Gene editing using the CRISPR/Cas9 system has become a routinely applied method in several plant species. The most convenient gene delivery system is *Agrobacterium*-mediated gene transfer with antibiotic selection and stable genomic integration of transgenes, including *Cas9*. For elimination of transgenes in the segregating progeny, selfing is applied in many plant species. This approach, however, cannot be widely employed in potato because most of the commercial potato cultivars are self-incompatible. Thus, the main goal of our project was the development of a transgene-free genome editing tool for potato based on *Agrobacterium*-mediated transformation and transient expression of *Cas9*. This goal was achieved via the experiments described below.

The efficiency of a transient *Cas9* expression system with positive/negative selection based on *codA-nptII* fusion was tested. The *PHYTOENE DESATURASE* (*PDS*) gene involved in carotenoid biosynthesis was targeted. A new vector, based on our earlier vector PROGMO carrying only the right border of T-DNA [Transgenic Res. 2006, 15:729-737], was constructed and designated PROGED::gPDS. Using only the positive selection function of PROGED::gPDS and the restriction enzyme site loss method in PCR of genomic DNA after digestion with the appropriate restriction enzyme, it was demonstrated that the new vector is as efficient in *Agrobacterium*-mediated gene editing as a traditional binary vector with right- and left-border sequences using either leaves or tubers for transformation. The frequency of mutation was notably high (47-88%). Two albino plants, indicating mutations in all four *PDS* alleles, with large deletions were obtained. One chimaeric plant characterised by white areas and a large deletion in the *PDS* region was also found. All other *PDS* mutant plants were green and did not differ from the non-transformed control in phenotype. In contrast to continuous positive selection, two weeks of positive selection followed by negative selection did not result in the isolation of *PDS* mutants.

It was shown that in *Arabidopsis thaliana*, the integration of T-DNA from *Agrobacterium* critically depends on polymerase theta (Polθ) [Nat. Plants 2016; 2:16164]. Thus, we isolated a fragment of the potato orthologue of *Polθ* by PCR and cloned it behind the *Rubisco* promoter in antisense orientation into PROGED::gPDS in front of 2x35S-Cas9. Transformation was carried out with the new construct with two weeks of positive selection followed by negative selection. However, no *PDS* mutants with lack of *Cas9* were obtained. It was also demonstrated that in the absence of DNA double-strand break repair pathways, integration of T-DNA into the *Arabidopsis* genome is severely compromised [Plant J. 2014, 77:511-543]. To repress the expression of those genes that may be involved in the integration of foreign genes in potato PCR fragments of potato *KU8*, *UVH1*, *XRCC2*, *CAK2* and *TOPOISOMERASE* were isolated and cloned separately into the binary vector pCP60 behind the CaMV 35S promoter in antisense orientation. A co-transformation experiment with a mixture of six *Agrobacterium* strains carrying PROGED::gPDS and the pCP60 antisense constructs, as well as another experiment with the PROGED::gPDS::Polθ and the pCP60 antisense constructs, were carried out. Nevertheless, neither experiment resulted in the generation of *PDS* mutants.

The negative results of 2-week selection experiments indicated that the transgenes are in an integrated stage by this period of time. Therefore, a considerably shorter, 3-day positive selection was applied followed by lack of selection or with counter-selection of emerging calluses and regenerating shoots carrying integrated transgenes derived from PROGED::gPDS. In parallel, a transformation experiment without any selection for the transformation event was carried out as a control. Due to the short time or even lack of selection, hundreds of regenerated shoots appeared both from tuber and leaf transformation either by *Agrobacterium* gv2260 or LBA4404. We hypothesised that the majority of the regenerated plants are non-transformed. Therefore, genomic DNA was isolated from groups of plants to search for *PDS* mutants.

