

1 **Title:**
2 **Mapping of transgenic alleles in plants using a Nanopore-based sequencing strategy**

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30 Running Title: Nanopore-based strategy to map transgene alleles

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41 **Abstract**

42 Transgenic technology was developed to introduce transgenes into various organisms to validate
43 gene function and add genetic variation for the development of beneficial input or output trait
44 over 40 years ago. However, the identification of the transgene insertion position in the genome,
45 while doable, can be cumbersome in the organisms with complex genomes. Here, we report a
46 Nanopore-based sequencing method to rapidly map transgenic alleles in the soybean genome.
47 This strategy is high-throughput, convenient, reliable, and cost-efficient. The transgenic allele
48 mapping protocol outlined herein can be easily translated to other higher eukaryotes with
49 complex genomes.

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72 **Introduction**

73 Transgenic technologies that introduce genetic variation into bacteria, animals and plants were
74 developed in 1972 (Cohen *et al.*, 1972), 1974 (Jaenisch and Mintz, 1974) and 1982 (Barton *et al.*,
75 1983), respectively. They have become a valuable resource to enhance genetic variations and to
76 gain insight of gene function. In higher plants, a single cope or multiple copies of transgenes are
77 randomly inserted into the genome (Kim *et al.*, 2007; Weising *et al.*, 1988). Expression levels of
78 a transgene are often influenced by the genomic context surrounding the transgenic allele and the
79 complexity of the genome (Butaye *et al.*, 2004; Day *et al.*, 2000; van Leeuwen *et al.*, 2001;
80 Weising *et al.*, 1988). Moreover, the transgene insertion position may also affect the function of
81 surrounding genes (Azpiroz-Leehan and Feldmann, 1997; Weising *et al.*, 1988). Importantly,
82 prior knowledge of map position of a transgenic allele is beneficial when breeding programs
83 begin to introgress the allele into elite germplasms. Consequently, there is a need to efficiently
84 and accurately characterize transgenic alleles in higher plants

85
86 Strategies have been developed for mapping of transgenic alleles (Guo *et al.*, 2016; Lepage *et al.*,
87 2013). Complexity of the transgenic locus can be estimated through multiple approaches
88 including Southern blot analysis (Southern, 1975), quantitative PCR (Ingham *et al.*, 2001) and
89 droplet PCR (Glowacka *et al.*, 2016). One of the first methods used to successfully map a
90 transgenic allele in higher plants was plasmid rescue. This strategy involves restriction enzyme
91 digestion of the host genome containing the transgenic allele, cloning the cleavage products into
92 plasmid and selection of the plasmid containing the transgene fragment (Nan and Walbot, 2009).
93 Subsequent methods for mapping transgenic alleles are also primarily PCR based, include
94 Thermal Asymmetric Interlaced PCR (TAIL-PCR) (Liu and Chen, 2007; Liu *et al.*, 1995),
95 Adaptor PCR which is sometimes referred to as anchored PCR (Singer and Burke, 2003; Thole
96 *et al.*, 2009), and T-linker PCR that utilizes a specific T/A ligation (Yuanxin *et al.*, 2003).
97 However, these methods are often challenging to scale-up for high-throughput (Guo *et al.*, 2016;
98 Ji and Braam, 2010). Moreover, failure to map transgenes can happen due to the complexity of
99 the transgenic locus and/or issues associated with the genomic context about the transgenic allele
100 (Wahler *et al.*, 2013). The next-generation Illumina sequencing technology is a method that can
101 map transgenic alleles in plants due to its depth of sequencing capacity (Guo *et al.*, 2016; Lepage
102 *et al.*, 2013; Polko *et al.*, 2012). However, because this method produces short reads, a high

103 degree of sequencing depth is needed, especially in crops that have large genomes that are rich in
104 repetitive sequence. This in turn, impacts the cost per transgenic locus mapped. In addition,
105 short-read sequencing data is challenging to resolve transgene insertion position in many plant
106 species, such as soybean, due to issues related with genome rearrangements and copy-number
107 variations, which may lead to inaccurate mapping locations.

108
109 Recently, single molecule real-time (SMRT) sequencing technologies have been developed that
110 provide long-read sequencing datasets. These SMRT platforms developed by Pacific Biosciences
111 (PacBio[®]) and Oxford Nanopore Technologies[®] offer significant attributes for genotyping plant
112 species. The most significant benefit is long read lengths, with Pacbio[®] platform generating up to
113 60 kb reads, and Nanopore[®] reads being up to ~ 1Mb (Jain *et al.*, 2018; Lu *et al.*, 2016). Both
114 technologies have been used in genome assembly (Badouin *et al.*, 2017; Jain *et al.*, 2018;
115 Michael *et al.*, 2018; Rhoads and Au, 2015; Schmidt *et al.*, 2017)). The MinION device, which
116 was developed by Nanopore[®] technology and entered the market in 2014, is a portable apparatus
117 with less than 100g in weight. Furthermore, it is compatible with a PC or laptop with USB 3.0
118 ports (Jain *et al.*, 2016) making it a flexibility attribute permitting use outside of a laboratory
119 setting (Castro-Wallace *et al.*, 2017). In addition, compared with PacBio[®], the Nanopore
120 Technology apparatus is affordable in most laboratories. Thus, the MinION platform provides
121 potential for a high-throughput, cost-effective strategy to map transgenic alleles in plant species
122 with complex genomes.

123
124 Described herein is a Nanopore Technology[®]-based platform pipeline designed for high-
125 throughput mapping of transgenic alleles in plant species. Employing a target enrichment
126 approach using a combination of oligo probes to capture DNA fragments containing the
127 transgenic allele, permitted the rapid identification of map position of 51 transgenic alleles in a
128 single 1D sequencing-run. The calculated cost incurred by the procedure to map 51 transgenic
129 alleles is estimated to be \$1,360, and the results are generated within one week. The reads with
130 the transgenic allele averaged in the hundred, for each sample, suggesting that pooling can be
131 further enlarged. These results demonstrate that this Nanopore[®]-based sequencing method is
132 rapid, convenient, reliable, cost-efficient and high-throughput.

133

134 **Materials and Methods**

135 **Soybean growth condition**

136 The soybean plants were grown in controlled greenhouse condition with 14 hour photoperiod
137 and 28/26°C day/night temperature. The soybean plants harboring the *Ds* element are in the
138 Thorne genetic background.

139

140 **DNA extraction and shearing**

141 DNA from of soybean leaves were extracted using CTAB method (Healey *et al.*, 2014) and
142 purified with DNeasy Plant Mini Kit (69104, QIAGEN). 6 µg genomic DNA in a total of 150 µl
143 nuclease free water was sheared into ~8 kb with g-TUBEs (520079, Covaris) by following
144 manufacturer's instruction.

