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IDENTIFICATION OF A SCAR MARKER LINKED TO A SHATTERING RESISTANCE TRAIT IN SESAME

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ABSTRACT

Sesame (*Sesamum indicum* L.) is one of the most important oil crops in temperate and tropical regions and is grown worldwide over an area of 5179 (hg ha^{-1}) to produce 5.469.024 tonnes of seed. Capsule shattering before or during harvest can cause yield losses of greater than 50%. The objectives of the present study were to evaluate the inheritance of resistance to capsule shattering in the F_2 derived from a cross between Cplus1, a sesame line with shattering-resistant capsules (*Sh1Sh1Sh2Sh2*), and KUAOX25, a line with shattering-susceptible capsules (*sh1sh1sh2sh2*$^-$); and use bulked segregant analysis and Amplified Fragment Length Polymorphisms (AFLPs) to identify Sequence Characterized Amplified Region (SCAR) markers associated with shattering resistance. After screening 192 AFLP primer combinations, nine polymorphic bands were identified, and one of these AFLPs was developed into a Si-SR-32-19 SCAR that could distinguish between shattering-susceptible and shattering-resistant phenotypes.

Keywords: AFLP, BSA, SCAR, *Sesamum indicum* L., shattering resistance

INTRODUCTION

Sesame (*Sesamum indicum* L.), a member of the Pedaliaceae family, is an important oilseed crop that is widely cultivated in tropical and subtropical areas (Ashri, 2010). Sesame seed has one of the highest oil contents of any seed (Tashiro et al., 1990; Arslan et al., 2007), and is a common ingredient in cuisines across the world. Sesame oil is highly prized because of its distinct flavor and nutritional value. Sesame is highly drought-tolerant, grows well in different kinds of soils and regions, and is thus well suited for different crop rotation practices. However, the main problems of its cultivation are seed-shattering at maturity preventing combine-harvest and indeterminate growth habit causing non-uniform capsule maturation among others (Cagirgan, 2006). The top three sesame producers, India, Myanmar and China account for 50 % of the world's sesame production. Sesame is cultivated mainly in the tropical and subtropical regions of Asia, Africa and South America (Ashri, 1998; Anilakumar et al., 2010). Currently, worldwide production of sesame is estimated at 5.469.024 tonnes, and the average annual yield worldwide is roughly 5179 (hg ha^{-1}) (FAOSTAT, 2014)

The production of sesame is limited due to capsule shattering that results in high yield losses. Up to 50% of sesame seeds are lost due to shattering before harvesting (Langham and Wiemers, 2002). The Sesaco Company (http://www.sesaco.com/about-us) has developed shattering-resistant varieties or non-dehiscent varieties. Induced closed capsule mutants in the *idid* gene were also obtained experimentally by gamma rays for the first time in Turkish sesame backgrounds (Cagirgan, 1996, 2001). The shattering resistance trait was developed by combining six capsule characteristics, most of which were identified by Langham et al. (1956). Kotcha et al. (2012) found that this shattering resistance was controlled by two genes with duplicate dominant epistasis (15:1) or duplicate recessive epistasis (9:7) in crosses between pairs of shattering-resistant lines or between a shattering-resistant line and a non-dehiscent line, respectively.

Langham et al. (1956) described the morphology of sesame capsules as they dried down and identified five important capsule characters related to shattering, including 1) capsule opening, 2) capsule splitting, 3) capsule constriction, 4) membrane completeness and 5) placenta attachment. The more details were explained: 1) Capsules should open at the tip, but if capsules are too open, seeds are more likely to be lost, whereas if they are only slightly open, mold could grow inside the capsule. 2) During capsule splitting, the capsule should dehisce at both sutures to expose the false membrane. This character is not important for seed retention but it is critical for seed release. 3) Capsule constriction can occur as the capsule dries. Sometimes the capsule walls shrink around the seed holding it in place. 4) The false membranes within the carpels should be as complete and attached as possible to hold the two halves of the capsule together. 5) Placenta attachment is also a variable character. In some lines, attachment of seeds to the placenta is incomplete or the seeds abscise from the placenta, and in other lines the seeds are firmly attached to the placenta.

