Title: The *Solanum tuberosum GBSSI* gene: a target for assessing gene and base editing in tetraploid potato

Authors: Florian Veillet[1*], Laura Chauvin[1], Marie-Paule Kermarrec[1], François Sevestre[2,3], Mathilde Merrer[1], Zoé Terret[4,5], Nicolas Szydlowski[2,3], Pierre Devaux[6], Jean-Luc Gallois[4], Jean-Eric Chauvin[1]

Adress: [1]INRA, Agrocampus Ouest, Université Rennes 1, UMR 1349 IGEPP, Domaine de Kéraïber, 29260 Ploudaniel, France; [2]Univ. Lille, CNRS, UMR8576 – UGSF – Unité de Glycobiologie Structurale et Fonctionnelle, Lille, France; [3]Univ. Lille, CNRS, USR 3290 – MSAP – Miniaturisation pour la Synthèse, l'Analyse et la Protéomique, Lille, France; [4]GAFL, INRA, Montfavet, France; [5]SYNGENTA SEEDS SAS - 346 Route des Pasquiers – 84260 Sarrians, France; [6]Germicopa Breeding, Kerguivarc'h, 29520 Chateauneuf du Faou, France

Keywords: Genome editing, CRISPR-Cas9, Cytidine base editor, Potato, GBSS, HRM

* Corresponding author: Florian Veillet, florian.veillet@inra.fr, +33 2 29 62 63 33

Florian Veillet ORCID ID: 0000-0002-6892-6825

François Sevestre ORCID ID: 0000-0002-3888-2278

Pierre Devaux ORCID ID: 0000-0002-7076-7624

Jean-Luc Gallois ORCID ID: 0000-0003-0451-1740

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Key Message

The *StGBSSI* gene was successfully and precisely edited in the tetraploid potato using gene and base editing strategies, leading to plants with impaired amylose biosynthesis.

Abstract

Genome editing has recently become a method of choice for basic research and functional genomics, and holds great potential for molecular plant breeding applications. The powerful CRISPR-Cas9 system that typically produces double-strand DNA breaks is mainly used to generate knockout mutants. Recently, the development of base editors has broadened the scope of genome editing, allowing precise and efficient nucleotide substitutions. In this study, we produced mutants in two cultivated elite cultivars of the tetraploid potato (*Solanum tuberosum*) using stable or transient expression of the CRISPR-Cas9 components to knockout the amylose-producing *StGBSSI* gene. We set up a rapid, highly sensitive and cost-effective screening strategy based on high-resolution melting analysis followed by direct Sanger sequencing and trace chromatogram analysis. Most mutations consisted of small indels, but unwanted insertions of plasmid DNA were also observed. We successfully created tetra-allelic mutants with impaired amylose biosynthesis, confirming the loss-of-function of the StGBSSI protein. The second main objective of this work was to demonstrate the proof of concept of CRISPR-Cas9 base editing in the tetraploid potato by targeting two loci encoding catalytic motifs of the StGBSSI enzyme. Using a cytidine base editor (CBE), we efficiently and precisely induced DNA substitutions in the KTGGL-encoding locus, leading to discrete variation in the amino acid sequence and generating a loss-of-function allele. The successful application of base editing in the tetraploid potato opens up new avenues for genome engineering in this species.

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Introduction

Most staple crops are harvested for their starch-storing organs, such as cereal grains, tubers and storage roots. They are mainly cultivated as food or feed for humans or livestock, but there is also an increasing demand of renewable resources for non-food applications (Zeeman et al. 2010). Originating from Latin America, potato (*Solanum tuberosum*) constitutes one of the most important crops for human consumption owing to its starch-rich tubers.

In higher plants, photosynthetic cells produce transitory starch in chloroplasts and also export sucrose to heterotrophic organs, where it is converted to starch for long-term storage in amyloplasts (Lemoine et al. 2013). Starch is an insoluble glucan composed of two polymers of glucose, the ratio of which strongly determines its physiochemical properties. Amylose is a nearly linear glucose polymer with α-1,4-linked residues, whereas amylopectin is made up of α-1,4-linked chains with α-1,6-linkages at branch points, conferring crystallinity to the starch granule. In higher plants, starch biosynthesis is mainly mediated by four classes of enzymes: ADP-glucose pyrophosphorylases, starch synthases (SSs), starch branching enzymes (SBEs) and starch debranching enzymes (SDBEs) (Zeeman et al. 2010). Starch synthases isoforms SSI, SSII, SSIII govern the elongation of the chains of amylopectin. SSIV is involved in starch initiation; the role of SSV and SSVI is still unclear (Helle et al. 2018; Roldan et al. 2007). In addition, granule-bound starch synthase (GBSS) binds to the starch granule and mediates amylose biosynthesis (Ball et al. 1998; Rongine De Fekete et al. 1960).

Amylose determines many physicochemical properties of starch, namely its pasting temperature and viscosity (Bull et al. 2018; Park et al. 2007). Therefore, modifying potato starch composition by decreasing the amylose (or amylopectin) content may be useful for industrial applications. For example, modulating GBSSI function directly *in planta* can lead to the reduction of post-harvest treatments (Sonnewald and Kossmann 2013). In most dicot species, such as potato, the GBSSI protein is encoded by a single nuclear locus (Cheng et al. 2012). This monogenic control has facilitated the production of amylose-free potato varieties through mutational breeding (Hovenkamp-Hermelink et al. 1987; Muth et al. 2008). In some studies, transgenic approaches have been used to silence the *GBSSI* gene through antisense (Kuipers et al. 1994; Visser et al. 1991) or RNAi technologies (Andersson et al. 2003). Although the genetically modified potato Amflora (BASF) has been commercialized for two years, to date the development of such transgenic crops for commercial purposes has been

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58   limited in the European Union, mainly due to regulatory policies (Zeeman et al. 2010).

59   In the past years, genome editing techniques have received much attention due to their
60   powerful applications in model plants and crops. These techniques rely on the precise
61   introduction of DNA double-strand breaks (DSBs) in the plant genome through a variety of
62   techniques (Ma et al. 2016). DSBs are readily recognized by the cell and repaired either
63   through non-homologous end joining (NHEJ) or homologous recombination (HR), the former
64   being the main pathway for the repair of DSBs in somatic cells (Puchta 2005). Contrary to
65   HR, NHEJ is error-prone and may lead to random small insertions or deletions (indels) at the
66   cut site. Since 2012, tremendous breakthroughs have been made using clustered regularly
67   interspaced short palindromic repeats (CRISPR)-associated nuclease Cas9 systems (Sternberg
68   et al. 2016). When expressed in eukaryotic cells, CRISPR-Cas9 systems fulfil their function
69   by forming a complex made up of a single-guide RNA molecule (sgRNA) and the Cas9
70   nuclease (Jinek et al. 2012). The latter recognizes a protospacer adjacent motif (PAM), mainly
71   NGG in the case of *Streptococcus pyogenes* Cas9 (*Sp*Cas9), which is found just downstream
72   of the target sequence. Recognition of the PAM likely destabilizes the adjacent double-
73   stranded DNA, allowing base pairing between a 17-21 bp target-dependent sequence from the
74   sgRNA and its matching target DNA sequence (Anders et al. 2014; Bortesi and Fischer 2015).
75   The Cas9 nuclease eventually induces DSB in DNA about 3 bp upstream of the PAM
76   sequence. Error-prone NHEJ may lead to small indels, potentially resulting in frameshift
77   mutations (Soyars et al. 2018). A truncated and/or non-functional protein will be translated,
78   possibly triggering the nonsense-mediated decay (NMD) pathway that leads to mRNA
79   degradation (Pauwels et al. 2018; Popp and Maquat 2016).

80   The CRISPR-Cas9 system has been successfully developed in potato in the past few
81   years (Hameed et al. 2018). For example, the *StIAA2* and the *StALS* genes have been
82   efficiently targeted in a double haploid cultivar and/or a tetraploid potato using
83   *Agrobacterium*-mediated stable transformation (Butler et al. 2015; Butler et al. 2016; Wang et
84   al. 2015). More recently, the full knockout of the *StGBSS* gene in the tetraploid potato cultivar
85   Kuras was obtained using transient expression of CRISPR-Cas9 components in protoplasts,
86   either as DNA plasmids or as ribonucleoprotein (RNP) complexes (Andersson et al. 2017;
87   Andersson et al. 2018).

