

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

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1 Key Message

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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

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

23
24

25 **Introduction**

26

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

33 In higher plants, photosynthetic cells produce transitory starch in chloroplasts and also
34 export sucrose to heterotrophic organs, where it is converted to starch for long-term storage in
35 amyloplasts (Lemoine et al. 2013). Starch is an insoluble glucan composed of two polymers
36 of glucose, the ratio of which strongly determines its physicochemical properties. Amylose is a
37 nearly linear glucose polymer with α -1,4-linked residues, whereas amylopectin is made up of
38 α -1,4-linked chains with α -1,6-linkages at branch points, conferring crystallinity to the starch
39 granule. In higher plants, starch biosynthesis is mainly mediated by four classes of enzymes:
40 ADP-glucose pyrophosphorylases, starch synthases (SSs), starch branching enzymes (SBEs)
41 and starch debranching enzymes (SDBEs) (Zeeman et al. 2010). Starch synthases isoforms
42 SSI, SSII, SSIII govern the elongation of the chains of amylopectin. SSIV is involved in
43 starch initiation; the role of SSV and SSVI is still unclear (Helle et al. 2018; Roldan et al.
44 2007). In addition, granule-bound starch synthase (GBSS) binds to the starch granule and
45 mediates amylose biosynthesis (Ball et al. 1998; Rongine De Fekete et al. 1960).

46 Amylose determines many physicochemical properties of starch, namely its pasting
47 temperature and viscosity (Bull et al. 2018; Park et al. 2007). Therefore, modifying potato
48 starch composition by decreasing the amylose (or amylopectin) content may be useful for
49 industrial applications. For example, modulating GBSSI function directly *in planta* can lead
50 to the reduction of post-harvest treatments (Sonnewald and Kossmann 2013). In most dicot
51 species, such as potato, the GBSSI protein is encoded by a single nuclear locus (Cheng et al.
52 2012). This monogenic control has facilitated the production of amylose-free potato varieties
53 through mutational breeding (Hovenkamp-Hermelink et al. 1987; Muth et al. 2008). In some
54 studies, transgenic approaches have been used to silence the *GBSSI* gene through antisense
55 (Kuipers et al. 1994; Visser et al. 1991) or RNAi technologies (Andersson et al. 2003).
56 Although the genetically modified potato Amflora (BASF) has been commercialized for two
57 years, to date the development of such transgenic crops for commercial purposes has been

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 (*SpCas9*), 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

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.

121

122 **Materials and methods**

123 **Plant material**

124 Potato cultivars Desiree (ZPC, the Netherlands) and Furia (Germicopa, France) were *in vitro*
125 propagated in 1X Murashige and Skoog (MS) medium (pH 5.8) including vitamins (Duchefa,
126 the Netherlands), 0.4 mg/L thiamine hydrochloride (Sigma-Aldrich, USA), 2.5% sucrose and
127 0.8% agar powder (VWR, USA). Plants were cultivated in a growth chamber at 19°C with a
128 16:8 h light:dark photoperiod. For the production of *in vitro* microtubers, plants were placed
129 in 5 mL of 1X MS culture medium for about one month at 19°C with a 16:8 h light:dark
130 photoperiod, until depletion of the medium. Then, 4 mL of half-strength MS medium
131 supplemented with 50 µg/mL coumarin and 4 µg/mL kinetin were added and the plants were
132 transferred to a dark room at 20°C for about five additional weeks, until microtubers were
133 sufficiently developed. The production of tubers in soil was carried out by transferring *in vitro*
134 plants to a greenhouse. Watering was stopped 2 weeks before the tubers were harvested.

135

136 **Target identification**

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

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

152 (Haeussler et al. 2016). In this study, four guides were designed upstream of the NGG PAM
153 and were named sgGBSS1 (5'-GTTGGTCCTTGGAGCAAAC-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 *SIDELLA* locus, the plasmid was digested by the FastDigest restriction enzymes
176 *BstXI* and *SpeI* 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 *BstXI* and
179 a *SpeI* 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

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

188 ***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

205 **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

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

223 **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

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>) (Hsiao et al. 2018).

