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16 # A space-saving visual screening method, *Glycine max* FAST, for generating
17 transgenic soybean
18

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26
27

28 ## Abstract

29 Soybean is an important crop plant for food and biofuel production, and there have been considerable efforts to
30 develop transgenic soybean lines with higher seed oil contents and/or seed yields. However, the process of
31 screening transgenic lines is laborious and requires a large amount of space. Here, we describe a powerful
32 screening method, *Glycine max* Fluorescence-Accumulating Seed Technology (GmFAST), which is based on a
33 seed-specific fluorescent marker. The marker is composed of a soybean seed-specific promoter coupled to the
34 *OLE1-GFP* gene, which encodes GFP fused to the oil-body membrane protein OLEOSIN1 of *Arabidopsis*
35 *thaliana*. We introduced the marker gene into cotyledonary nodes of *G. max* Kariyutaka via
36 *Agrobacterium*-mediated transformation and regenerated heterozygous transgenic plants. OLE1-GFP-expressing
37 soybean seeds can be selected nondestructively using a fluorescence stereomicroscope. Among T2 seeds, the
38 most strongly fluorescent seeds were homozygous. GmFAST uses one-tenth of the growing space required for
39 the conventional method. This space-saving method will contribute to facilitating transformation of soybean.
40 OLE1-GFP was localized specifically to oil bodies in the cotyledon cells of seeds, but it did not affect oil content
41 per seed, the size and density of the oil bodies, or oil composition. One of the homozygous lines (line #8)
42 showed a 44% increase in the seed pod number, which resulted in 41% and 30% increases in seed yield and total
43 oil production, respectively, compared with the wild type. In line #8, *OLE1-GFP* was inserted into the intron of
44 Glyma13g30950, causing its overexpression. An increase in seed pod number was confirmed in *Arabidopsis*
45 *thaliana* plants that overexpressed the Arabidopsis ortholog of Glyma13g30950, *E6L1*. These results suggest
46 that line #8 is a valuable resource for agricultural and industrial applications. Taken together, GmFAST provides
47 a space-saving visual and non-destructive screening method for soybean transformation, thereby increasing the
48 chance of developing useful soybean lines.
49
50

51 **Keywords:** *Arabidopsis thaliana*, *Glycine max*, GmFAST, oil body, oleosin, seed production

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52 ## Introduction

53 Soybean (*Glycine max*) is an agriculturally important crop that produces oil-rich and protein-rich seeds (19% oil
54 and 35%–40% protein) (Tidke et al. 2015). Therefore, soybean seeds are used as a source of food and
55 nutraceuticals. Oil seeds have recently gained attention as biofuel resources with the increasing demand for
56 energy in emerging countries. The total world production of soybean reached 276 million tons in 2013 and is
57 increasing (FAOSTAT; http://faostat3.fao.org).

58 Soybean oil is composed of five fatty acids; palmitic acid (C16:0), stearic acid (18:0), oleic acid (18:1),
59 linoleic acid (18:2), and linolenic acid (18:3)(Clemente and Cahoon 2009). These fatty acids are sequestered in a
60 specific organelle, the oil body, which is 0.5 to 2.0 µm in diameter and surrounded by a lipid monolayer and
61 membrane proteins including oleosin (Shimada and Hara-Nishimura 2010). Oleosin modulates the size of oil
62 bodies (Abell et al. 1997; Shimada et al. 2008; Siloto et al. 2006) and the seed oil contents (Hu et al. 2009b;
63 Shimada et al. 2008; Siloto et al. 2006). *Arabidopsis thaliana* has 16 oleosins, of which five (OLE1, OLE2,
64 OLE3, OLE4, and OLE5) are expressed in seeds (Kim et al. 2002). Genetic engineering approaches based on
65 oleosin function have been used to increase the seed oil content of rice; overexpression of a soybean oleosin
66 increased the oil content of rice by 37%–46% compared with that of wild-type rice (Liu et al. 2013). However,
67 whether this approach would also work in soybean seeds, where oil biosynthetic genes are reported to increase
68 the oil content (Lardizabal et al. 2008; Rao and Hildebrand 2009), remains unknown.

69 The generation of transgenic plants involves two processes; introduction of the gene into plants, and
70 screening of transgenic plants. Genes of interest are usually introduced via *Agrobacterium tumefaciens*-mediated
71 transformation. The subsequent screening of transgenic plants is generally based on antibiotics or herbicides,
72 since antibiotic- and herbicide-resistance genes are introduced into plants as selectable markers together with the
73 gene(s) of interest. However, there are several problems associated with the screening process. Firstly,
74 antibiotics/herbicides can inhibit plant growth. Secondly, screening requires aseptic techniques to prepare sterile
75 seeds and agar plates containing antibiotics/herbicides, making the selection process time consuming and labor
76 intensive.

77 To overcome these disadvantages, we previously established a method to select transgenic *Arabidopsis* and
78 rice (*Oryza sativa*), designated as the FAST (Fluorescence-Accumulating-Seed Technology) method (Shimada
79 et al. 2011; Shimada et al. 2010). This method relies on the FAST marker, in which a translational fusion gene
80 encoding *Arabidopsis* OLE1 and green fluorescent protein (GFP), *OLE1-GFP*, is driven by a seed-specific
81 promoter. The FAST marker is expressed in seeds, and the emission of GFP fluorescence in transgenic seeds
82 allows for their easy and non-destructive isolation under a fluorescence stereomicroscope or blue LED
83 handy-type instrument. Unlike conventional methods, the FAST method requires no aseptic techniques. This
84 method reduced the time required to obtain homozygous transgenic plants from 7.5 months to 4 months in

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85 *Arabidopsis* (Shimada et al. 2010). The objective of the present study was to establish the FAST method in
86 soybean. To this end, a GmFAST marker was generated, in which *OLE1-GFP* was expressed under the control
87 of the *GLYCININ* promoter of *G. max* (Fischer and Goldberg 1982; Nielsen et al. 1989; Scallon et al. 1985). This
88 screening system was used in combination with *Agrobacterium*-mediated transformation of *G. max* cotyledonary
89 nodes (Yamada et al. 2010). Since the 11S globulin GLYCININ is highly expressed in seeds (Meinke et al.
90 1981; Schmidt et al. 2011), we examined the effect of OLE1-GFP on oil body organization and oil content in
91 soybean seeds.

