

1 **Running Title:** Epigenetic repression of the SHATTERPROOF 1 gene is
2 associated with pod shatter resistance

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4 **An Epigenetic LTR-retrotransposon insertion in the upstream**
5 **region of *BnSHP1.A9* controls quantitative pod shattering**
6 **resistance in *Brassica napus***

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53 Abstract

54 Seed loss resulting from pod shattering is a major problem in oilseed rape
 55 (*Brassica napus* L.) production worldwide. However, the molecular
 56 mechanisms underlying pod shatter resistance are not well understood. Here
 57 we show that the pod shatter resistance at quantitative trait locus, qSRI.A9.1
 58 is controlled by a *SHATTERPROOF1* (*SHP1*) paralog in *B. napus*
 59 (*BnSHP1.A9*). Expression analysis by quantitative RT-PCR showed that
 60 *BnSHP1.A9* was specifically expressed in flower buds, flowers and
 61 developing siliques in the oilseed rape line (R1) carrying the qSRI.A9.1 allele
 62 with negative effect, but not expressed in any tissue of the line (R2) carrying
 63 the positive effect qSRI.A9.1 allele. Transgenic plants constitutively
 64 expressing *BnSHP1.A9* alleles from pod resistant and pod shattering
 65 parental lines showed that both alleles are responsible for pod shattering via
 66 promoting lignification of *enb* layer, which indicated allelic difference of
 67 *BnSHP1.A9* gene *per se* is not the causal factor of the QTL. The upstream
 68 sequence of *BnSHP1.A9* in the promotor region harboring highly methylated
 69 long terminal repeat retrotransposon insertion (LTR, 4803bp) in R2
 70 repressed the expression of *BnSHP1.A9*, and thus contributed to the positive
 71 effect on pod shatter resistance. Genetic and association analysis revealed
 72 that the *copia* LTR retrotransposon based marker *BnSHP1.A9*-R2 can be
 73 used for breeding for pod shatter resistant varieties and reducing the loss of
 74 seed yield in oilseed rape.

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77 **Keywords:** Oilseed rape, pod shattering, genetic analysis, natural
 78 variation, LTR-retrotransposon, gene expression

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81 Introduction

82 Oilseed rape (*Brassica napus* L.) is not only a major source of edible
83 vegetable oil for human consumption, but also provides an important energy
84 resource for stock-feed and biodiesel production. Upon maturity, siliques
85 (pods) of oilseed rape open, dehiscing seed and causing significant yield
86 loss; particularly if oilseed is harvested after the full maturity (BBCH scale 95).
87 Pod shattering usually accounts for an approximately 10% yield loss on an
88 average, however in certain environments it can cause yield loss up to 50%
89 (Kadkol et al., 1984; Wang et al., 2007). In order to reduce such loss, oilseed
90 rape is either harvested manually or mechanically before full maturation of
91 seeds (windrowing). Premature harvest can reduce yield loss, but immature
92 seed can lead to lower oil content (Østergaard et al., 2006) and higher
93 chlorophyll content. In recent years, due to the shortage and high cost of
94 labour, broad-acre cropping of oilseed rape has been preferentially combine
95 harvested by farmers. Varieties with improved pod shatter resistance,
96 amenable for mechanical harvesting provide a cost-effective solution for
97 commercial oilseed rape production worldwide.

98 Genetic variation for pod shatter resistance exists in *Brassica napus*,
99 *Brassica rapa*, *Brassica juncea* and *Brassica carinata* germplasm (Hu et al.,
100 2012; Liu et al., 2016; Raman et al., 2014; Raman et al., 2017; Kadkol et al.,
101 1984), and can be exploited to breed commercial varieties with improved pod
102 shatter resistance. Genetic studies have revealed that pod shatter resistance

103 is controlled by multiple genes. Several quantitative trait loci (QTL)
104 associated with this trait have been localized on genetic and physical maps
105 of oilseed rape (Hu et al., 2012; Raman et al., 2014; Liu et al., 2016).
106 However, the identification of corresponding genes underlying QTL for pod
107 shatter resistance in *B. napus* has not been reported yet.

108 In the model plant *Arabidopsis thaliana*, which belongs to the same
109 Brassicaceae family as oilseed rape, gene network involved in pod
110 development and dehiscence has been elucidated (Liljegren et al., 2004).
111 For example, MADS-box transcription factors *SHATTERPROOF1* (*SHP1*)
112 and *SHATTERPROOF2* (*SHP2*) (Liljegren et al., 2000), basic helix-loop-helix
113 (bHLH) gene *INDEHISCENT* (*IND*) (Liljegren et al., 2004), and *ALCATRAZ*
114 (*ALC*) (Rajani and Sundaresan, 2001) regulate the differentiation of the
115 dehiscence zone. The activity of valve margin identity genes is repressed by
116 *FRUITFULL* (*FUL*) in the valves (Gu et al., 1998), and *REPLUMLESS* (*RPL*)
117 in the replum (Roeder et al., 2003). Several phytohormones such as auxin,
118 cytokinin and gibberellin are also essential for pod development and the
119 expression of valve margin identity and *IND* genes (Larsson et al., 2014;
120 Simonini et al., 2017; Arnaud et al., 2010; Zuniga-Mayo et al., 2014). Braatz
121 et al (2018a and 2018b) have shown that induced mutations in *IND* and *ALC*
122 homologues are linked with pod shatter resistance in oilseed rape. Ectopic
123 expression of *FUL* gene from *Arabidopsis* has been shown to result in pod
124 shatter resistance via inhibiting *SHP* expression in *B. juncea* (Ostergaard et

125 al., 2006). However for commercial production of 'conventional' oilseed rape,
126 a fine tuning of these genes is required to develop a desirable level of
127 dehiscence.

128 Comparative mapping studies have shown that the *SHATTERPROOF*
129 paralogs of *A. thaliana* (*SHP1* and *SHP2*) are located in the vicinity of the
130 QTL associated with pod shatter resistance on chromosome A9 (designated
131 as *BnSHP1.A9* and *BnSHP2.A9*) in Australian and Chinese oilseed rape
132 populations, including the R1/R2 DH population utilized in this study (Liu et
133 al., 2016; Raman et al., 2014). *SHP1* and *SHP2* are MADS-box genes,
134 which regulate cell differentiation of the valve margin and promote
135 lignification (Liljegren et al., 2000). Both genes are highly homologous and
136 functionally redundant.

137 In the present study, we cloned the *BnSHP1.A9* gene underlying the
138 resistant QTL qSRI.A9.1 for pod shatter resistance and characterized its
139 functional role by employing comparative expression analysis using
140 quantitative RT-PCR, anatomical and transgenic approaches. Our findings
141 suggested that the LTR retroelement insertion silences the expression of
142 *BnSHP1.A9* epigenetically via DNA methylation, and contributes to the pod
143 shatter resistance in oilseed rape.

144 **Materials and Methods**

145 **Plant materials and evaluation of resistance to pod shattering**

146 For genetic analysis we used parental lines, R1 (pod shatter resistant), R2
147 (pod shatter prone) and a mapping population comprising of 96 DH lines that
148 showed segregation for pod shatter resistance, developed from a cross
149 between R1 and R2 (Liu et al., 2013). In addition, we used a total of 135
150 diverse accessions, comprised of four winter type, 119 semi-winter type and
151 12 spring type (Supplemental table 1) to validate the association between
152 pod shatter resistance index (PSRI) and the *BnSHP1.A9* promoter specific
153 marker. All accessions were evaluated for pod shatter resistance using
154 random impact test as described previously (Liu et al., 2016).

