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Identification of Simple Sequence Repeat Markers Linked to Sudden Death Syndrome Resistance in Soybean

Jirawat Sanitchon,[a] Apichart Vanavichit,[a] Sontichai Chanprame,[a] Theerayut Toojinda,[b] Kanokporn Triwitayakorn,[c] Victor N. Njiti[d] and Peerasak Srinives[a,*]

[a] Department of Agronomy, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
[b] National Center for Genetic Engineering and Biotechnology, Klong Luang, Pathum Thani 12120, Thailand.
[c] Institute of Molecular Biology and Genetics, Mahidol University, Salaya, Nakhon Pathom 73170,Thailand.
[d] Department of Plant, Soil and General Agriculture, Southern Illinois University, Carbondale, IL 62901-4415, USA.

* Corresponding author, E-mail: agrpss@ku.ac.th

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Abstract: Sudden Death Syndrome (SDS) caused by the soil borne fungus *Fusarium solani* is a major soybean disease. Resistance to this fungus is controlled by quantitative trait loci (QTLs). This study was aimed at identifying simple sequence repeat (SSR) markers linked to the QTLs conditioning SDS resistance. A mapping population was constructed by crossing the resistant soybean line GC87018-12-2B-1 with the susceptible line GC89045-13-1. One hundred and four recombinant inbred lines (RILS) were obtained via the single seed descent technique. Fifteen-linkage groups were constructed from data derived from 106 SSR markers. Inoculation was accomplished by the infested soil technique in the greenhouse conditions and phenotypic data were recorded. Broad-sense heritability of disease severity was 56% of the total variation. A QTL with LOD score of 2.53 was identified to locate on linkage group J as a cluster of four SSR markers. This QTL was previously unidentified for conferring resistance to the SDS.

Keywords: soybean sudden death syndrome, *Fusarium solani*, simple sequence repeat markers, quantitative trait loci.

Introduction

Sudden death syndrome (SDS), caused by the soilborne fungus *Fusarium solani* (Mart.) Sacc. f.sp. *glycines* (Burk.) Snyd. & Hans, is an important disease devastating major soybean production countries such as the US, [1] Argentina, [2] Brazil, [3] and Thailand .[4] Plants infected with *Fusarium solani* show leaf chlorosis and necrosis. In more severely infected plants, the leaflets drop off, leaving the petioles attached. Pod abortion subsequently may occur in the late reproductive stage of growth, resulting in heavy yield loss.

Evaluation of SDS resistance can be accomplished by field testing and greenhouse inoculation. The more popular greenhouse techniques are the infested oat grain technique[5]and sorghum seed technique. [6]

Stephen *et al*[5] reported that SDS resistance in soybean was controlled by a single dominant gene *Rfs1* in greenhouse conditions. In contrast, studying in a different mapping population, Njiti *et al*[7] found that the resistance was conditioned by quantitative trait loci. In addition, Hnetkovsky *et al*[8] reported multigenic inheritance of SDS resistance in the soybean population 'Essex' x 'Forrest'.

In the last two decades, DNA markers, both hybridization based and PCR based, were developed and applied to assist in plant breeding programs. Two applications of DNA markers are as genetic milestones for mapping and tagging traits of interest, and as indicators of genetic diversity.[9] Simple sequence repeats (SSRs) are PCR based markers that were first introduced to evaluate human genetics and later applied to plant breeding. SSR markers are potential breeding tools due to their abundance and high polymorphism. Additionally, the markers distribute well throughout the soybean genome.[10,11] They have been used in

soybean research for investigation of genetic similarity and relationship[12], DNA fingerprinting[13], and genetic mapping[14]. The objective of this experiment was to verify the QTLs underlying SDS resistance and identify SSR markers linked to them.

Materials and Methods

Mapping Population

The mapping population in this experiment was constructed from the cross between GC89045-13-1 (the susceptible parent) and GC87018-12-2B-1 (the resistant parent). Seeds of the parents were kindly provided from the Asian Vegetable Research and Development Center (AVRDC) through its Asian Regional Center located at Kasetsart University, Kamphaeng Saen, Thailand. The F_1 seeds were sown to produce F_2 seeds for subsequent plantings through F_6 seeds via single seed descent technique. Individual F_6 plants were harvested and treated as recombinant inbred lines. Finally, 104 recombinant inbred lines (RILs) were obtained and used for gene mapping.