92

93

94 ## Materials and Methods

95 **Plant materials and growth conditions of soybean plants**

96 The Japanese soybean variety Kariyutaka was used as the wild type (WT), which was never placed in culture,
97 and the OLE1-GFP-expressing plants were generated in the Kariyutaka background. Seeds were sown on
98 compost and grown at 25°C under a 16-h light (white light at 100–150 μmol m^{-2} s^{-1}), 8-h dark photoperiod. After
99 germination, the position of plant pots relative to the light source was changed daily to ensure that all plants
100 received the same amount of light.

101

102 **Plant materials and growth conditions of *Arabidopsis thaliana***

103 *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild type. Seeds, which had been
104 surface-sterilized with 70% ethanol and dried, were sown on Murashige-Skoog (MS) agar plates (Wako) and
105 grown at 22°C under continuous 100 μmol m^{-2} s^{-1}white light for 2–3 weeks. Plants were then transferred to
106 vermiculite and further grown at 22°C under the light.

107

108 **Plasmid DNA construct for the GmFAST-G marker**

109 The *G. max* 11S globulin (GLYCININ) promoter (proGm11S, 1100 bp, accession AB353075) (Nakano et al.
110 2014) was cloned and a genomic fragment of proGm11S was amplified by PCR using the following primers:
111 forward, 5′-GCAAGCTTGCGAATTCTCTCTTATAAAACACAAACAC-3′ and reverse,
112 5′-GCTCTAGAGCGTTTAAGGACCAATGGAGAGAATG-3′. Genomic DNA from *G. max* was used as the
113 template for PCR. The amplified fragment was purified and amplified with Taq polymerase with the addition of
114 adenine. The PCR product was inserted into the pGEM-T-Easy (Promega, Madison, WI, USA) vector to produce
115 pGEM-proGm11S. Then, pGEM-proGm11S was treated with *Hind*III and *Xba*I to cut out the proGm11S
116 fragment. pMDC123-IG (Yamada et al. 2010) was also treated with *Hind*III and *Xba*I to remove the 35S
117 promoter region. The two fragments were ligated to produce the pMDC123-Gm11SIG vector.
118 A DNA fragment containing proGm11S, the *OLE1-GFP* gene, and the 35S terminator (ter35S) was
119 amplified by overlapping PCR. In the first PCR, the proGm11S fragment was amplified using the following
120 primers: forward, 5′-CACCGAATTCTCTCTTATAAAACACAA-3′ and reverse,
121 5′-TGTATCCGCCATGTTTAAGGACCAATGGAG-3′. The template for PCR was pMDC123-Gm11SIG. The

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122 *OLE1-GFP*::ter35S fragment was amplified using the following primers: forward,
123 5′-TGGTCCTTAAACATGGCGGATACAGCTAGA-3′ and reverse,
124 5′-GAATTCCGACGTCGCATGCCTGCAGGTCA-3′. The template for PCR was
125 pBGWF7-proOLE1::OLE1-GFP (Karimi et al. 2002; Shimada et al. 2010). In the second PCR, the
126 proGm11S::*OLE1-GFP*::ter35S fragment was amplified using the following primers: forward,
127 5′-CACCGAATTCTCTCTTATAAAACACAA-3′ and reverse,
128 5′-GAATTCCGACGTCGCATGCCTGCAGGTCA-3′. The first PCR products were used as the templates for
129 PCR. The proGm11S::*OLE1-GFP*::ter35S fragment was inserted into pENTER/D-TOPO (Invitrogen, Carlsbad,
130 CA, USA) by the TOPO reaction to produce pENTR/proGm11S::*OLE1-GFP*::ter35S. The
131 proGm11S::*OLE1-GFP*::ter35S fragment was amplified for use in the In-Fusion cloning system (Clontech
132 Laboratories, Mountain View, CA, USA) using the following primers: forward,
133 5′-CCAAGCTTGCGAATTCTCTCTTATAAAACACAAACACAATT-3′ and reverse,
134 5′-CCATGATTACGAATTCCGACGTCGCATGCCTGCAGGTCACT-3′. The template for PCR was
135 pENTR/proGm11S::*OLE1-GFP*::ter35S. Finally, the fragment was inserted into the *Eco*RI site of pMDC123
136 (Curtis and Grossniklaus 2003) using the In-Fusion cloning system to produce the expression vector
137 GmFAST-G.
138
139 **Plasmid DNA construct for *E6L1* overexpression**
140 The full-length genomic fragment of *A. thaliana E6L1*, starting from nucleotide 1 (corresponding to A of the
141 start codon ATG) to nucleotide 804, was amplified by PCR using the primer set:
142 5’-GCAGGCTCCGCGGCCATGGCTTTTTCCACTAGCTC-3’ (forward) and
143 5’-AGCTGGGTCGGCGCGTCATGGAGTGATCTGATCTC-3’ (reverse). The PCR product was subcloned
144 with In-Fusion HD Cloning Kit (Takara Bio Inc.) into pENTR/D-TOPO (Invitrogen) which was digested by
145 *Not*I and *Asc*I. The subcloned sequence was then transferred into the pH2GW7 vector (Karimi et al. 2002) via a
146 recombination reaction with LR Clonase (Invitrogen), yielding the pH2GW7-E6L1 vector.
147
148 **Transformation of soybean plants**
149 *Agrobacterium*-mediated transformation was performed as described previously (Yamada et al. 2010). The
150 cotyledonary nodes of Kariyutaka were prepared for infection with *A. tumefaciens* EHA105 harboring
151 GmFAST-G. The T0 plants were selected based on resistance to glufosinate-ammonium (Sigma-Aldrich, St.
152 Louis, MO, USA). The inheritance of the transgene was confirmed based on seedling resistance to Basta (Bayer
153 Crop Science, Monheim, Germany). In addition, mature seeds emitting GFP were selected under a fluorescence
154 stereomicroscope (MVX10, Olympus, Tokyo, Japan). Three independent lines (#30, #2, and #8) were
155 established, and homozygous T3 plants were used in further analyses.
156
157 **Stable transformation of Arabidopsis plants**
158 The binary vector pH2GW7-E6L1 was introduced into *A. thaliana* (Col-0) plants with *Agrobacterium*
159 *tumefaciens* (strain GV3101) using the floral dip method (Bechtold and Pelletier 1998) to generate *E6L1*