155 **Sequence analysis of *BnSHP1.A9***

156 Total DNA was extracted from the leaves of 4 weeks old seedlings by CTAB
157 method (Stein et al., 2001). The reference sequence of *BnSHP1.A9*
158 (BnaA09g55330D) from the *B. napus* cv. Darmor genome
159 (www.genoscope.cns.fr/brassicanapus) was used as a template to design
160 specific primer pairs (Table1) for cloning the genomic sequence and open
161 reading frame of *BnSHP1.A9*. DNA amplification was performed in a 20 µl
162 reaction system comprising 2 µl (10 µM) of forward and reverse primers, 4 µl
163 5× TransStart®FastPfu Fly buffer, 2 µl 2.5mM dNTPs, 2 µl 5× PCR stimulant,
164 0.4 µl 50mM MgSO₄, 1 U TransStart® FastPfu Fly DNA Polymerase (Beijing
165 TransGen Biotech Co., Ltd., Product Code: AP231), and 20-30 ng template
166 DNA. After initial denaturation of template DNA at 95 °C for 3 min, PCR was

carried out following 34 cycles of 20 seconds at 94 °C , 30 seconds at about 5 °C lower than melting temperature of the primer pair used, 1 min/kb at 72 °C with a final extension of 5 min at 72 °C. The PCR products were separated by electrophoresis in 0.8% low melting agarose gels, the target bands were purified, cloned into the pEASY-Blunt Zero cloning vector (Beijing TransGen Biotech Co. Ltd., Product Code: CB501-02) and then transformed into *Trans1-T1* competent *E. coli* cells by the heat shock method (Beijing TransGen Biotech Co. Ltd., Product Code: CB501, CT101). Four to six positive clones were randomly selected and sequenced with M13 sequencing primers at Shanghai Sangon Biotech Co. Ltd (<http://www.sangon.com/>). Sequences were then analyzed by MultAlin online software (Corpet, 1988). To determine the physical location and sequence similarities of *BnSHP1.A9* clones, the BLASTn was used against the Darmor reference sequence (<http://www.genoscope.cns.fr/brassicnapus/>). To clone the upstream sequence of *BnSHP1.A9*, we developed two specific primer pairs for R1 (BnSHP1.A9p1) and R2 (BnSHP1.A9p2) that are listed in Table 2.

183 **Development and assay of *BnSHP1.A9*-specific marker**

184 The genomic sequences of *BnSHP1.A9* from R1 and R2 were aligned in
185 MEGA7 (Kumar et al., 2016) using both the reference genomic and coding
186 sequences (BnaA09g55330D) of Darmor-*bzh*. Based on the sequence
187 differences we developed a co-dominant Indel marker (*BnSHP1.A9-IF4*)

targeting the indel difference of the first intron of *BnSHP1.A9* between the parental lines of the R1/R2 population for genetic mapping and the allelic diversity analysis (Table 1). PCR was performed in a volume of 10 µl system, including 5 µl 2×Taq MasterMix, 1 µl (10 µM) of each primer, 2 µl ddH₂O, 1 µl genomic DNA (20-30 ng). The PCR condition used was as following: 3 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 30 s at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were examined on either 3% agarose gel by electrophoresis at 130 V for 1 hour or using capillary electrophoresis on an automated CEQ2000 system (Beckman-Coulter) as described previously (Raman et al., 2005). The gels were stained with Syber-green and visualized under UV light. The PCR yielded a 267-bp product from R1 (pod shatter resistance allele) and a 290-bp product from R2 (pod shatter prone allele).

QTL mapping

The 96 DH lines derived from R1/R2 cross were genotyped with an InDel marker *BnSHP1.A9-IF4* and other new InDel and SSR makers developed based on sequence variation between the two parental lines in the vicinity of qSRI.A9 region (Supplemental table 2). This polymorphic data was integrated with the Illumina Brassica 60K Infinium ® SNP array and pod shatter resistance data generated in our previous study (Liu et al., 2016). To determine putative QTL for pod shatter resistance, we performed the

209 composite interval mapping (CIM) using WinQTL Cartographer 2.50 (Zeng,
210 1994; Wang et al., 2006). The genome scan was performed at every 2 cM to
211 estimate the likelihood of a QTL and its corresponding phenotypic effect (R^2).
212 The empirical threshold was computed using 1,000 permutations (overall error
213 level 5%) as described in Churchill and Doerge (1994).

214 **Analysis of *BnSHP1.A9* transcript levels**

215 To detect the dynamic expression patterns of the *BnSHP1.A9* gene, tissues
216 of root, stem and young leaf were collected from 4 weeks old plants, while
217 bud, fully-open flower, and developing pods were collected at 10, 20, 30 and
218 40 days after flowering (DAF) from R1 and R2 parental and DH lines (DH56
219 and DH82), and snap frozen in liquid nitrogen. For each sample, including
220 pods at different developmental stages, three biological replicates were used
221 for semi-quantitative reverse transcription PCR (RT-PCR). Total RNA was
222 extracted using the RNAPrep Pure Plant Kit (TIANGEN Biotech Beijing Co.,
223 Ltd. Product code: DP432). DNase I-treated RNA was reverse transcribed
224 using the cDNA synthesizing kit following the manufacturer's instructions
225 (TIANGEN Biotech Beijing Co., Ltd. Product code: KR106-02). To eliminate
226 interference from other paralogues, an allele-specific primer pair
227 *BnSHP1.A9*-RT (F/R) was designed (Supplemental table 3) and the
228 specificity was validated by PCR product sequencing. The *actin* gene was

229 used as an internal control for semi-quantification of relative expression
230 values.

231 **DNA methylation analysis**

232 McrBC endonuclease (NEB, M0272S) was firstly employed to analyze the
233 DNA methylation status of *BnSHP1.A9* promoter region and genic region. 0.5
234 µg genomic DNA extracted from 20 DAF siliques of R1 and R2 was digested
235 respectively with 5 U McrBC overnight at 37 °C, the methylated plasmid DNA
236 in the product was used as positive control. The McrBC-digested DNA was
237 then used as template to amplify *BnSHP1.A9* promoter and genic region with
238 allele specific primer pairs (Supplemental table 4). The Chop PCR products
239 were then checked by agarose gel electrophoresis and stained with Syber-
240 green and visualized under UV light.

241 Detailed analysis of promoter DNA methylation was then performed with
242 bisulfite sequencing method (Gruntman et al., 2008). Genomic DNA of R1
243 and R2 from 20 DAF siliques was bisulfite treated using EpiTect Bisulfite Kit
244 (Qiagen 59104) following the instructions of manufacturer. Bisulfite
245 sequencing primers were designed with MethPrimer online tool
246 (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) and primer
247 designing tool in NCBI. The bisulfate-treated DNA was used as template to
248 amplify the target fragments of R1/R2 promoter with specific bisulfite primers
249 (Supplemental table 5) and the resulting PCR fragments were cloned into
250 pTOPO-T simple vector (Aidlab CV1501). At least 24 positive clones were

251 sequenced from each fragment. Vector sequences together with primer
252 sequences of sequencing results were first trimmed out and the remaining
253 sequences were then blasted against *Brassica napus* reference genome
254 database (<http://www.genoscope.cns.fr/brassicanapus/>) to confirm the
255 specificity. The methylation status of the specifically amplified fragments was
256 then analyzed by Kismeth online tool
257 (<http://katahdin.mssm.edu/kismeth/revpage.pl>). The data of methylated
258 cytosines (CG, CHG and CHH) between the two parental lines were collected
259 and compared by t-test.

260 **Generation and analysis of transgenic lines overexpressing *BnSHP1.A9***

261 To examine the function of different *BnSHP1.A9* alleles from R1 and R2
262 parental lines, two overexpression vectors driven by CAMV35S promoter,
263 35S::*BnSHP1.A9*_{-R1} and 35S::*BnSHP1.A9*_{-R2}, were constructed by cloning
264 *BnSHP1.A9* coding sequence from R1 and R2 into the pCAMBIA-1301
265 vector using *Nco* I-*Bst* E II cloning sites. *BnSHP1.A9* cDNA was amplified
266 from developing pods using primer pair BnSHP1.A9orf (Table 2). The two
267 overexpression vectors were then transformed into *Agrobacterium*
268 *tumefaciens* GV3101 strain individually and then used for transformation of
269 the *B. napus* line R1 (resistant to pod shatter) by *A. tumefaciens* mediated
270 method (Hood et al., 1993). Transgenic T0 and T1 plants were confirmed by
271 PCR detection of hygromycin resistance gene with primer pair *HptII*-F/R

(Supplemental table 6). The expression level of *BnSHP1.A9* in transgenic plants was evaluated by RT-PCR. Since CAMV35S promoter could drive constitutive expression of downstream target genes in all tissues of transgenic plant, for the convenience of RNA extraction and early detection, we used leaf tissue for determining the expression level of *BnSHP1.A9* in transgenic plant. Transgenic lines of the T1 generation derived from both constructs were evaluated for pod shatter resistance using the random impact test as described previously (Liu et al., 2016).