255

256 **Amylose assay**

257 For a visual assay, 20 μ L 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 μ m 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, \approx 1-2 mg of starch were dissolved in 500 μ L of 10 mM
266 NaOH and loaded on a CL-2B column (0.5 cm x 65 cm). 300 μ L fractions were collected at a
267 flow rate of 12 mL/h prior to measuring the OD and λ_{\max} 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

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

280 **Results**

281 **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 PSRFPCGL 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

296 **Highly efficient *StGBSSI* gene knockout using *Agrobacterium*-based stable** 297 **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

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).

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 *nptIII* amplicon successfully grew on a medium containing kanamycin, confirming the
394 presence of a functional *nptIII* 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

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 *StGBSSI* 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 chromosomal 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 PSRFEP CGL 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

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

459 Discussion

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

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

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

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

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

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-G₁₇ 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

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 *SpCas9* nickase can be replaced by Cas9 orthologues from
569 other bacterial and archaeal species that display alternative PAM compatibilities. For example,
570 *SpCas9* 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 *SpCas9* and *SaCas9* nickase
573 variants that considerably expand the targetable sites in the rice genome. Great efforts have
574 been made recently in engineering expanded PAM *SpCas9* 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

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

592 **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

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601

602

603 **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.

607

608

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610

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624

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 PSRFEPGL 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

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 (\approx 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 (\approx 25, 50, 75 or

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 C₁₇. **(b)** Sanger sequencing results and high-resolution melting (HRM) outputs for two
695 edited plants at the sgGBSS3 target harbouring a perfect C₁₇-to-G₁₇ 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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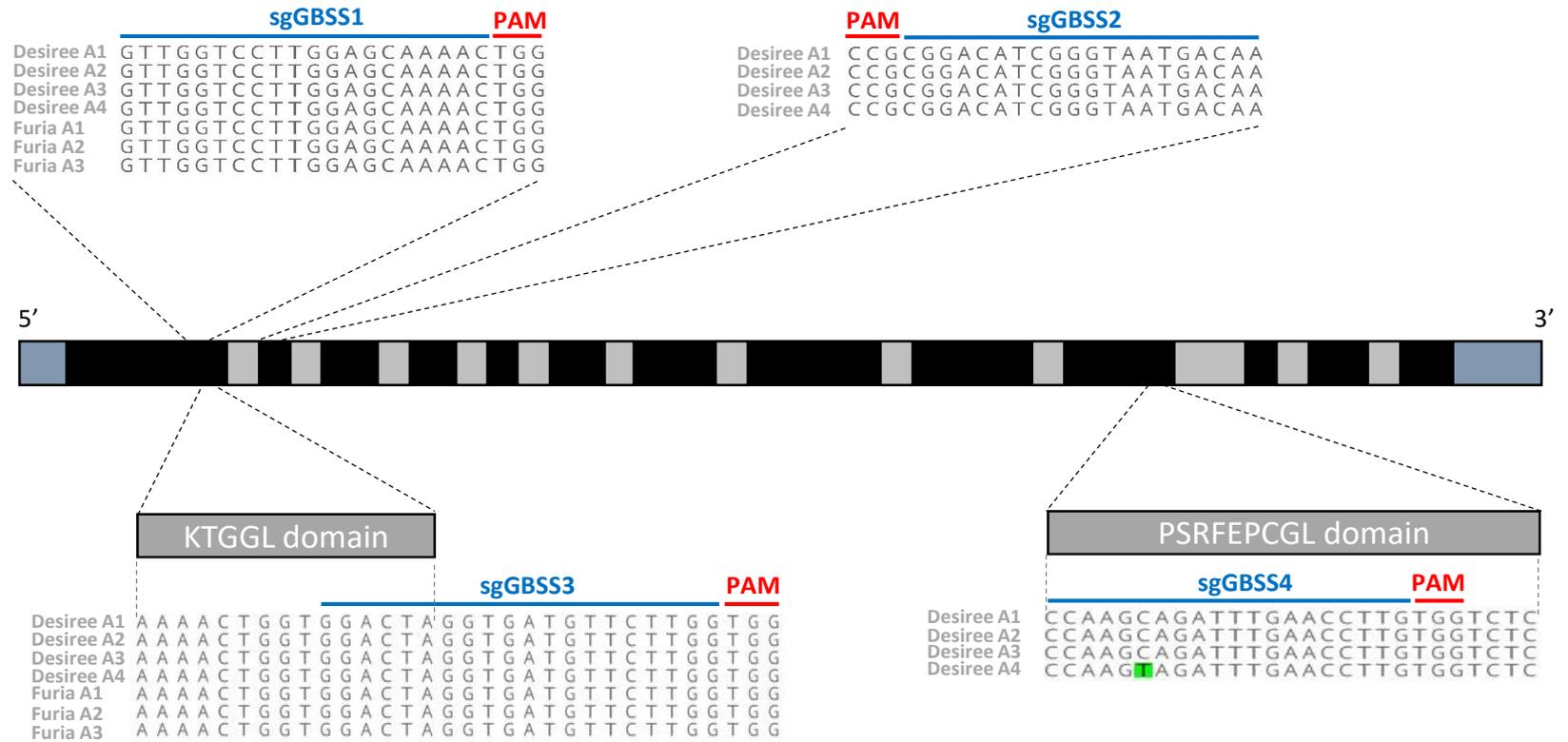