Silique dehiscence has been studied from mechanistic and genetic standpoints in *Arabidopsis thaliana*. Several traits, including the development of the margins of the halves, or valves, of siliques have been identified that affect the shattering phenotype (Cosio and Dunand, 2010). Analyses of mutations in *Arabidopsis* genes encoding the transcription factors *SHATTERPROOF1* (*SHP1*), *SHATTERPROOF2* (*SHP2*), *NAC* (*NST1* and *NST3*) and the basic helix-loop-helix protein genes *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) have shown that these genes are involved in valve-margin development (Liljegren et al., 2000; Rajani and Sundaresan, 2001; Østergaard et al., 2006; Mitsuda et al., 2007; Ogawa et al., 2009; Girin et al., 2010). The *BEL1*-like homeobox gene *REPLUMLESS* (*RPL*) and the *FRUITFULL* (*FUL*) gene, which are expressed in valves, repress the expression of genes that control the identity of cells forming the margins of the valves (Ferrandiz et al., 2000). Analyses of ectopic expression of *IND*, *PG* (polygalacturonase) and *FUL* indicate that these genes regulate resistance to pod shatter in *B. oleracea*, *B. napus* and *B. juncea* (Jenkins et al., 1999).

Amplified fragment length polymorphism (AFLP) (Vos et al. 1995) technology requires no prior sequence information to identify markers linked to target genes. It can be particularly effective when used in combination with bulked segregant analysis (BSA) (Michelmore et al. 1991) and is an efficient approach for detecting markers closely linked to genes controlling important traits in various crops Ashutosh et al. (2007), Dussle et al. (2003), Hayashi et al. (2011), Ke et al. (2004), Negi et al. (2000).

In the present study, we focused on 1) evaluating the inheritance of shattering resistance in a *S. indicum* L. cross between the shattering-resistant Cplus1 line and the shattering-susceptible KUAOX25 line, and 2) developing molecular markers linked to the genes controlling shattering resistance in *S. indicum* L.

MATERIALS and METHODS

Plant materials

A set of 324 segregating F_2 progenies was developed from a cross between the shattering-resistant (*Sh1Sh1Sh2Sh2*) Cplus1 sesame line and the shattering-susceptible (*sh1sh1sh2sh2*) KUAOX25 sesame line as parents (Fig. 1). The parent lines, and the F_1 and F_2 generations were grown in the greenhouse conditions at the Department of Agronomy at Kasetsart University, Thailand from February to May 2016. The shattering-resistant and shattering-susceptible plants were harvested when seeds were mature in roughly 75% of the capsule-bearing zone of the plants and phenotypes were visually evaluated when plants had dried for 15 days after harvest.

DNA extraction and preparation of bulked DNA samples

Genomic DNA was isolated from fresh young leaves of parental and F_2 plants following the modified CTAB method (Doyle and Doyle, 1990). Concentrations of the purified DNAs were measured using a Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and adjusted to 100 ng μl^{-1}. Two bulked DNA samples for subsequent bulked segregant analysis (BSA) (Michelmore et al., 1991) were prepared from F_2 individuals by pooling equal amounts of total DNA from each of 10 shatter-resistant or 10 shatter-susceptible F_2 plants.

