Plant Biotechnology Journal
Open Access

Plant Biotechnology Journal (2024), pp. 1–3 doi: 10.1111/pbi.14400

Brief Communication

Mutation of the *Vinv* 5′ UTR regulatory region reduces acrylamide levels in processed potato to reach EU food-safety standards

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Received 28 November 2023;
revised 27 January 2024;
accepted 15 May 2024.
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Keywords: potato, 5′ UTR editing, Cold induced sweetening, CRISPR/Cas9, Vacuolar invertase.

The recent prohibition of Chlorpropham (CIPC) in the EU (Commission Implementing Regulation(EU) 2019/989) is prompting the potato processing industry to search for alternative and safer anti-sprouting approaches. Storage at cold temperature (i.e. 4 °C) has emerged as a valuable option for long term storage of potato without the use of CIPC. However, most commercial potato varieties accumulate high levels of reducing sugars (RS) during cold storage, a phenomenon called cold-induced sweetening (CIS). During high temperature processing of potatoes into products such as crisps and French fries, the RS react with asparagine and peptides to produce the neurotoxin acrylamide, whose presence is evidenced by a brown-to-black coloration of the processed products (Bhaskar *et al.*, 2010). The challenges of potato storage are graphically depicted in Figure 1a.

Because of the difficulty in breeding CIS-resistant potato varieties to replace the ones that are CIS-susceptible, New Genomic Techniques (NGTs) are emerging as useful approaches to rapidly introgress the CIS-resistant trait into commercial varieties used by the processing industry. Despite the versatility of CRISPR-base approaches to target any selected sequence in a plant genome, the technology has so far been mainly used to target protein-coding sequences in plants.

In the present work, we exploited editing of a 5′ UTR sequence to engineer CIS resistance in an industry-preferred potato variety. Vacuolar invertase (VInv) has been identified as a key enzyme for conversion of sucrose into RS. Previous studies have demonstrated that silencing of the *VInv* gene is a suitable approach to lower the accumulation of RS upon cold storage of potato (Bhaskar *et al.*, 2010; Zhu *et al.*, 2016).

We designed a short guide RNA (sgRNA) targeting the 5′ UTR region of the *VInv* gene. Its activity was assessed in an *in vitro* cleavage assay using an amplicon from potato DNA as template (Figure S1). We next transformed potato variety Lady Rosetta (LaRo), a CIS-susceptible variety used in the crisp industry, with the construct PC2300-sgRNA1–pcoCas9–eGFP. Based on the PAM sequence, the selected sgRNA was expected to target two of the four 5′ UTR allelic sequences. Thirty-two independent potato lines were generated. Eleven lines showed the desired genetic profile in a PCR–restriction enzyme (RE) assay and their mini-tubers (T0 tubers) were vegetatively propagated to produce T1 tubers. T1 tubers of wild-type LaRo and Verdi (CIS-resistant) varieties were also produced for use as controls. Further analyses were performed on the T1 tubers.