145

146 **DNA barcodes and Enrichment of the *Ds* element-containing fragments**

147 1 µg sheared DNA fragments were end-repaired with Ultra II End-prep enzyme mix (E7546L,
148 NEB) for 5 minutes at 20°C and 5 minutes at 65°C using a thermal cycler, followed by
149 purification with the AMPure XP beads in a 1.5 ml DNA LoBind Eppendorf tube. After end-
150 repaired, DNA fragments were ligated to the Barcode Adapter from the barcode Kit 1D (EXP-
151 PBC001, Nanopore) using Blunt/TA Ligase Master Mix (M0367L, NEB). Following purification
152 with AMPure XP beads, the DNAs were ligated to the Barcode (EXP-PBC001, Nanopore) using
153 LongAmp Taq (M0287S, NEB). The barcoded DNA library was then purified with AMPure XP
154 beads. After barcoding, the library was purified with pheno/chloroform method, and diluted with
155 4.8ul H₂O+8.5ul xGen 2X Hybridization buffer, then add 2.7ul xGen Hybridization enhancer
156 (1072281, Integrated DNA Technologies, IDT) and 1ul probe. Then hybridization was
157 performed at 65°C for 4h in a thermal cycler. After hybridization, the targets were captured by
158 the Dynabeads M-270 Streptavidin beads (65-305, Thermo Fisher Scientific) that recognize the
159 dualbiotinylated probe. After washing with Stringent Wash Buffer and Wash Buffer I, II, III by
160 following the manufacture's protocol, the captured target fragments were amplified for 12 cycles
161 with primers recognizing the barcode using LongAmp Taq at the PCR condition: 15 seconds at
162 98°C, 30 seconds at 60°C, 6 minutes at 72°C. The resulting PCR products were purified with
163 AMPure XP beads, which were subjected to second round enrichment (step 3 and 4), or library

164 construction following manufacturer's instruction. The 5' dual biotinylated probe was
165 synthesized from IDT and its sequence is shown probe in Supplementary Table S1.

166

167 **Library Construction and Sequencing**

168 Following target enrichment, barcoded libraries were pooled and 1 µg samples were end-repaired
169 with the Ultra II End-prep enzyme, purified with the AMPure XP beads and then ligated to the
170 sequencing adaptor (SQK-LSK108, Nanopore) with the Blunt/TA Ligation Master Mix. After
171 purification with the AMPure beads, the adapted DNA libraries were sequenced in the flow cells
172 (R9.4 version, FLC-MIN106, Nanopore). After 20-24 hours, the sequencing was stopped.

173

174 **Assessment of target enrichment efficiency**

175 To assess the target enrichment, 2% of samples were used as templates to perform quantitative
176 PCR (qPCR) using SYBR Green PCR Master Mix (Bio-Rad) with primers recognizing the *Ds*
177 element or an unrelated intergenic region in soybean chromosome 7. The primer sequences are
178 shown in Supplementary Table S1.

179

180 **PCR validation**

181 PCR reaction was performed with primers listed in Supplementary Table S1 using the condition:
182 95 °C 2 min; 95 °C 30 sec, 50 °C 30 sec, 72 °C 1:20 min for 34 cycles; 72 °C 5 min. The PCR
183 products were isolated with 1% agarose gel and visualized by Ethidium bromide staining.

184

185 **Bioinformatics analysis**

186 All barcoded reads were de-multiplexed and adapters were trimmed off using the Porechop
187 version 0.2.1 (<https://github.com/rrwick/Porechop>) with default parameters. To identify reads
188 with the *Ds* target sequence, the *Ds* target sequence was searched against trimmed reads for each
189 sample with E-value $\leq 10^{-3}$. For all hits with the *Ds* target sequence, the 5' end and 3' end
190 sequences of the *Ds* target sequence were scanned on each read to identify long reads with one or
191 two complete ends of the *Ds* target sequence. Sequences on 5' end and/or 3' end sequences of
192 long reads beyond the *Ds* target sequence, if length > 20bp, were recorded as flanking sequences,
193 which come from soybean genome. The flanking sequences were undergone blast searches
194 against the soybean genome (v1.0). Uniquely aligned hits with aligned length > 200bp and >

195 80% sequence identity were kept. The genomic location for each flanking sequence were
196 determined based on its alignment. The insertion sites were determined based on statistically
197 enriched flanking sequences. The zero-inflated Poisson regression was used to model count data
198 that has an excess of zero counts. All read counts were fitted into the Zero-inflated Poisson
199 regression model with the R package, ZIM. For each peak of read counts, to determine if it was
200 a significant peak, a P-value was calculated as the probability of observing a count value equally
201 as extreme, or more extreme, than the given read count based on the fitted distribution

202

203 **Results**

204

205 **Mapping of maize *Ds* transpositions in the soybean genome through MinION sequencing** 206 **without target enrichment**

207 To evaluate the potential application of MinION sequencing to map transgenic alleles, a soybean
208 line, which contains a transgene stack harboring the maize Activator (*Ac*)/Dissociation (*Ds*)
209 transposon system were used. The *Ac* transposase is controlled by the 35S CaMV promoter, and
210 the *Ds* element harbors the cassava vein mosaic virus promoter (CsVMV) as an activation tag.
211 The selected soybean lines were previously genotyped via Southern blot analysis to ascertain the
212 presence of the *Ds* loci and the absence of *Ac* allele, along with mapping of the *Ds* allele using
213 TAIL-PCR (Fig. 1A and Supplementary Fig. S1). To assess the power of MinION sequencing to
214 map transgenic alleles, genomic DNA isolated from one of the selected genotyped soybean lines
215 carrying the *Ds*-activation tag was sequenced on the FLO-MIN106 flow cell following the 1D
216 sequencing protocol without DNA fragmentation (Fig. 1B). A 24-hour sequencing run produced
217 approximately one million reads, resulting in about 2.8 Gb of sequence data (Table 1). Mining
218 the sequence data for *Ds* element revealed two reads containing the *Ds* element (Table 1). One
219 read was 957 bp covering partial *Ds* element flanked by 370 bp sequence at 3' end, and the other
220 was 6,806 bp, containing the full-length *Ds* element flanked by 2,347 bp 5' upstream sequences
221 and 3,047 bp downstream flanking the *Ds* sequence (Fig. 1C). The identified *Ds* junction
222 fragment sequences were mapped to the soybean Glyma.15g128600 gene (Fig. 1C), in
223 agreement to the TAIL-PCR results. To further validate the sequencing and TAIL-PCR
224 outcomes, PCR reactions were carried out with a primer set designed to span the *Ds*/junction
225 about the insertion site (Fig. 1A). The data revealed a 360 bp PCR product amplified from the

226 endogenous Glyma.15g128600 gene when control DNAs were used as templates, and a 1526 bp
227 fragment predicted to carry the *Ds*/junction target sequence amplified from DNAs of the
228 transgenic soybean plants (Fig. 1D). These results demonstrate the potential of MinION
229 sequencing to map a transgenic allele in the soybean genome. However, given the few reads that
230 contain the *Ds*, refinement in the genomic DNA processing steps would be required for a high
231 throughput/cost effective mapping pipeline with this technology.