Results

Sequence variation of *BnSHP1.A9* underlying pod shatter resistance

We isolated the full length of *BnSHP1.A9* gene from both the R1 and R2 parental lines of the DH mapping population. The size of *BnSHP1.A9* genomic sequences varied from 2,737 bp in R1 to 2,757 bp in R2, with 7 exons and 6 introns (Figure 1C). Sequence alignments revealed polymorphisms in the form of SNPs and InDel between the two parental lines. There were 9 SNPs in the exon sequences, including 2 nonsynonymous SNPs in exon1 at # 25 (T/G) and # 203 (G/T), leading to tyrosine (Y) to aspartic acid (D) and arginine (R) to leucine (L) amino acid variation, respectively. The point mutation in exon 3 (# 1751,T/C) causes Y to H (histidine) amino acid alteration, while A/T mutation at # 2480 in exon 6 leads to D to valine (V) amino acid change (Supplemental table 7). However, the most abundant sequence divergence

293 was found in the first intron including 21 SNPs and 10 InDels between R1 and
294 R2, which enabled us to develop a gene specific marker for *BnSHP1.A9*.
295 In order to determine whether the polymorphism in *BnSHP1.A9* is associated
296 with pod shatter resistance in a DH population derived from R1 and R2, we
297 developed a primer pair (IF4) for specific amplification of *BnSHP1.A9* alleles
298 (Table 1). IF4 amplified a 267-bp fragment from R1 and a 290-bp fragment
299 from R2. Genotypic analysis of all the 96 DH lines showed a segregation for a
300 single locus, with 35 lines containing the R1 allele and 55 lines containing the
301 R2 allele ($\chi^2 = 4.44$, $P = 0.035$) (Supplemental table 8). These marker data
302 were integrated with previously obtained SNP data from the R1/R2 DH
303 population (Liu et al., 2016). QTL analysis identified two genomic regions,
304 *qSRI.A9.1* and *qSRI.A9.2* for pod shattering resistance on chromosome A9,
305 explaining 11.26% - 12.07% and 27.58% - 38.11% phenotypic variation,
306 respectively (Figure 1A; Table 2). At *qSRI.A9.1*, the R2 allele contributed a
307 positive effect to pod shatter resistance, whereas the R1 allele contributed a
308 negative effect. In contrast, at *qSRI.A9.2*, the R1 allele contributed a positive
309 effect to pod shatter resistance, whereas the R2 allele contributed a negative
310 effect. Linkage analysis revealed the order of markers to be ni113 –
311 *BnSHP1.A9* – BrEMS4; the *BnSHP1.A9* gene was mapped within the
312 *qSRI.A9.1* genomic region for pod shatter resistance (Table 2, Figure 1A, B).

313 **A LTR retrotransposon is inserted in *BnSHP1.A9* promoter of R2**

314 To gain an understanding on promoter region of the two *BnSHP1.A9* alleles,
315 we sequenced and analyzed the upstream region of *BnSHP1.A9* from both
316 parental lines of the mapping population. We identified a 4,803 bp long
317 terminal repeat (LTR)/copia retrotransposon insertion at 252 bp upstream the
318 start codon of *BnSHP1.A9* in R2 in opposite orientation (Figure 1C). This
319 LTR retrotransposon contains the typical retro-transposable element
320 structure, including a predicted 4,356 bp single open reading frame with
321 conserved *gag*, *prot*, *int*, *RT* and *RNaseH* domains, two identical 169 bp 5'
322 and 3' LTRs flanked by 5 bp direct repeat sequence (5'-GAGGT-3') (Cao et
323 al., 2015). Sequence alignment of this LTR retrotransposon against the NCBI
324 database revealed 100% sequence identity with a *B. rapa* A9 scaffold
325 (LR031568.1), which suggested this LTR retrotransposon insertion may have
326 been originated from *B. rapa* in R2 paternal line of R1R2 DH mapping
327 population.

328 **Overexpression of *BnSHP1.A9* alleles promoted pod shattering in pod** 329 **shatter resistant 'R1' line**

330 Sequence analysis of *BnSHP1.A9* revealed 4 nonsynonymous SNPs in the
331 coding sequence of *BnSHP1.A9* alleles of the two parental lines and three of
332 them are located in the predicted conserved MADS-box and K-box domains.
333 To verify whether these SNPs have any 'phenotypic' effect on gene function,
334 we overexpressed *BnSHP1.A9* coding sequence (CDS) under the control of

335 constitutive promoter from either R1 or R2 in pod shatter resistant R1
336 background respectively (named *BnSHP1.A9-R1* and *BnSHP1.A9-R2* hereafter).
337 A total of 71 transgenic plants were generated and subsequently assessed for
338 pod shatter resistance index by RIT. The PSRI of the transgenic T1 plants
339 varied from 0.35-0.53, in comparison with the 'wild-type (untransformed)' R1
340 plants, which averaged 0.83 (n = 8). However, no statistically significant
341 difference was found for PSRI between the transgenic *BnSHP1.A9-R1* and
342 *BnSHP1.A9-R2* overexpression lines (OE). For example, four OE lines T18,
343 T24, T26 and T33 of *BnSHP1.A9-R1* had similar PSRI as that of the T9 line of
344 *BnSHP1.A9-R2* (Figure 2).

345 Expression of *BnSHP1.A9* in developing pods in 15 selected transgenic T1
346 plants (three from each T1 line) were examined using semi-quantitative RT-
347 PCR. All the tested T1 plants exhibited elevated level of *BnSHP1.A9*
348 expression (Supplemental figure 1). These results proved that both alleles of
349 *BnSHP1.A9* gene (R1 and R2) are functional in promoting pod shattering in *B.*
350 *napus*. To explore whether overexpression of *BnSHP1.A9* causes dehiscence
351 zone differentiation in siliques, we analyzed pod anatomical structure of
352 *BnSHP1.A9-R1* and *BnSHP1.A9-R2* OE lines as described in Raman et al.
353 (2017). A more compact arrangement of the 'enb' layer at the valve margin
354 was observed in pod cross sections of the *BnSHP1.A9-R1* and *BnSHP1.A9-R2*
355 OE T1 lines than in the wild type (Figure 3). Our results indicate that
356 *BnSHP1.A9* gene promotes pod shattering through the increase of the

357 number of lignified cells in the 'enb' layer , thus enhancing tension caused by
358 differential contraction of the fruit wall tissue in oilseed rape.

359 **Expression of *BnSHP1.A9* is repressed in R2**

360 To test the dynamic expression of *BnSHP1.A9*, we investigated its
361 expression pattern in root, stem, leaf, bud, flower, and developing siliques in
362 R1 and R2 parental lines of the mapping population. Since there is a highly
363 homologous copy of *SHP1* on chromosome C08 (BnaC08g29520D), to
364 eliminate potential nonspecific amplification, we designed a specific RT-PCR
365 primer pair for *BnSHP1.A9* based on SNPs between coding sequences of
366 these two homologues. The result showed that *BnSHP1.A9* was expressed
367 exclusively in bud, flower and developing siliques in R1. In contrast, almost
368 no expression in either vegetative organs or reproductive organs could be
369 detected in R2 (Figure 4A).