Figure 1

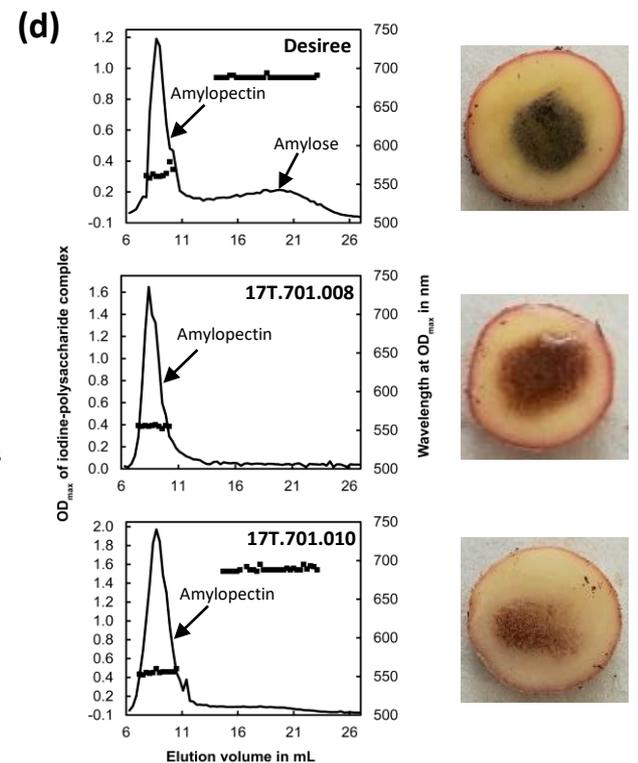
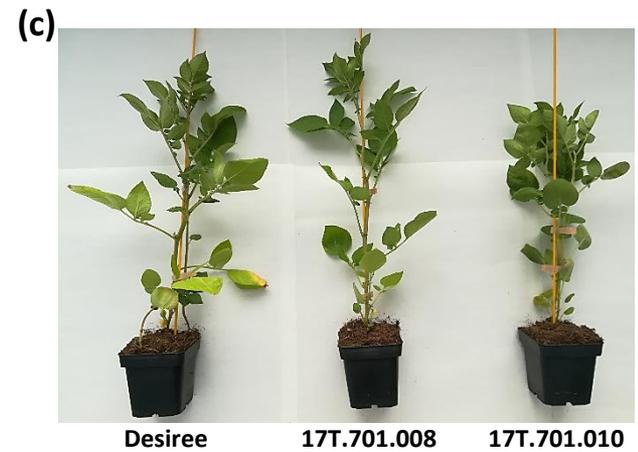