Disease Scoring

A soil infested technique[6] was employed to determine the reactions of the RILs to the pathogen. The experiment was laid out in a Completely Randomized Design with three replications, having one plant grown in a 3-inch styrofoam cup as an experimental unit. *Fusarium solani* inoculum was prepared from macrospores collected from cultured plates. The concentration of inoculum was diluted with distilled water to 2,500 spores/ml. The bottom half of each styrofoam cup was filled with sterilized soil and overlaid with 0.5 ml of inoculum. The remaining soil was topped up and overlaid with another 0.5 ml inoculum. Five to six seeds from each RIL were sown in a 5" plastic pot containing sterilized mixed soil. Each two-week-old seedling was transplanted to a styrofoam cup and kept in a plastic bucket which can accommodate up to 106 cups, making a replicate. There were altogether 4 buckets in this experiment, viz. 3 buckets with infested soil and a control bucket with no inoculation. The buckets were filled with water up to 1.5 inches deep to saturate the soil. A solution of complete fertilizer was applied weekly with irrigation water. Susceptible plants began to show foliar disease symptoms three weeks after inoculation. Disease severity was scored as phenotypic data following Njiti *et al*.[7] In their system, higher scores represent more susceptibility.

RILs genotyping

The young leaves from RILs were collected. DNA extraction was done with DNeasy Plant Kits, according to manufacturer's instructions (QIAGEN Inc., Valencia, CA, USA). The DNA concentration was adjusted to 25 ng/ml. SSR markers, as surveyed from soybean genomic markers[15], were purchased from Research Genetics, Inc. (Huntsville, AL, USA). The 20 ml reaction PCR mixtures contained 25 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.15 *m*M of 5' and 3' end primers, 200 mM of each nucleotide, 1x PCR buffer and 0.5 unit of *Taq* DNA polymerase. DNA amplification was carried out by holding the temperature at 94°C for 2 min, followed by denaturation at 94°C for 30 sec, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 35 cycles, and a final extension at 72°C for 2 min. PCR products were separated on 4% MetaPhor agarose (FMC BioProducts, Rockland, ME, USA) gels and run for 1 hour in a 100V electrophoresis set. The DNA bands were stained by ethidium bromide to identify the PCR products.

QTL AnalYsis and Heritability Estimation

Genotypic scores obtained from all primers were used as genetic data for linkage construction. Linkage analysis was accomplished using MAPMAKER/Exp version 3.0.[16] LOD score and percent of recombination allowed for map construction were 3.00 and 50, respectively. Mapping and QTL analysis were completed by the mQTL program. The association between each marker and disease scoring was confirmed by group comparison t-test with unequal variances,[17] between the plants carrying marker from resistant vs. susceptible parents.

Broad-sense heritability (h^2), a ratio of genotypic variation over phenotypic variation of disease severity was estimated from the equation, $h^2 = \sigma^2_t/(\sigma^2_t+\sigma^2)$.[18] Where σ^2_t and $(\sigma^2_t+\sigma^2)$ are genotypic and phenotypic variances of soybean disease scoring, respectively (Table 1). Genotypic variance was calculated by σ^2_t = (MSB-MSW)/k. Where MSB and MSW are the mean square between lines and within lines, respectively. MSE itself is the estimate of σ^2. The denominator, k, is calculated by the formula $k = (n. - \Sigma n_i^2/n.)/(S - 1)$. S is the number of RILs (104 in this case), n_i is the number of plants in the i^{th} RIL (1-3 in this case), and n. is the total number of plants in the RILs (307 in this case).

Results

Polymorphism of SSR Markers and Linkage Construction

The SSR primers from the soybean genomic map covering 20 linkage groups of soybean were surveyed upon DNA of the two parents. One hundred and six SSR primers were identified as polymorphic markers and then used to amplify and map on DNA of the 104 RILs and their parents. Upon linkage analysis of all

Table 1. Analysis of variance of disease severity in the RILs.

SOV	DF	Mean square	F-value	EMS
Between lines	103	MSB = 1.052	4.68**	$\sigma^2 + k\sigma^2_t$
Within lines	203	MSW = 0.225		σ^2
Total	306			

** significant difference at the probability ≤ .01

polymorphic markers, eighty-one were assigned into 15 linkage groups. The other 25 markers were found unlinked. All grouped markers are listed in Table 2. Based on the constructed linkage map[15], the names of linkage groups of the markers were given. On average, five markers were located on each linkage group and the average genetic distance between two adjacent markers was 22.82 cM. An example of DNA bands in Fig 1 was amplified by marker Satt183. The upper band in the first lane obtained from GC89045-13-1 represents the susceptible band and was scored as A. The lower band in the second lane derived from GC87018-12-2B-1 represents the resistant band and was scored as B. The bands in the other lanes belong to the RILs.