232

233 **Target enrichment of transgenic allele to improve mapping throughput with MinION** 234 **sequencing.**

235 To improve read counts around the junction of a transgenic allele, a PCR-based method to enrich
236 the target sequences in the DNA library (Fig. 2) was developed. To test the enrichment protocol,
237 DNA from two soybean lines carrying a *Ds* activation tag allele, previously characterized via
238 Southern blot and mapped by TAIL-PCR, were used. The enrichment protocol incorporated steps
239 for fragmentation of DNA to approximate 8 kb, end-repairing and dT-tailing, with subsequent
240 ligation to barcode adapters and PCR-barcoding (Fig. 2). The resultant reaction products were
241 subjected to a 120nt 5'-dual biotinylated probe designed to capture the transgenic *Ds* allele (Fig.
242 2). Following the probe capture step, the probe-captured fraction was re-amplified by PCR and
243 products were pooled for sequencing (Fig. 2). Total readings obtained were 357765 and 326189
244 for Line 2 and Line 3, respectively (Table 2). The average read length of Line 2 was 2426 bp
245 with the longest read of 20453 bp (Table 2), while the average read length of Line 3 was 2445 bp
246 with the longest read of 48971 bp (Table 2). Among the reads obtained implementing the
247 enrichment steps, 203 and 438 contained the *Ds*-allele sequence, for lines 2 and 3, respectively,
248 which correctly mapped to gene calls, Glyma.19g105100 and Glyma.11g247400, respectively
249 (Fig. 3A, 3B, and Table 2). The map positions were re-confirmed using PCR analyses
250 incorporating a primer set designed to amplify *Ds*/junction fragment region (Fig. 3C).

251

252 Given the high number of reads containing the *Ds* element, following the targeted enrichment
253 approach, the method appeared to be amendable for higher throughput by increasing sample pool
254 size. To this end, 15 soybean lines previously ascertained to harbor a single *Ds* element (Line 4-
255 Line 18) were selected for integrating a pooling strategy with the targeted enrichment method.
256 Here five DNA pools, each of which contained DNAs from three soybean lines (Table 3 and

257 Supplementary Fig. S2), were prepared. Following the first target enrichment step, the pools
258 were subjected to an additional round of purification to increase coverage of the *Ds*-containing
259 DNA fragments (Fig. 2). Subsequent to each purification step, a quantitative PCR (qPCR) was
260 used to estimate the relative enrichment level of target fragment compared with an unrelated
261 DNA region that served as an internal control. After one round of enrichment, the ratio of target
262 fragments to the unrelated region was enriched 132-1120x across all DNA pools (Fig. 4A).
263 Following two rounds of purification the enrichment ratio ranged from 7469 to 238193 times in
264 the pools (Fig. 4B). MinION sequencing of the double enriched products resulted in total number
265 of reads ranged from 117266 to 523192 across the pools (Table 3), with reads containing the *Ds*
266 sequence ranging from 1856 to 36388 in the pools (Table 3). These results were translated to
267 ratios of reads containing the target sequence per total read counts for each pool in the range of
268 0.53 to 6.95% (Table 3). The average length of these *Ds*-containing reads was longer than 2Kb
269 and the majority (>99%) of these readings were longer than 1.2 Kb (Fig. S3). The *Ds*-containing
270 reads of each DNA pool were successfully mapped to three positions in the soybean genome
271 (Supplementary Table S2; Fig. 4C showing a position of readings at soybean genome from Pool
272 4), reflecting that the pools each contained three independently integrated *Ds* elements within the
273 soybean genome. The predicted mapped locations identified in Pool 4 were subsequently verified
274 by PCR using primer sets that spanned the *Ds* element/soybean genome junction (Line 13-Line
275 15; Fig. 4D).

276

277 **MinION sequencing provides a platform for high-throughput method to identify map** 278 **position of transgenic alleles in plants**

279 Reads containing sequences of the target allele in soybean, a *Ds*-activation tag element, averaged
280 in the hundreds (Table 3) from non-enriched genomic DNA, reflecting the power of MinION
281 sequencing technology as a cost effective tool that could be translated as a high-throughput
282 method to map a transgenic allele in the soybean genome. To further test its throughput, an
283 expanded pooling was performed with the enrichment steps, wherein 51 independent soybean
284 lines containing a single *Ds* element were divided into six pools, each of which contained eight
285 to ten lines (Table 4), for minion sequencing. The outcome from this expanded throughput
286 evaluation resulted in total read counts ranging from 19758 to 282690 across the pools, with
287 reads containing the *Ds* sequence ranging from 212 to 16146 (Table 4). These data were

288 sufficient to successfully map the transgenic allele in each of the 51 soybean lines analyzed
289 (Supplementary Table S3).

290
291 To further validate if this method is suitable to map potential multiple transgene insertions, we
292 selected 18 transgenic soybean lines harboring one to 3 copies of the original Ds transgene (~
293 5Kb), which were determined by southern blot (Figure S4 and Supplementary Table S4). We
294 divided these plants into 4 pools, and performed MinIon sequencing after target enrichment. We
295 were able to identify 29 transgenic insertion loci (Supplementary Table S3), which agree with
296 the southern blot result. This result suggests that our method can be used to map transgene loci
297 with known insertion numbers.

298 299 **Discussion**

300 Communicated herein is a long read and affordable sequencing-method suitable for high-
301 throughput mapping of transgenic alleles in higher plants. This method has at least five
302 advantages. First, it provides reliable information of sequences flanking the insertion position. In
303 most scenarios, over a hundred reads contain the target transgenic allele and associated junction
304 sequences. Second, the method is scalable, by coupling pooling with enrichment steps prior to
305 sequencing the transgenic allele in 51 independent lines were successfully mapped in a single
306 sequencing run. Importantly, the reads containing the target allele are sufficient to accurately
307 map a transgenic allele back to a reference genome. Thus, it is likely that sample pools can be
308 further enlarged. Moreover, the target enrichment method still has potential for additional
309 refinement given the ratios of reads containing the target sequences per total read count are still
310 low (ranging from ~0.5 to 10%). The current enrichment step only incorporates one probe to the
311 target allele. A refinement in the enrichment step might include the use of multiple probes that
312 recognize different regions of the target allele thereby improving specificity and efficiency of
313 capture. Third, the cost per map is relatively low, estimated at \$1360 per 51 samples, excluding
314 labor. If pooling can be expanded, the cost will be further reduced. In addition, after one round
315 purification, we may also use primers that recognize the target and adaptor to amplify the target
316 containing fragments, which will eliminate second round purification and improve specificity,
317 and thereby reduce the cost and allow pooling more samples. Fourth, it is rapid with the
318 timeframe from DNA fragmentation to mapped transgenic allele being approximately one week.