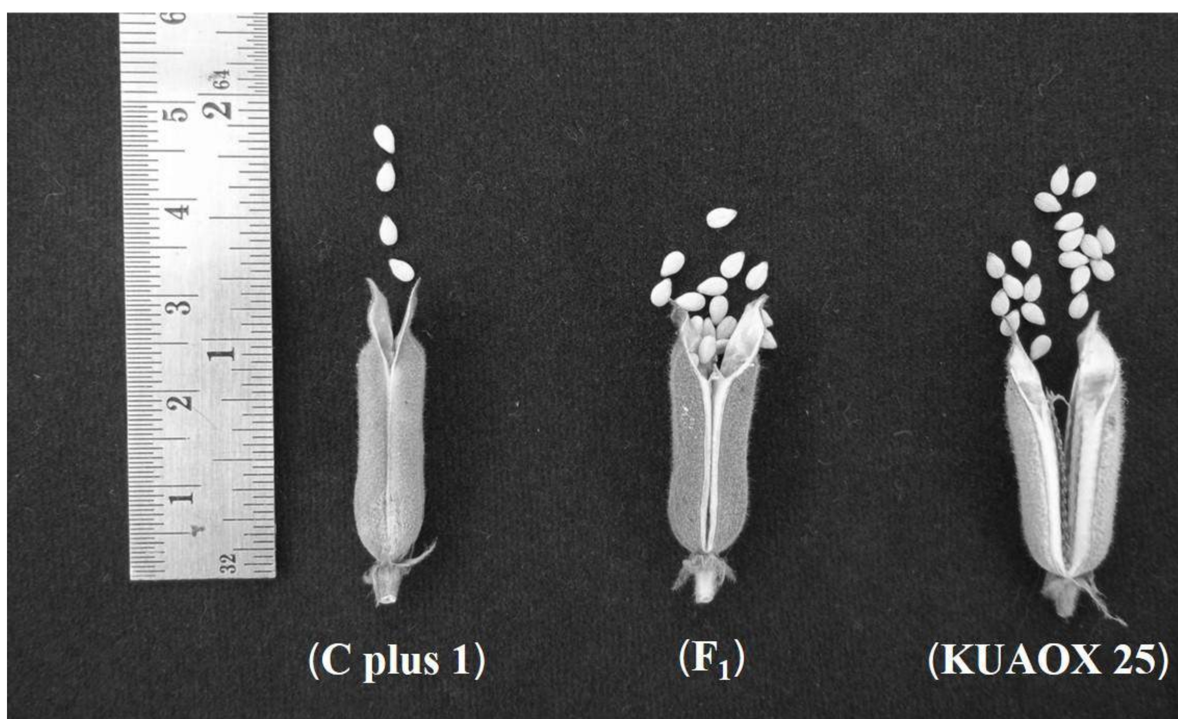

Figure 1. Capsule of the shattering-resistant female sesame line Cplus1 is shown on the left, the shattering-susceptible male sesame line KUAOX25 is shown on the right and the F_1 is shown in the center.

AFLP analysis

AFLP analysis was performed using the bulked shattering-resistant or shattering-susceptible DNA samples following the method of Vos et al. (1995) with minor modifications. A set of 192 primer combinations was used to screen for polymorphisms (Table 1). For each sample, the AFLP template DNAs were prepared by digesting 250 ng DNA with the restriction enzyme combination *Eco*RI-*Mse*I (New England BioLabs, Beverly, MA, USA) and then ligating to the corresponding oligonucleotide adaptors (*Eco*RI adapter and *Mse*I adapter (Table 1) with 1 U T4 DNA ligase (Fermentas, Burlington, Ontario, Canada) in a total volume of 10 µ*l*. The ligation products were diluted 10-fold with sterile deionized H_2O. Pre-selective PCR amplification with primers corresponding to the *Eco*RI and *Mse*I adapter core sequences (Table 1) was performed in a 25-µ*l* reaction containing 2.5 µ*l* (62.5 ng) of AFLP template DNA. Each PCR contained 1× PCR buffer (20 mM Tris-HCl pH 8.8 at 25°C), 20 mM $(NH_4)2SO_4$ 1.5 mM $MgCl_2$, (Fermentas, Burlington, Ontario, Canada), 0.2 µM of each primer, 0.4 mM dNTPs and 1 U Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada) with temperature cycling performed in a T1 Thermocycler (Biometra, Göttingen, Germany). The temperature profile for pre-amplification consisted of 20 cycles of 30 s at 94°C for DNA denaturation, 1 min at 56°C for annealing and 1 min at 72°C for extension, followed by incubation for 5 min at 72°C before storing the sample at 4°C. Amplification products were diluted 10-fold with sterile deionized H_2O, and 5 µ*l* of each reaction was used for selective amplification in a total volume of 20 µ*l* containing 1× PCR buffer (20 mM Tris-HCl pH 8.8 at 25°C, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, Fermentas, Burlington, Ontario, Canada), 0.4 mM dNTPs, 1 U Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada) and 0.25 µM of each *Eco*RI-XXX and *Mse*I-XXX adapter primer (Table 1) extended by each of three selective nucleotides (X). PCR was performed using a touchdown protocol with initial denaturation of 30 s at 94°C and annealing for 30 s at 65°C for the first cycle, after which the annealing temperature was lowered by 0.7°C per cycle until reaching 56°C, with extension for 1 min at 72°C, followed by 30 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C. Finally, selective amplification products were separated on 6% denaturing polyacrylamide gels and visualized using the silver nitrate staining method (Benbouza et al., 2006).