88   In addition to generating gene knockout through the introduction of indels, CRISPR-
89   Cas9 can precisely replace nucleotides, allowing the study of specific domains within a

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90   protein or also providing polymorphism within a gene to confer valuable agronomic traits in
91   elite varieties. Such modifications can be carried out using HR and the insertion of a DNA
92   template bearing the polymorphism. However, precise and efficient base editing via HR in
93   plants suffers from low efficiency and the delivery of template DNA is still challenging
94   (Schindele et al. 2018). A new CRISPR-Cas9-based genome editing system has recently been
95   developed based on fusing either a cytidine or an adenine deaminase to a Cas9 nickase (nCas9
96   D10A), leading to a C-to-T or an A-to-G conversion on the edited strand, respectively
97   (Schindele et al. 2018). The sgRNA directs the nCas9/deaminase fusion to the target locus,
98   enabling edition on the non-complementary strand. Cytidine or adenine is converted to uracil
99   or inosine, respectively, without introducing DSBs. Uracil and inosine are then converted
100   through DNA replication to thymine and guanine, respectively, although C-to-G and C-to-A
101   conversions have also been reported (Nishida et al. 2016). The edition has been shown to take
102   place in a 3-8 bp deamination window distal from the PAM on the non-complementary
103   strand, with some variations according to the base editor (Gaudelli et al. 2017; Kang et al.
104   2018; Shimatani et al. 2017; Zong et al. 2017). The nCas9 nicks the opposite strand of the
105   deamination site to direct DNA repair mechanisms to the G-or-T-containing DNA strand
106   using the edited strand as a template for mismatch repair, thus preserving the edit and
107   increasing the mutation rate (Nishida et al. 2016). To date, base editors have been
108   successfully used in some crops, including rice, tomato, wheat, maize, oilseed rape, potato
109   and watermelon (Hua et al. 2018a; Kang et al. 2018; Li et al. 2018; Shimatani et al. 2017;
110   Tian et al. 2018; Yan et al. 2018; Zong et al. 2018; Zong et al. 2017).

111   In this work, we targeted the *StGBSSI* gene to assay various CRISPR-Cas9 tools using
112   stable and transient expression in different varieties of the tetraploid potato *Solanum*
113   *tuberosum*. We identified single- and multi-allelic edited plants, and mutations in all four
114   alleles were observed, resulting in amylose biosynthesis impairment. To induce this
115   modification, we used protoplast transformation, which can produce non-transgenic plants.
116   Finally, we assessed the efficiency of the cytidine base editor (CBE) in two loci encoding
117   catalytic motifs of the StGBSSI enzyme, resulting in precise base conversion and amino-acid
118   substitution. Our results highlight that CRISPR-Cas9 gene and base editing can be efficiently
119   developed in a genetically complex and vegetatively propagated crop such as potato to
120   modify agronomic traits.

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Materials and methods

Plant material

Potato cultivars Desiree (ZPC, the Netherlands) and Furia (Germicopa, France) were *in vitro* propagated in 1X Murashige and Skoog (MS) medium (pH 5.8) including vitamins (Duchefa, the Netherlands), 0.4 mg/L thiamine hydrochloride (Sigma-Aldrich, USA), 2.5% sucrose and 0.8% agar powder (VWR, USA). Plants were cultivated in a growth chamber at 19°C with a 16:8 h light:dark photoperiod. For the production of *in vitro* microtubers, plants were placed in 5 mL of 1X MS culture medium for about one month at 19°C with a 16:8 h light:dark photoperiod, until depletion of the medium. Then, 4 mL of half-strength MS medium supplemented with 50 µg/mL coumarin and 4 µg/mL kinetin were added and the plants were transferred to a dark room at 20°C for about five additional weeks, until microtubers were sufficiently developed. The production of tubers in soil was carried out by transferring *in vitro* plants to a greenhouse. Watering was stopped 2 weeks before the tubers were harvested.

Target identification

Genomic sequences of the *StGBSSI* gene (Gene ID from NCBI: 102577459) were obtained from leaf DNA extracted using the NucleoSpin Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's instructions. Primers were designed using Primer3 (Untergasser et al. 2012) and Netprimer (www.premierbiosoft.com/netprimer) from the reference genome (https://plants.ensembl.org/Solanum_tuberosum/Info/Index), and are listed in Supplementary Table S1. Amplification was carried out on about 10 ng of DNA using Invitrogen Platinum SuperFi DNA polymerase (Thermo Fisher Scientific, USA) following the supplier's instructions. PCR products were cloned into the pCR4-TOPO TA vector (Thermo Fisher Scientific, USA) and transformed by heat shock into One Shot™ TOP10 Chemically Competent *E. coli* (Thermo Fisher Scientific). Bacteria were grown overnight at 37°C on LB plates with 50 µg/mL kanamycin and plasmids from randomly selected positive clones were purified using QIAprep Spin Miniprep kit (QIAGEN, Germany) and Sanger sequenced (Genoscreen, Lille, France).

Target loci in the *StGBSSI* gene were selected manually based on their distance from the KTGGL and PSRFEPCGL motifs, and then analysed using the CRISPOR software

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152   (Haeussler et al. 2016). In this study, four guides were designed upstream of the NGG PAM

153   and were named sgGBSS1 (5'-GTTGGTCCTTGGAGCAAAAC-3'), sgGBSS2 (5'-

154   TTGTCATTACCCGATGTCCG-3'), sgGBSS3 (5'-GGACTAGGTGATGTTCTTGG-3') and

155   sgGBSS4 (5'-CCAAGCAGATTTGAACCTTG-3'). Guide design was performed according

156   to the predicted efficiency and the off-target potential from the potato reference genome,

157   selecting guides with no off-target site with less than two mismatches and with no mismatch

158   in the seed region adjacent to the PAM.

159

160   **Vector construction**

161   For the sgGBSS1 and sgGBSS2 targets, guide sequences were respectively placed

162   downstream a *StU6* (Z17301.1) (Guerineau and Waugh 1993) or a *StU3* (NW_006239017.1)

163   promoter (Supplementary Fig. S1) and upstream a sgRNA scaffold previously described

164   (Shimatani et al. 2017). The constructs were synthesized (Genscript, USA) with Gateway

165   AttB1 and AttB2 sequences on both sides to perform a BP reaction with the pDONR207

166   plasmid. For stable transformation using *Agrobacterium tumefaciens*, the pDONR207-

167   containing the sgGBSS1 construct was LR-recombined with the pDe-Cas9 (Fauser et al.

168   2014) harbouring a *nptII* resistance cassette. The resulting plasmid was transferred into *A.*

169   *tumefaciens* (C58pMP90) strain by heat shock. For transient expression in protoplasts, the

170   pDeCas9, pDONR207-sgGBSS1 and pDONR207-sgGBSS2 were purified using the

171   QIAGEN Plasmid Plus Midi Kit (QIAGEN, Germany), followed by a sodium acetate

172   precipitation.

173   For the sgGBSS3 and sgGBSS4 targets, guide sequences were cloned into the

174   pDicAID_nCas9-PmCDA_NptII_DELLA (Shimatani et al. 2017). To replace the guide

175   targeting the *SlDELLA* locus, the plasmid was digested by the FastDigest restriction enzymes

176   *Bst*XI and *Spe*I in the presence of Fast Alkaline Phosphatase (Thermo Fischer Scientific,

177   USA). The sgGBSS3 and sgGBSS4 guide sequences were synthesized (Genscript, USA)

178   together with a portion of the AtU6 promoter and the sgRNA scaffold, flanked by a *Bst*XI and

179   a *Spe*I restriction site. This construct was cloned into the pDONR207 plasmid using a BP

180   reaction (Gateway) and digested as described above, without the phosphatase treatment.

181   Guide sequences were then ligated into the digested binary vector using T4 DNA ligase (New

182   England Biolabs, USA). Reaction mixture was transformed into One Shot™ TOP10

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183　Chemically Competent *E. coli* (Thermo Fisher Scientific, USA) and bacteria were grown

184　overnight at 37°C on LB plates containing 100 µg/mL spectinomycin. Plasmids were Sanger

185　sequenced (Genoscreen, Lille, France) and transferred into *A. tumefaciens* C58pMP90 strain

186　by heat shock.