319 Lastly, since MinION is a portable device that can run at a laptop or desktop computer,
320 permitting utilization of this tool to modestly equipped laboratories globally, it has unrivaled
321 convenience and broad usability.

322

323 The introduction of novel genetic variation into higher plants through the tools of transgenic
324 technology offers a powerful way to complement plant breeding programs. Prior knowledge of
325 transgene insertion position facilitates breeding decisions. The MinION-based sequencing
326 strategy outlined here is a powerful, high-throughput tool to determine the insertion position of
327 transgenic alleles in higher plants. The average length of reads containing the Ds element here
328 was ~ 2.1 Kb and the longest reads was ~ 10 kb in the 51 sample-sequencing. This length should
329 be sufficient to cover a portion of a longer transgene with flanking sequencing at one end. Indeed,
330 we used this method to determine a population of soybean lines containing a ~5kb transgene.
331 The average reads containing the Ds elements are more than one hundred, which should be
332 sufficient to identify multiple insertion events in the genome. However, it may still be a
333 challenge to identify transgene copy numbers with the current target enrichment method when
334 multiple copies of the transgene exist in the same location of the genome. In this scenario, the
335 average read length needs to be improved. A possible solution is to perform size selection after
336 each round of target enrichment or after adapter addition to eliminate the short DNA fragments,
337 and thereby to improve the read length, although this may reduce the numbers of reads
338 containing the transgene.

339

340 Although this method is developed to examine a population of soybean lines containing the same
341 transgene, it can be adapted to map the transgene insertion from plants containing different
342 transgenes that do not share common fragments using probes targeting individual transgenes. We
343 noticed variations of reading within a barcode. This may due to the difference of DNAs
344 surrounding the insertion positions, which results in variations in efficiency of ligation or PCR.
345 Moreover, there are reading variations among different pools. This is likely due to that different
346 barcodes may have different optimal PCR conditions, as we currently use the same PCR
347 amplification condition for all pools.

348

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354

355 **Conflict of Interest**

356 The authors declare Conflict of Interest

References:

- Azpiroz-Leehan R, Feldmann KA.** 1997. T-DNA insertion mutagenesis in Arabidopsis: going back and forth. *Trends Genet* **13**, 152-156.
- Badouin H, Gouzy J, Grassa CJ, Murat F, Staton SE, Cottret L, Lelandais-Briere C, Owens GL, Carrere S, Mayjonade B, Legrand L, Gill N, Kane NC, Bowers JE, Hubner S, Bellec A, Berard A, Berges H, Blanchet N, Boniface MC, Brunel D, Catrice O, Chaidir N, Claudel C, Donnadiou C, Faraut T, Fievet G, Helmstetter N, King M, Knapp SJ, Lai Z, Le Paslier MC, Lippi Y, Lorenzon L, Mandel JR, Marage G, Marchand G, Marquand E, Bret-Mestries E, Morien E, Nambeesan S, Nguyen T, Pegot-Espagnet P, Pouilly N, Raftis F, Sallet E, Schiex T, Thomas J, Vandecasteele C, Vares D, Vear F, Vautrin S, Crespi M, Mangin B, Burke JM, Salse J, Munos S, Vincourt P, Rieseberg LH, Langlade NB.** 2017. The sunflower genome provides insights into oil metabolism, flowering and Asterid evolution. *Nature* **546**, 148-152.
- Barton KA, Binns AN, Matzke AJ, Chilton MD.** 1983. Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. *Cell* **32**, 1033-1043.
- Butaye KM, Goderis IJ, Wouters PF, Pues JM, Delaure SL, Broekaert WF, Depicker A, Cammue BP, De Bolle MF.** 2004. Stable high-level transgene expression in Arabidopsis thaliana using gene silencing mutants and matrix attachment regions. *Plant J* **39**, 440-449.
- Castro-Wallace SL, Chiu CY, John KK, Stahl SE, Rubins KH, McIntyre ABR, Dworkin JP, Lupisella ML, Smith DJ, Botkin DJ, Stephenson TA, Juul S, Turner DJ, Izquierdo F, Federman S, Stryke D, Somasekar S, Alexander N, Yu G, Mason CE, Burton AS.** 2017. Nanopore DNA Sequencing and Genome Assembly on the International Space Station. *Sci Rep* **7**, 18022.
- Cohen SN, Chang AC, Hsu L.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of Escherichia coli by R-factor DNA. *Proc Natl Acad Sci U S A* **69**, 2110-2114.
- Day CD, Lee E, Kobayashi J, Holappa LD, Albert H, Ow DW.** 2000. Transgene integration into the same chromosome location can produce alleles that express at a predictable level, or alleles that are differentially silenced. *Genes Dev* **14**, 2869-2880.
- Glowacka K, Kromdijk J, Leonelli L, Niyogi KK, Clemente TE, Long SP.** 2016. An evaluation of new and established methods to determine T-DNA copy number and homozygosity in transgenic plants. *Plant Cell Environ* **39**, 908-917.
- Guo B, Guo Y, Hong H, Qiu LJ.** 2016. Identification of Genomic Insertion and Flanking Sequence of G2-EPSPS and GAT Transgenes in Soybean Using Whole Genome Sequencing Method. *Front Plant Sci* **7**, 1009.
- Healey A, Furtado A, Cooper T, Henry RJ.** 2014. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods* **10**, 21.
- Ingham DJ, Beer S, Money S, Hansen G.** 2001. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *Biotechniques* **31**, 132-134, 136-140.
- Jaenisch R, Mintz B.** 1974. Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc Natl Acad Sci U S A* **71**, 1250-1254.
- Jain M, Koren S, Miga KH, Quick J, Rand AC, Sasani TA, Tyson JR, Beggs AD, Dilthey AT, Fiddes IT.** 2018. Nanopore sequencing and assembly of a human genome with ultra-long reads. *Nature Biotechnology*.