370 **The LTR insertion correlates with *BnSHP1.A9* repression in DH lines**

371 Since the LTR element insertion locates in the upstream of the start codon of
372 the R2 *BnSHP1.A9* allele, it may play a role in the repression of *BnSHP1.A9*
373 expression. To further test this hypothesis, we investigated the 96 R1/R2 DH
374 lines using promoter specific primers of *BnSHP1.A9* and selected two DH
375 lines (DH56 and DH82 lines) that had the same genotype of *BnSHP1.A9*
376 allele as R1 and R2, respectively. We then analyzed *BnSHP1.A9* expression
377 level in 10, 20, 30 DAF siliques of DH56 and DH82 lines by RT-PCR. A weak

band was visible in the siliques of all three developing stages of DH56, which does not contain the LTR insertion (LTR⁻). There was no amplification in any of the three silique samples in DH82 containing the LTR insertion (LTR⁺) (Figure 4B). On the basis of these data together with the expression pattern of *BnSHP1.A9* in the two parental lines, our results suggest that the LTR insertion in upstream of *BnSHP1.A9* is responsible for repression of *BnSHP1.A9* transcript.

DNA methylation of LTR retroelement insertion is responsible for the repression of *BnSHP1.A9*

To understand the basis of LTR insertion mediated repression of *BnSHP1.A9*, we performed site-specific chop-PCR and bisulfite sequencing of genomic DNA extracted from 20 DAF silique of R1 and R2 to analyze DNA methylation status of the upstream and genomic regions of *BnSHP1.A9*. Chop-PCR amplified no bands from the LTR insertion fragments (b3, b4, b5 and b6) of R2 (Figure 5C and 5E) because of hypermethylation of the sequences, whereas same bands were amplified from other fragments of R2 as those from R1 (Figure 5B and 5D). These observations hinted that DNA methylation mainly occurred in the LTR insertion. The cytosine residues of LTR inner region were also decayed along far away the central insertion region. The bisulfite sequencing results showed that the LTR retrotransposon and

the transcription start region of *BnSHP1.A9* in R2 was hypermethylated, while the promoter region of *BnSHP1.A9* in R1 was hypomethylated (Figure 6B). The methylation level of mF4 region in R2 located at about 1.8 kb upstream the LTR element and the corresponding region of mF3 in R1 are much lower than the downstream regions close to the start codon (Fig. 6B and 6C). In mF1 region, we found the 100% cytosine (C) residues in all sites of CG and CHH (H represents any residues other than G) were methylated in R2 because of the LTR insertion, whereas only 7.84% cytosine residues of the corresponding sites were methylated in R1. The mF2, mF3 and mF4 regions in R1 were hypomethylated from 2.37% to 6.10%. We investigated the methylation level of mF2 and mF3 of the LTR region in R2. In the mF2 region, 97.49% of cytosine residues was hypermethylated, while only 50.47% of cytosine residues were methylated in mF3 region. Among these cytosine residue containing sites in mF3 region, cytosine residues of CG were still hypermethylated, however the methylation of cytosine residues of CHG and CHH were significantly decreased to 43.85% and 17.53%. For mF4, the upstream region of about 2 kb away from the LTR and 7 Kb away from the transcription start codon of *BnSHP1.A9*, the methylation level was significantly decreased to a normal level of about 6.10% in both lines. Thus, methylation by the LTR retrotransposon in the vicinity of *BnSHP1.A9* promoter region in R2 may directly affect in the down-regulation of *BnSHP1.A9* expression.

421 **The LTR insertion correlates with pod shatter resistance in diverse** 422 **oilseed rape germplasm**

423 To verify the linkage between this LTR retrotransposon insertion and pod
424 shatter resistance, we tested 135 diverse accessions with an allele specific
425 diagnostic marker for LTR detection (BnSHP1.A9p1/p2, Table 1). The
426 homozygous *BnSHP1.A9* (R1) allele (R1 specific, LTR⁻) and homozygous
427 *BnSHP1.A9* (R2) allele (R2 specific, LTR⁺) were detected in 63.7% (86) and
428 15.6% (21) accessions, respectively, and heterozygous *BnSHP1.A9* (H) was
429 found in 11.9% (16) accessions (Supplemental table 1). A significant
430 association between the *BnSHP1.A9* (R2, LTR⁺) promoter allele and pod
431 SRI was observed among the tested oilseed rape accessions (Figure 7). The
432 average pod SRI of the lines with *BnSHP1.A9* (R2, LTR⁺) genotype was
433 significantly higher than that of lines with *BnSHP1.A9* (R1, LTR⁻), indicating
434 that *BnSHP1.A9* (R2, LTR⁺) allele could increase PSRI compared to
435 *BnSHP1.A9* (R1, LTR⁻) allele.

436

437 **Discussion**

438 ***SHATTERPROOF 1* gene, *BnSHP1.A9* underlies pod shatter resistance**

439 In this study, we delineated two QTL; *qSRI.A9.1* and *qSRI.A9.2* with large
440 allelic effects, on chromosome A9 in a DH population from R1/R2. QTL
441 accounting for large phenotypic variance of pod shatter resistance have been

442 identified in various *B. napus* populations (Hu et al., 2012; Raman et al., 2014;
443 Liu et al., 2016). Since a *SHP1* paralog of *A. thaliana* is located in the vicinity
444 of the significant SNP markers associated with pod shatter resistance on
445 chromosome A9 (Raman et al., 2014; Liu et al., 2016, this study), we were
446 interested in determining whether *SHP1* indeed contributes to the genetic
447 variation for pod shatter resistance in *B. napus* germplasm. Through genetic
448 analyses (using a DH population from R1/R2 and a set of 135 diverse lines of
449 oilseed rape breeding germplasm) and functional characterization via
450 comparative expression analysis and transgenic approaches, we showed that
451 the *SHP1* paralog, *BnSHP1.A9* underlies pod shatter resistance at *qSRI.A9.1*
452 in oilseed rape.

453 In this study, we revealed that *BnSHP1.A9* is the functional gene regulating
454 pod shatter resistance in R1/R2 DH population and in diverse *B. napus* lines.
455 By overexpressing *BnSHP1.A9* cDNA from both R1 and R2 alleles, an
456 average of 50% of PSRI decrease was found in T1 lines, thus confirming the
457 function of *BnSHP1.A9* in pod shatter regulation. This functional analysis
458 result is in accordance with the expression pattern of *BnSHP1.A9* in the R1
459 and R2 parental lines. In R2 which contributed positive effect to PSRI on this
460 locus, the expression of *BnSHP1.A9* was repressed, indicating that the down-
461 regulation of the target gene enhances PSRI. Overexpression of *BnSHP1.A9*
462 only partly decreased the pod shatter resistance of R1. This can be attributed
463 to other loci known to contribute positive effect in R1 for pod shatter

464 resistance, such as the *qSRI.A9.2* as well as other *SHP1* or *SHP2*
 465 homologues which are known to act redundantly and control dehiscence zone
 466 differentiation (Liljegren et al., 2000). Although the expression difference of
 467 *BnSHP1.A9* exists in lines with contrast genetic effects of this locus, the allelic
 468 variation of *BnSHP1.A9* *per se* is not the causal factor for the phenotypic
 469 variation of pod shatter resistance, as overexpression of both alleles of the
 470 *BnSHP1.A9* gene facilitated pod shattering in *B. napus*.

471 **LTR retrotransposon insertion in the upstream region regulates** 472 ***BnSHP1.A9* epigenetically**

473 Comparative and association analyses revealed that the LTR
 474 retrotransposon insertion was significantly associated with pod shatter
 475 resistance among 135 collected accessions, and in R1/R2 DH population.
 476 The *BnSHP1.A9* (R2 allele) promoter region, including LTR retrotransposon
 477 insertion was found to be highly methylated, which is responsible for the
 478 depression of *BnSHP1.A9* expression and the positive effect to pod shatter
 479 resistance phenotype. Transposable elements (TEs) are well known to play
 480 positive roles in generating genomic novelty and diversity in plants (Song et
 481 al., 2017). TEs are frequent found in *B. napus* genomes (Chalhoub et al.,
 482 2014; Sun et al., 2017) and have been implicated in DNA methylation and
 483 H3K9me2 modification (Eichten et al., 2012; Gent et al., 2013), altering gene
 484 expression both genetically and epigenetically (Cui and Cao, 2014). In fact,

several oilseed rape genes related to morphological or physiological traits have evolved from TE insertions (Hou et al., 2012; Zhang et al., 2015; Gao et al., 2016; Shi et al., 2019). The current study implies that DNA methylation of the Copia-LTR insertion spreads to *BnSHP1.A9* cis-regulatory region. This epigenetic modification may change the accessibility of RNA polymerase II and transcription factors to the *BnSHP1.A9* promoter, and ultimately altering transcription patterns (Zhao et al., 2013).