187

Agrobacterium-mediated transformation

189　Stem and petiole explants were cut from the top of 3 to 5 week-old *in vitro* plants, and placed

190　overnight in a growth chamber on 1X Murashige and Skoog (MS) medium (pH 5.8) including

191　vitamins (Duchefa, The Netherlands), 2.5% sucrose, 0.4 mg/L thiamine hydrochloride

192　(Sigma-Aldrich, USA), 1 mg/L indole-3-acetic acid (Sigma-Aldrich, USA), 1 mg/L zeatin-

193　riboside (Sigma-Aldrich, USA), 1 mg/L gibberellin A3 (Sigma-Aldrich, USA) and 0.7% agar

194　powder (VWR, USA). *A. tumefaciens* C58pMP90 strain containing CRISPR-Cas9 plasmids

195　was grown overnight at 28°C at 250 rpm in LB medium with 50 µg/mL rifampicin, 25 µg/mL

196　gentamicin and 50 µg/mL spectinomycin. The bacterial optical density (OD) was set to ≈ 0.2

197　in the MS medium without antibiotics. Potato explants were co-cultured with *A. tumefaciens*

198　for 48 h at 25°C in the dark, and were then washed with sterile water and placed on the

199　culture medium described above, supplemented with 250 µg/mL cefotaxime, 100 µg/mL

200　timentin® and 50 µg/mL kanamycin. After two weeks, explants were transferred onto a fresh

201　culture medium with reduced indole-3-acetic acid (0.1 mg/L), and were then maintained by

202　subculturing every three weeks. Regenerating shoots were transferred to the culture medium

203　containing 50 mg/L kanamycin and/or tested for the presence of the T-DNA by PCR.

204

PEG-mediated protoplast transfection

206　Protoplasts were isolated from leaves of 3-5 week-old plants propagated *in vitro*. Plants were

207　kept in the dark for at least 18 h before the start of the digestion. Protoplast digestion and

208　isolation were mainly performed as previously described by Yoo et al. (2007) with some

209　modifications. Digestion was performed overnight in the dark at 25°C using 0.2% cellulase

210　Onozuka R10 (Yakult Pharmaceutical Industry, Japan) and 0.2% macerozyme R10 (Yakult

211　Pharmaceutical Industry, Japan), without shaking. The next day, the protoplast solution was

212　gently shaken at 70 rpm for 30 min to release round protoplasts. The solution was filtered

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213   with a 40 μm cell strainer before the washing steps. Round-bottomed tubes were used during

214   all the procedure. Transient expression of pDeCas9, pDONR207-sgGBSS1 and/or

215   pDONR207-sgGBSS2 was performed by a PEG-mediated transfection using 3.6×10^5

216   protoplasts in 300 μL. A total amount of 10 μg of plasmid DNA was added to the protoplasts,

217   followed by 300 μL of 25% PEG4000 (Merck, Germany) for 2 min. The transfection solution

218   was gradually diluted in MaMg medium (0.4 M mannitol, 15 mM $MgCl_2$, pH 5.8) and kept at

219   4°C for 15 min in the dark. Protoplasts were embedded in 3 mL of alginate solution (Sigma,

220   USA) as described by Andersson et al. (2017). Plant regeneration was essentially performed

221   following the protocol described by Masson et al. (1987).

222

Detection of mutations

224   For the detection of CRISPR-Cas9-induced deletions and/or insertions, PCR genotyping was

225   performed across sgGBSS1 and sgGBSS2 target sequences. Amplification was carried out

226   using the GoTaq G2 Flexi DNA polymerase (Promega, USA) and the PCR products were run

227   on a 1.5% agarose gel.

228   The high-resolution melting (HRM) curve analysis was performed as described in Veillet et

229   al. (2016). Genomic DNA from leaf tissue was extracted using the NucleoSpin Plant II kit

230   (Macherey-Nagel) according to the manufacturer's instructions. Primers were designed to

231   obtain amplicons of about 100 bp (Supplementary Table S1) and tested for their specificity

232   and dimer formation on an agarose gel. PCR amplification was carried out in 12 μL volumes

233   containing 6 μL of High Resolution Melting Master (Roche Applied Science, Germany), 0.24

234   μL of each 10 μM primer, 1.44 μL of 25 mM $MgCl_2$ solution, and 5-30 ng of genomic DNA.

235   PCR was performed using 96-well white PCR plates using the LightCycler® 480 II system

236   (Roche Applied Science, Germany). The amplification started with an initial denaturation step

237   at 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 63-59°C for 10 s and 72°C for 10

238   s. HRM was immediately performed with a denaturation step at 95°C for 1 min followed by

239   an incubation at 40°C for 1 min. The melting curve was generated over a 65-95°C range, with

240   a 0.2°C/s increment and 25 acquisitions per °C. Results were analysed with LightCycler® 480

241   Gene Scanning software (Roche Applied Science, Germany). To detect mutations, all samples

242   were spiked with 10-20% of wild type DNA. For each mutated plant, a new HRM run was

243   carried out with and without spiking to identify putative homozygous mutated plants. All

9

244 plants with a mutated profile were Sanger sequenced (Genoscreen, Lille, France), as well as
245 some plants with a wild-type profile to check for the sensitivity of the HRM. Some plants
246 harbouring both an HRM profile and a Sanger chromatogram of interest were then analysed
247 by cloning the PCR products into the pCR4-TOPO TA vector (Thermo Fisher Scientific,
248 USA) followed by Sanger sequencing. Using the natural polymorphism found in wild-type
249 Desiree, each sequencing read was paired to a particular allele.

250

251 **Inference of CRISPR editing (ICE) analysis**

252 Chromatograms from Sanger sequencing were analysed using the Inference of CRISPR
253 Editing (ICE) software (Synthego, USA), an open-source and free-to-use web-based tool
254 (https://ice.synthego.com) (Hsiau et al. 2018).

255

256 **Amylose assay**

257 For a visual assay, 20 µL of a half-strength Lugol's solution (Sigma-Aldrich, USA) was
258 directly dropped onto the surface of a sliced tuber.

259 For size-exclusion chromatography analysis of starch polysaccharides, potato starch was
260 isolated and purified according to Helle et al. (2018). Washed and peeled tubers were cut into
261 0.5 cm x 0.5 cm pieces and ground in a mortar with 10 mL of ultrapure water. Samples were
262 then filtered through a nylon net (100 µm mesh) and starch granules were left to sediment.
263 Starch suspensions were then washed three times prior to size-exclusion chromatography
264 analysis. Starch polysaccharides were separated on a Sepharose CL-2B column as described
265 in Delvalle et al. (2005). Briefly, ≈1-2 mg of starch were dissolved in 500 µL of 10 mM
266 NaOH and loaded on a CL-2B column (0.5 cm x 65 cm). 300 µL fractions were collected at a
267 flow rate of 12 mL/h prior to measuring the OD and λmax of the iodine-polysaccharide
268 complexes with the use of a microplate spectrophotometer.

269

270 **Structural analysis of the GBSS mutation**

271 Eight GBSS templates were selected using the Modeller 9.18 programme (Webb and Sali

10

272 2016) based on sequence identity (>20%) from *Hordeum vulgare* (PDB:4HLN),
273 *Saccharomyces cerevisiae* (PDB:1YGP), *Oryza sativa* (PDB:3VUE), *Corynebacterium*
274 *callunae* (PDB:2C4M), *Oryctolagus cuniculus* (PDB:2GJ4), *Streptococcus* (PDB:4L22),
275 *Cyanophora paradoxa* (PDB:6GNG) and *Cyanobacterium sp.* (PDB:6GNF). The best model
276 was chosen using the discrete optimized protein energy (DOPE) method (Shen and Sali 2006)
277 and/or the GA341 method (John 2003; Melo et al. 2002). The model was optimized using
278 energy minimization protocols available in Yasara software (Krieger et al. 2009).

279

Results

Design of CRISPR-Cas9 targets

282 In potato, the StGBSSI protein is encoded by a single gene that contains 13 exons (Cheng et
283 al. 2012). The loci of interest were sequenced in two cultivars, Desiree and Furia, to assess
284 inter-allelic polymorphism and to design targets (Fig. 1 and Supplementary Fig. S2 and S3).
285 The locus encoding the catalytic domain KTGGL (amino acids 95 to 99), likely involved in
286 ADP-glucose binding (Nazarian-Firouzabadi and Visser 2017), was completely conserved in
287 both cultivars (Fig. 1). Based on these sequences, two different strategies were implemented
288 to target the *StGBSSI* gene using CRISPR-Cas9 editing methods. We designed two sgRNAs
289 in exon 1 and 2, named sgGBSS1 and sgGBSS2 (Fig. 1), to knock out the *StGBSSI* gene
290 using the pDeCas9 construct (Fauser et al. 2014). We also designed one sgRNA in exon 1
291 (sgGBSS3) and another one in exon 10 (sgGBSS4) to target the loci encoding the KTGGL
292 and the PSRFEPCGL catalytic domains, respectively (Fig. 1), using a CBE. The sgGBSS4
293 spanned a region of synonymous allelic variation at its 5' end in one of the four alleles in the
294 Desiree cultivar (Fig. 1).