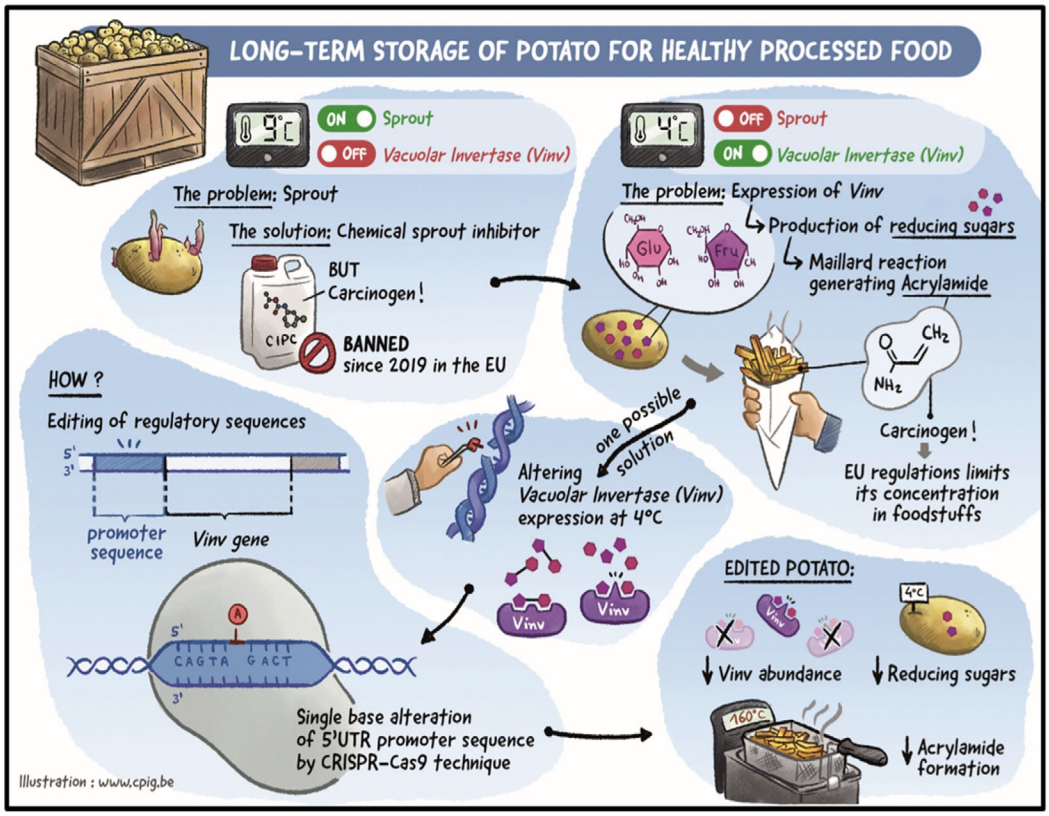
Illumina sequencing of the target region revealed four lines carrying one adenine insertion between positions −34 and −35 in the two editable alleles (P4, P6, P24 and P26), one transformed line without edits (P1), and six lines with different types and percentages of deletions in the two editable alleles (Figure 1b).

Quantification of RS (glucose + fructose) in the selected lines after storage at 4 °C for 1 month revealed that the four lines with the single A-insertion contained significantly lower levels of RS compared with the non-edited control line (P1), and the other lines displaying various editing profiles of the 5′ UTR (Figure 1c). Lines P6 and P26 had significantly lower levels of RS relative to the control CIS-resistant potato variety Verdi. There were no statistically significant differences in the levels of RS amongst the wild-type LaRo potatoes, the non-edited line (P1) and the edited lines. In line with previous observations (Bhaskar *et al.*, 2010; Shumbe *et al.*, 2020), sucrose contents in all samples inversely correlated with the contents of RS (Figure 1c). These results indicate that the single A-insertion between positions −34 and −35 of the 5′ UTR of *VInv* gene was sufficient to engineer CIS resistance.

We assayed VInv enzymatic activity in selected lines and control varieties (Figure 1d) and observed a similar trend to that of RS (Figure 1c) and that of the transcript levels of *VInv* assayed in selected CIS-contracting T1 tubers after 1-month storage at 4 °C (Figure S2a). These results indicate that a single A-insertion in the 5′ UTR region cause a consistent and significant down-regulation of the *VInv* transcripts and a reduction of VInv activity upon cold storage. After 1-month storage at RT, no statistically significant

Please cite this article as: Shumbe, L., Soares, E., Muhovski, Y., Smit, I. and Vanderschuren, H. (2024) Mutation of the *Vinv* 5′ UTR regulatory region reduces acrylamide levels in processed potato to reach EU food-safety standards. *Plant Biotechnol. J.*, https://doi.org/10.1111/pbi.14400.