B. napus is an allotetraploid originated from natural hybridization of *B. rapa* and *Brassica oleracea*. The LTR insertion in *BnSHP1.A9* promoter region showed 100% sequence identities with *B. rapa*. This suggests that the insertion of LTR retrotransposon event took place before the generation of *B. napus* as a species. However, only 15.6% of the natural *B. napus* population was found to contain the LTR insertion, which indicates that the LTR insertion might be lost in the process of domestication or breeding of rapeseed. As pod shattering is beneficial to seed release, the loss of LTR insertion is evolutionarily advantageous. This is further consistent with the theory that pod shatter resistance was selectively against during natural evolution or domestication.

We thus propose a simple model to explain the *BnSHP1.A9* dependent pod shatter resistance in *B. napus*. The non-LTR *BnSHP1.A9* (in R1, LTR⁻) is in a transcriptionally active state. DNA methylation of the LTR insertion in *BnSHP1.A9* promoter (in R2, LTR⁺) spreads to the transcription initiation

507 region, thus converting *BnSHP1.A9* from an active state to a silenced state.
508 In this study, hypermethylation of the *BnSHP1.A9* promoter region, mainly
509 through CG and CHH methylations, appears to be the major epigenetic
510 factor in the regulation of gene expression. It seems reasonable for plants to
511 evolve such an epigenetic regulatory mechanism to gain functional variation
512 for pod shatter resistance.

513 **Application of *BnSHP1.A9* gene for oilseed rape breeding**

514 In this study we investigated the molecular basis of pod shatter resistance in
515 oilseed rape utilizing natural variation in *B. napus* germplasm and showed that
516 a single gene, *BnSHP1.A9*, controls genetic variation for pod shatter resistant
517 at *qSRI.A9.1* locus. Our research provides two gene-specific markers, one co-
518 dominant marker (BnSHP1.A9IF4) detecting sequence difference within the
519 CDS of *BnSHP1.A9*, and the other co-dominant marker (BnSHP1.A9P1/P2)
520 detecting presence/absence variation (PAV) of LTR insertion in *BnSHP1.A9*
521 promoter region. Both markers can be applied for the efficient selection of this
522 QTL for pod shatter resistance in *B. napus* breeding programs starting from
523 early generations. Previously, there were only linked markers available for
524 marker assisted selection for pod shatter resistance in *B. napus* to PSRI QTL
525 reported and could be used for breeding (Raman et al., 2014; Liu et al., 2016).
526 Our two gene-specific markers developed in this study could be used to
527 further improve the resistance potential by introducing the *BnSHP1.A9* (R2)

528 allele with positive effect into lines containing the *BnSHP1.A9* (R1) allele for
529 genetic improvement. These markers could be easily assayed via
530 conventional agarose gel electrophoresis and high throughput capillary
531 electrophoresis platforms. The sources of pod shatter resistance identified
532 herein can be used for introgression of favorable alleles and enrichment of
533 alleles in the breeding germplasm.

534 Recently CRISPR-Cas9 genome editing and other genetic transformation
535 platforms have become available for oilseed rape improvement (Zaman et al.,
536 2019). Editing IND and ALC genes has improved the pod shatter resistance in
537 *B. napus* (Braatz et al., 2018a; b). Our results clearly revealed that down-
538 regulation of *BnSHP1.A9* could increase PSRI. Genome editing can not only
539 mutate *BnSHP1.A9*, but also other functionally redundant homologous of
540 *SHP1*, as well as the homologous of *SHP2*. Knockdown the multiple copies of
541 functional redundant genes or homologues has more effect on phenotypic
542 variation. However, these approaches are difficult to deploy commercially due
543 to the legal restrictions on GMO crops in some countries, such as Europe. In
544 countries that are open to gene edited crops, our finding on the down-
545 regulation of the *BnSHP1.A9* having a positive effect on pod shatter
546 resistance can be used to manipulate the gene expression by mutation of this
547 gene and its homologs for the improvement of pod shatter resistance in *B.*
548 *napus*.

549 In conclusion, we showed that a *SHP1* paralog, controls pod shatter

550 resistance at qSRI.A9.1 QTL in the DH population from R1/R2 and in a
551 diversity panel of oilseed rape. Our results suggest that upstream promoter
552 region of *BnSHP1.A9* controls pod shatter resistance, rather than the coding
553 sequence. This study provides a novel source of germplasm, gene-specific
554 markers and insights on molecular basis of *SHP1* mediated resistance to pod
555 shatter in oilseed rape. These resources will facilitate the genetic
556 improvement of pod shattering resistance in oilseed rape.

557

558

559 **DECLARATIONS**

560 **Ethics approval and consent to participate**

561 Not applicable

562

563 **Consent for publication**

564 Not applicable

565

566 **Competing interests**

567 The authors declare that they have no competing interests.

568

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578

Authors' contributions

QH and HR designed the research. JL, RZ, WH, WW, DM and TC performed
genetic analysis and field research. RZ performed genetic transformation
and DNA methylation analysis, JL and RZ analyzed data. YQ and RR
performed SHP1 analysis using capillary electrophoresis, JL wrote the first
version of the manuscript. All authors reviewed the manuscript.

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- 729

1 **Table 1. Primer pairs used for *BnSHP1.A9* gene cloning and genetic analyses**

Primer name	Amplification objective	Forward/Reverse (5'-3')	Products length (bp)
BnSHP1.A9g	Genetic analysis	F: AGAAGTGTCTGAAATCAAA GTGGTA	57 R1: 1928
		R: CCTTA ACTATGAATAAGAAT GTCGC	57 R2: 1953
BnSHP1.A9p1	Promoter of R1	F: CCGAGTCCCACGCAAATA G	59 R1: 2264
		R: CGGCCACGAGTGGAAAAG AT	61
BnSHP1.A9p2	Promoter of R2	F: TCATTGAAAACGCCGAAGG GTT	61 R2: 6122
		R: AGCATACCTGTTGCTGGCG	62

		T		
IF4	InDel marker analysis	F:		
		ACTTGGGACATAGCCTAAT	58	R1: 267
		GATG		
		R:		
		TCGTACCACTTTGATTTC	58	R2: 290
		GACA		

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19 **Table 2. QTL associated with pod shatter resistance in a doubled haploid**
20 **population from R1 (pod shatter resistant)/R2 (pod shatter susceptible). DH lines**
21 **were evaluated for resistance using random impact test. R² refers to phenotypic**
22 **variance explained**

		Confident Interval		Additive Effect	
QTL	Year		LOD score	R2 (%)	(parental allele)
		(cM)			

qSRI.A9. 1	2013	86.5-93.2	4.91	11.26	-0.07 (R2)
	2014	88.1-96.6	4.76	12.07	-0.15 (R2)
qSRI.A9. 2	2013	109.6-113.5	13.43	38.11	0.12 (R1)
	2014	109.0-112.1	10.32	27.58	0.23 (R1)

23

24

Figure legend

Figure 1. Genetic mapping of the QTL; qSRI.A9.1 and underlying the candidate SHATTERPROOF1paralog of *A. thaliana* in *B. napus*, BnSHP1.A09. A. The schematic diagram of the LOD curve for the QTL of pod SRI of the A9 linkage group. The abscissa coordinates represent to the genetic linkage group, and the ordinate represents to the LOD value. B. Partial linkage map of chromosome A09, illustrating of the genetic position (cM) of *BnSHP1.A9* locus underlying qSRI.A9.1 for pod shatter in R1/R2 mapping population. The left indicates the tag name (the newly developed Indel marker and SSR markers) and corresponding genetic map on the linkage group, corresponding to the location of the corresponding QTL for the pod SRI. C. The structural variations present in the *BnSHP1.A9* gene between the parental lines of the DH mapping population R1 and R2. QTL was mapped using gene specific InDEL markers.