Table 1. Nucleotide sequences of adapters and primers used for AFLP analysis and SCAR marker amplification

Adapter/primer	Sequences		
*Eco*RI adapter	5′-CTCGTAGACTGCGTACC-3′		
	3′-CATCTGACGCATGGTTAA-5′		
*Mse*I adapter	5′-GACGATGAGTCCTGAG-3′		
	3′-TACTCAGGACTCAT-5′		
Preselective amplification primers			
*Eco*RI-A	5′GACTGCGTACCAATTCA-3′		
*Eco*RI-T	5′GACTGCGTACCAATTCT-3′		
*Eco*RI-C	5′GACTGCGTACCAATTCC-3′		
*Eco*RI-G	5′GACTGCGTACCAATTCG-3′		
*Mse*I-A	5′-GATGAGTCCTGAGTAAA-3′		
*Mse*I-T	5′-GATGAGTCCTGAGTAAT-3′		
*Mse*I-C	5′-GATGAGTCCTGAGTAAC-3′		
*Mse*I-G	5′-GATGAGTCCTGAGTAAG-3′		
Selective amplification primers *Eco*RI	5’-GACTGCGTACCAATTCXXX-3′		
*Eco*RI-AAC	*Eco*RI-TAA	*Eco*RI-CAT	*Ec*oRI-GCA
*Eco*RI-AAG	*Eco*RI-TAG	*Eco*RI-CAA	*Eco*RI-GAG
*Eco*RI-ACA	*Eco*RI-TCG	*Eco*RI-CGA	*Eco*RI-GAT
*Eco*RI-AGG	*Eco*RI-TGC	*Eco*RI-CAC	*Eco*RI-GTA
Selective amplification primers *Mse*I	5’-GATGAGTCCTGAGTAAXXX-3′		
*Mse*I -AAG	*Mse*I -TAG	*Mse*I-CTA	*Mse*I-GAG
*Mse*I -ATG	*Mse*I -TGA	*Mse*I-CAT	*Mse*I-GCA
*Mse*I -ACT	*Mse*I -TAC	*Mse*I-CAG	*Mse*I-GTA
*Mse*I -ACC	*Mse*I -TGT	*Mse*I-CAC	*Mse*I-GTC
SCAR primers (Si-SR-32-19)	Forward 5’-CGTCATCCTCAAGAATCG-3’ Reverse 3’-TCGGAAGGTCTTAGCTAG-5’		

Conversion of AFLP markers into SCAR markers

The amplified bands corresponding to AFLP markers were sliced from the gel and boiled for 5 min in 20 μ*l* of sterile water. After centrifugation, a 5-μ*l* aliquot of the supernatant was amplified again with the corresponding selective primer combination and the same PCR conditions that were used for the selective amplification. The amplicons were separated on a 1.0% agarose gel, and the PCR products were then purified and sent to Macrogen Inc. (Seoul, Rep. of Korea) for DNA sequencing. The raw sequences were confirmed and edited using Sequence Scanner Software v1.0 (Applied Biosystems, Foster City, USA) and then compared against nucleotide sequence information using blast queries (Altschul et al., 1997) at the public nucleotide sequence data repositories GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Phytozome (http:// www.phytozome.net/search.php). Primer pairs for developing SCAR markers were designed based on the two terminal sequences of AFLP markers using the Primer3 program (Rozen and Skaletsky, 2000). PCR conditions were optimized with the pre-amplification products from the two parental lines and the two DNA bulks as templates.

