Comparative analysis of CRISPR/Cas9 delivery approaches for polyphenol oxidase 2 gene editing in potato

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Abstract

- *StPPO2* gene editing was analyzed by *Agrobacterium*-mediated transformation with CR-PPO vector, ribonucleoprotein complexes (RNP-PPO) transfection to protoplasts, and CR-PPO transient expression in protoplasts, yielding efficiencies of 9.6%, 18.4%, and 31.9%, respectively.
- Transient expression of CR-PPO in protoplasts resulted in tetra-allelic edited lines, observed in 46% of total edited lines.
- On-target DNA insertions were found in lines from all three approaches.
- Loss of function of the StPPO2 protein was confirmed in a tetra-allelic edited line.

Introduction

Delivery of the CRISPR/Cas9 components to the plant cells, and further regeneration of edited lines, are key steps in the use of this technology. In potato, genomic integration of Cas9 and sgRNAs coding sequences via *Agrobacterium tumefaciens*-mediated transformation is the most commonly used approach (Van Eck 2018). As an alternative, CRISPR/Cas9 components can be delivered to protoplasts (Nadakuduti et al. 2018). Such a strategy allows the delivery of the components encoded in DNA molecules but, also, assembled as ribonucleoprotein complexes (RNPs). The use of RNPs is of particular importance when using this technology to obtain improved commercial crops (Zhang et al. 2020). In this study, all three approaches were analyzed to direct the CRISPR/Cas9 components for *StPPO2* gene editing. *StPPO2* is the principal contributor to the polyphenol oxidase activity in the tuber, responsible for enzymatic browning in potato (González et al. 2020).

Methods and Materials

Components design: sgRNA1 and sgRNA2 (Figure 1A) were designed with RGEN Cas-Designer (http://www.rgenome.net/cas-designer/). CR-PPO vector was constructed (Figure 1B), and RNP-PPO (Figure 1C) were obtained with GeneArt Precision sgRNA Synthesis Kit (Thermo Fisher Scientific). ***Agrobacterium*-mediated transformation:** potato explants were transformed as described in Kumar (1995) (Figure 2). **Protoplasts transfection and plant regeneration:** Performed according to Nicolia et al. (2015), with the modifications described by Andersson et al. (2017) (Figure 2). **Mutations screening:** Performed by High Resolution Fragment Analysis (HRFA, Andersson et al. 2017) of the region spanning both sgRNAs target sites. Results were confirmed by Sanger sequencing. **Phenotypic characterization**: Tubers were analyzed for enzymatic browning and PPO activity, as previously described (González et al. 2020).

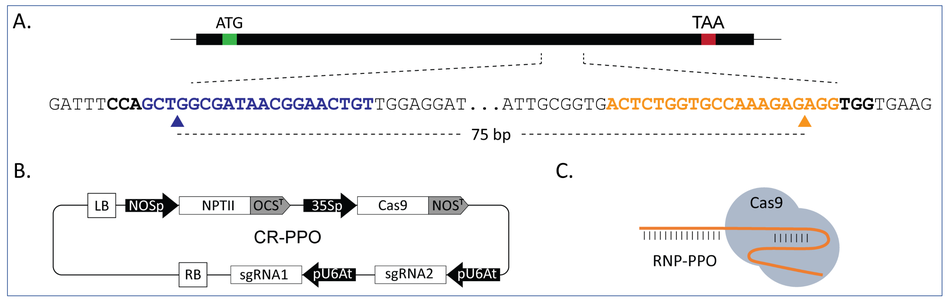

Figure 1. A. sgRNA 1 (blue) and sgRNA2 (orange) target sites on *StPPO2.* PAMs are indicated as bold letters. *Cas9* cut sites are indicated with colored arrow heads, separated by 75 bp. **B.** CR-PPO binary vector scheme. **C.** RNP-PPO scheme, obtained by in vitro transcription of sgRNA1 and sgRNA2 and assembly with Cas9 nuclease.

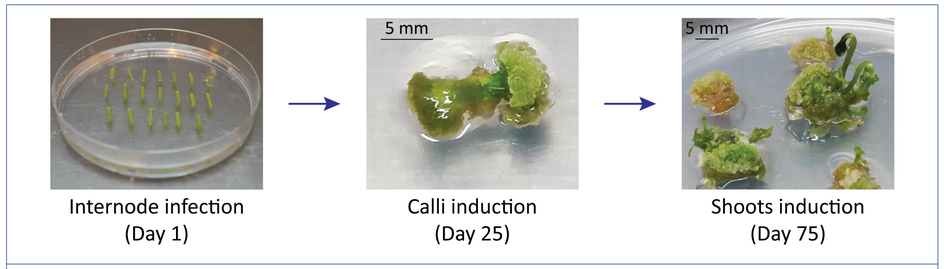

Figure 2. CRISPR/Cas9 components delivery approaches. *Agrobacterium*-mediated transformation with CR-PPO (top) and protoplasts transfection and regeneration with CR-PPO and RNP-PPO (bottom).