Figure 2. Pod shatter resistance index (PSRI) of different transgenic plants (T1) and two parental lines. PSRI was assessed with random impact test and 20 pods/each line were assessed with two replications. Standard deviation of each line is also shown.

Figure 3. Histological analysis of 30-DAP silique cross-sections of 35S:*BnSHP1.A9* transgenic rapeseed oil lines.

(A and B) Microscopy observations of cross-sections of R1 siliques. Bars, 200 µm and 40 µm. (C and D) Microscopy observations of cross-sections of 35S:*BnSHP1.A9* transgenic rapeseed oil siliques. Scale bars, 200 µm and 40 µm.

Figure 4. Expression status of *BnSHP1.A9* correlates with LTR retrotransposon insertion in parents and DH lines. A, *BnSHP1.A9* expression analysis of different tissues and silique, taken at different developmental stages of R1 and R2. *BnActin* gene was used as an internal control for relative expression analysis. 1: Root; 2: Stem; 3: Leaf; 4: Bud; 5: Flower; 6: 10d silique; 7: 20d silique; 8: 30d silique; 9: 40d silique. B, *BnSHP1.A9* expression status in DH56 and DH82 lines. 1: 10d silique; 2:

58 20d silique; 3: 20d silique. **C:** Genotyping of R1, R2, DH56 and DH82 with specific
59 promoter primers of proR1, proR2 and InDel marker of *BnSHP1.A9*.

60

61 **Figure 5. Cross validation of DNA methylation status of *BnSHP1.A9* in**
62 **R1/R2 silique genomic DNA.**

63 **A,** amplified fragments illustrated in *BnSHP1.A9* DNA from R1/R2

64 **B, D:** chop-PCR with *BnSHP1.A9* gDNA template and McrBC digested gDNA of 20 DAF
65 silique from R1 without McrBC digestion; a1: -2412/-1542, a2: -1616/-882, a3: -902/-30,
66 a4: -49/+1413, a5: +1034/+2007

67 **C, E:** chop-PCR with *BnSHP1.A9* gDNA template and McrBC digested gDNA of 20 DAF
68 silique from R2. b1: -7412/-6823, b2: -6753/-5765, b3: -3191/-2285, b4: -2538/-1788, b5:
69 -1707/-1027, b6: -1035/-102, b7: -49/+436, b8: +1035/+2012, b9: +1883/+2793

70 M: Trans 2k plus.

71

72 **Figure 6. Methylation analysis of upstream promoter region of the**
73 ***BnSHP1.A9* comparison between R1 and R2**

74 **A** The positions of BSP analysis in the upper 5' UTR region of *BnSHP1.A9* (black line) and
75 4803 bp *copia* LTR-retrotransposon insertion (red box) are illustrated. Red lines indicate
76 the position of the bisulphite sequencing (BSP) analyses.

77 **B** Methylation of cytosine residues in CG, CHG and CHH sites (purple, green and blue
78 lines, respectively) was revealed by bisulphite sequencing of the four BSP regions.

79 **C** Methylation data of cytosine residues in CG, CHG and CHH sites from the four BSP
80 regions

81

82 **Figure 7. The distribution of *BnSHP1.A9* promoter allele in natural population**
83 **and corresponding PSRI in 2012 and 2013 environments.**

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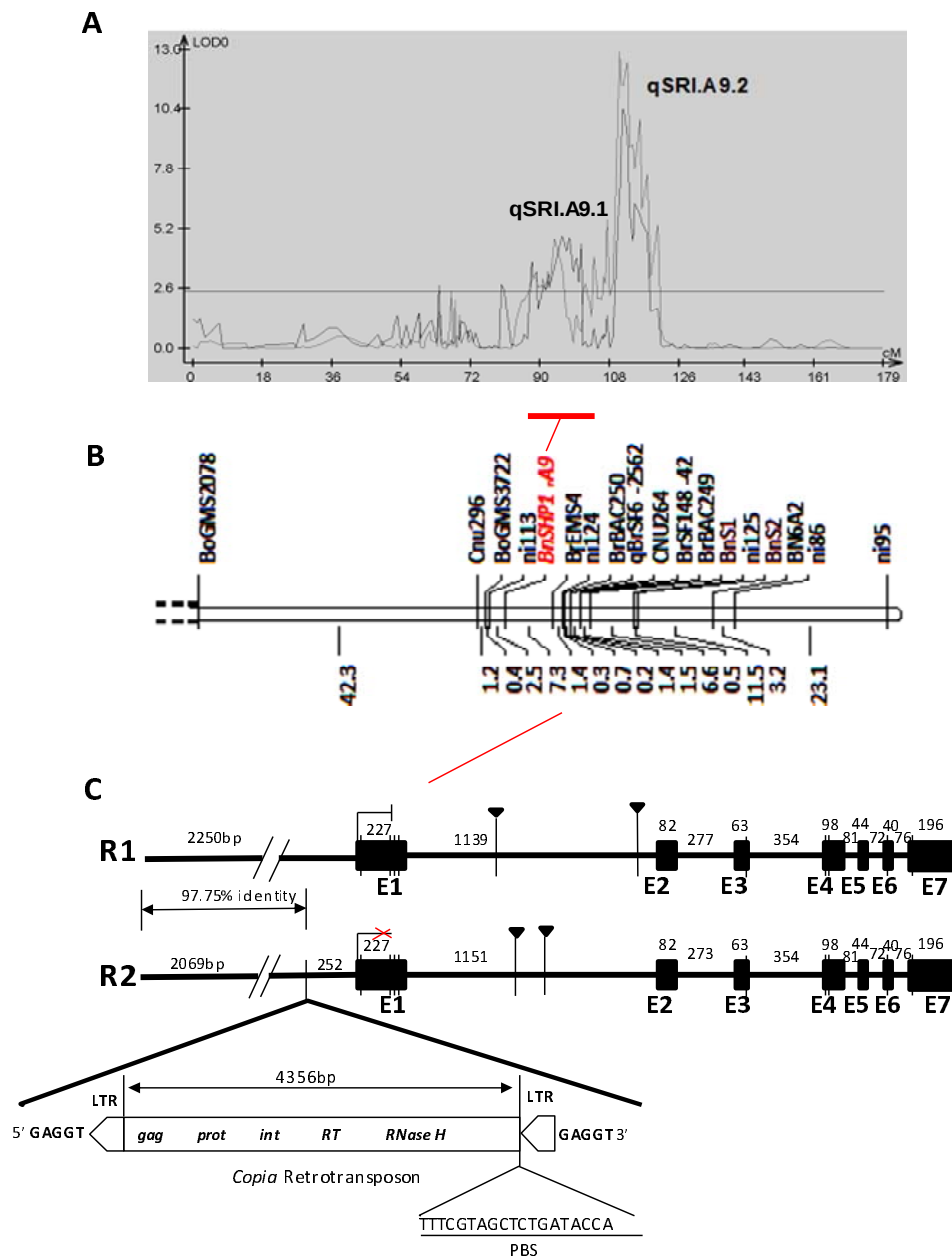


Fig. 1.