- Jain M, Olsen HE, Paten B, Akeson M.** 2016. The Oxford Nanopore MinION: delivery of nanopore sequencing to the genomics community. *Genome Biol* **17**, 239.
- Ji J, Braam J.** 2010. Restriction site extension PCR: a novel method for high-throughput characterization of tagged DNA fragments and genome walking. *PLoS One* **5**, e10577.
- Kim IH, Nagel J, Otten S, Knerr B, Eils R, Rohr K, Dietzel S.** 2007. Quantitative comparison of DNA detection by GFP-lac repressor tagging, fluorescence in situ hybridization and immunostaining. *BMC Biotechnol* **7**, 92.
- Lepage E, Zampini E, Boyle B, Brisson N.** 2013. Time- and cost-efficient identification of T-DNA insertion sites through targeted genomic sequencing. *PLoS One* **8**, e70912.
- Liu YG, Chen Y.** 2007. High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *Biotechniques* **43**, 649-650, 652, 654 passim.
- Liu YG, Mitsukawa N, Oosumi T, Whittier RF.** 1995. Efficient isolation and mapping of Arabidopsis thaliana T-DNA insert junctions by thermal asymmetric interlaced PCR. *Plant J* **8**, 457-463.
- Lu H, Giordano F, Ning Z.** 2016. Oxford Nanopore MinION Sequencing and Genome Assembly. *Genomics Proteomics Bioinformatics* **14**, 265-279.
- Michael TP, Jupe F, Bemm F, Motley ST, Sandoval JP, Lanz C, Loudet O, Weigel D, Ecker JR.** 2018. High contiguity Arabidopsis thaliana genome assembly with a single nanopore flow cell. *Nat Commun* **9**, 541.
- Nan GL, Walbot V.** 2009. Plasmid rescue: recovery of flanking genomic sequences from transgenic transposon insertion sites. *Methods Mol Biol* **526**, 101-109.
- Polko JK, Temanni MR, van Zanten M, van Workum W, Iburg S, Pierik R, Voeselek LA, Peeters AJ.** 2012. Illumina sequencing technology as a method of identifying T-DNA insertion loci in activation-tagged Arabidopsis thaliana plants. *Mol Plant* **5**, 948-950.
- Rhoads A, Au KF.** 2015. PacBio Sequencing and Its Applications. *Genomics Proteomics Bioinformatics* **13**, 278-289.
- Schmidt MH, Vogel A, Denton AK, Istace B, Wormit A, van de Geest H, Bolger ME, Alseikh S, Mass J, Pfaff C, Schurr U, Chetelat R, Maumus F, Aury JM, Koren S, Fernie AR, Zamir D, Bolger AM, Usadel B.** 2017. De Novo Assembly of a New Solanum pennellii Accession Using Nanopore Sequencing. *Plant Cell* **29**, 2336-2348.
- Singer T, Burke E.** 2003. High-throughput TAIL-PCR as a tool to identify DNA flanking insertions. *Methods Mol Biol* **236**, 241-272.
- Southern EM.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-517.
- Thole V, Alves SC, Worland B, Bevan MW, Vain P.** 2009. A protocol for efficiently retrieving and characterizing flanking sequence tags (FSTs) in Brachypodium distachyon T-DNA insertional mutants. *Nat Protoc* **4**, 650-661.
- van Leeuwen W, Ruttink T, Borst-Vremsen AW, van der Plas LH, van der Krol AR.** 2001. Characterization of position-induced spatial and temporal regulation of transgene promoter activity in plants. *J Exp Bot* **52**, 949-959.
- Wahler D, Schauer L, Bendiek J, Grohmann L.** 2013. Next-Generation Sequencing as a Tool for Detailed Molecular Characterisation of Genomic Insertions and Flanking Regions in Genetically Modified Plants: a Pilot Study Using a Rice Event Unauthorised in the EU. *Food Analytical Methods* **6**, 1718-1727.
- Weising K, Schell J, Kahl G.** 1988. Foreign genes in plants: transfer, structure, expression, and applications. *Annu Rev Genet* **22**, 421-477.

Yuanxin Y, Chengcai A, Li L, Jiayu G, Guihong T, Zhangliang C. 2003. T-linker-specific ligation PCR (T-linker PCR): an advanced PCR technique for chromosome walking or for isolation of tagged DNA ends. *Nucleic Acids Res* **31**, e68.

Figure legends:

Fig. 1. MinION sequencing without *Ds*-enrichment. (A) The schematic diagram of *Ds* insertion in soybean genome. The length of *Ds* insertion is 1166bp. The positions of forward (F) and reverse (R) primers used for PCR genotyping are shown. (B) Workflow of direct genome sequencing without target-enrichment. Genomic DNA was end-repaired and dA-tailed, ligated with sequencing adapters and sequenced on the FLO-MIN106 flow cell. (C) The schematic diagram of the *Ds* insertion in Glyma.15g128600 gene. Two reads are shown. The first one

covers 2347bp in the 5' flanking region and 3047bp in the 3' flanking region. The second one contains 370bp flanking sequence in the 3' region. **(D)** PCR validation of the *Ds* insertion in Line 1. Thorne was used as control plant. The length of DNA fragment without the *Ds* element in control plant is 360 bp, while the fragment length from *Ds*-containing Line 1 is 1526 bp.

Fig. 2. The workflow of the enrichment of *Ds*-containing fragments in DNA libraries

(A) The schematic diagram of oligo probe used to capture the *Ds* element. The probe is dual biotinylated at 5' end (green diamond). **(B)** The workflow of sequencing the enriched *Ds*-containing DNA fragments. Genomic DNA was sheared and ligated to PCR barcode adapters. The *Ds*-containing fragments were enriched one or two rounds. The enriched fragments were pooled and sequenced.

Fig. 3. Sequencing results after one-round enrichment of the *Ds*-containing fragments.

(A) and **(B)** Schematic diagram of the flanking sequences of Line 2 (A) and Line 3 (B). Partial sequences of reads were shown. **(C)** PCR validation of the *Ds* insertion in Line 2 and Line 3. Thorne was used as control plant. The lengths of the DNA fragment without the *Ds* element are 612bp for Line 2 and 689 bp for Line 3. With the *Ds* elements, the lengths of the DNA fragments are 1778 bp for Line 2 and 1855 bp for Line 3.

Fig. 4 Sequencing results after two-round enrichment of the *Ds*-containing fragments. **(A)**

and **(B)** Efficiency of one-round (A) and two-round (B) enrichment of the *Ds* element-containing fragments. 2% of samples before and after probe-enriching were used to perform qPCR. The amount of target fragments was normalized to that of internal control. **(C)** Schematic diagram of the flanking sequences of Line 15. Partial sequences of reads were shown. **(D)** PCR validation of the *Ds* insertion in Line 13, Line 14, and Line 15. The three individual lines were examined with three pairs of primers, respectively. Each primer pair (labeled above the picture) recognizes a potential insertion position of the *Ds* element, identified by sequencing. Line 13 containing a *Ds* insertion in Glyma15G128600 gene produced a 1719 bp fragment, while Line 14 and Line 15 without insertions in this gene generated ~ 559 bp fragments (indicated as arrows). Line 14 containing a *Ds* insertion in Glyma05G163800 gene produced a 1628 bp fragment, while Line 13 and Line 15 without insertions in this gene generated 462 bp fragments (indicated as arrows).