SCAR primers for conversion of AFLP markers into SCAR markers were designed and synthesized (Macrogen, Seoul, Rep. of Korea) and are shown in Table 1. The PCR amplification reactions were set up with 20 ng of genomic DNA and 10 μM each of the forward- and reverse-specific primers, 1 mM of each dNTP, 25 mM $MgCl_2$, 1 μ*l* of 10× reaction buffer (1× concentration: 10 mM Tris-HCl pH 8.3 and 50 mM KCl), and 1 U Taq DNA polymerase (5 U μl^{-1}; Fermentas). Template DNA was initially denatured at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, annealing at 58°C for 30 sec and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min on a PTC-225 Peltier Thermal Cycler (MJ Research). The PCR products were visualized by electrophoresis on 1.5 % agarose gels.

Linkage analysis

Chi-square tests were performed using an Excel spreadsheet (Microsoft Office 365 (2016) Microsoft, Redmond, WA) to analyze the phenotypic segregation of 167 F_2 plants and to perform linkage analysis between markers and the *Sh1Sh1Sh2Sh2* genotype.

RESULTS

Inheritance of shattering resistance

The Chi-square value for a segregating F_2 population of 323 individuals derived from the cross between Cplus1 and KUAOX25 showed significant ($\chi^2 = 53.48$; d.f. = 1; $p = 0.05$) deviation of the observed segregation ratio from the expected 3:1 ratio of shattering-resistant to shattering-susceptible individuals (Table 2). Two other segregation ratios of 9:7 and 15:1 shattering-resistant to shattering-susceptible individuals were also tested (Table 2). The significant Chi-square value for the 9:7 ratio ($\chi^2 = 173.89$; d.f. = 1; $p = 0.05$) indicated that the F_2 was not segregating in this ratio and that duplicate recessive epistasis was unlikely. However, the non-significant Chi-square value calculated for the 15:1 ratio ($\chi^2 = 0.74$; d.f. = 1; $p = 0.05$) confirms reports of two independent genes in sesame segregating for shattering resistance with dominant duplicate epistatic gene action.

Table 2. Segregation of shattering and shattering-resistant phenotypes in the F_2 generation of a cross between Cplus1 and KUAOX25

Cross	Total no. of plants	Shattering	Shattering resistant	Segregation ratio	χ^2
Cplus1 × KUAOX25	324	300	24	15:1	0.74

$\chi^2_{0.05,\,1} = 3.84$

Identification of an AFLP marker linked to shattering resistance

From the 192 AFLP primer combinations tested in AFLP analysis, only one polymorphic fragment was obtained using the primer combination E-CAA/MGTA. This fragment could only be amplified from the shattering-resistant DNA pool and shattering-resistant individuals. This AFLP marker was converted into a SCAR marker to facilitate more precise identification of the linkage relationship between the marker and the shattering resistance genes and phenotype.

Conversion of AFLP markers into SCAR markers

The AFLP marker E-CAA/MGTA was converted into a 219-bp SCAR marker designated as Si-SR-32-19. A blast search using Si-SR-32-19 as a query identified a 79% match to the ocpggb0_0384_H06.ab1 mRNA sequence (GenBank accession: JK076838.1; Fig 2) from a *Sesamum indicum* seed EST library (Ke et al., 2011).

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        Si-SR-32-19R
        >>>>>>>>>>>>>>>>>>>
1       TCGGAAGGTCTTAGCTAGTCGTCATCCTCAAGAATCGGGAGGTTTCCTGC
51      TGATCGGGTTGAAATTCAGGCGACCTTCTCGGTCAGGCAAGCTGGTCCTC
101     CAAATGGTTCGAGATCGATACCTTTCACAAATTAGTAATTGGGTATTGAG
151     AAAAGAAGCAGATCGTTAGGCTTGTTGGGGTGGCCCCTAGCTAAGACCTT
201     CCGATTCTTGAGGATGACG
         <<<<<<<<<<<<<<<<<<<
                 Si-SR-32-19F
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Figure 2. Nucleotide sequence of the 219-bp Si-SR-32-19 fragment. Left-pointing arrows at the end of the sequence indicate forward primer and right-pointing arrows at the beginning of the sequence indicate reverse primers.

