последующую регенерацию растений из модифицированных клеток. Применение этой технологии в селекции ограничено тем, что генотипы в разной степени подвержены генетической трансформации и различаются по способности к регенерации *in vitro*. Генотип-зависимость эффективности биотехнологических манипуляций особенно ярко выражена у культурных зерновых злаков. В настоящей работе была проведена оценка эффективности регенерации растений *in vitro* из клеток незрелых зародышей десяти сибирских сортов ячменя. Было показано, что только один из исследуемых сортов сопоставим с модельным для биотехнологических и генно-инженерных работ сортом Golden Promise. Сорт Алей продемонстрировал самую высокую эффективность регенерации среди сибирских сортов ячменя и был выбран для проведения эксперимента по модификации генома в протопластах мезофилла листа. Для проведения модификации генома было выбрано два целевых гена, которые контролируют хозяйственные признаки. Ген *Nud* контролирует признак голозерности или пленчатости, ген *Vrs1* – признак двурядности или шестирядности. Были сконструированы генетические век-

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торы, несущие систему модификации генома, направленную на три сайта в двух целевых генах. Конструкции были введены в протопласты методом полиэтиленгликоль-зависимой трансформации, детекция мутаций осуществлялась методом глубокого секвенирования целевых последовательностей, амплифицированных с геномной ДНК трансформированной клеточной популяции. Мутации были выявлены в 6–20 % популяции трансформированных клеток. Делеции разного размера обнаружены в трех целевых сайтах, однонуклеотидные инсерции обнаружены только в одном из сайтов. Результаты, полученные в работе, демонстрируют возможность сайт-специфической модификации генома сибирского ячменя. Дальнейшие шаги по развитию технологии сайт-направленной модификации геномов культурных злаков потребуют разработки «генотип-независимых» методов генетической трансформации клеток и последующей регенерации растений из модифицированных клеток.

Ключевые слова: CRISPR/Cas; *Cas9*; ячмень; протопласты; *Nud*; *Vrs1*; регенерация; трансфекция; сайт-направленный мутагенез; культивирование *in vitro*.

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Targeted genome modification is a modern, particularly versatile tool to genetically engineer plants (amongst other organisms) (Gerasimova et al., 2017). There are a lot of reports of successful crop improvement using different applications of RNA-guided endonucleases (RGEN) which is also known as Cas endonuclease technology or CRISPR/Cas technology (Korotkova et al., 2017). Most of the published studies on barley targeted genome modification were performed on the model cultivar Golden Promise (Holme et al., 2017; Kapusi et al., 2017; Holubova et al., 2018; Kumar et al., 2018). To take full advantage of this technology for agricultural production, one needs to establish protocols for elite germplasm. In cereals however, the feasibility of genetic engineering is highly dependent on the genotype used (Kumlehn, Hensel, 2009). In the West Siberia region of the Russian Federation, barley occupies an area of three million hectares. The introduction of Cas endonuclease technology for breeding of Siberian varieties is a challenge, because effective protocols for *in vitro* regeneration, transformation and genome modifications of elite lines are yet to be established. The present study aims to select candidate barley cultivars for targeted genome modification and to demonstrate the modification of valuable trait-controlling genes in the genome of Siberian barley using Cas endonuclease technology. As first criterion for cultivar selection, recommendations of local breeders were considered. Next, a representative panel of pre-selected local cultivars was tested for efficient *in vitro* regeneration from immature embryos, which constitute the explant of choice for barley genetic engineering procedures. One cultivar from the Russian spring barley collection was eventually selected to conduct a genome modification experiment using mesophyll protoplasts. As targets, two previously known barley domestication genes controlling valuable traits in monogenic fashion were selected. The *Nud* gene controls hulled *vs*. naked barley (Taketa et al., 2008) and the *Vrs1* gene controls two-rowed *vs*. six-rowed barley (Pourkheirandish et al., 2015).