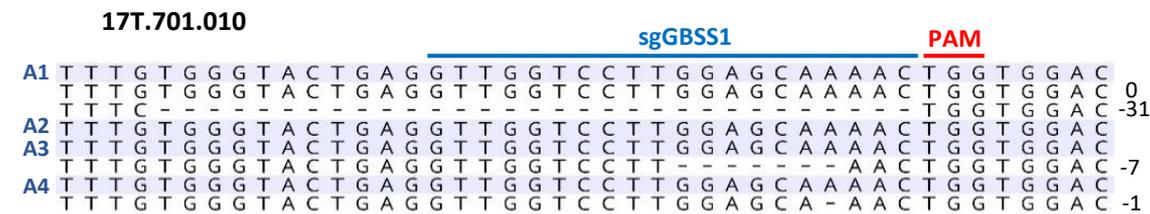
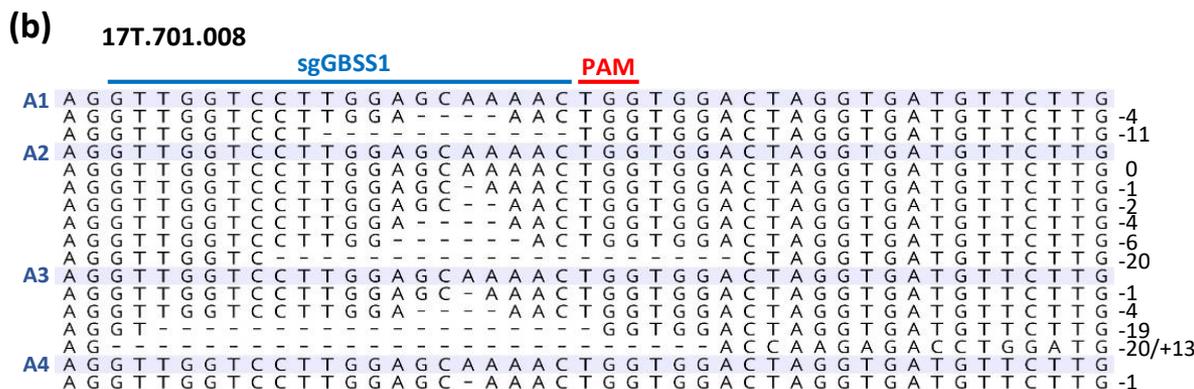
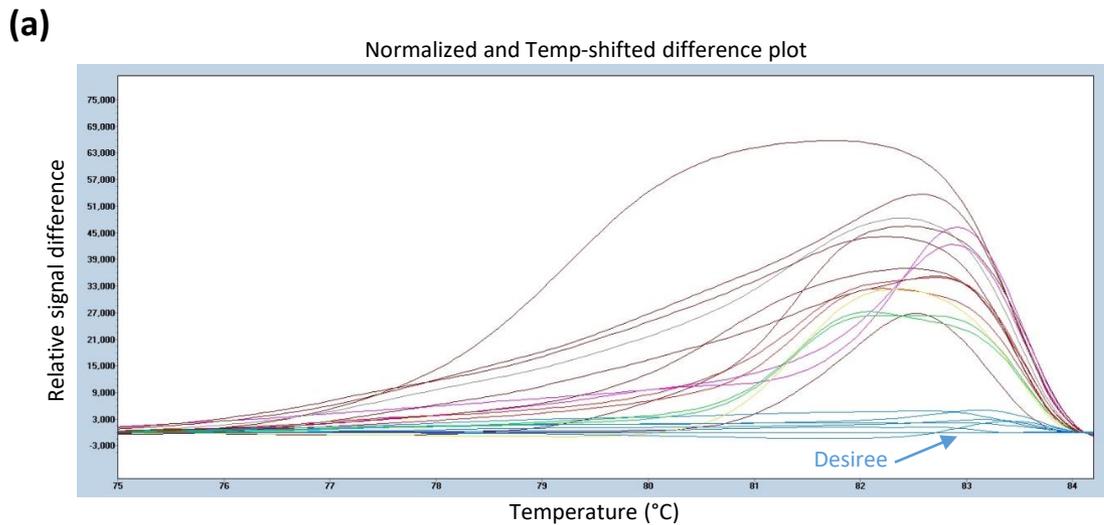