When 167 F2 plants were screened using the Si-SR-32-19 marker, a 219-bp band could be amplified in DNA samples from 152 shattering-resistant F2 plants and the maternal parent, but no band could be amplified from the DNA samples of 15 shattering-susceptible plants or the paternal parent (Fig. 3). These results indicated the dominant nature of the markers. The Chi-square value for segregation of the SCAR marker ($\chi^2 = 2.12$, d.f. = 1, $p = 0.05$) was not significant, thus the observed segregation ratio of this marker in the shattering-resistant and shattering-susceptible phenotypes (Table 3) was consistent with a 15:1 ratio. However, the observed segregation of this marker was not consistent with 3:1 or 9:7 ratios expected for the segregation of a single gene or duplicate recessive genes, respectively. The absence of an amplified band for the Si-SR-32-19 marker from any of the 15 shattering-susceptible plants (*sh1sh1sh2sh2*) combined with linkage analysis indicated that the Si-SR-

32-19 SCAR marker was tightly linked to the shattering resistance genes and phenotype with a 0% recombination frequency.

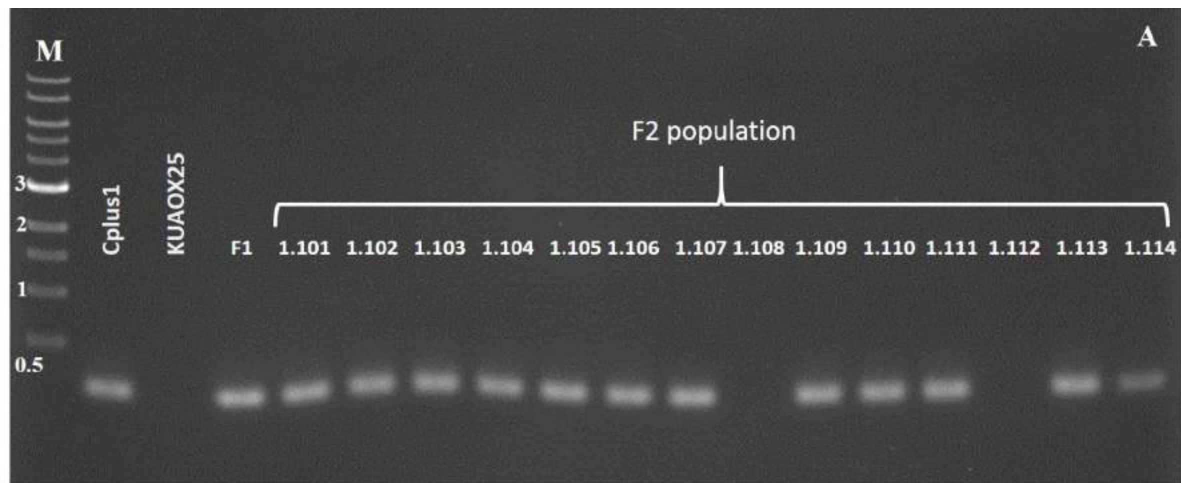

Figure 3. Amplification patterns of the Si-SR-32-19 marker in Cplus1, KUAOX25, the F_1 and F_2 individuals; M:1-kb DNA Ladder (New England BioLabs) used as molecular weight marker.