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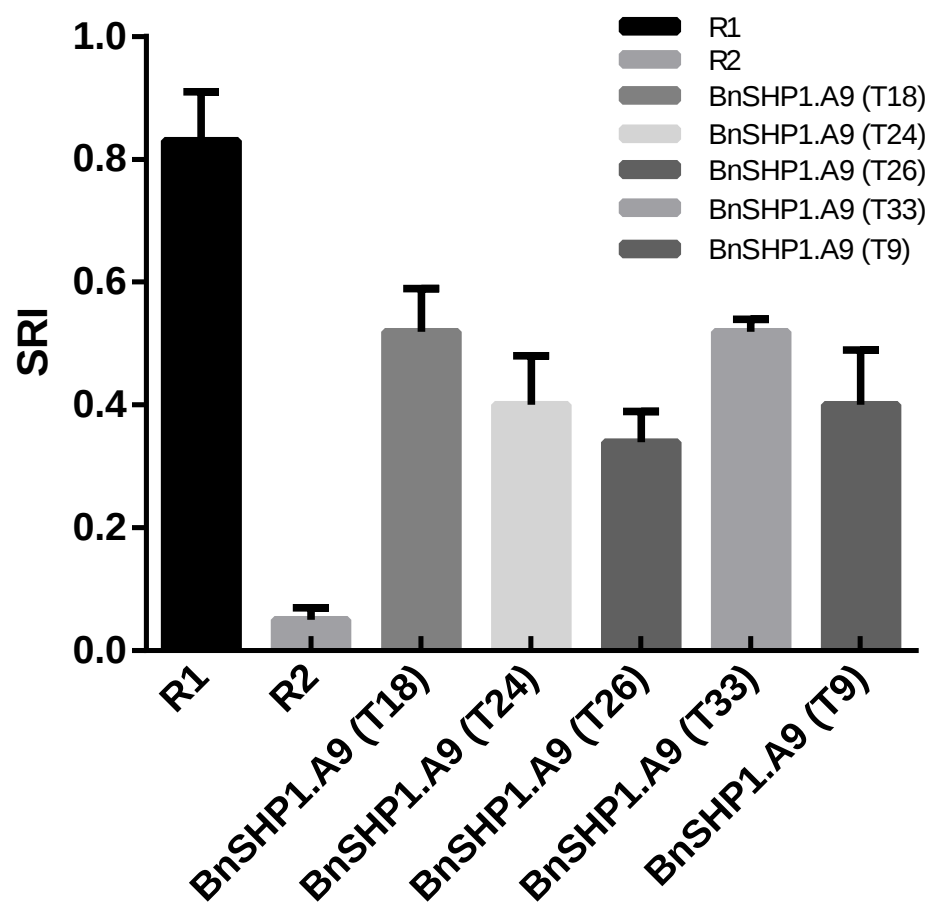


Fig. 2.

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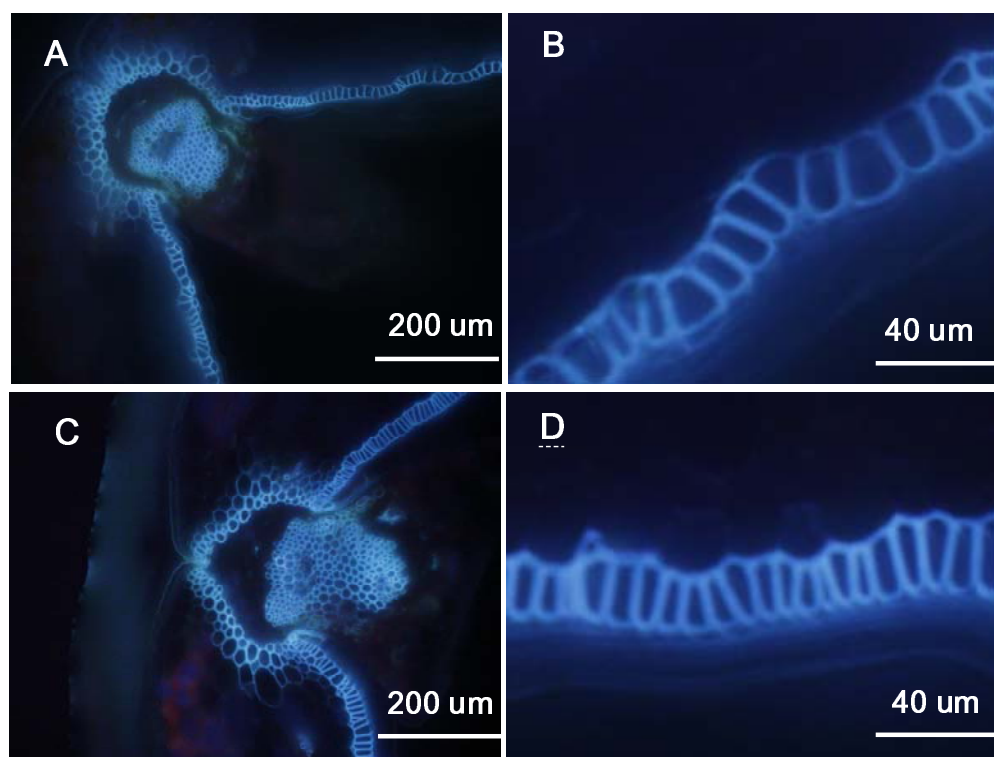


Fig. 3.

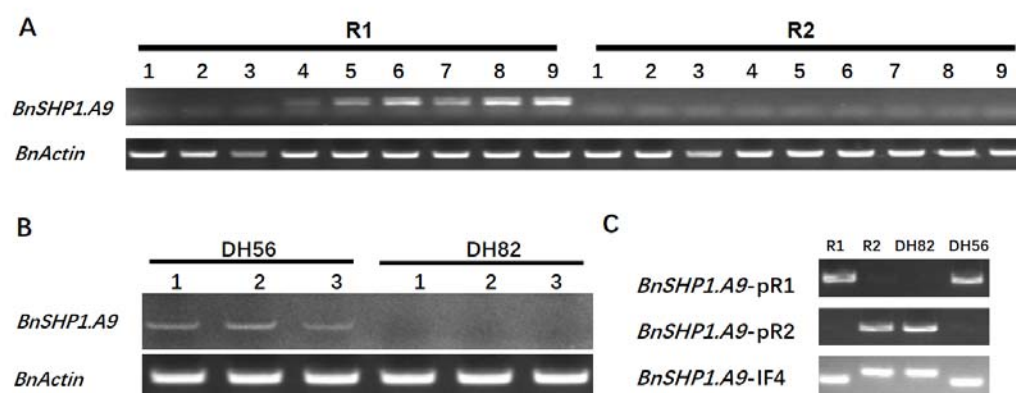


Fig. 4.