295

Highly efficient *StGBSSI* gene knockout using *Agrobacterium*-based stable transformation

298 Our objective was to knockout the *StGBSSI* gene through the creation of small indels at the
299 locus encoding the KTGGL domain, targeted by sgGBSS1, following *Agrobacterium*-
300 mediated transformation. After transformation of Desiree explants, 21 kanamycin-resistant

11

301 transgenic plantlets were regenerated. Mutations were screened using HRM analysis: 15 out

302 of 21 Desiree transgenic plantlets showed a distinct melting-curve shape, resulting in a 71%

303 editing efficiency at the sgGBSS1 target (Fig. 2a). Because the frequency of mosaic plants is

304 often high in primary transformants (Fauser et al. 2014; Pan et al. 2016; Peng et al. 2017), we

305 cloned PCR amplicons before sequencing to obtain individual sequences. Two primary

306 transformants were selected, for which 18 independent sequences were analysed. In both

307 plants, small deletions were detected in the *StGBSS* targeted site a few bp upstream of the

308 PAM (Fig. 2b). In some cases, deletions downstream of the PAM were also observed, as

309 previously reported in potato (Butler et al. 2015; Wang et al. 2015). Mutations were

310 characterised for four and three alleles for the 17T.701.008 and 17T.701.010 plants,

311 respectively. Interestingly, several different sequences (up to six) were observed for a single

312 allele from the same plant, demonstrating that these primary transformants were mosaic (Fig.

313 2b). Depending on the chimerism level, non-mosaic mutants may be obtained in clonally

314 propagated progenies. In all cases, the mutation was predicted to induce a frameshift or to

315 alter the KTGGL domain, likely leading to a loss of function of the encoded protein.

316 To explore the phenotypic consequences of the targeted mutations, the two mutated plants

317 were transferred to a greenhouse with a natural photoperiod to produce tubers in soil. We did

318 not notice any obvious deleterious effects of the mutation to overall plant growth (Fig. 2c).

319 Tubers were harvested after three months and assessed for their amylose content. First, the

320 iodine solution was directly dropped onto a tuber slice for a quick qualitative analysis,

321 staining brown for the two mutated plants but dark-blue for wild-type plants (Fig. 2d), as

322 expected for starch containing amylose. Starch polysaccharides were then separated by size-

323 exclusion chromatography using a Sepharose CL-2B matrix. Although the amylopectin peak

324 (high mass fraction) was unaffected in the two mutants compared to Desiree, amylose

325 accumulation (low mass fraction) was totally abolished or strongly impaired in the

326 17T.701.008 and 17T.701.010 plants, respectively (Fig. 2d). The residual amylose content in

327 17T.701.010 may result from the presence of a sufficient amount of cells with unedited alleles

328 (Fig. 2b). Taken together, these data clearly show that *StGBSSI* can be efficiently knocked-out

329 in the tetraploid potato by CRISPR-Cas9, leading to modifications in tuber starch quality.

330

331 **Successful gene editing in regenerated potato plants using protoplast transfection**

332   *Agrobacterium*-based gene editing is associated with the integration of the *Cas9*-harbouring

333   transgene. To generate transgene-free edited plants through transient expression of CRISPR

334   components, Desiree protoplasts were transfected using plasmid DNA. In this case, we

335   simultaneously applied two sgRNAs (sgGBSS1 and sgGBSS2) on opposite DNA strands and

336   spaced 135 bp apart, aiming at generating larger deletions. Four months after transfection, 444

337   plantlets were regenerated and their DNA was extracted from leaf samples.

338   Despite the use of the two sgRNAs, only 2 regenerated plants out of 269 (0.7%) showed a

339   PCR band shift consistent with the expected deletion for at least one allele (Fig. 3a). This

340   result indicates that simultaneous cutting by both guides is low. However, 6 out of 269 plants

341   (2.2%) were detected with a clear insertion in the targeted region (Fig. 3a). We also analysed

342   the 17T.716.146 plant, which displayed no wild-type alleles, but both smaller and larger band

343   shifts in the targeted locus. The locus was cloned and sequenced, revealing the expected

344   deletion of 142 bp as well as two insertions of 116 bp and 211 bp, originating from the

345   plasmids used in the experiment (data not shown). This is similar to previous observations

346   made in potato (Andersson et al. 2017). In line with the absence of the wild-type *StGBSSI*

347   allele, the amylopectin content was not affected in soil-grown tubers from 17T.716.146 plants

348   (no growth penalties observed), and amylose accumulation was completely abolished (Fig.

349   3b). This result confirms the loss of function of the *StGBSSI* gene in 17T.716.146 plants.

350   Due to the low efficiency of inducing large deletions using the two-guide strategy, we first

351   used HRM analysis to detect small indels at the sgGBSS1-cutting site: 38 plants out of 444

352   (8.6%) were identified as differing from Desiree at the *StGBSSI* locus (Fig. 3a). For these 38

353   plants, the expected polymorphism was assessed by simultaneously sequencing the four loci

354   by direct Sanger sequencing. The resulting sequences were analysed with ICE software,

355   which determines the rate of CRISPR-Cas9 editing at a specific locus, allowing us to

356   reconstruct the mutated alleles. Most of the mutations consisted of small indels (-23 to +1 bp)

357   leading to frameshifts or amino-acid deletions (Fig. 3a). The region targeted by sgGBSS2

358   could not be similarly analysed because the high natural polymorphism downstream the target

359   prevented us from designing suitable HRM primers (Supplementary Fig. S4).

360   In a tetraploid species, one single allele is sufficient to produce amylose (Andersson et al.

361   2017). Using ICE software, the mutants can be theoretically classified in four categories in a

362   tetraploid species like potato: ICE 25% (one allele likely to be mutated), ICE 50% (2 alleles

363   likely mutated), ICE 75% (3 alleles likely mutated) and ICE 100% (4 alleles likely mutated).

13

364 We therefore screened the 38 *StGBSSI* mutant plants to identify plants with all four alleles

365 mutated. As a control, we cloned and sequenced 10 individual amplicons from the 17.716.302

366 plant, which displayed a 21% ICE score: in agreement with this score, only one allele was

367 modified, associated with a 8 bp deletion. Accordingly, tubers from this plant were not

368 affected in amylose accumulation (Fig. 3c). Most of the confirmed edited plants were

369 similarly mutated in a single allele (29 plants, 77%), but we focused on three plants (9%)

370 mutated at all four alleles (Fig. 3a and Supplementary Fig. S5), including the aforementioned

371 17T.716.146 plant, identified as knocked-out. For the two putative tetra-allelic mutants

372 (17T.716.439 and 17T.716.542), we also cloned and sequenced the sgGBSS1 target locus. No

373 wild-type sequence could be detected among the 20 sequencing reads obtained for each plant.

374 The mutations identified were in accordance with those predicted by the ICE software, with 5,

375 4 and 1 bp deletions for 17T.716.439, and 7, 5 and 4 bp deletions for 17T.716.542 (Fig. 3c

376 and Supplementary Fig. S5). We detected two different mutations for the same allele in

377 17T.716.439 and 17T.716.542, indicating that these mutants may be mosaic (Fig. 3c).

378 Surprisingly, in both plants, sequences corresponding to only three different natural allelic

379 variants could be detected, and the A2 and A1 alleles were not detected in 17T.716.439 and

380 17T.716.542, respectively (Fig. 3c). One possible explanation may be a very large insert in

381 the cutting site of one allele, preventing PCR detection. Alternatively, a change in

382 chromosome number/structure due to somaclonal variation, which is common during plant

383 regeneration from protoplasts (Fossi et al. 2019), potentially explaining the stunted growth of

384 these two plants. The rapid amylose assay performed on an *in vitro* microtuber from

385 17T.716.542 indicates that this mutant was strongly impaired in amylose biosynthesis (Fig.

386 3c).