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160 overexpression (E6L1-OX) plants.
161
162 **SDS-PAGE and immunoblotting for soybean**
163 Three dry seeds were broken into small pieces with a hammer and ground to a powder with a mortar and pestle
164 in liquid nitrogen. Fifty milligrams of each powdered sample was diluted in 10 ml sample buffer [50 mM
165 Tris-HCl, pH 6.8, 2% w/v sodium dodecyl sulfate (SDS), 6% v/v β-mercaptoethanol, and 10% v/v glycerol],
166 mixed well, boiled at 99°C for 5 min, and then centrifuged at 9,000 × *g* for 1 min. A 1-mL aliquot of the
167 supernatant was mixed with 50 µl bromophenol blue, and then the extracted proteins were separated by
168 SDS-PAGE on a precast gel (Bio-Rad, Hercules, CA, USA) at a constant voltage of 200 V for 45 min. The gel
169 was stained with Coomassie Brilliant Blue (CBB).
170 For immunoreaction experiments, gel-separated proteins were transferred onto a polyvinylidene fluoride
171 membrane using the iBlot system (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with skim milk
172 in TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween-20). The primary antibody reaction
173 was performed with anti-OLE1 diluted 1:2000 or anti-GFP diluted 1:5000 in TBST buffer for 1 h. The
174 secondary antibody reaction was performed with anti-mouse IgG-HRP diluted 1:5000 or anti-rabbit IgG-HRP
175 diluted 1:5000 for 30 min. After the reaction of HRP-conjugated secondary antibody with ECL (GE Healthcare
176 Life Sciences, Buckinghamshire, United Kingdom), chemiluminescence was visualized using a LAS-3000
177 LuminoImage analyzer (FujiFilm, Tokyo, Japan).
178
179 **DNA isolation and PCR analysis for soybean**
180 Dry seeds were broken into small pieces with a hammer and then ground to a powder with a mortar and pestle in
181 liquid nitrogen. Genomic DNA was purified from seeds of *G. max* with a DNeasy plant mini kit (Qiagen, Hilden,
182 Germany) as per the manufacturer's instructions. The primer sets used to amplify the OLE1, GFP, and
183 OLE1-GFP fragments were as follows: 5′-ACCCACAGGGATCAGACAAG-3′ (OLE1 forward) and
184 5′-GTTCCCCACCAGTATGTTGC-3′ (OLE1 reverse), and
185 5′-CACCATGGTGAGCAAGGGCGAGGAGCTGTT-3′ (GFP forward) and
186 5′-TCAGAGATCTCCCTTGTACAGCTCGTCCAT-3′ (GFP reverse). The PCRs were performed with
187 MightyAmp DNA polymerase (Takara, Otsu, Japan) under the following conditions: 40 cycles of 98°C for 10 s,
188 55°C for 30 s, and 72°C for 60 s.
189
190 **Fluorescence microscopy for soybean**
191 Dry seeds were fixed in fixation buffer (50 mM PIPES, 10 mM EGTA, and 5 mM $MgSO_4$, pH 7.0) containing
192 2% formaldehyde and 0.3% glutaraldehyde overnight. The GFP fluorescence of seed cotyledons was observed
193 under a fluorescence stereomicroscope (Zeiss, Oberkochen, Germany) or a confocal laser scanning microscope
194 (LSM780 META; Zeiss). The mean GFP fluorescence level of dry seeds was determined using Image J software
195 (http://rsb.info.nih.gov/ij).
196 To visualize lipids, the fixed seed cotyledons were stained with 5 µg/ml Nile red and observed under a
197 confocal laser scanning microscope (Zeiss LSM780 META).

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198

199 **Transmission electron microscopy for soybean**

200 Dry seeds were cut into approximately 1 × 1 mm pieces using a razor blade and fixed with 4% paraformaldehyde
201 and 2% glutaraldehyde in 0.05 M cacodylate buffer, and then with 2% osmium tetroxide in 0.05 M cacodylate
202 buffer. After dehydration, samples were embedded in Quetol 651 resin (Electron Microscopy Sciences, Hatfield,
203 PA, USA). Ultrathin sections (80 nm) were prepared, stained with uranyl acetate in lead stain solution, and
204 observed under a transmission electron microscope (JEM-1400Plus; JEOL, Tokyo, Japan).

205

206 **Number of seeds and seed pods, and seed weight measurements for soybean**

207 The T3 plants were grown under the conditions described above until the seed pods were completely dry, and
208 then the seeds were harvested together with their pods. The number of seeds per plant was counted and the total
209 seed weight per plant was measured with a digital electronic balance (XS105DU; Mettler Toledo, Greifensee,
210 Switzerland). The number of pods per plant was also counted, and the percentage of pods with one, two, or three
211 seeds on each plant was calculated.

212

213 **Fatty acid content analysis for soybean**

214 The fatty acid content of T4 dry seeds was analyzed as described previously (Yamada et al. 2014). Mature seeds
215 were ground using a mortar and pestle. The fatty acid content (palmitic, oleic, linoleic, and linolenic acids) was
216 determined by gas chromatography based on the ratio of the areas of respective peaks to that of heptadecanoic
217 acid. Lipid content was calculated as the total fatty acid content.

218

219 **An adapter ligation-mediated PCR for soybean**

220 The site of *OLE1-GFP* insertion in the genome of OLE1-GFP transgenic line #8 was determined by an adapter
221 ligation-mediated PCR as reported previously (O'Malley et al. 2007). Briefly, total DNA was isolated from seeds
222 with a DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total DNA was digested
223 with restriction enzymes (Hind III and Eco RI), and the digested DNA was ligated to the following primers:
224 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGC-3' (long strand of adapter 1
225 for Hind and Eco), 5'-AGCTGCAGCCCG-3' (short strand of adapter Hind), and 5'-AATTGCAGCCCG-3'
226 (short strand of adapter Eco). The DNA was amplified by a nested PCR using the following primer sets: 5'-
227 AGTCGACCGTGTACGTCTCC-3' (pMDC123_LB1) and 5'-GTAATACGACTCACTATAGGGC-3' (AP1)
228 for 1st PCR; 5'-GTTTCTGGCAGCTGGACTTC-3' (pMDC123_LB2) and 5'- TGGTCGACGGCCCGGGCTGC
229 -3' (AP2) for 2nd PCR, 5'-AAAAACGTCCGCAATGTGTT-3' (pMDC123_LB3) and 5'-
230 TGGTCGACGGCCCGGGCTGC -3' (AP2) for 3rd PCR. The 3rd PCR products were subjected to agarose gel
231 electrophoresis, and DNA fragments of interest were excised from the gel and purified. The DNA fragments
232 were sequenced using a genetic analyzer (3130xl; ABI). Insertion of OLE1-GFP into Glyma13g30950 was
233 confirmed by genotyping PCR using the following primer sets: 5'-ACTGTAGGCTTGATGCCACT-3'
234 (forward) and 5'-TCCTCTACGTCATTCGATGG-3' (reverse); 5'-AAAAACGTCCGCAATGTGTT-3'
235 (pMDC123_LB3) and 5'-TCCTCTACGTCATTCGATGG-3' (reverse).