Line 15 containing a *Ds* insertion in Glyma11G181700 gene produced a 1560 bp fragment, while Line 13 and Line 14 without insertions in this gene generated 394 bp fragments (indicated as arrows).

Table 1. Sequencing result of one line without enrichment

Table 2. Sequencing result of two lines with one-round enrichment

Table 3. Sequencing result of the 15-sample pools

Table 4. Sequencing result of the 50-sample pools

Supplementary data

Fig. S1. The diagram of the *Ds* system.

Fig. S2. Agarose gel electrophoresis of sheared DNA. 8 ug genomic DNA samples in 150 ul ddH₂O were fragmented to 8 kb

Fig. S3. Size distribution of readings containing the *Ds* elements.

Fig. S4. Copy numbers in various soybean transgenic lines determined by Southern Blot.

Table S1. Oligo DNAs used in this study

Table S2. Positions of *Ds* insertion identified in the 15-sample sequencing

Table S3. Positions of *Ds* insertion identified in the 50-sample sequencing

Table S4. Positions of *T-DNA* insertion identified in the 18-sample sequencing

Table 1. Sequencing result of one line without enrichment

	Total reads number	Longest read (bp)	Target reads number	Percent of target reads	Longest read with targets (bp)
Line 1	1061117	351899	2	0.00019	6806

Note: Longest read indicates the longest read in all readings.

Table 2. Sequencing result of two lines with one-round enrichment

	Total reads number	Longest read (bp)	Target reads number	Percent of target reads	Longest read with targets (bp)
Line 2	357765	20453	203	0.057	6524
Line 3	326189	48971	438	0.134	6725

Note: Longest reads indicate the longest reads in the individual barcoded lines.

Table 3. Sequencing result of the 15-sample pools

	Line number	Total reads number	Longest read (bp)	Target reads number	Percent of target reads	Longest read with targets (bp)
DNA pool 1	Line 4-6	351722	16352	1856	0.53	5100
DNA pool 2	Line 7-9	490852	21457	30937	6.30	9317
DNA pool 3	Line 10-12	117266	8000	3165	2.70	5770
DNA pool 4	Line 13-15	523192	25983	36388	6.95	13213
DNA pool 5	Line 16-18	234809	14215	5412	2.30	6008

Note: Line number indicates the pooled Ds-containing lines. Longest reads indicate the longest reads in each pool.

Table 4. Sequencing result of the 51-sample pools

	Line number	Total reads number	Longest read (bp)	Target reads number	Percent of target reads	Longest read with targets (bp)
DNA pool 1	Line 19-26	20317	8512	2104	10.36	6096
DNA pool 2	Line 27-34	47257	9995	212	0.45	6042
DNA pool 3	Line 35-42	19758	6953	1569	7.94	5476

DNA pool 4	Line 43-50	181763	10577	14485	7.97	8178
DNA pool 5	Line 51-59	63227	10137	5698	9.01	7007
DNA pool 6	Line 60-69	282690	10397	16146	5.71	8691

Note: Line number indicates the pooled Ds-containing lines.
Longest reads indicate the longest reads in each pool.

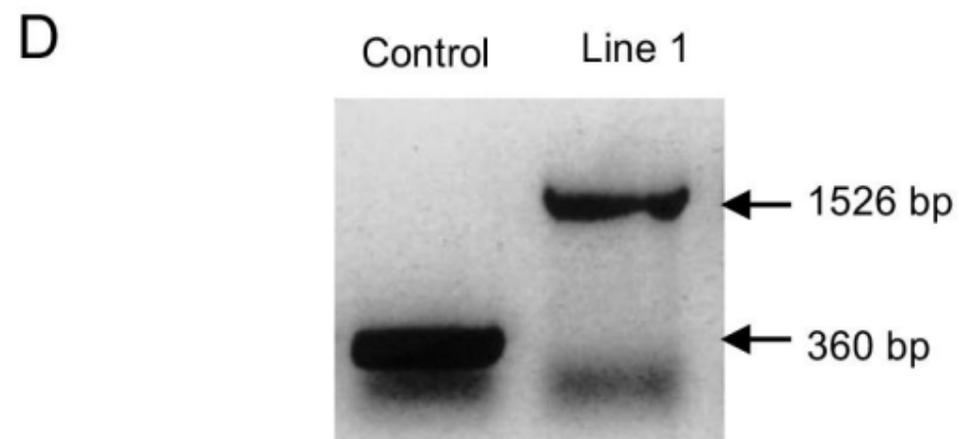
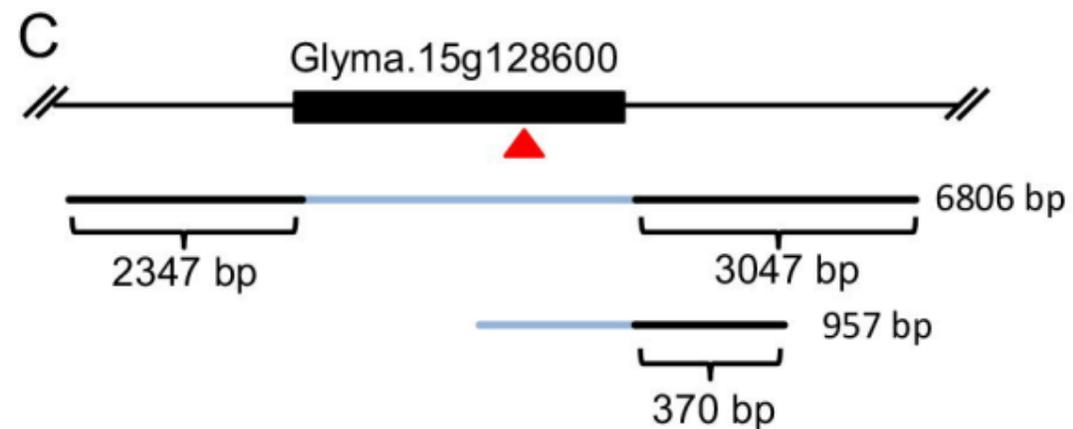
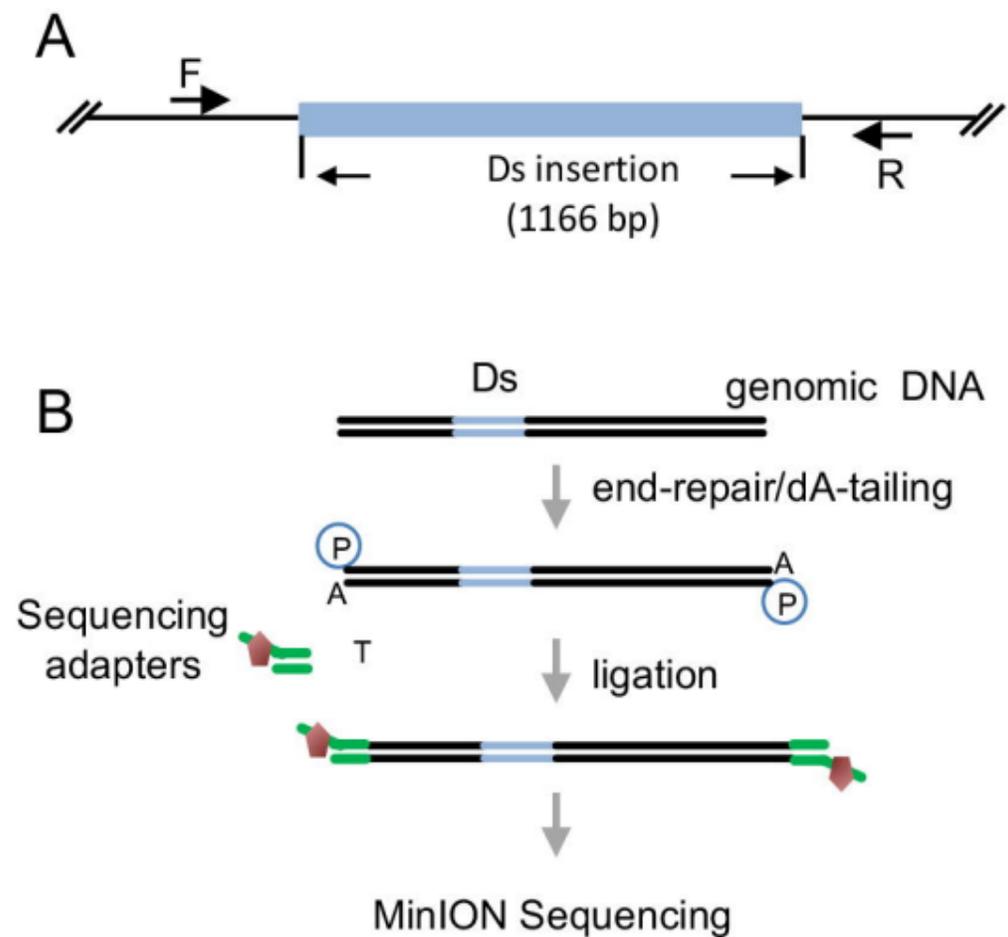