Figure 2

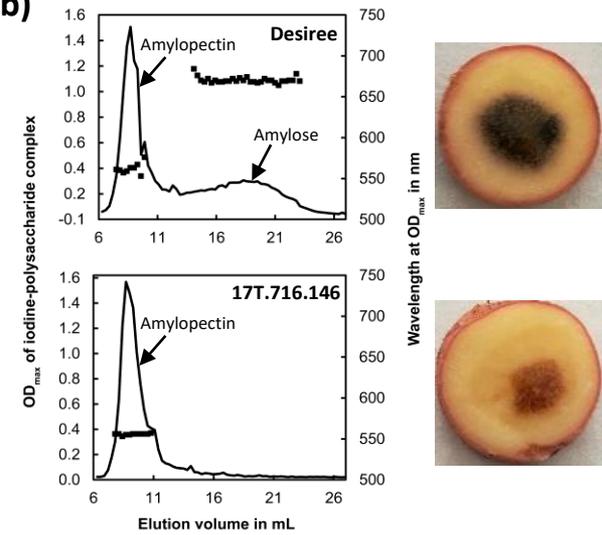
(a)



	sgGBSS1-sgGBSS2				sgGBSS1	
	Plants analyzed	Deletion(s)	Insertion(s)	Plants analyzed	Mutated plants	
Number of plants	269	2 (0.7%)	6 (2.2%)	444	38 (8.6%)	

Mutations	-1	-2	-4	-5	-6	-7	-8	-9	-12	-13	-18	-23	+1	SNP	Band shift	ICE ≈ 25%	ICE ≈ 50%	ICE ≈ 75%	ICE ≈ 100%	ND
Number of plants	14	1	4	9	2	2	2	1	1	1	1	1	4	1	6	27	2	3	3	3

(b)



(c)