Material and methods

Plant material. The ten following West Siberian barley cultivars have been selected from the Russian spring barley collection according to the advice of local barley breeders: Biom, Talan, Vorsinskiy2, Aley, Acha, Signal, L-421, Kolchan, V-1, Krasnoyarskiy91. The model cultivar Golden Promise was used as a control with a high *in vitro* regeneration potential. Plants were grown under greenhouse conditions until milk or wax ripening stage of the caryopses.

Evaluation of *in vitro* regeneration efficiency. Milk or wax ripening stage spikes (stage 75–83 of BBCH scale) were harvested; grains were isolated from spikes and sterilized by 96 % ethanol for thirty seconds and bleach-surfactant solution (1:1 water dilution of Domestos, Unilever) for four minutes with subsequent washing in sterile water. Analysis was performed separately for five plants of each cultivar. For analysis, one-three spikes were taken from each plant, 10 to 13 immature embryos per plant were isolated. As a control the model barley cultivar Golden Promise was used.

After dissection and removal of the embryonic axis, embryos were plated scutellum side up on callus induction medium (4.3 g/l Murashige & Skoog plant salt base, 30 g/l maltose·H_2O, 1.0 g/l casein hydrolysate, $CuSO_4 \cdot 5H_2O$ 160 mg/l, 350 mg/l myo-inositol, 690 mg/l proline, 1.0 mg/l thiamine·HCl, 2.5 mg/l Dicamba, 3.5 g/l Phytagel, pH adjusted to 5.8 with NaOH) and incubated at 22 °C in the dark (Harwood et al., 2009). After four weeks of cultivation on callus induction medium, calli were transferred to transition medium (2.7 g/l Murashige & Skoog modified plant salt base [without NH_4NO_3], 20 g/l maltose·H_2O, $CuSO_4 \cdot 5H_2O$ 160 mg/l, 165 mg/l NH_4NO_3, 750 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine·HCl, 2.5 mg/l 2,4-dichlorophenoxy acetic acid, 0.1 mg/l 6-benzylaminopurine, 3.5 g/l Phytagel, pH adjusted to 5.8) and were cultivated for another four weeks at 22 °C under low light. Then, calli were transferred to regeneration medium (2.7 g/l Murashige & Skoog modified plant salt base [without NH_4NO_3], 20 g/l maltose·H_2O, 165 mg/l NH_4NO_3, 750 mg/l

glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine·HCl, 3.5 g/l Phytagel, pH 5.8) at 22 °C under full light (16/8 hours day/night). The number of explants giving rise to regenerated plants was counted after four weeks of growing at regeneration medium. The *in vitro* regeneration efficiency was calculated as percent of explants producing regenerating plants. The Mann–Whitney U test was performed for each Siberian cultivar for pairwise comparison to the control Golden Promise with regards to *in vitro* regeneration efficiency.

Target gene sequencing, guide-RNA design and vector construction. Genomic DNA was isolated from leaves of barley cv. Aley. A two cm-long piece of leaf was cut, transferred to the tube with 200 µl of warm (60 °C) buffer (100 mM Tris-HCl pH 7.5–8.0, 500 mM NaCl, 50 mM EDTA, 1.25 % SDS, 0.38 % $Na_2S_2O_5$) and ground in a homogenizer. Then, 500 µl of buffer was added and incubated at 60 °C for 30 minutes with subsequent addition of 700 µl of chloroform-isoamyl alcohol mixture (24:1) and centrifugation at 12000 g for 25 minutes. The upper fraction was collected and mixed with 1.4 ml of 96 % ethanol. DNA was precipitated by centrifugation again at 12000 g for 25 minutes, washed with 70 % ethanol and dissolved in TE buffer. Fragments corresponding to first exons of *Nud* (HORVU7Hr1G089930) and *Vrs1* (HORVU2Hr1G092290) barley genes were amplified from genomic DNA using the primers Hv_Nud_1exF and Hv_Nud_1exR for the *Nud* gene and Hv_Vrs1_F1 and Hv_Vrs1_R1 for the *Vrs1* gene (Suppl. Table)[1]. PCR fragments were subjected to Sanger sequencing. To confirm the identity of target sites in the Aley genome to known reference sequences (morex_contig_43456 CAJW010043456 carma=7HL, morex_contig_135757 CAJW010135757 carma=2HL, barke_contig_396030 CAJV010295100 carma=7HL, barke_contig_69176 CAJV010052191, bowman_contig_850766 CAJX010845871 carma=7HL, bowman_contig_16990 CAJX010016287), a pairwise alignment of sequenced *Nud* and *Vrs1* first exons was performed.