Table 3. Segregation of a SCAR marker in the F_2 generation of a cross between Cplus1 and KUAOX25

Marker name	Segregation pattern (Present:Absent)	Expected ratio	χ^2	Recombination frequency (%)
Si-SR-32-19	152:15	15:1	2.12	0
		9:7	82.03	
		3:1	22.85	

$\chi^2_{0.05,1} = 3.84$

Several genes related to abscission or dehiscence phenomena in plants have been isolated. We designed primers (Table S1) based on the nucleotide sequences encoding the *Lepidium campestre* SHATTERPROOF1-like protein (*shp*1 gene) (Lenser et al., 2013), the *Capsella bursa-pastoris* SHATTERPROOF1-like protein (*SHP1b*) and the *Brassica napus* SHATTERPROOF1 (*BnSHP1*) gene (Tan et al., 2009), and tested them in sesame but found no polymorphisms between shattering-resistant and shattering-susceptible plants (data not shown). The genes that control shattering in sesame (*sh1sh2*) may function differently than the genes that control shattering or dehiscence phenomena in the plant species mentioned above.

Table S1. Nucleotide sequences of primers related to abscission or dehiscence phenomena in three plant species

	Plant species	Gene name	Gen Bank Accession No.	Primer name	Sequences
1	*Lepidium campestre*	SHATTERPROO F1-like (*SHP1b*)	FR727235.1	SiSPH1_1F	5′-ATGGAWGAAGGHGGRAGTAG-3′
				SiSPH1_1R	5′-TRACRGCATCRGAACAAGCT-3′
2	*Capsella bursa-pastoris*	SHATTERPROO F1-like (*SHP1b*)	EU551771.1	SiSPH1_2F	5′-TGTGARRGGTACAATTGAAAGG-3′
				SiSPH1_2R	5′-AKCGGACRCGGCTKATTCCT-3′
3	*Brassica napus*	SHATTERPROO F1 (*BnSHP1*)	AY036062.1	SiSPH1_3F	5′-AACCTHGAAGGACGKCTTGA-3′
				SiSPH1_3R	5′-TTAMACAAGYTGAAGAGGAGG-3′

DISCUSSION

Despite of its attributes, plant architecture of sesame is poorly adapted to modern farming due to seed shattering at maturity, indeterminate growth habit (Cagirgan, 2006) and sensitivity to wilting (Silme and Cagirgan, 2010) under intensive management. Shattering resistance in sesame is of different mechanism than the indehiscence or closed capsule (Langham, 1946; Cagirgan, 2001) trait, and it was developed by combining six capsule characters (Langham and Wiemers, 2002). Langham (1946) reported a sesame spontaneous mutation that prevented the shattering of capsules controlled by a recessive allele of the *indehiscent* (*id*) gene. In 1986, Sesaco discovered a second closed-capsule trait controlled by a recessive allele of the *seamless* (*gs*) gene (Ashri, 1998; Langham, 2001). Cagirgan (2001) obtained closed capsule induced mutants by gamma-rays for the first time in 1995. Cplus1 is a shattering-resistant *S. indicum* line developed by the sesame breeding project in the Agronomy Department at Kasetsart University and was released in 2003 (Wongyai et al., 2003). Chi-square analysis by Kotcha et al. (2012) indicated that shattering resistance in Cplus1 was not controlled by two genes with duplicate recessive epistasis in crosses between shattering-resistant and shattering line.

In the present study, we investigated the inheritance of shattering resistance in *S. indicum* due to the segregation of the *Sh1* and *Sh2* genes and described the linkage of an AFLP/SCAR marker to these genes using a combination of BSA, AFLP and SCAR methodologies. The genetic analysis in this study revealed that shattering resistance is controlled by the action of either of the duplicate dominant alleles of two genes, *sh1* and *sh2*, and that the shattering phenotype results from the dominant alleles, *Sh1* and *Sh2*. These results are distinct from those of Kotcha et al. (2012), in which the same parental lines were used, and suggests that the shattering trait may controlled by two genes with duplicate recessive epistasis.

No recombination was identified between the 219-bp SCAR marker Si-SR-32-19 and the loci controlling shattering because the marker could not be amplified in any of the fifteen shattering-susceptible plants. The SCAR marker Si-SR-32-19 developed here is closely linked to the genes controlling shattering, but because it is dominant, it will not be useful for screening heterozygotes. To our knowledge, this is the first report of control of the shattering trait in sesame by dominant alleles at two loci.

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