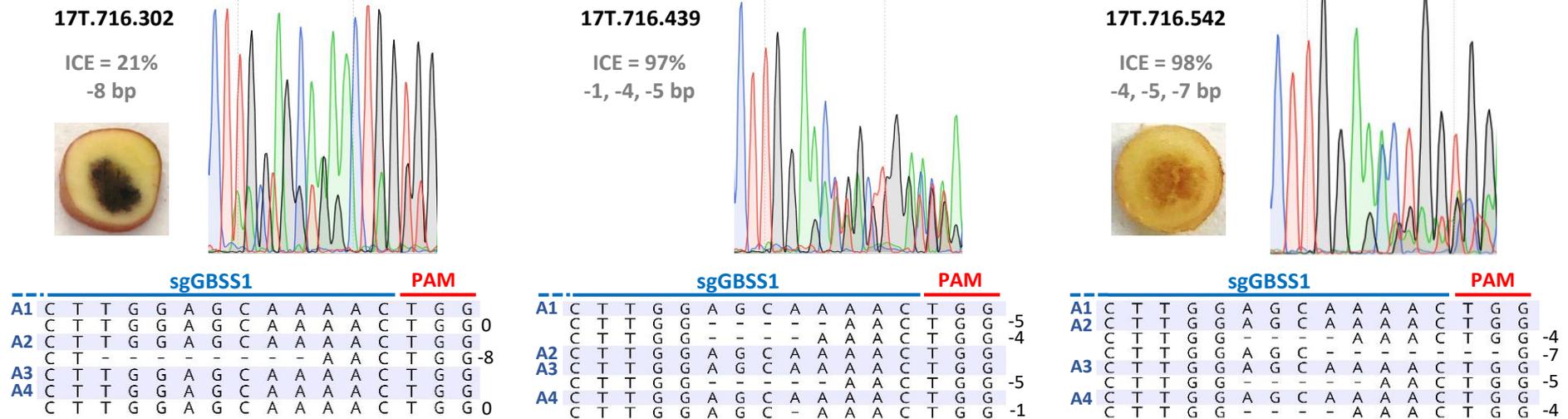
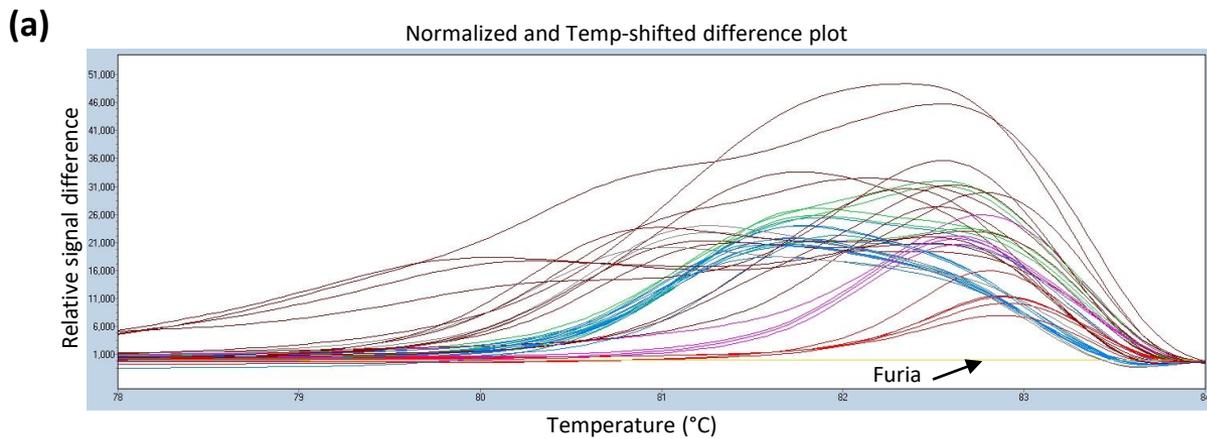