Target site selection was performed using the online tools DESKGEN (https://www.deskgen.com/landing/; Doench et al., 2016) and WuCRISPR (Wong et al., 2015). Guide-RNA secondary structures were modelled using the RNAfold tool (Gruber et al., 2008).

The generic vector pSH121 harboring maize codon-optimized Cas9 under control of *Zea mays Ubiquitin-1* promoter and a guide-RNA scaffold preceded by the rice *U3* promoter was used as a backbone for final RGEN vector construction. To this end, pSH121 had been generated from pSH91 (Budhagatapalli et al., 2016) using a Gibson assembly approach (Gibson et al., 2009) in order to remove the necessity for a Guanine as starting nucleotide of the gRNA. The DNA molecules for the Gibson assembly were generated as followed: pSH91 was digested by *Hind*III restriction enzyme; DNA fragments were separated in agarose gel and the 10166 bp fragment containing the Cas9 expression cassette was isolated from the gel. Two additional DNA molecules with overhangs each compatible to the end of another DNA molecule used, were created in separate PCRs using pSH91 as template and either the oligonucleotide pair OsU3-1_GibF- OsU3-1_GibR or OsU3-1_GibF- OsU3-1_GibR (see Suppl. Table). The resulting 487 and 1633 bp amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific). Gibson assembly of the three described DNA molecules was performed using the NEBuilder HiFi DNA Assembly Master Mix (NEB) according to the manufacturers instructions.

The target-specific ca. 20 bp region of the gRNA can be inserted into pSH121 vector between two *Bsa*I restriction sites as a double-stranded oligonucleotide with 5′-overhang tails. Double-stranded oligos were created by melting and reannealing the following pairs of oligonucleotides: Nud_ex1-45-F and Nud_ex1-45-R, Nud_ex1-50-F and Nud_ex1-50-R, Vrs1_ex1-33-F and Vrs1_ex1-33-R (see Suppl. Table). pSH121 was digested by *Bsa*I restriction enzyme; DNA fragments were separated in agarose gel. The 10972 bp fragment was eluted from a gel and used in three ligation reactions with double-stranded oligonucleotides. As a result of ligation, three RGEN vectors VRS1-33RGEN, NUD-45RGEN and NUD-50RGEN were assembled. The accuracy of construct assembly was confirmed by Sanger sequencing.

Protoplast isolation and transformation. Grains were germinated in the dark at 23 °C. Leaf samples from seven days-old etiolated seedlings were used for protoplast isolation. Protoplasts were isolated according to Q. Shan et al. (2014). Protoplast sample quality and cell population density were estimated in a counter chamber slide using a microscope. A total amount of 20 µg plasmid DNA consisting of gRNA/Cas9 transformation vector and GFP expression vector pYF133 (Fang et al., 2002) was taken to transform 5×10^5 protoplasts in 220 µl volume. PEG-mediated transfection was performed according to Q. Shan et al. (2014). After two days of incubation at 21 °C, the transformed protoplasts were observed using an inverted fluorescence microscope (Zeiss Axiovert 200M, filter set 13 with excitation wavelength BP 470/20 and emission wavelength BP 505–530). The proportion of GFP-positive cells was calculated per counting field of the chamber slide to provide a metric for transformation efficiency.