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236

237 **Reverse transcription PCR for soybean**

238 Total RNA was isolated from soybean leaves using RNeasy Plant Mini Kit (Qiagen) according to the
239 manufacturer's instructions. Total RNA was subjected to first-strand cDNA synthesis using Ready-To-Go
240 RT-PCR Beads (GE Healthcare), and the cDNA was amplified by PCR using the following primer sets:
241 5'-TTTTCCTCACCACCCTTTTG -3' (forward) and 5'-CGGAATGAAAGGTGGTTGTT-3' (reverse);
242 5'-ATCTTGACTGAGCGTGGTTATTCC-3' (forward) and 5'-GCTGGTCCTGGCTGTCTCC-3' (reverse) for
243 *ACTIN11*(Hu et al. 2009a). The size of PCR products of Glyma13g30950 and *ACTIN11* was 164 and 126 bp,
244 respectively.

245

246 **Reverse transcription PCR for Arabidopsis**

247 Total RNA was isolated from Arabidopsis seedlings using RNeasy Plant Mini Kit (Qiagen). The RNA was
248 used for cDNA synthesis using ReverTra Ace qPCR RT Master Mix (Toyobo), followed by PCR using the
249 following primer sets: 5'-ATGGCTTTTTCCACTAGCTC-3' (forward) and
250 5'-TCATGGAGTGATCTGATCTC-3' (reverse) for *E6L1*, and 5'-ACTGGAGGTTTTGAGGCTGGTAT-3'
251 (forward) and 5'-GCACCGTTCCAATACCACCAATC-3' (reverse) for *EF1a*. The size of PCR products of
252 *E6L1* and *EF1a* was 804 and 494 bp, respectively.

253

254 **Number of seeds and seed pods, and seed weight measurements for Arabidopsis**

255 Wild-type and E6L1-OX plants (T2 generation) were grown until the seed pods were completely dried. For
256 confirming whether the T2 plants contained the transgene, the following primer sets were used for genotyping by
257 PCR: 5'-ATGAAAAAGCCTGAACTCACCGC-3' (forward) and 5'-CTATTCCTTTGCCCTCGGACGAG-3'
258 (reverse) for hygromycin-resistant gene. The seed pods in each plant were counted before seed harvesting. The
259 seeds were harvested in each plant, and then the total seed weight per plant was measured with a digital
260 electronic balance (GH-120; A&D Company, Tokyo, Japan). The weight of 100 seeds were measured with the
261 digital electronic balance (GH-120) for calculating the weight of one seed. According to the data, the number of
262 seeds per plant and per pod were calculated.

263

264

265 **Results and Discussion**

266 **Identification of transgenic soybean seeds by GmFAST**

267 The FAST system for soybean was established by constructing the GmFAST-G vector, which contains the
268 *OLE1-GFP* fusion gene driven by the *G. max* 11S globulin promoter (Fig. 1A). The GmFAST-G vector was
269 introduced into cotyledonary nodes of *G. max* Kariyutaka through *Agrobacterium*-mediated transformation.
270 After the regeneration of transgenic plants from independent cotyledonary nodes, T1 mature seeds were
271 harvested and grown to obtain the T2 seed population, which contained homozygous, heterozygous, and WT

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272 seeds. Next, we carried out T2 seed selection based on the GmFAST method. The GFP fluorescence levels are
273 reflective of the GmFAST-G vector copy numbers: two and one in homozygotes and heterozygotes, respectively
274 (Shimada et al. 2010). Therefore, we selected strongly fluorescent seeds from independent T2 seed populations
275 using a fluorescence stereomicroscope and finally obtained T3 seeds from three independent transgenic lines
276 designated as #2, #8, and #30 (Fig. 1B). The GFP fluorescence intensity levels of lines #2 and #8 were twice that
277 of WT, whereas the GFP fluorescence of line #30 was lower and similar to that of WT (Fig. 1C). The
278 homozygosity of these lines was confirmed by a non-destructive segregation analysis of the T3 seeds using a
279 fluorescence stereomicroscope. As expected, all the T3 seeds from each line emitted GFP fluorescence,
280 indicating that they were homozygous. The insertion of the *OLE1-GFP* gene in all three transgenic lines was
281 also confirmed by genotyping (Fig. 1D, upper). An immunoblot analysis showed that the OLE1-GFP protein
282 was expressed in lines #2 and #8, but was not expressed at detectable levels in line #30 (Fig. 1D, middle). Thus,
283 the GmFAST method enables the isolation of homozygous plants in the T2 generation.

284

285 **Intracellular localization of OLE1-GFP in transgenic soybean seeds**

286 An analysis of the localization of OLE1-GFP in transgenic seeds showed that it was exclusively expressed in
287 seed cotyledons in lines #2 and #8 (Fig. 2A; #2 and #8). In the palisade mesophyll cells of cotyledons,
288 OLE1-GFP showed a meshwork-like expression pattern (Fig. 2B, left), similar to the distribution pattern of the
289 oil bodies stained with Nile red (Fig. 2B, right). These results suggested that OLE1-GFP was functional in oil
290 bodies.

291 Next, we examined the effects of OLE1-GFP on oil body organization in transgenic seeds using
292 transmission electron microscopy. As previously reported (Herman and Larkins 1999; Schmidt et al. 2011;
293 Yamada et al. 2014), seed cotyledon cells contained many oil bodies and several protein storage vacuoles (PSVs)
294 in all the plants examined (Fig. 3, left). No differences in the size and density of oil bodies were observed
295 between WT seeds and seeds of lines #30 and #2 (Fig. 3, right). This could be attributed to the low expression
296 levels of OLE1-GFP. However, compared with WT seeds, line #8 seeds had oil bodies that were relatively small
297 and densely distributed (Fig. 3, right). Line #8 also exhibited an altered PSV organization (Fig. 3, left). These
298 results were consistent with previous reports that the oleosin contents affects both oil-body sizes and PSV
299 organization in *Arabidopsis* and soybean (Schmidt and Herman 2008; Shimada et al. 2008; Siloto et al. 2006).