387 To assess the effectiveness of the protoplast strategy, we then wanted to ensure that no foreign

388 DNA was inserted elsewhere in the plant genome. We performed PCR on the mutated plants

389 with four couples of primers matching the CRISPR/Cas9 plasmids. Interestingly, although we

390 did not observe any amplification for most of the plants (32 plants, 84%), four of them (11%)

391 had integrated at least one large plasmid fragment (>350 bp) (Supplementary Fig. S6). This

392 was confirmed by the sequencing of some of the amplicons. Two of the plants that harboured

393 the *nptII* amplicon successfully grew on a medium containing kanamycin, confirming the

394 presence of a functional *nptII* resistance cassette (Supplementary Fig. S7). We postulate that

395 at least some of these insertions may result from random integration into the genome,

396 although we cannot exclude the insertion of a very large fragment into the target site

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397   (preventing PCR amplification) or into an off-target site.

398   To summarize, the two-guide strategy did not improve the efficiency of mutagenesis, but we
399   were able to edit and knockout the *StGBBSI* gene in regenerated plantlets by transiently
400   expressing CRISPR-Cas9 components into potato protoplasts. In comparison with the
401   *Agrobacterium*-mediated knockout approach, we were able to regenerate plants without large
402   insertions of T-DNA fragments, although foreign DNA insertions could be detected in a
403   subset of plants. However, smaller insertions may be present as part of the substantial
404   chromosomic reshuffling caused by protoplast regeneration(Fossi et al. 2019). Finally, a high
405   number of regenerated plants displayed stunted growth (20 to 40%), confirming the major
406   drawback of protoplast-based regeneration.

407   As a direct application of our work on Desiree, a cultivar commonly used in laboratories for
408   many years, we targeted the *StGBSSI* gene through protoplast transfection in the Furia
409   cultivar, a recently registered starch potato. We applied a single-guide approach with
410   sgGBSS1 only and, four months after transfection, we detected 42 out of 259 regenerated
411   plants (16%) as mutated at this locus using HRM (Fig. 4a). In total, eight plants (19% of the
412   mutants) were identified by Sanger sequencing as potential tetra-allelic mutants (Fig. 4b, c).
413   This suggests that our strategy can be readily developed in other cultivars for plant molecular
414   breeding, but with the same potential drawbacks of somaclonal variation and residual foreign
415   DNA integration (data not shown).

416

417   **Precise and efficient base editing using a CRISPR-Cas9 cytidine deaminase fusion**

418   Recently, substitution of nucleotides without DSBs has been successfully carried out on
419   plants using base editing tools. Here, we used a cytidine base editor (Shimatani et al. 2017) to
420   target two loci encoding catalytic domains of the StGBSSI enzyme. One suitable guide
421   sequence in each target locus was designed, named sgGBSS3 (targeting the KTGGL encoding
422   locus) and sgGBSS4 (targeting the PSRFEPCGL encoding locus) (Fig. 1). We carried out two
423   independent *Agrobacterium*-mediated explant transformations in Desiree, leading to the
424   regeneration of 48 and 15 transgenic plants targeting sgGBSS3 and sgGBSS4 sequences,
425   respectively. HRM analysis suggests that both sgGBSS3- and sgGBSS4-targeted sites were
426   efficiently mutated with 43 out of 48 transgenic plants and 13 out of 15 transgenic plants
427   edited, respectively. Direct Sanger sequencing confirmed these results, demonstrating a very

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428   high mutation efficiency, close to 90% (Fig. 5a).

429   The sgGBSS4-targeted site had been selected to assess the edition efficiency of three closely

430   located Cs in the edition window (C_{-20}, C_{-19} and C_{-15}, counting from the PAM). Sanger

431   sequencing showed that these 3 Cs can be substituted with a T, and to a lesser extent with a G

432   or an A, but strikingly indels were very frequently found (77%), mainly in the edition window

433   (data not shown). Moreover, we confirmed that, consistent with the presence of a mismatch

434   with one allele at the distal end of the guide (Fig. 1), complete editing of the four alleles could

435   not be characterized in the sgGBSS4 target zone.

436   For the sgGBSS3-targeted site, Sanger sequencing revealed that only C_{-17} in the editing

437   window was most frequently substituted with a G or a T, and to a much lesser extent with an

438   A (Fig 5a-b). In all edited plants, we observed a C_{-17}-to-G_{-17} substitution, while a C_{-17}-to-T_{-17}

439   transition occurred in 30 mutated plants (70%) (Fig. 5a and Supplementary Fig. S8). C_{-17}-to-

440   A_{-17} conversion was detected in only four plants (9%). Among the 43 mutants, indels were

441   observed in nine plants (21%), with an initiation site mainly located in the vicinity of the C_{-17}

442   (Supplementary Fig. S8). As expected, we did not observe base substitution at positions

443   surrounding the edition window. In particular, we identified a perfect C_{-17}-to-G_{-17} conversion

444   in two plants (18T.511.039 and 18T.511.073) (Fig. 5a-b). Interestingly, the complete

445   homozygous conversion of the target base hinders detection by HRM, a fact that can be

446   circumvented by the addition of a small amount of wild-type DNA into the samples (spiking)

447   to clearly alter the melting shape (Fig. 5b). The cloning and sequencing of the targeted region

448   in both plants confirmed that these plants were tetra-allelic for the mutation and non-mosaic

449   (Fig. 5b). This C_{-17}-to-G_{-17} base substitution leads to the replacement of the leucine (L) by a

450   valine (V) in the KTGGL motif, potentially leading to a loss of function of the protein. The

451   StGBSSI 3D protein structure was modelled based on available 3D structures (see Methods)

452   and the mutated residue L99V was reported on the GBSSI 3D model (Fig 5c). It is located

453   within an α-helix and is likely to impair its structure, subsequently leading to abnormal

454   GBSSI protein folding and/or impacting the glucose binding. The amylose assay on *in vitro*

455   microtubers supports this assumption, because amylose accumulation was totally impaired in

456   the two base-edited plants compared with wild-type Desiree (Fig. 5d), indicating that the

457   leucine residue in the KTGGL is essential for StGBSSI catalytic activity.

458

16

Discussion

Gene editing using CRISPR-Cas9 is efficient in elite cultivars of tetraploid potato

Our work demonstrates that the delivery of CRISPR-Cas9 components either through stably integrated transgene(s) or transient expression of plasmids can efficiently induce targeted mutations into the *StGBSSI* gene in elite cultivars of the tetraploid potato. Using *Agrobacterium*-mediated transformation, we successfully obtained, in the first generation, tetra-allelic mutants with impaired amylose biosynthesis, confirming the loss of function of the StGBSSI enzyme (Fig. 2). For fundamental research purposes, the persistence of the transgene(s) may not be a problem because Tang et al. (2018) showed that the continued presence of CRISPR-Cas9 reagents in rice does not cause off-target mutations if sgRNAs are rigorously designed.

In sexually propagated species, the stably integrated transgene can be eliminated through Mendelian segregation, generating transgene-free lines (Ricroch et al. 2017). However, the segregation of the transgene is not feasible in the vegetatively propagated cultivated potato, because selfing would change the cultivar characteristics of this highly heterozygous species. Thus, regenerated mutants lacking unwanted insertion of foreign DNA are of utmost interest, especially for commercial applications. With this goal, we used the transient expression of CRISPR-Cas9 plasmids to generate transgene-free knockout mutants. While we conducted these experiments, two studies were published showing how the *StGBSSI* gene can be knocked-out in plantlets regenerated from protoplasts of the Kuras cultivar, using both plasmid DNA and RNPs, respectively (Andersson et al. 2017; Andersson et al. 2018). Our results are consistent with these findings, extending them to other cultivars and using different transfection and screening strategies. Although being less efficient than *Agrobacterium*-mediated stable transformation, we demonstrated that PEG-mediated transformation of Desiree and Furia protoplasts is efficient for the generation of tetra-allelic edited plants at a reasonable rate (8 to 19% of all mutated plants), producing plants impaired in amylose biosynthesis.

At the same time, our work highlights caveats associated with genome editing approaches in the highly heterozygous tetraploid potato. Rigorous analysis of the target locus must be carried out to avoid polymorphism that will impair tetra-allelic editing (as shown here for sgGBSS4) or genotyping (as exemplified here by the locus downstream from sgGBSS2).