DNA isolation, library preparation, deep amplicon sequencing analysis. Genomic DNA was isolated from protoplast samples by the method described by W. Wang et al. (2016). For each gene, specific primers were designed to amplify target loci from protoplast genomic DNA. Target fragments were amplified using primer pairs Hv_Nud_F4-Hv_Nud_1exR and Hv_Vrs1_1exF- Hv_Vrs1_1exR for *Nud* and *Vrs1* genes, respectively (see Suppl. Table). Amplicon barcoding and library preparation was performed according to recommendations of the service provider for amplicon sequencing (https://en.novogene.com/next-generation-sequencing-services/microbial-genome/amplicon-sequencing/). Sequencing was performed using the Illumina HiSeq platform generating paired-end 250 bp reads. After filtration, the sequence depth ranged from 6000 to 8000 reads per amplicon sample. The indel frequency and patterns induced by VRS1-33RGEN, NUD-45RGEN and NUD-50RGEN vectors were analyzed. The threshold for mutation detection was adjusted to the level of one percent. Indel frequency was normalized to the control GFP vector co-transformation efficiency.

Results

Cultivar selection. The regeneration test was performed in order to select the candidate cultivars for further establishment

[1] Supplemantary Materials are available in the online version of the paper: http://www.bionet.nsc.ru/vogis/download/pict-2018-22/appx16.pdf

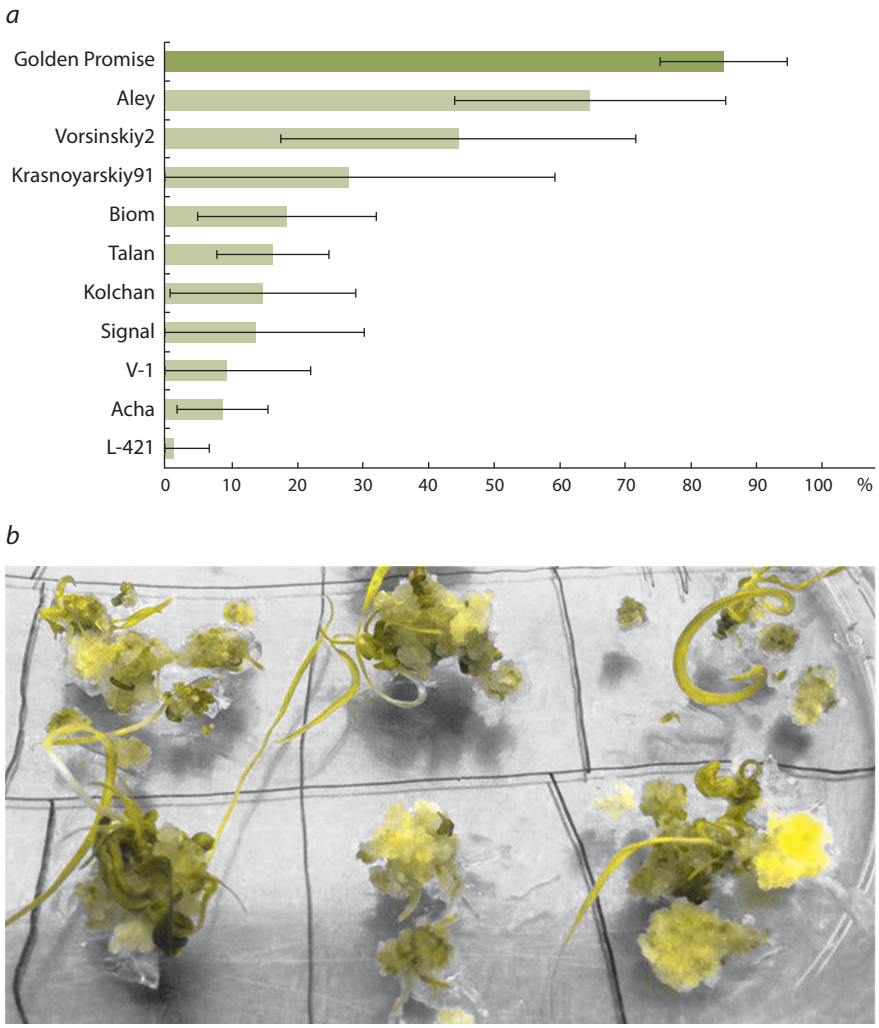

Fig. 1. *In vitro* regeneration from immature embryos of 10 Siberian barley cultivars in comparison to the model cultivar Golden Promise.