300

301 **Seed production characteristics and seed oil contents in transgenic soybean plants**

302 The seed production of each transgenic line was examined. Under our experimental conditions, line #8 showed a
303 44% increase in seed pod number per plant compared with WT (Fig. 4A). Line #8 also showed 41% increases in

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304 total seed weight per plant (Fig. 4B) and 47% seed grain number per plant compared with WT (Fig. 4C).
305 However, the average weight per seed (Fig. 4D) and the proportions of one-, two-, and three-seed pods (Fig. 4E)
306 did not differ significantly between WT and line #8. Lines #2 and #30 did not show any changes in the above
307 parameters compared with WT (Fig. 4). Considering the comparable expression levels of OLE1-GFP in lines #2
308 and #8 (Fig. 1C), the higher seed production of #8 might not be related to OLE1-GFP function. Instead, the
309 insertion position of the *OLE1-GFP* gene in the genome might be responsible for the higher seed production in
310 line #8.

311 Next, we examined the fatty acid contents of transgenic seeds. Line #8 showed a 30% increase in total oil
312 production per plant compared with WT. The amounts of palmitic acid (C16:0), stearic acid (C18:0), and
313 linolenic acid (C18:3) were higher in seeds of line #8 than in seeds of WT (Fig. 5A). However, the total fatty
314 acid content based on seed weight was similar among all three transgenic lines (including line #8) and WT (Fig.
315 5B). Consequently, line #8 may be a valuable resource for agricultural and industrial applications.

316 In a previous study, the oil content increased by ~40% in the transgenic rice grains that expressed soybean
317 oleosin under the control of the RICE EMBRYO GLOBULIN-2 PROTEIN (REG-2) promoter (Liu et al. 2013).
318 However, no increase in the oil content based on seed weight was observed in soybean seeds that expressed the
319 oleosin gene *OLE1* under the control of the soybean 11S globulin (GLYCININ) promoter (Fig. 5B), possibly
320 because soybean seeds inherently have much higher oil contents than rice grains.

321

322 **Site of the *OLE1-GFP* insertion in the transgenic soybean line #8 genome**

323 Because the phenotype of line #8 could be result from an insertion mutation of *OLE1-GFP*, we performed an
324 adapter ligation-mediated PCR (O'Malley et al. 2007) to locate the insertion site of *OLE1-GFP* in its genome.
325 *OLE1-GFP* was inserted in the intron of Glyma13g30950, and this was confirmed using genotyping PCR (Fig.
326 6A and 6B). Intriguingly, the OLE-GFP insertion enhanced the transcript level of Glyma13g30950 in #8,
327 compared with the wild type, #2, and #30 (Fig. 6C).

328

329 **The effects of E6L1 on seed pod number in Arabidopsis**

330 To support our hypothesis that Glyma13g30950 is involved in seed pod formation, we investigated the function
331 of the Glyma13g30950 ortholog in Arabidopsis. From a BLAST analysis using the amino acid sequence of
332 Glyma13g30950.2, a candidate for an Arabidopsis orthologs, an *E6-like1* gene (*E6L1*; At2g33850), was found.
333 Then, we generated transgenic Arabidopsis plants overexpressing *E6L1* (E6L1-OX) (Fig. S1). Of three lines
334 analyzed (#2, #6, and #12), line #2 exhibited a statistically significant increase in seed pod number compared
335 with WT (Fig. 7A), although there were no remarkable changes between WT and the three E6L1-OX lines in the

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336 total seed weight per plant, total seed grain number per plant, the weight per seed, or the seed number per pod
337 (Fig. 7B–E). These results suggest that E6L1 is involved in regulating the seed pod number. According to the
338 Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), *E6L1* is expressed weakly at the
339 inflorescence shoot apex that includes the shoot apical meristem producing floral meristem. In E6L1-OX,
340 overexpressed *E6L1* may stimulate the shoot apical meristem to produce more flowers than WT plants, resulting
341 in an increase of seed pod numbers.

342

343 Conclusions

344 The results of this study showed that GmFAST, combined with *Agrobacterium*-mediated transformation using
345 cotyledonary nodes, is an efficient and powerful method to establish homozygous soybean lines. Compared with
346 the conventional method, which requires a large space to grow soybean plants for the selection of homozygous
347 transgenic plants and for segregation analyses, the GmFAST method simply requires the selection of the most
348 strongly fluorescent T2 seeds based on the expression of a fluorescent marker using a fluorescence
349 stereomicroscope. This reduces the space requirement to at least one-tenth of that required for conventional
350 methods (Table 1). We also generated a transgenic line with an increased seed yield. This line could be valuable
351 in agriculture and industry. Thus, the space-saving GmFAST method will facilitate soybean transformation,
352 which will increase the chance of developing useful soybean lines.

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353 ## Acknowledgments

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363

364

365 ## Author contributions

366 K.I., T.L.S., and I.H.-N. conceived the study. K.I. and T.Y. performed experiments with *Glycine max*.
367 TLS performed experiments with *Arabidopsis thaliana*. K.I., T.Y., T.L.S., and I.H.-N. analyzed whole
368 data and wrote the manuscript.

369

370

371 ## Additional information

372 Supplementary information is available online.

373

374

375 **Competing interests:** The authors declare no competing financial or non-financial interests.

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436 ## Figure legends

437 **Fig. 1. Expression of OLE1-GFP in transgenic soybean seeds.** (A) Schematic diagram of the GmFAST-G
438 vector. *Arabidopsis OLE1* was fused to *GFP* and expressed under the control of the soybean 11S globulin
439 promoter. (B) Representative GFP fluorescence images of dry mature seeds of wild type (WT) and OLE1-GFP
440 transgenic lines (#2, #8, and #30). (C) Quantification of GFP fluorescence determined using data from (B). The
441 mean fluorescence of 10 seeds was determined for each plant. Data represent means ± SEMs (n = 6 plants; $^{**}P$
442 < 0.01, Student's t test). (D) Expression of OLE1-GFP in dry seeds of WT and OLE1-GFP transgenic lines (#2,
443 #8, and #30). Genotyping was performed with specific primers for *OLE1*, *GFP*, and *OLE1-GFP* (upper), and
444 immunoblotting was performed with anti-GFP and anti-OLE1 (middle). CBB staining was conducted as a
445 loading control for immunoblotting (lower). Arrowheads indicate α and β subunits of 7S globulin.
446

447 **Fig. 2. Localization of OLE1-GFP in transgenic soybean seeds.** (A) Representative images of seed cotyledons
448 of wild type (WT) and OLE1-GFP lines (#2, #8, and #30) after fixation. Top (upper panels) and side (lower
449 panels) views are shown. (B) Side-view images of adaxial epidermal and palisade mesophyll cells of cotyledons
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451 (left panels) and lipid droplets using Nile red staining (right panels).
452