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490   Similarly, we confirm here that a dual sgRNA approach on opposite DNA strands does not

491   necessarily lead to an efficient deletion rate of a large segment of the gene, probably because

492   the cutting efficiencies at the two sgRNA-targeted sites are not similar. For an efficient and

493   cost-effective screening of mutants, we present a streamlined approach with an HRM analysis

494   followed by direct Sanger sequencing of positive plants and accompanied by sequence

495   analysis mediated by the ICE algorithm.

496   During the protoplast transfection process, the delivery of CRISPR-Cas9 components through

497   plasmids may result in the insertion of degraded DNA fragments into the target sequence

498   (Salomon and Puchta 1998), but also into random sites (Kim et al. 2017; Liang et al. 2017).

499   Accordingly, we found that a substantial rate of mutants harboured insertions in the target

500   sequence, which may originate from plasmid DNA and/or host genome. These results

501   corroborate previous studies using transient transfection in potato protoplasts, revealing a high

502   rate of DNA insertions (Andersson et al. 2018; Clasen et al. 2016). Moreover, the

503   unpredictable insert sequence can impair the detection of foreign DNA insertions into the

504   plant genome, likely underestimating the rate of random and unwanted insertions. Whole-

505   genome sequencing of the mutated line(s) may be an exhaustive - and expensive - option, as

506   done in tomato (Nekrasov et al. (2017) and in rice (Tang et al. (2018). Another strategy for

507   CRISPR-Cas9 expression relies on the use of CRISPR-Cas9 ribonucleoproteins (RNP). This

508   method has been successfully developed in some plant species, including potato, completely

509   avoiding the risk of foreign DNA insertion(s) into the host genome (Andersson et al. 2018;

510   Liang et al. 2017; Woo et al. 2015). Recently, Chen et al. (2018) developed a method using an

511   *Agrobacterium*-mediated transient CRISPR-Cas9 gene expression system to generate

512   transgene-free mutants without the need for sexual segregation, which holds great potential

513   for vegetatively propagated crops. Furthermore, we developed a strategy for the production of

514   T-DNA free edited plants using the *Agrobacterium*-mediated delivery of a CBE, opening up

515   new perspectives for genome engineering, especially in vegetatively propagated species like

516   potato (Veillet et al. 2019). The use of nanomaterials for CRISPR components delivery also

517   holds great promises for genome editing without foreign DNA integration (Demirer et al.

518   2019). These promising strategies, which directly generate transgene-free edited plants

519   without the need for transgene segregation, may provide science-based evidences for

520   decision-makers and may also help to reduce public concerns about gene edited crops.

521   Somaclonal variation in regenerated plantlets from protoplasts or *Agrobacterium*

522   transformation constitutes another bottleneck for an efficient gene editing strategy, especially

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523 for commercial purposes. For example, this type of variation can result in changes in the

524 number of chromosomes from polyploidy to aneuploidy, chromosome rearrangements and

525 DNA base deletions and substitutions (Krishna et al. 2016). In our conditions, we observed a

526 substantial rate of plants with stunted growth or abnormal development, which may be due to

527 somaclonal/epigenetic variation (Fossi et al. 2019). Collectively, our results suggest that many

528 regenerated plants need to be screened to isolate clean edited plants suitable for commercial

529 purposes. Therefore, the improvement of the transformation/regeneration method is crucial to

530 obtain a higher ratio of tetra-allelic mutants.

531

532 **CRISPR-Cas9 cytidine deaminase fusion precisely and efficiently edits targeted bases**

533 Base editing using CBE has been recently developed in some plant species, allowing the

534 precise modification of cytidine residues in a small edition window. In this study, we

535 successfully applied CBE to the tetraploid potato using *Agrobacterium*-mediated

536 transformation. We obtained a high rate of edited plants for the two targeted-sites, close to

537 90% of the transgenic plants. By targeting the active site KTGGL, we achieved a perfect C_{-17}-

538 to-G_{-17} substitution in all the alleles of two independent plants, leading to a L99V substitution

539 in this motif. This amino acid change, which is predicted to alter the function of StGBSSI,

540 effectively led to amylose biosynthesis impairment. Although this mutation resulted in a loss-

541 of-function allele, such precise genome editing approach is very likely to help create new

542 allelic variants with potentially modified activity. This is a promising result for the

543 characterization of specific protein motifs in potato, but also for crop improvements through

544 the production of gain-of-function mutants, for example exhibiting resistance to plant

545 pathogens at no yield loss (Bastet et al. 2019). Based on our results, we suggest that further

546 studies are needed to draw up the guidelines for designing more efficient sequence guides.

547 One such avenue to explore is whether C-rich regions are likely to be associated with indels,

548 which should be avoided for precise gene editing. Similarly, instead of the expected C-to-T

549 mutation, C-to-G/-A conversion may be attractive for the diversity of amino-acid

550 substitutions, but unwanted in some cases. To avoid such undesired products due to uracil

551 excision and downstream repair systems, the addition of a uracil DNA glycosylase inhibitor

552 protein (UGI) to the construct is a promising approach, because this strategy has been shown

553 to result in a majority of C-to-T conversions and a reduced rate of indel formation (Nishida et

554 al. 2016). Furthermore, the recent application of base editing in potato using a fusion of

19

555   human APOBEC3A, nCas9 and UGI demonstrated that C-to-T conversion is efficiently

556   mediated in a 17 bp edition window, independently of the sequence context and with a very

557   low frequency of indels or undesired edits (Zong et al. 2018).

558   Base editing technology will benefit from the expanding base editing toolbox. In particular,

559   an adenine base editor (ABE) has been developed, mediating clean A-to-G substitutions

560   (Schindele et al. 2018). Together with CBEs, this new ABE opens up further possibilities for

561   fine-tuning allele variation. Nevertheless, a recent study pointed out that CBEs, but not ABEs,

562   can generate genome-wide off-target mutations (Jin et al. 2019), highlighting the need for an

563   optimization of CBEs and/or the use of delivery methods reducing the expression of CRISPR

564   components to a few days. Finally, CRISPR-based base editing requires the presence of a

565   PAM sequence that places the target base(s) within a small base-editing window. This

566   requirement limits the number of sites that can be targeted in plant genomes, as experienced

567   in our work for the loci encoding catalytic domains of the StGBSSI. To overcome this

568   limitation, the commonly used *Sp*Cas9 nickase can be replaced by Cas9 orthologues from

569   other bacterial and archeal species that display alternative PAM compatibilities. For example,

570   *Sp*Cas9 orthologues from *Streptococcus thermophilus* and *Staphylococcus aureus* have been

571   successfully used for gene editing in *Arabidopsis* (Steinert et al. 2015). Furthermore, (Hua et

572   al. 2018b) developed new CBEs and ABEs with engineered *Sp*Cas9 and *Sa*Cas9 nickase

573   variants that considerably expand the targetable sites in the rice genome. Great efforts have

574   been made recently in engineering expanded PAM *Sp*Cas9 variants (xCas9 and Cas9-NG)

575   with broadened PAM compatibility in mammalian and plant cells (Endo et al. 2018; Hu et al.

576   2018; Nishimasu et al. 2018; Wang et al. 2018), opening new exciting avenues for base

577   editing.

578

579   **Conclusion**

580   Our present work confirms that the *StGBSSI* gene can be successfully targeted and altered in

581   elite potato cultivars using gene and base editing systems. Therefore, the *StGBSSI* appears to

582   be a very good - and economically feasible - gene model to assess genome editing in potato,

583   as well as its potential future technological improvements. Furthermore, these results can be

584   transferred to other starch-producing crops: the targeted mutagenesis of *GBSS* was recently

585   shown to be efficient in modifying amylose content in cassava (Bull et al. 2018). With the

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586 extraordinarily rapid development of genome editing tools in both animals and plants,
587 researchers are now able to efficiently characterize genes underlying important agronomic
588 traits directly into crops, at the base-pair level. Along with the development of plant
589 transformation and regeneration processes, there is no doubt that genome editing will have a
590 tremendous impact on basic research and on molecular crop improvement.

591

Author contributions

593 FV, LC and JEC designed the experiments. FV, LC, MPK, ZT, FS, MM and NS performed
594 the experiments. FV and JLG wrote the article. FV, LC, FS, NS, PD, JLG and JEC discussed
595 the data and revised the article. All authors approved the final manuscript.