Fig. 1

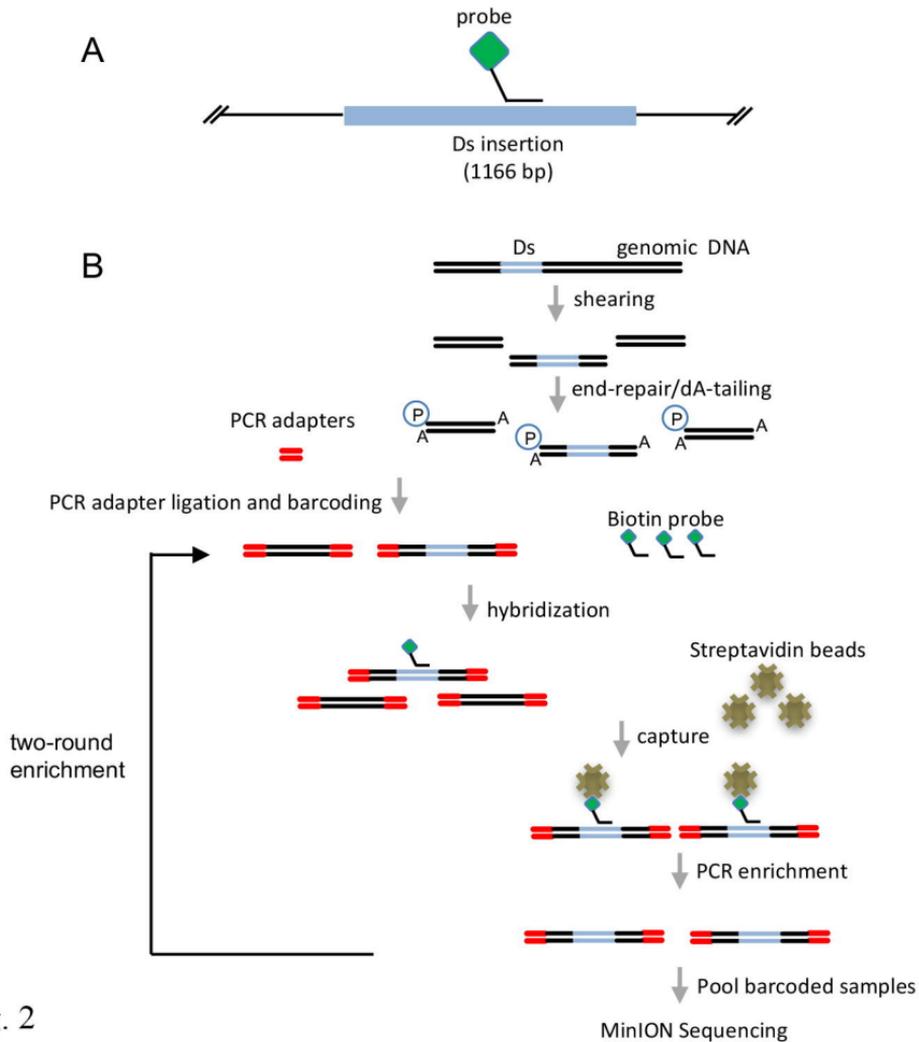


Fig. 2

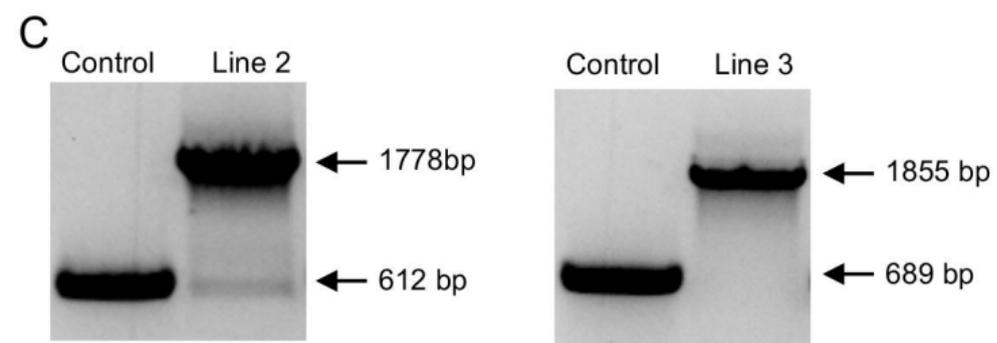