Table 1. *StPPO2* gene editing in regenerated lines.

CRISPR/Cas9 system	Delivery method	Analyzed lines	*StPPO2* genotype		Efficiency	Tetra-allelic edited lines
			WT	Edited (at least one allele)		
CR-PPO	A. tumefaciens	93	84	9	9.6%	0% (0)
CR-PPO	Protoplasts	47	32	15	31.9%	46.6% (7)
RNP-PPO	Protoplasts	76	62	14	18.4%	0 % (0)

Table 2. On-target DNA insertions.

Line ID	CRISPR/Cas9 system	Delivery method	Insertion length (target site)	Insert origin
4.2.3	CR-PPO	A. tumefaciens	21 bp (sgRNA2)	n/d
CR1053	CR-PPO	Protoplasts	380 bp (sgRNA2)	CR- PPO vector (fragments of NPTII gene and OCS terminator)
RNP6173	RNP-PPO	Protoplasts	152 bp (sgRNA2)	DNA template used for sgRNA1 in vitro transcription

n/d indicates ¨no determined¨

Results

- The CR-PPO transient expression in protoplasts resulted in higher editing efficiency and a high frequency of lines edited in all four *StPPO2* alleles (Table 1).
- Only two lines (obtained by CR-PPO transient expression) carried deletions of around 75 bp as expected for Cas9 inducing cuts at both target sites.
- On-target DNA insertions were observed in 1.1% (1 line), 8.5% (4 lines), and 3.9% (3 lines) of analyzed lines from *Agrobacterium*-mediated transformation, CR-PPO, and RNP-PPO transfections in protoplasts, respectively (Table 2).
- The tetra-allelic edited line CR1037 displayed a reduced PPO activity and enzymatic browning in tubers when compared to that of the wild type (Figure 3).

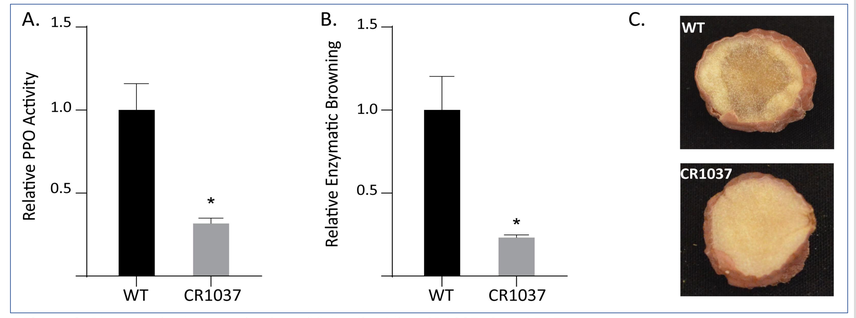

Figure 3. Phenotypic characterization of full edited line CR1037. **A** and **B**. Error bars represent the standard error from three plants per line. Significant differences according to the t-test ($p < 0.05$) is denoted *. **C.** Appearance of representative tubers after cut and 48 h air exposure

Discussion

Transient expression of CR-PPO resulted in a higher *StPPO2* gene editing efficiency in comparison to the *Agrobacterium*-mediated transformation with the same system. Even though selection pressure was applied during the whole transformation process, we cannot disregard the possibility of transgene silencing (Gelvin 2017). For protoplasts transfection, *StPPO2* editing efficiency was higher with CR-PPO than RNP-PPO. This supports the concept that RNPs are rapidly subjected to degradation in the cell (Kumlehn et al. 2018). All three approaches resulted in on-target DNA insertions. Insertions of T-DNA into the target sites seem to occur at low frequencies in plants obtained via *Agrobacterium*-mediated transformation (Banakar et al. 2019). Here, the small length of the 21 bp insertion found in one of such a line made it complex to determine its origin. In concordance with our results, transient expression of DNA in protoplasts has been reported to produce on-target insertions in potato (Andersson et al. 2017, 2018; Tuncel et al. 2019; Veillet et al. 2019) and wheat (Arndell et al. 2019), and RNPs obtained by in-vitro transcription of sgRNAs has been reported to produce on-target DNA insertions in potato (Andersson et al. 2018; González et al. 2020).

Conclusions

Gene editing efficiency in potato is affected by the delivery approach of CRISPR/Cas9 components. For potato breeding applications, RNPs transfection into protoplasts represents a promising approach, although a careful design of the CRISPR/Cas9 components must be performed to accomplish a high editing efficiency and avoid unwanted DNA insertions.

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