(a)

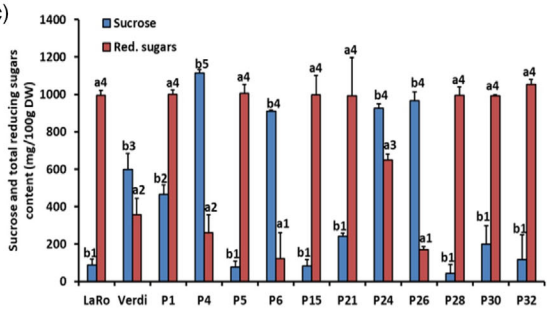


(b)

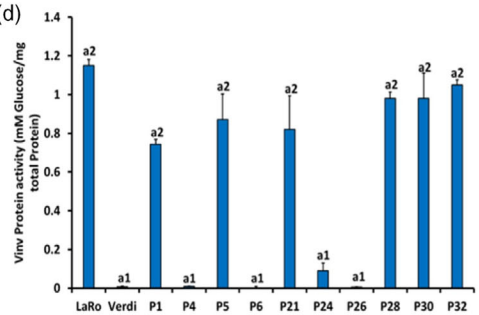
Line ID	Target region Sequence and % edited/editable alleles	In/Del
LaRo	CACCTATATATAAAGCAGTA GACTGGTAGC -50% CCCCTATATATAAAGCCGTA GACTAGTTGC -25% CCCCTTTATATAAAGCAGTA GACTAGTTGC -25%	0
Verdi	CACCTATATATAAAGCAGTA GACTGGTAGC -100%	0
P1	CACCTATATATAAAGCAGTA GACTGGTAGC -50%	0
P4	CACCTATATATAAAGCAGTAAGACTGGTAGC -50%	+1
P5	CACCTATATATAAAGCAG- -ACTGGTAGC -25% CACCTATATATAAAGCAG- GACTGGTAGC -25%	-3 -2
P6	CACCTATATATAAAGCAGTAAGACTGGTAGC -50%	+1
P15	CACCTATATATAAAGCAG- -ACTGGTAGC -19% CACCTATATATAAAGCAG- GACTGGTAGC -18% CACCTATATATAAAGCAGT GACTGGTAGC -12%	-3 -2 -1
P21	CACCTATATATAAAGCAG- -ACTGGTAGC -25% CACCTATATATAAAGCAG- GACTGGTAGC -25%	-3 -2
P24	CACCTATATATAAAGCAGTAAGACTGGTAGC -50%	+1
P26	CACCTATATATAAAGCAGTAAGACTGGTAGC -50%	+1
P28	CACCTATATATAAAGCAG- -ACTGGTAGC -20% CACCTATATATAAAGCAG- GACTGGTAGC -19% CACCTATATATAAAGCAGT GACTGGTAGC -11%	-3 -2 -1
P30	CACCTATATATAAAGCAG- -ACTGGTAGC -25% CACCTATATATAAAGCAG- GACTGGTAGC -25%	-3 -2
P32	CACCTATATATAAAGCAG- -ACTGGTAGC -21% CACCTATATATAAAGCAGT GACTGGTAGC -20% CACCTATATATAAAGCAG- GACTGGTAGC -8%	-3 -1 -2

PAM

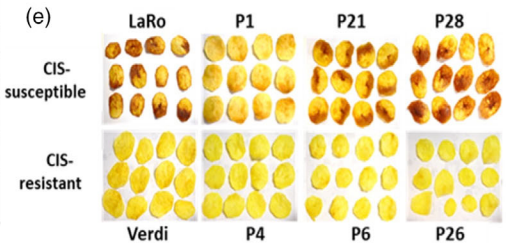
(c)



(d)



(e)



(f)

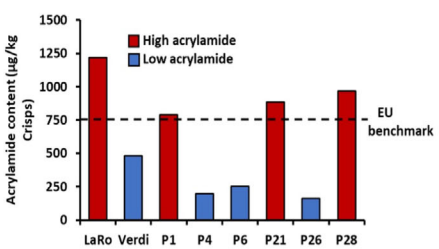


Figure 1 (a) Graphical presentation of the challenges for long term storage of potato in the EU processing industry and the genome editing solution presented in the article (b) target region from Illumina sequenced Lady Rosetta (LaRo) edited lines showing In-dels and the percentages of reads corresponding to the percentage of edited alleles (c) Contents of sucrose and reducing sugars (RS) from tubers of non-transformed control, selected edited lines after 1 month storage at 4 °C (d) Activity of Vacuolar Invertase (VInv) quantified from potato tubers after 1 month of storage at 4 °C (n = 3). a1, a2, a3 and a4 indicate statistically significant differences in RS and VInv protein activity, and b1, b2, b3, b4, b5 represent statistically significant difference in sucrose content, at $P < 0.05$ computed by Scott-Knott test. (e) Crisps produced from CIS-contrasting tubers after cold storage. (f) Acrylamide content of the crisps and indication of the EU benchmark level (n = 2).