596

Funding

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Conflict of interest statement

604
605 The authors declare that the research was conducted in the absence of any commercial or
606 financial relationships that could be construed as a potential conflict of interest.
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Acknowledgments

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617   the BrACySol BRC (INRA Ploudaniel, France) that provided us with the plants that were

618   used in this study and Gisèle Joly and Catherine Chatot from Florimond Desprez/Germicopa

619   (France) for helpful discussions and choice of the starch elite cultivar Furia. We are grateful

620   to Jean-Louis Rolot from CRAW (Belgium) for providing us with the procedure to obtain *in*

621   *vitro* microtubers as well as to Marie-Ange Dantec and all the INRA Ploudaniel greenhouse

622   staff for acclimation and cultivation of the regenerated plantlets. Finally, we are thankful to

623   Carolyn Engel-Gautier and Marina Perez Benitez for their help in correcting the manuscript.

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625
626
627   **Figure legends**

628
629   **Fig. 1**

630
631   *StGBSSI* **structure and CRISPR-Cas9 targets.** The *StGBSSI* gene structure is composed of

632   13 exons (in black) and 12 introns (in grey), with 5'UTR and 3'UTR on both sides (in blue).

633   The localization and sequences of the four targeted loci used in this study are indicated, as

634   well as the allelic variation (in green) in the loci encoding the PSRFEPCGL catalytic domain.

635   The guide sequences (sgGBSS) are depicted in blue and the PAM sequence in red.

636   Polymorphism between allelic variants is not shown.

637

638   **Fig. 2**

639   **Generation and identification of CRISPR-Cas9-mediated mutations in primary**

640   **transformants of potato cultivar Desiree. (a)** High-resolution melting (HRM) analysis of

641   *Agrobacterium*-transformed potato plants. Desiree sample was defined as the base curve (blue

642   line). Non-mutated plants are shown in blue and the mutated ones are shown in different

643   colours, according to the shape of their melting curve. **(b)** Alignment of sequencing reads

644   from *StGBSSI* target locus of two independent mutated primary transformants with the

645   sequences from their respective wild-type alleles (named A1/2/3/4, and highlighted in blue).

646   Natural polymorphism between the four Desiree allelic variants is outside of the represented

647   window. The length of deletion (-) or insertion (+) is indicated on the right of each read. The

648   PAM motif is shown in red and the sgGBSS1 target locus in blue. **(c)** Picture of about 2

649   month-old potato plants grown in soil in a greenhouse with a natural photoperiod. **(d)**

650   Determination of amylose content in tubers harvested from soil-grown potato with a rapid

22

651   iodine test (pictures) and with size-exclusion chromatography (graph). Amylopectin and

652   amylose peaks are indicated with black arrows.

653

654   **Fig. 3**

655   **Generation and identification of CRISPR-Cas9-mediated *StGBSSI* mutations in**

656   **regenerated plants from PEG-mediated transfection of potato cultivar Desiree**

657   **protoplasts. (a)** Genotyping strategies for selecting deletions in *StGBSSI*. The PCR band shift

658   assay using primers on both sides of the targeted region (sgGBSS1 and sgGBSS2) is shown in

659   red, and the HRM analysis at the sgGBSS1-targeted site is given in blue. Exons and introns

660   are shown in dark and grey, respectively. The frequency of mutated plants was calculated

661   based on the number of regenerated and analysed plants. The nature of mutations was

662   determined after Sanger sequencing using both manual analysis and ICE software. Most

663   plants could be assigned an ICE score (≈ 25, 50, 75 or 100%). **(b)** Determination of amylose

664   content in tubers harvested from soil-grown potato plants using a rapid iodine test (pictures)

665   and with size-exclusion chromatography (graph). Amylopectin and amylose peaks are

666   indicated with black arrows. **(c)** Sanger sequencing chromatograms (C in blue, G in dark, A in

667   green and T in red) and Sanger sequencing reads from a few regenerated plants at the

668   sgGBSS1-targeted locus. The ICE score and the mutation are indicated in grey. The wild-type

669   allelic sequences are highlighted in blue (A1/2/3/4). Polymorphism between Desiree allelic

670   variants is not located within the sequencing window. The length of deletion (-) is indicated

671   on the right of each read. The PAM motif is shown in red and the sgGBSS1-targeted locus in

672   blue. Determination of amylose content with a rapid iodine test in tubers harvested from soil-

673   grown (17T.716.302) or from *in vitro*-propagated (17T.716.542) potatoes is shown on the left

674   of the chromatogram.

675

676   **Fig. 4**

677   **Generation and identification of CRISPR-Cas9-mediated *StGBSSI* mutations in**

678   **regenerated plants from PEG-mediated transfection of potato cultivar Furia**

679   **protoplasts. (a)** High-resolution melting (HRM) analysis of regenerated potato plants. Furia

680   sample was defined as the base curve (yellow). Mutated plants are shown in different colours,

681   according to the shape of their melting curve. **(b)** Summary of the mutation detection strategy.

682   Frequency of mutated plants was calculated based on the number of regenerated and analysed

683   plants. The nature of mutations was determined after Sanger sequencing using a manual

684   analysis and ICE software. Most plants could be assigned an ICE score (≈ 25, 50, 75 or

23

685   100%). **(c)** Sanger sequencing chromatograms from Furia and two mutated plants at the

686   sgGBSS1-targeted locus (C in blue, G in dark, A in green and T in red). The ICE score and

687   the mutation are indicated in grey for each callus. The PAM motif is indicated in red and the

688   sgGBSS1-targeted locus in blue.

689

690   **Fig. 5**

691   **Generation and identification of CBE-mediated mutations in primary transformants of**

692   **potato cultivar Desiree. (a)** Base-editing efficiencies at sgGBSS3 target in primary

693   transformants. The number of plants harbouring a specific nucleotide conversion is indicated

694   for C_{-17}. **(b)** Sanger sequencing results and high-resolution melting (HRM) outputs for two

695   edited plants at the sgGBSS3 target harbouring a perfect C_{-17}-to-G_{-17} substitution. The HRM

696   analysis was performed without or with the addition of a small amount of Desiree DNA in the

697   samples (spiked). Non-mutated profiles are shown in blue and the mutated ones are shown in

698   red, according to the shape of the melting curve. The chromatograms are the result of direct

699   sequencing of all four alleles together. Grey arrows indicate the location of base substitutions.

700   Sanger reads were aligned with the wild-type allelic sequences (in blue). Natural

701   polymorphism between Desiree allelic variants is not shown. Base substitutions are

702   highlighted in yellow. The PAM motif is indicated in red and the sgGBSS3-targeted locus in

703   blue. Amino-acid residues for all four alleles of the edited plants are indicated below the

704   Sanger reads. Changes in amino-acid residues are highlighted in red. K: Lysine, T: Threonine,

705   G: Glycine, L: Leucine, V: Valine, D: Aspartic acid. **(c)** Localisation of mutated residue in the

706   3D model of StGBSSI. Overall 3D model of StGBSSI (i), zoom on L99 (ii) and the L99V

707   mutation (iii). The StGBSSI consists of 24 α-helix (in red) and 19 β-strands (in yellow). L99

708   is shown in blue (ii). L99 (situated in α-helix5) is mutated in V99 and the clash between the

709   side chain from V99 and other residues is indicated by a red square (iii). **(d)** Determination of

710   amylose content in microtubers harvested from *in vitro* plants with a rapid iodine test (picture)

711   and with a size-exclusion chromatography (graph). Amylopectin and amylose peaks are

712   indicated with black arrows.