453 **Fig. 3. Morphology of protein storage vacuoles and oil bodies in transgenic soybean seeds.** Representative
454 transmission electron micrographs of mature seeds in wild type (WT) and OLE1-GFP transgenic lines (#2, #8,
455 and #30) showing protein storage vacuoles (PSVs) and oil bodies (OBs). High-magnification images of OBs are
456 shown in the right panels.
457

458 **Fig. 4. Seed production in transgenic soybean plants.** Dry mature seeds were harvested from wild type (WT)
459 and OLE1-GFP lines (#2, #8, and #30). (A) Number of seed pods per plant. (B) Total weight of seeds per plant.
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462

463 **Fig. 5. Fatty acid composition in transgenic soybean seeds.** The fatty acid contents of total seeds (A) and a
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467

468 **Fig. 6. Site of the *OLE1-GFP* insertion in the transgenic soybean line #8 genome.** (A) Schematic
469 representation of Glyma13g30950.2, showing the site of the *OLE1-GFP* insertion. Gray boxes, untranslated
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471 wild type (WT) and OLE1-GFP-expressing lines, #2, #8, and #30. G, genomic fragments amplified with a pair
472 of forward and reverse Glyma13g30950-specific primers (L and R in A); I, an inserted fragment amplified with
473 a left border primer for pDMC123 vector (LB in A) and Glyma13g30950 reverse primer (R in A). (C) RT-PCR

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474 of Glyma13g30950 and *ACTIN11* (control) transcripts in leaves of the WT and OLE1-GFP-expressing lines, #2,
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476
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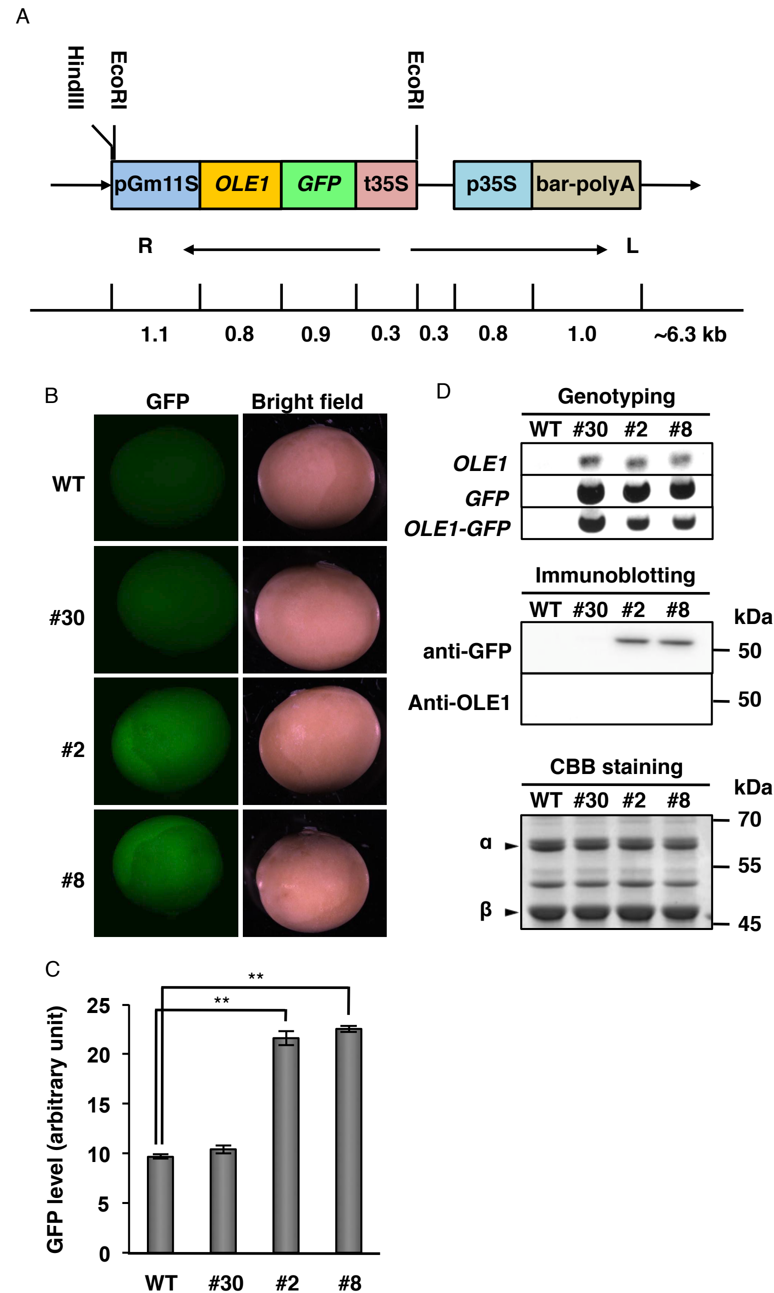