difference was observed in the levels of RS between all the lines while sucrose levels appeared lower in the selected CIS-resistant lines and Verdi (Figure S2b). Furthermore, the levels of RS in the CIS-resistant lines and variety (P4, P24, P26 and Verdi) were consistently significantly lower than the levels in the CIS-susceptible lines and variety after two generations of propagation (T2) and after 1-month storage at 4 °C (Figure S2c).

Finally, cold stored T1 tubers of selected CIS-contrasting lines and control varieties (Figure S3) were processed into crisps. Crisps from CIS-susceptible lines (P1, P21 and P28) and the control LaRo variety displayed brown-to-black colour, whereas those from the CIS-resistant A-insertion lines (P4, P6 and P26) and the control Verdi variety consistently appeared pale yellow (Figure 1e). The acrylamide contents from crisps of the CIS-resistant lines and variety were well below the benchmark value of 750 μg/kg set by the European Union. Conversely the levels in the CIS-susceptible lines and variety were higher than the benchmark value (Figure 1f).

Taken together, our findings demonstrate that a single adenine insertion in the 5′ UTR region of 50% of the LaRo *VInv* alleles is sufficient to alter the CIS phenotype of the LaRo variety, and thereby to reduce the acrylamide content in the processed products to an acceptable level within EU requirements. VInv is known to regulate several aspects of growth and development in plants (Wang *et al.*, 2010; Wang and Ruan, 2016); however, no abnormal phenotype was observed for the CIS-contrasting lines (Figure S3), nor any significant differences in the mean tuber size (Figure S2d).

Because of its key function in the initiation of transcription, the 5′ UTR region offers an alternative to the traditional exon targeting for genome editing (Si *et al.*, 2020). While trait engineering usually relies on targeting all allelic coding sequences in potato, our study demonstrates that a stable CIS-resistance trait can be achieved by differential allele targeting of the *VInv* 5′ UTR. In some specific cases, differential allele targeting might also represent a more suitable approach to reduce the possible pleiotropic effects associated with silencing or knocking out of all alleles in polyploid crop species such as potato (Andersson *et al.*, 2017). With the newly proposed regulation on genome editing in the European Union (Vanderschuren *et al.*, 2023), our results provide a timely solution to convert commercial CIS-susceptible potato varieties widely used by the European potato industry into CIS-resistant varieties for the subsequent production of safe and healthy crisps and French fries.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1 Material and methods.
Figure S1 *In vitro* cleavage activity of target sgRNA on PCR-amplified DNA from potato. The lowest two bands in the first and second lanes represent monomers and dimers of the sgRNA respectively.
Figure S2 Characterization of selected CIS-contrasting lines and varieties. (a) Fold change of VInv gene expression in tubers stored for 1 month at 4 °C relative to tubers stored at room temperature for the same duration. (b) Sucrose and reducing sugars content quantified from CIS-contrasting potato lines and controls after storage for 1 month at room temperature. (c) Reducing sugars quantified from CIS-contrasting lines multiplied after 3 generations (T2) and stored at 4 °C for 1 month (d) mean diameter of four largest tubers from selected T1 generation lines and varieties. a1, a2, a3… a7 indicate statistically significant differences in reducing sugars and VInv gene expression between the different lines and controls, and b1, b2 represent statistically significant difference in sucrose content between the different lines and controls, at $P<0.05$ computed by Scott-Knott test.
Figure S3 Phenotype of selected CIS-contrasting T1 tubers from edited lines and control varieties.