a – numbers represent percent of explants producing regenerated plants. Horizontal bars represent standard deviation; *b* – plantlet regeneration from callus of the Siberian barley cultivar Aley.

of targeted genome modification protocols. A standard protocol for *Agrobacterium*-mediated barley transformation using immature embryos (Harwood et al., 2009) was adopted and control regeneration tests with neither *Agrobacterium* inoculation nor use of selective agents were performed. Fig. 1 (*a*) shows the percentage of explants which gave rise to regenerated plants. Nine out of ten Siberian barley cultivars showed significantly ($p < 0.05$) lower regeneration efficiency than the model cultivar Golden Promise, whereas only one cultivar from the experimental group did not differ significantly ($p > 0.05$) from this control. Aley evidently performed best across all experimental repetitions showing an overall efficiency over 60 % (see Fig. 1). Consequently, this cultivar was chosen for the following genome modification experiment.

Target site selection and vector construction. The barley genes *Nud* and *Vrs1* were selected for targeted mutagenesis. To reveal the sequence of these genes in the Siberian cultivar Aley, specific primers were designed based on the barley reference sequence and a fragment containing the first exon of the respective gene was amplified and sequenced. Alignment to *Nud* and *Vrs1* sequences available in databases showed high identity of the Aley gene variants to the reference (Suppl. Figure). Target sites were selected in the first exon of each gene, two sites for *Nud* and one for *Vrs1*. The generic vector pSH121 (Fig. 2, *a*) was used to generate the three target-specific vectors used for transformation experiments (Table 1).

Evaluation of genome modifications in protoplasts. Protoplasts were isolated from seedlings of barley cv. Aley and co-transformed with two vectors, more specifically, any one of the RGEN vectors and the control vector pYF133 harboring a GFP expression cassette (see Fig. 2, *a* and *b*). Anticipating that the transformation efficiency of the co-transformed GFP plasmid is equal to that of the gRNA/Cas9 vector, the proportion of green fluorescing cells was used to normalize the cleavage activity of the respective guide-RNA tested (Lin et al., 2018). Genomic DNA was isolated from protoplasts and used as a template for the amplification of target sequences. Mutations were detected in each target site and occurred with different frequency (Table 2, Fig. 2, *c*). The Nud-45 guide-RNA showed the highest efficiency in this experiment (22 %). There were only few mutation types detected for each target site (see Fig. 2, *c*). Three different deletion sizes were detected for the Nud-45 target site (–1, –2 and –9 nucleotides), two deletion sizes for the Nud-50 target site (–5 and –6 nt) as well as one deletion and one insertion size for the Vrs1-33 target site (–3 and +1 nt).

Discussion

Little is known about the mechanisms which are causative for the high genotype dependence of genetic engineering. Most available reports of targeted barley genome modifications describe mutations in the Golden Promise genome. We aimed to demonstrate successful targeted genome modifications in cells of a non-model elite Siberian barley cultivar. The protoplast system is a well established tool for assessing the efficiency of targeted genome modifications in plants. The evaluation of RGEN cleavage efficacy *in vivo* allows one to select the best variants of target site/guide RNA pairs (Budhagatapalli et al., 2016).