Figure 3



(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

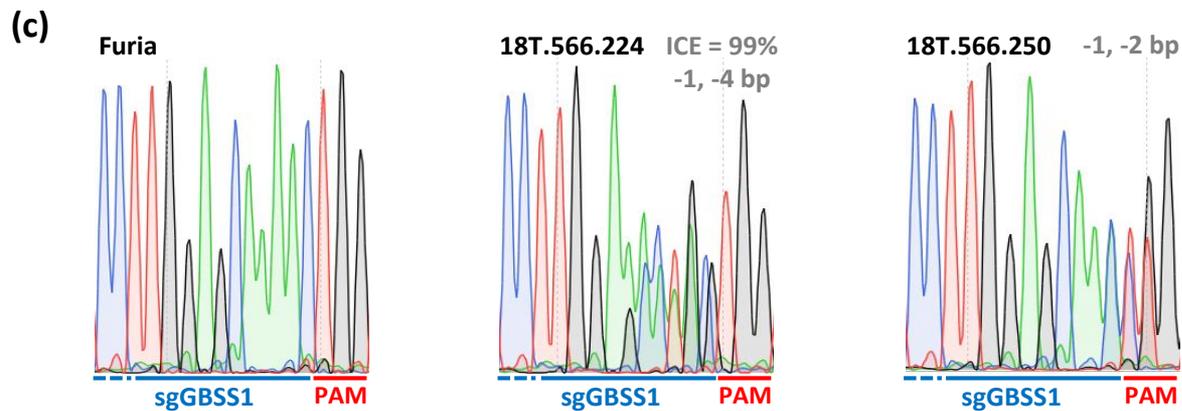


Figure 4

(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