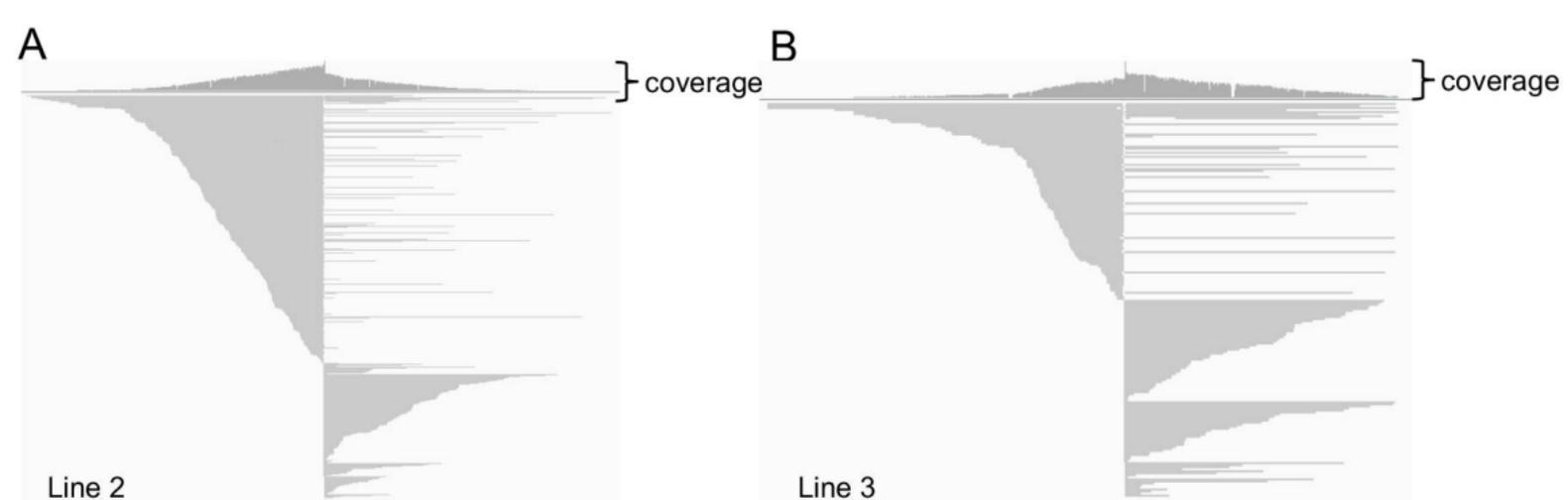


Fig. 3

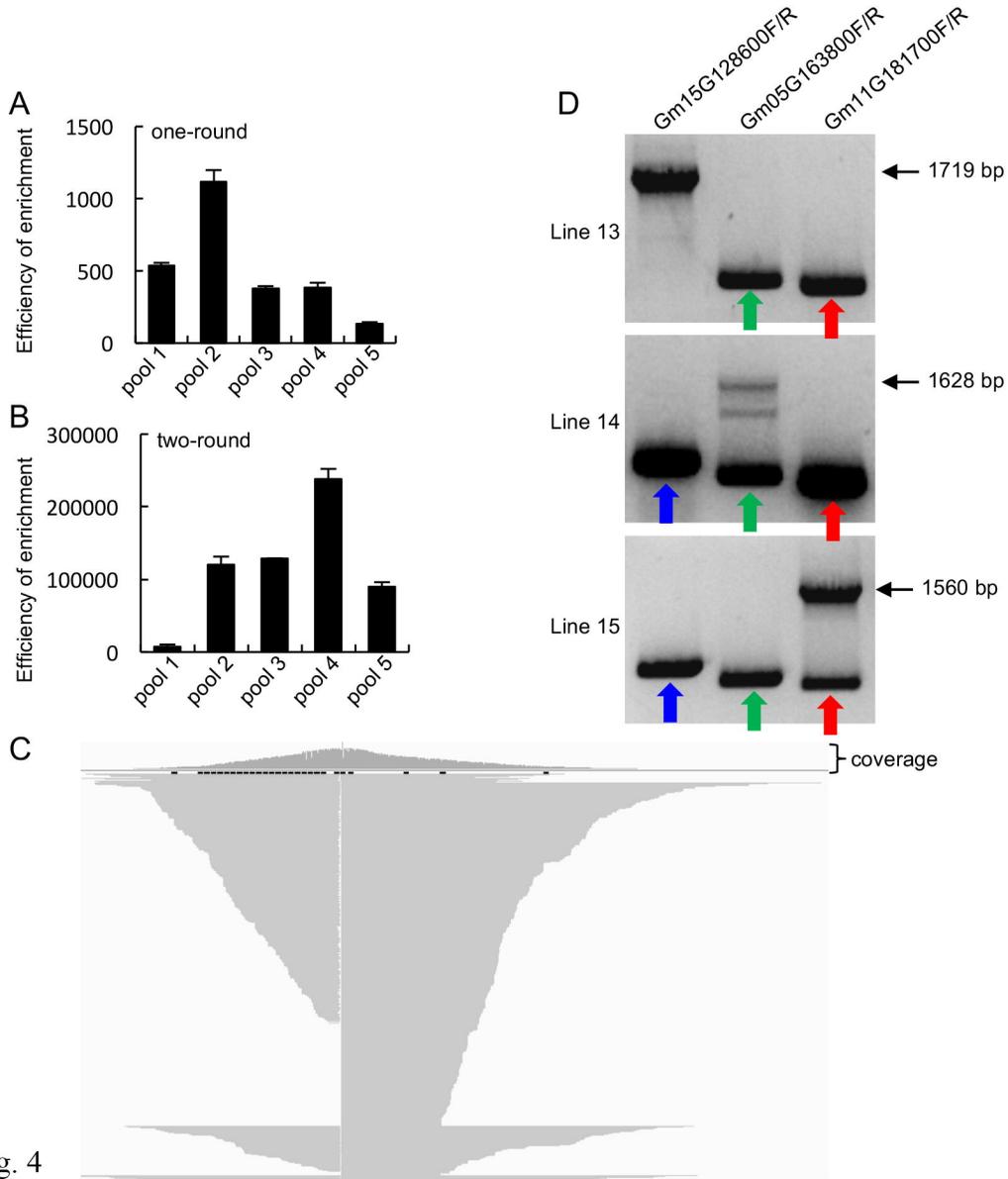


Fig. 4