We selected two target genes controlling domestication traits in barley. The *Nud* and *Vrs1* genes control hulled *vs.* naked and two rowed *vs.* six rowed barley variants, respectively. The selected cv. Aley features a hulled and two-rowed phenotype and its genome contains wild-type alleles of both genes. Two sites in the *Nud* and one site in the *Vrs1* coding sequence were targeted.

Table 1. Target sites, target-specific parts of guide-RNAs and transformation vectors
(PAM, protospacer-adjacent motif; CDS, coding sequence)

Gene	Target site with PAM (underlined)	Target position in the CDS	Target-specific part of guide-RNA	Final vector
Nud	GGAGACCCAGGAGCCCCAGTGG	45–64, exon 1	GGAGACCCAGGAGCCCCAG	NUD-45RGEN
Nud	GCTCCTGGGTCTCCGAGATCAGG	50–69, exon 1	GCTCCTGGGTCTCCGAGATC	NUD-50RGEN
Vrs1	GTGGACACGACTTTCTTCGCGG	33–52, exon 1	GTGGACACGACTTTCTTCG	VRS1-33RGEN

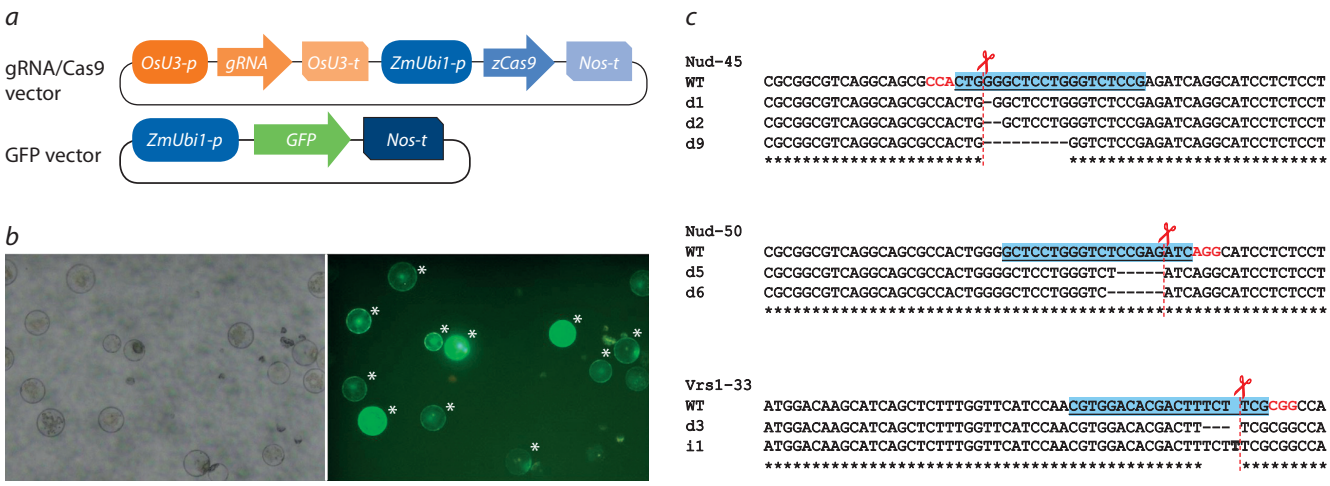

Fig. 2. Protoplast transformation and detection of site-directed mutations.

a – vector architectures used for protoplast transformation; gRNA/Cas9 vector corresponds to pSH121; GFP vector – to pYF133; *OsU3-p* – rice *U3* promoter; *gRNA* – chimeric guide RNA; Os*U3-t* – rice *U3* terminator element; *ZmUbi1-p* – maize *Ubiquitin-1* promoter; *zCas9* – maize codon-optimized Cas9 endonuclease; *Nos-t* – 3′-signal of nopaline synthase gene; *b* – GFP detection in transformed protoplasts; bright field (left) and GFP filter (right). GFP-positive cells are marked with asterisk; *c* – mutation types obtained in Aley protoplasts for the three target sites (marked blue, PAM in red).