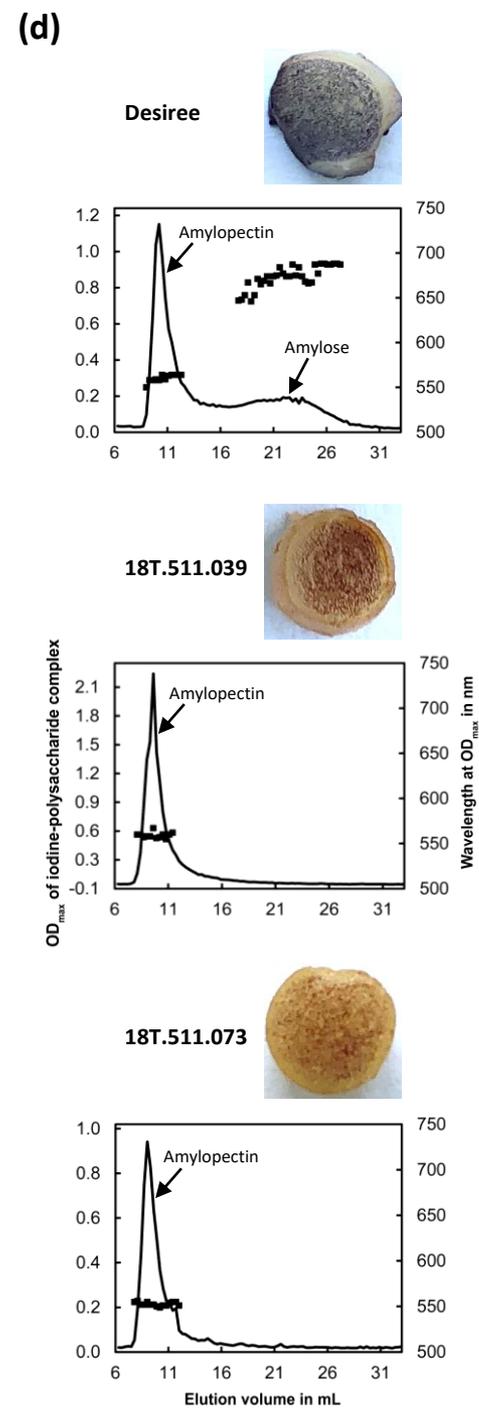
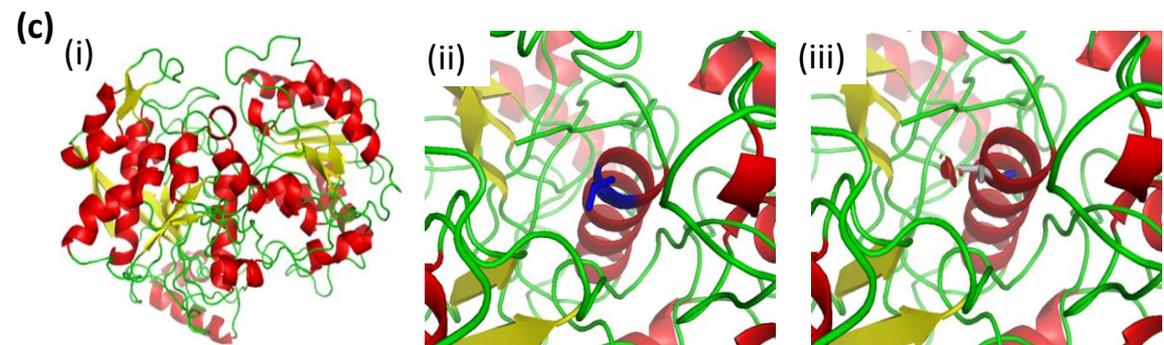
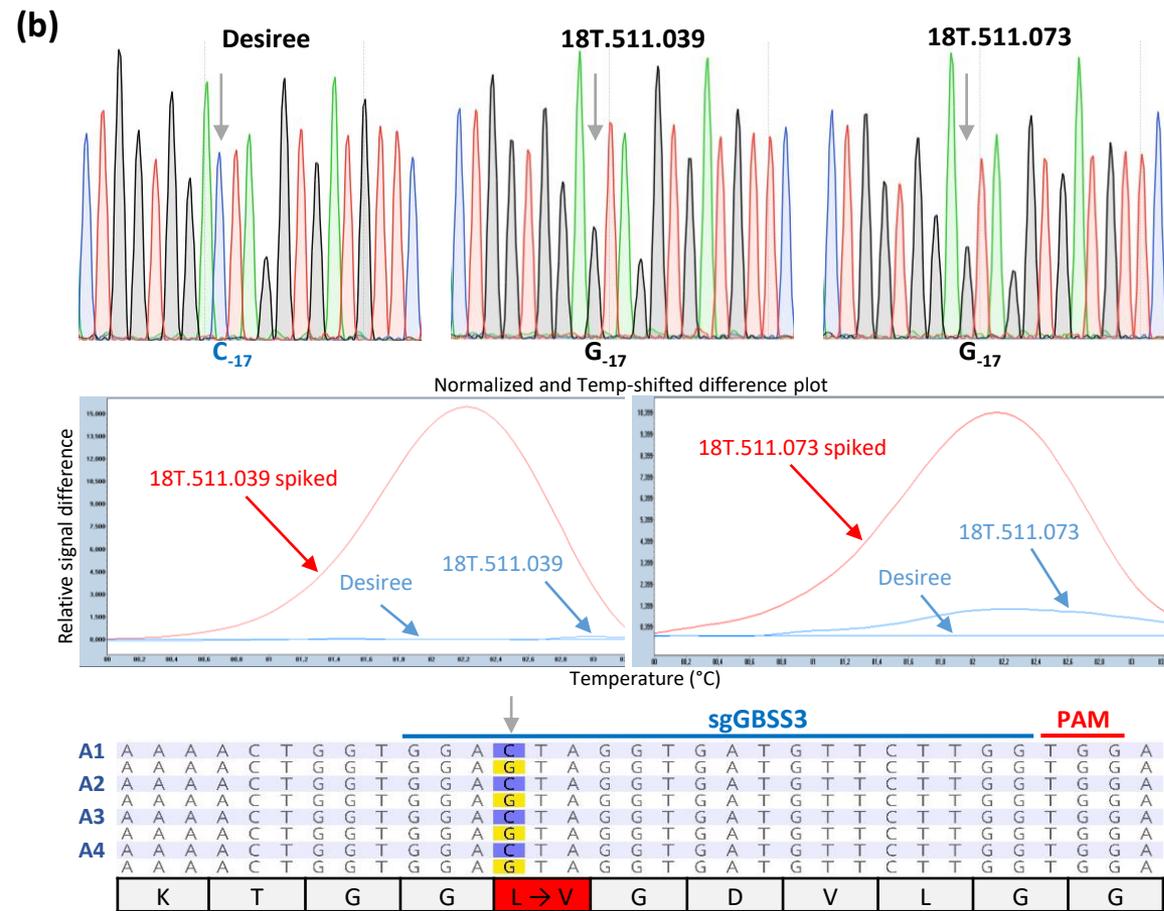


Figure 5