Individual testing of plants of positive groups by PCR resulted in the identification of 22 *PDS* mutants appearing with a frequency of 2-10% with no substantial difference between counter-selected and non-counter-selected plant populations. In contrast with the majority of the plants with integrated transgenes, none of the mutant plants had a deletion large enough to be visible on a gel in the PCR test. Two chimaeric plants with a green-white mosaic phenotype were obtained. From the experiment with a lack of selection, 32 groups were tested. None of the groups harboured a *PDS* mutant. Thus, it was concluded that the chance to find a gene-edited mutant in a regenerated potato plant population without any selection is less than 0.5%.

Ten lines isolated after 3 days of selection, including the two chimaeras, were further investigated by PCR. Using primer pairs specific for the *Cas9-scfRNA* fragment, *Cas9* and *nptII*, it was concluded that the *PDS* mutations in these plants were generated from transient expression of *Cas9* and *gPDS*, as no PCR fragments derived from the above listed parts of PROGED::gPDS were detected on agarose gels.

The region surrounding *gPDS* was PCR-amplified, cloned into pGEM-T Easy and Sanger-sequenced from the wild-type and 15 *PDS* mutants. The DNA sequence analysis supported the result of the previous PCR test because large deletions extending from 35 to 166 bp were detected in mutants obtained by continuous positive selection, while with temporary selection, deletions of only a few bp (6 mutants) or a few bp and 1-bp substitution (3 mutants) were obtained. The mutations were extended to all four alleles of *PDS*. Wild-type and mutant sequences or two different mutations in the same allele were detected in eight out of ten green plants indicating that not only the plants with green-white phenotype, but several green plants were also chimaeras.

Upon vegetative propagation from stem segments *in vitro*, the phenotype of the transgenic plants obtained by continuous antibiotic selection and carrying the *Cas9* construct did not change, suggesting that the expression of *Cas9* and *gPDS* is silenced or that the DNA repair system is highly active during the vegetative growth phase in potato. Low amounts of *Cas9* mRNA could be detected in these lines by RT PCR. Nevertheless, as we do not know the level of mosaicism, we cannot reach conclusions regarding the level of *Cas9* expression in those cells that harbour *Cas9*. The frequency of chimaeric mutations also cannot be calculated from our experiments because plants carrying mono-, bi-, or triallelic mutations in the *PDS* gene may be as green as wild-type plants and can be either chimaeric or non-chimaeric. Several publications demonstrated that chimaeras are a highly frequent phenomenon observed after *Agrobacterium*-mediated transformation. Crossing out the mutations from our *PDS* mutant plants would be necessary to get information on the frequency of chimaeras in *PDS*-edited population of potato generated by *Agrobacterium*-mediated transformation.

In conclusion, our work showed that *Agrobacterium*-mediated gene editing in potatoes is an efficient method either by tuber or leaf transformation and using either *A. tumefaciens* strain gv2260 or LBA4404. Large deletions can be generated by continuous positive selection for stable transgene integration, while small deletions and nucleotide substitutions are derived from the transient expression of the *Cas9* system. Stable integration of *Cas9* and *sgRNA* can generate mutations in all four copies of a gene in the tetraploid genome of potato, as demonstrated by the appearance of albino plants in the *PDS* mutant population. Nevertheless, irrespective of the selection method, chimaeras appear in the mutant population. Despite the presence of *Cas9* and *sgRNA* in the genome, the T0 plants propagated from stem segments had a stable phenotype, suggesting that the expression of *Cas9* and *sgRNA* is silenced or that the DNA repair system is highly active during the vegetative growth phase in potato. Gene function studies in potatoes have always been performed on the T0 generation of transgenic plants. Since the phenotype of

the gene-edited transgenic lines propagated *in vitro* is stable, these plants are suitable for gene-function studies.

Parts of the results described above were presented in two national and two international conferences and submitted for publication in BMC Biotechnology in September, 2019 and accepted for publication on 23 April, 2020. Results generated by our research group not described above, but also obtained in potato, were published in PLoS ONE. The publication fee was covered by the project budget with indication of the project ID: K-120641.