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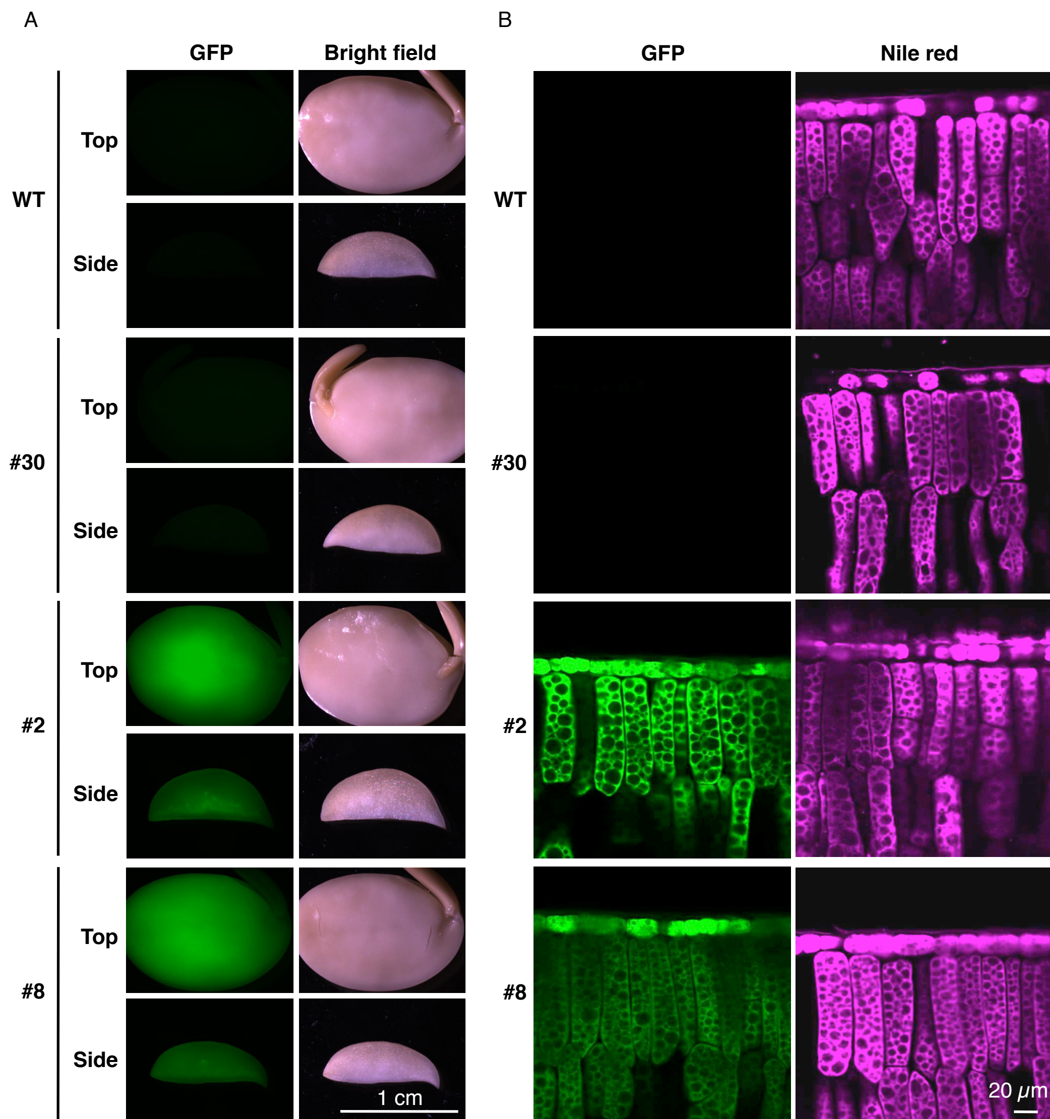

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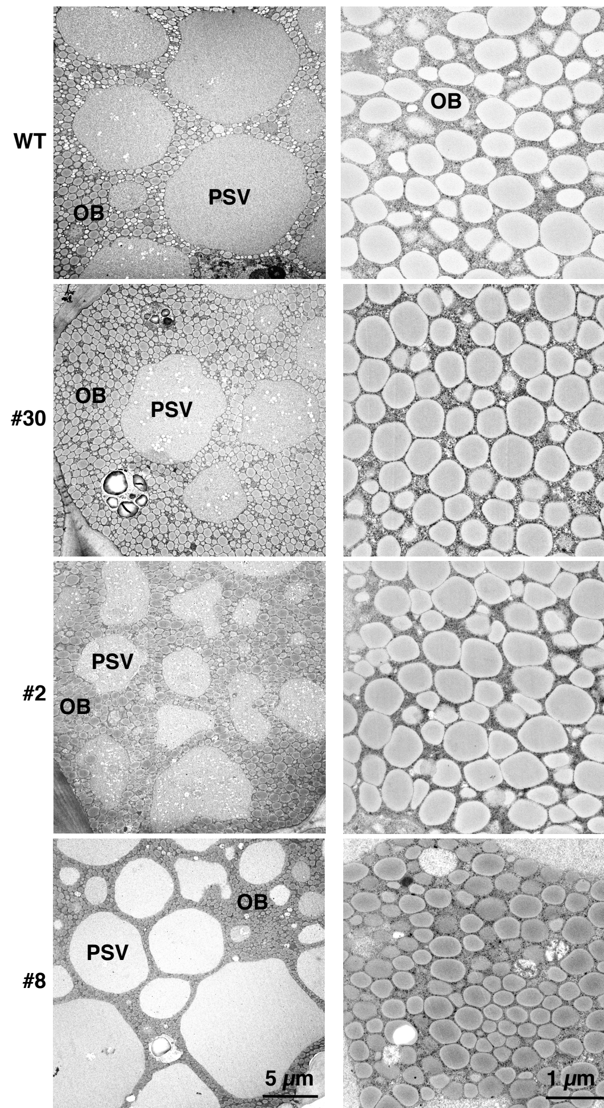

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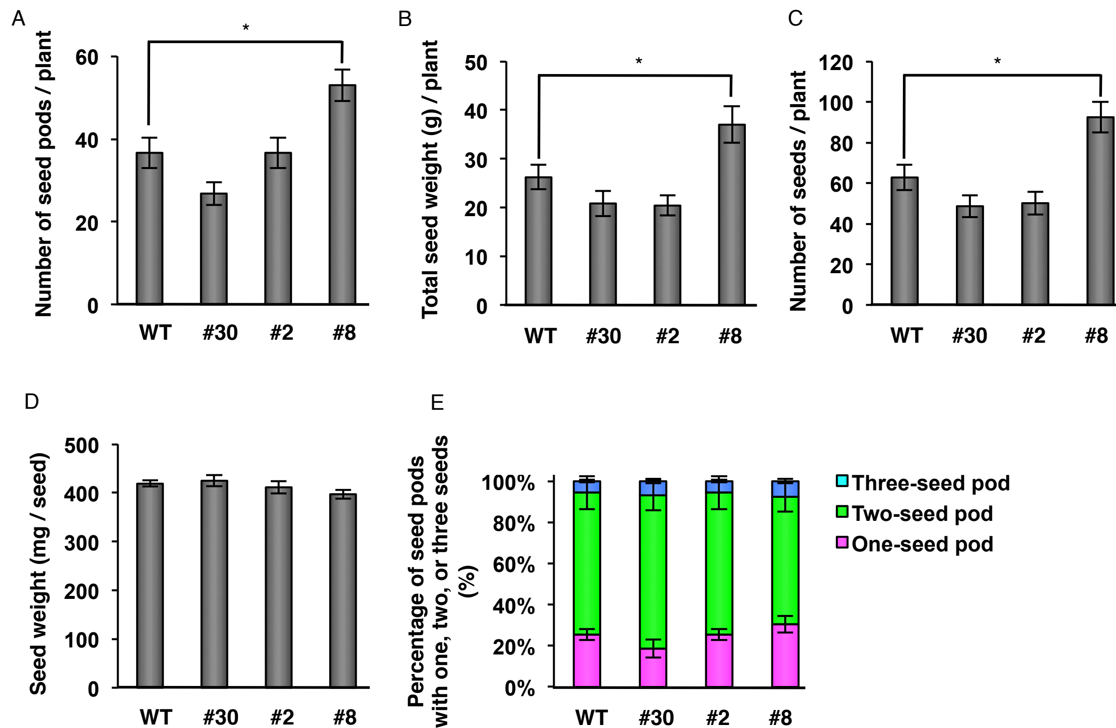