Table 2. Mutation detection in protoplasts with the given mutation frequencies being normalized to transformation efficiency

Guide-RNA	Total number of reads	Transformation efficiency	Mutation type*	Number of reads with mutation	Mutation frequency, %
Nud-45	6758	0.66	–G (–1)	691	15.5
			–GG (–2)	210	4.7
			–GGGCTCCTG (–9)	81	1.8
Nud-50	7821	0.46	–CTCGGA (–6)	158	4.4
			–CTCGG (–5)	112	3.1
Vrs1-33	6553	0.35	–TTC (–3)	74	3.2
			+T (+1)	66	2.9

* Number of inserted or deleted nucleotides is indicated in parenthesis.

Aley protoplast transformation efficiency was estimated from the proportion of GFP-positive cells which ranged from 0.35 to 0.66. This result is well comparable with previously published data (Bai et al., 2014; Lin et al., 2018). For all three selected target sites, mutated variants were obtained at frequencies of 6 to 22 % (normalized to transformation efficiency). There is no published data of targeted genome modification efficiency in barley protoplasts, but in comparison to other Poaceae family species, comparable mutation frequencies were obtained (Lin et al., 2018). However, the diversity of mutation types seen in the present study is comparatively low. There was no predominant type of mutations common for all target sites, i.e. mutation patterns were different for each particular case. We assume that the mutation type depends on target site properties; for example, the 9-nucleotides deletion of the Nud-45 site and the three nucleotides deletion for Vrs1-33 site might be due to sequence-specific microhomologies, and the insertion of T to Vrs1-33 site could be due to the reconstruction of a short CTTT repeat within the target site.

Our data show that RGEN-mediated genome modification is possible in cells of Siberian barley. To obtain plants of desirable genotypes with predefined genome modifications,

the genotype dependency of gene transfer mechanisms and *in vitro* regeneration has to be considerably reduced.

The number of genotypes hitherto engineered using Cas endonuclease technology is highest in rice (45 genotypes), which is followed by tomato (10), wheat (7), potato (4), oilseed rape (3), and further by switchgrass, soybean, maize, grapes and cucumbers with two genotypes per crop, and finally by barley, orange, grapefruit, apple, flax and cotton with only one genotype per crop (Korotkova et al., 2019). Further development of Cas endonuclease technology and its broad application potential for crop improvement requires involvement of more genotypes and varieties. Our results reveal a wide range of *in vitro* regeneration efficiency for a preselected group of 10 Siberian barley cultivars. Only one out of ten cultivars is comparable in regeneration efficiency with model cultivar Golden Promise. Aley is our current prime candidate cultivar for further establishment of stable genetic transformation. Identification of transformation amenability loci in the genome of Golden Promise and introduction of respective alleles into genomes of elite cultivars via marker-assisted selection has been suggested to create elite germplasm with increased transformation amenability (Hisano et al., 2017). Another approach may involve a transient overexpression of genes which enhance cellular totipotency. The maize *Baby boom* (*Bbm*) and *Wuschel2* (*Wus2*) genes were successfully used to stimulate transformation in maize and sorghum (Lowe et al., 2016, 2018). Further studies on the genetic control of barley transformation and regeneration ability and the utilization of known "transformation amenability" genes in genetic engineering experiments may facilitate the transition from fundamental research to practical applications.

Conclusion

Our study demonstrates the technical limitations of RGEN application for local cultivars. We showed that only a small portion of Siberian elite barley cultivars has useful *in vitro* regeneration ability. At the same time, we demonstrated at the cellular level that important genes of a local Siberian cultivar can readily be modified at predefined target motifs by Cas endonuclease technology. These results indicate that further work in the field of crop improvement has to be focused on developing efficient genetic engineering and *in vitro* regeneration systems and/or straightforward and universally useful methods for Cas9/gRNA delivery and targeted genome modification without the necessity of *in vitro* regeneration.

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Conflict of interest

The authors declare no conflict of interest.

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