713

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sgGBSS1　　**PAM**

Desiree A1	G T T G G T C C T T G G A G C A A A A C T G G
Desiree A2	G T T G G T C C T T G G A G C A A A A C T G G
Desiree A3	G T T G G T C C T T G G A G C A A A A C T G G
Desiree A4	G T T G G T C C T T G G A G C A A A A C T G G
Furia A1	G T T G G T C C T T G G A G C A A A A C T G G
Furia A2	G T T G G T C C T T G G A G C A A A A C T G G
Furia A3	G T T G G T C C T T G G A G C A A A A C T G G

PAM　　**sgGBSS2**

Desiree A1	C C G C G G A C A T C G G G T A A T G A C A A
Desiree A2	C C G C G G A C A T C G G G T A A T G A C A A
Desiree A3	C C G C G G A C A T C G G G T A A T G A C A A
Desiree A4	C C G C G G A C A T C G G G T A A T G A C A A

5'　　　　　　　　　　　　　　　　　　　　　　　　　　3'

KTGGL domain

sgGBSS3　　**PAM**

Desiree A1	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Desiree A2	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Desiree A3	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Desiree A4	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Furia A1	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Furia A2	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G
Furia A3	A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G

PSRFEPCGL domain

sgGBSS4　　**PAM**

Desiree A1	C C A A G C A G A T T T G A A C C T T G T G G T C T C
Desiree A2	C C A A G C A G A T T T G A A C C T T G T G G T C T C
Desiree A3	C C A A G C A G A T T T G A A C C T T G T G G T C T C
Desiree A4	C C A A G T A G A T T T G A A C C T T G T G G T C T C

Figure 1

(a)

Normalized and Temp-shifted difference plot

Relative signal difference vs Temperature (°C)

Desiree

(b)

17T.701.008

sgGBSS1   PAM

```
A1  A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C T A G G T G A T G T T C T T G
    A G G T T G G T C C T T G G A - - - A A C T G G T G G A C T A G G T G A T G T T C T T G    -4
    A G G T T G G T C C T T - - - - - - - - - - - - T G G T G G A C T A G G T G A T G T T C T T G    -11
A2  A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C T A G G T G A T G T T C T T G    0
    A G G T T G G T C C T T G G A G C - A A A A C T G G T G G A C T A G G T G A T G T T C T T G    -1
    A G G T T G G T C C T T G G A G C - A A A C T G G T G G A C T A G G T G A T G T T C T T G    -2
    A G G T T G G T C C T T G G A - - - A A C T G G T G G A C T A G G T G A T G T T C T T G    -4
    A G G T T G G T C C T T G G - - - - - A C T G G T G G A C T A G G T G A T G T T C T T G    -6
    A G G T T G G T C - - - - - - - - - - - - - - - - C T A G G T G A T G T T C T T G    -20
A3  A G G T T G G T C C T T G G A G C - A A A A C T G G T G G A C T A G G T G A T G T T C T T G    -1
    A G G T T G G T C C T T G G A G C - A A A C T G G T G G A C T A G G T G A T G T T C T T G    -4
    A G G T T G G T - - - - - - - - - - - - - - - - T G G T G G A C T A G G T G A T G T T C T T G    -19
    A G - - - - - - - - - - - - - - - - - - - - - - - A C C A A G A G A C C T G G A T G    -20/+13
A4  A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C T A G G T G A T G T T C T T G
    A G G T T G G T C C T T G G A G C - A A A C T G G T G G A C T A G G T G A T G T T C T T G    -1
```

17T.701.010

sgGBSS1   PAM

```
A1  T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C    0
    T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C    -31
    T T T C - - - - - - - - - - - - - - - - - - - - - - - - - - - - T G G T G G A C
A2  T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C
A3  T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C
    T T T G T G G G T A C T G A G G T T G G T C C T T G - - - - - - A A C T G G T G G A C    -7
A4  T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A A A A C T G G T G G A C
    T T T G T G G G T A C T G A G G T T G G T C C T T G G A G C A - A A C T G G T G G A C    -1
```

(c)

Desiree   17T.701.008   17T.701.010

(d)

Desiree
Amylopectin
Amylose

17T.701.008
Amylopectin

17T.701.010
Amylopectin

OD_{max} of iodine-polysaccharide complex

Wavelength at OD_{max} in nm
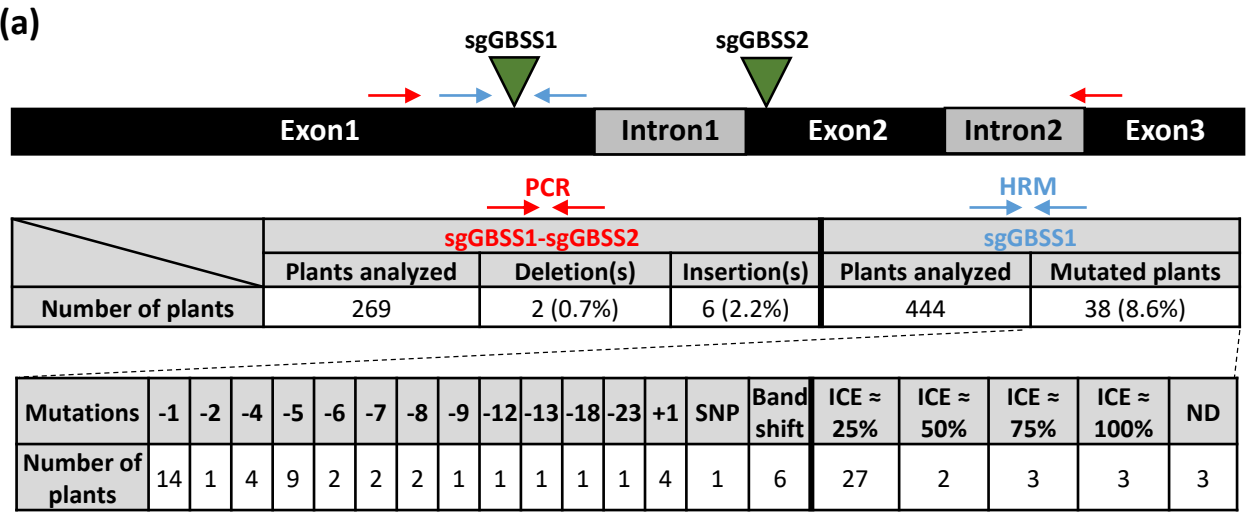
Elution volume in mL

Figure 2

Figure 3

(a)

Normalized and Temp-shifted difference plot

(b)

	sgGBSS1	
	Plants analyzed	Mutated plants (HRM)
Nb of plants	259	42 (16%)

Mutations	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10	-11	-15	-18	-22	+1	Band shift	ICE ≈ 25%	ICE ≈ 50%	ICE ≈ 75%	ICE ≈ 100%	ND
Nb of plants	13	8	3	6	10	4	2	3	2	2	2	1	1	1	2	24	19	2	7	5	9

(c)
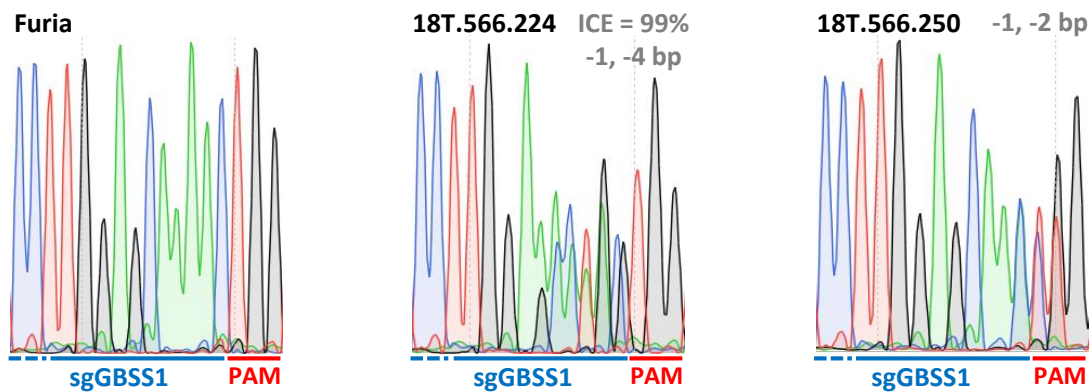
Figure 4

(a)

	Nb of plants	Edited plants	Base composition at N$_{-17}$ position							
			G	T,G	C,G	A,T,G	C,G,A	C,G,T	C,G,T,A	Indels
sgGBSS3	48	43 (90%)	2	11	2	2	1	15	1	9

(b)

Desiree   18T.511.039   18T.511.073

C$_{-17}$ G$_{-17}$ G$_{-17}$

Normalized and Temp-shifted difference plot

Relative signal difference

18T.511.039 spiked   18T.511.073 spiked

Desiree   18T.511.039   Desiree   18T.511.073

Temperature (°C)

sgGBSS3   PAM

A1 A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G A
A2 A A A A C T G G T G G A G T A G G T G A T G T T C T T G G T G G A
A3 A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G A
A4 A A A A C T G G T G G A C T A G G T G A T G T T C T T G G T G G A

K T G G L→V G D V L G G

(c) (i) (ii) (iii)

(d)

Desiree

Amylopectin   Amylose

18T.511.039

OD$_{max}$ of iodine-polysaccharide complex   Amylopectin   Wavelength at OD$_{max}$ in nm

18T.511.073

Amylopectin

Elution volume in mL

Figure 5