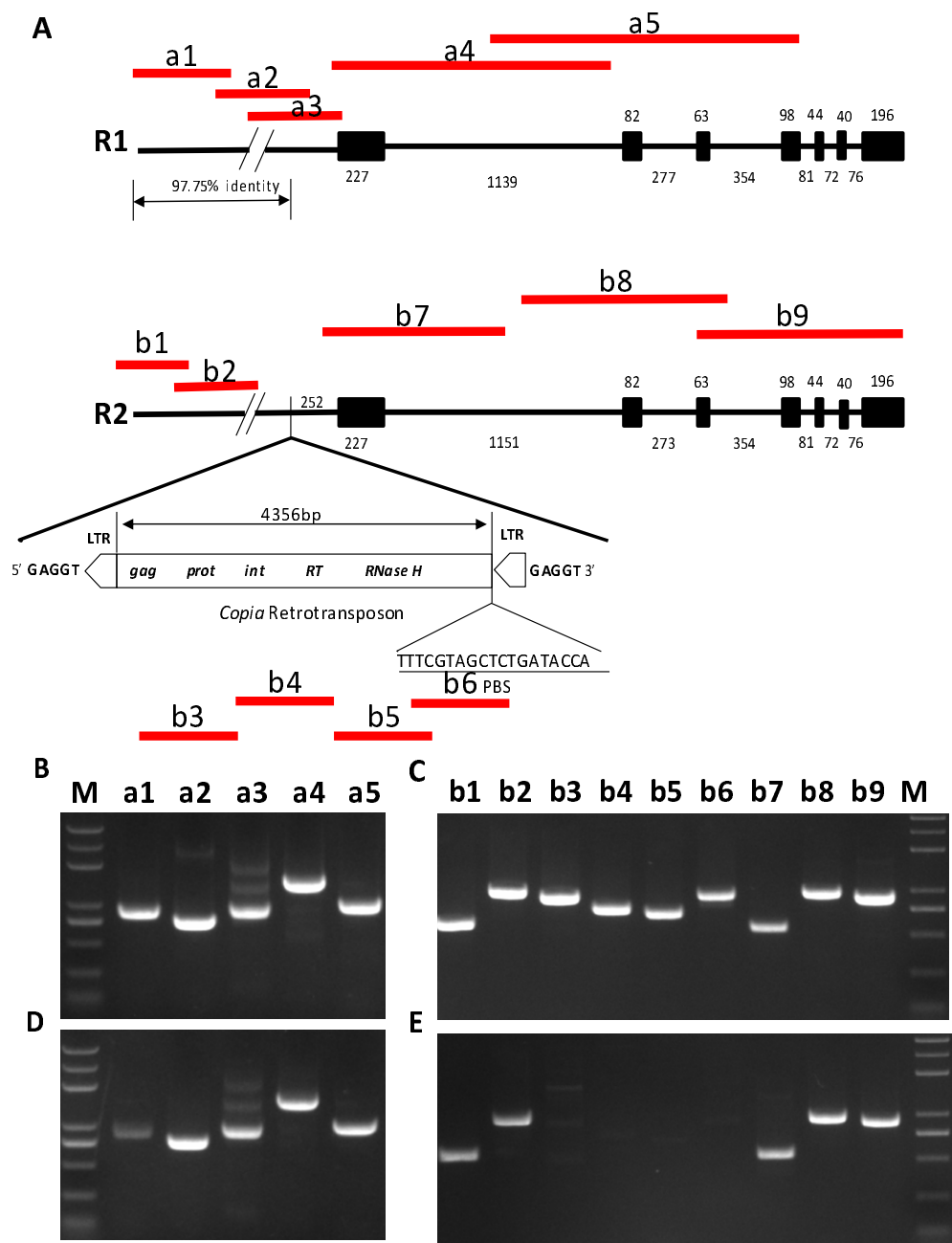


Fig. 5.

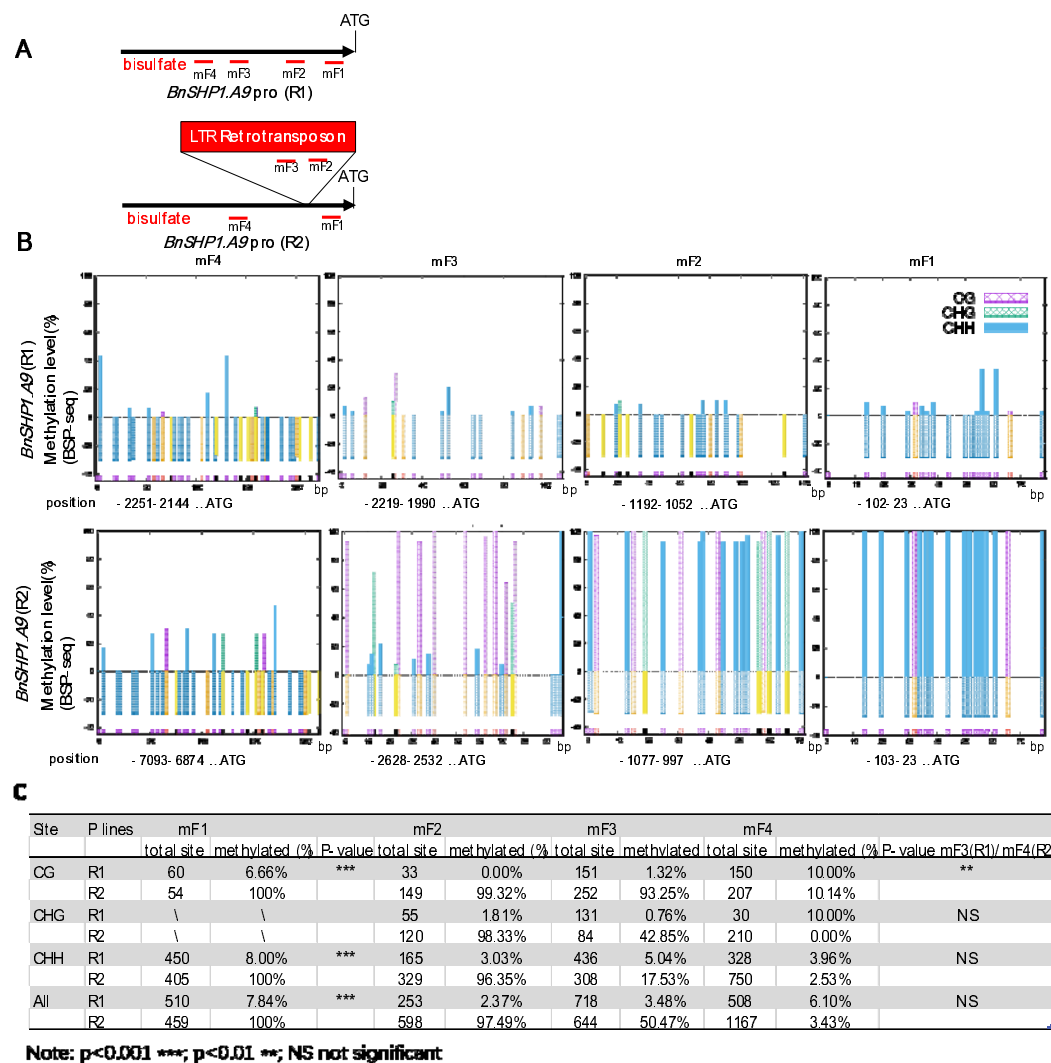


Fig. 6.

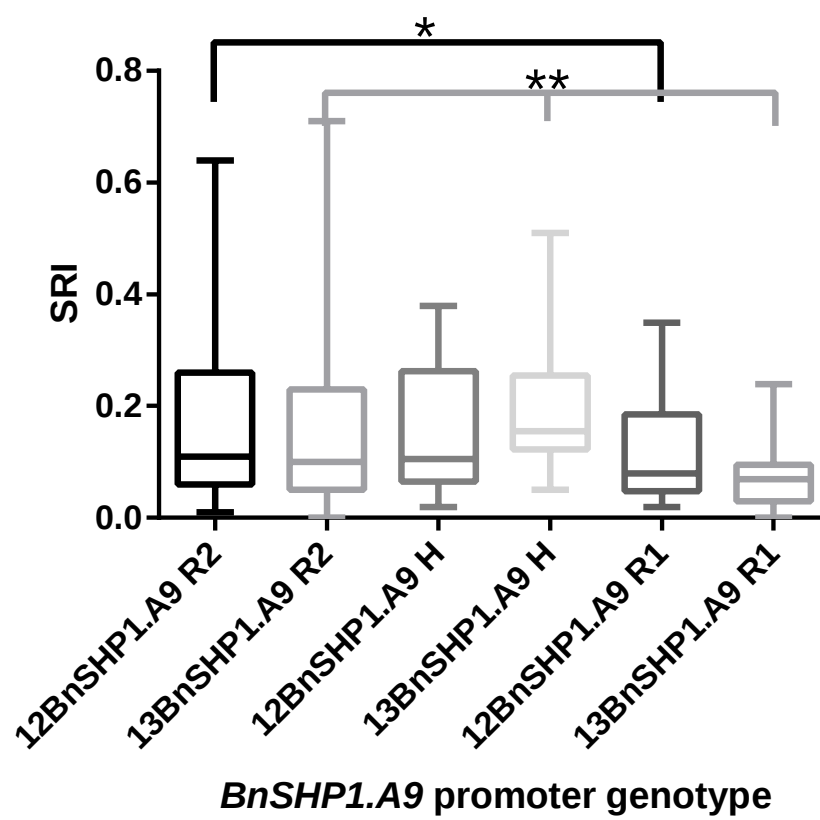


Fig. 7.