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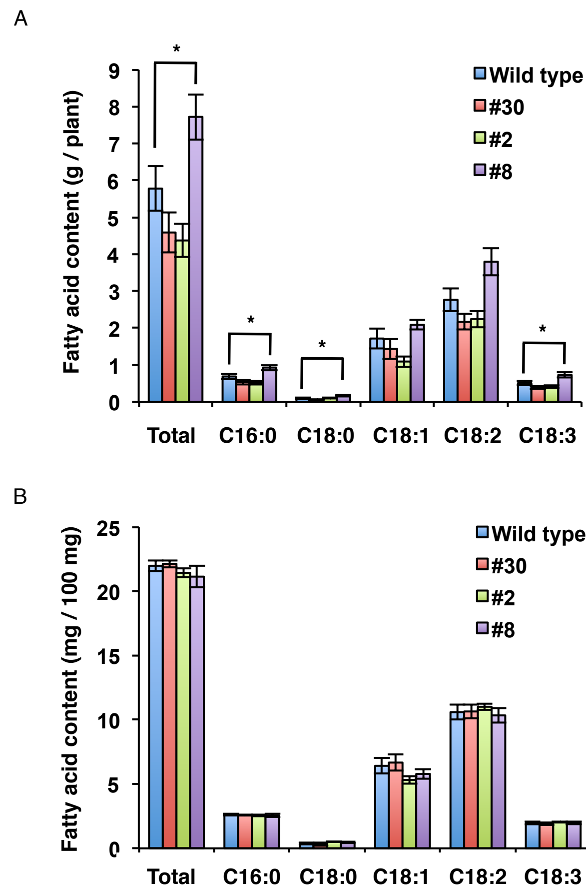

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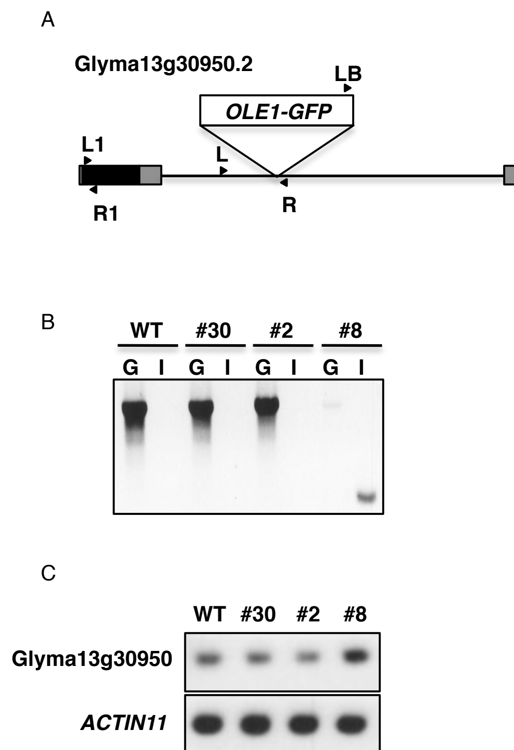

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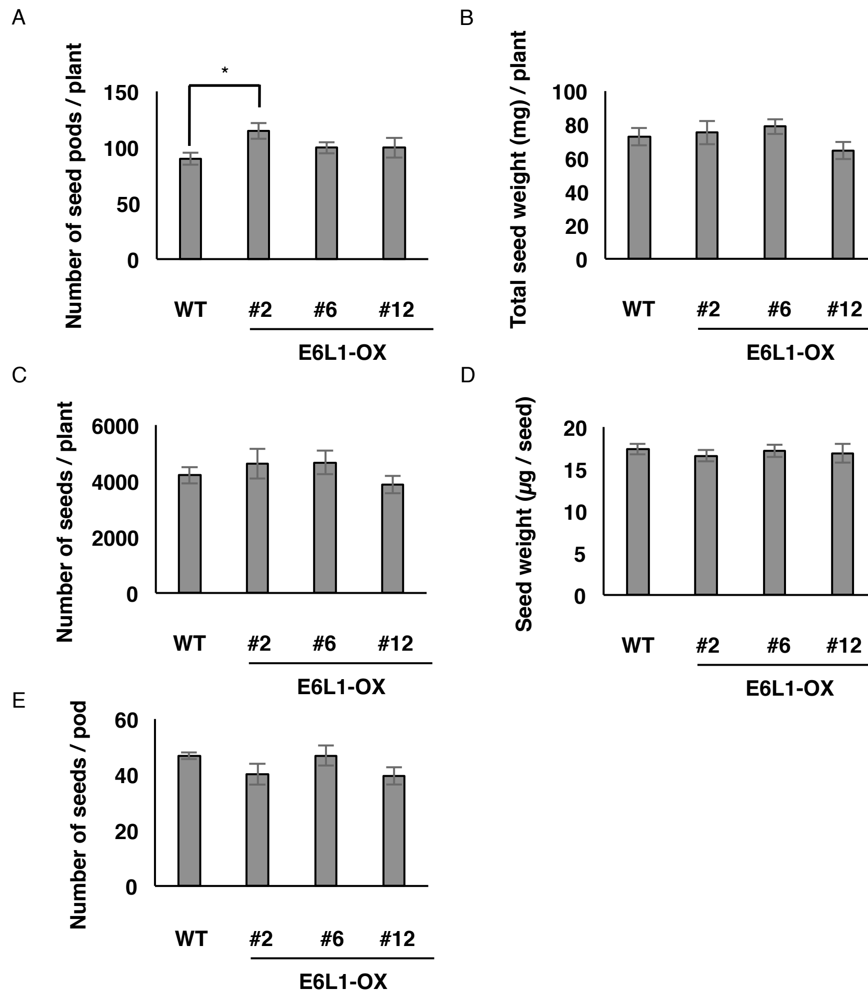

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Table 1. Comparison between GmFAST and the conventional transgenic soybean selection method.

Factors	GmFAST method	Conventional method
Transformant identification manner	GFP fluorescence of seeds	Drug resistance of seedlings
Homozygote identification timing	T2 generation (seeds)	T3 generation (seedlings)
Segregation analysis treatment	Not required (in principle)	Required (sowing seeds in plug trays for drug selection)
Relative cultivation area	0.1	1.0