Heritability Estimation

Analysis of variance for severity of the disease score is given in Table 1. Significant difference was detected at $P \leq 0.01$. Highly significant mean contrast between the two parents was also detected from the same analysis. The mean disease score of GC87018-12-2B -1, the resistant parent was 1.5 whereas the mean of GC89045-13-1, the susceptible parent was 4.5. The $LSD_{0.01}$ value for this mean contrast was 1.007. Broad-sense heritability was estimated as $h^2 = 0.56$, indicating that this QTL is moderately heritable and revealed a high likelihood of success in improvement for SDS resistance in this population.

Markers Associated with SDS Sesistance

The identified QTL underlying SDS resistance was located on linkage group J. Four SSR markers were

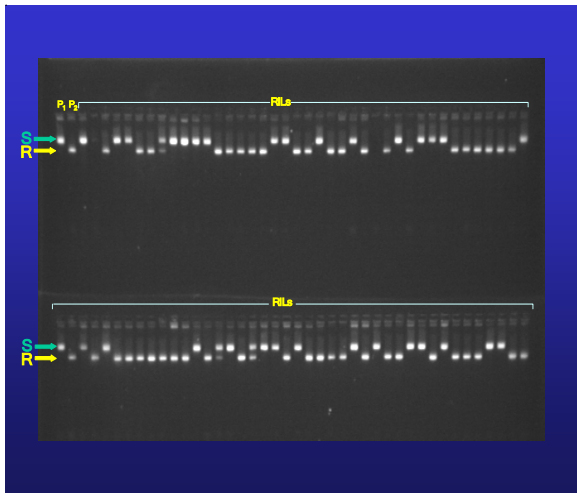

Fig 1. DNA bands obtained from amplification using marker Satt183, separated by 4% metaphore gel and stained by ethidium bromide. Lanes are as follow; lane 1: susceptible parent GC89045-13-1; lane 2: resistant parent GC87012-12-2B-1; the other lanes (above and below) are RILs.

Table 2. The consensus soybean linkage groups based upon alignments with those reported by Cregan *et al.* (1999).

Group	Consensus linkage group	SSR markers
1	A1	satt155, satt276, satt454, satt300, satt174, satt200, satt545
2	B1	satt359, sat453
3	B2	satt066, satt304
4	C2	satt100, satt134, satt202, satt277, satt286, satt289, satt307, satt365, satt460
5	D1b+w	sat089, sat135, satt141, satt350, satt546, satt157, satt542, satt549
6	D2	satt301, satt389, satt397, satt514
7	E	sat124, satt263, satt452, satt483, satt598
8	F	satt133, satt114, sct033, satt146, satt269
9	G	sat094, satt012, sat064, satt115, satt138, satt191, satt199, satt288, satt394, satt472, satt517, satt566, satt594
10	H	sat118, satt222, satt469, satt541,satt181, satt302, satt434
11	I	satt062, satt148, satt330, satt292
12	J	satt183, satt456, sct001, sct065, satt285, sct046
13	K	satt167, satt349
14	M	sat121, satt250, satt308, satt323, satt463
15	O	sat173, satt259
Unlinked		satt605, satt509, satt339, sat087, sat113, sat115, satt126, satt129, satt180, satt187, satt192, satt197, satt254, satt257, satt294, satt354, satt422, satt431, satt436, satt488, satt560, satt570, satt571, sat583, satt601

Table 3. Comparison of disease scores on the RILs carrying markers from the resistant vs the susceptible parents.

Markers	RILs carrying resistant band	RILs carrying susceptible band	Prob. of F-test[a]	Prob. of t-test[b]
Satt183	1.19 ± 0.31	1.49 ± 0.63	0.000	0.0031
Satt456	1.19 ± 0.32	1.45 ± 0.62	0.021	0.0117
Sct065	1.17 ± 0.29	1.44 ± 0.64	0.015	0.0055
Sct001	1.26 ± 0.40	1.42 ± 0.60	0.000	0.0073

[a] F-test for equality of variances between the RILs carrying SSR markers from the resistant vs the susceptible parents.
[b] t-test for difference in mean disease scores between the RILs carrying SSR markers from the resistant vs the susceptible parents.

found associated with the QTL, Satt183, Satt456, Sct065 and Sct001 (Fig 2). The peak of the QTL with LOD of 2.53 was 0.2 cM below the most tightly linked marker Satt 183. Data obtained from regression of the disease score on the marker score revealed that Satt183 had the strongest effect on the QTL, with a probability of 0.006. Markers Satt456 and Sct065 were located at the same position at 1.2 cM above Satt183. Satt456 showed an association probability of 0.012, while Sct065 linked to QTL at the probability of 0.015. The other linked marker, Sct001, was 2.0 cM below Satt183 (1.8 cM below the peak) with P = 0.015.

The significance of the linked markers was individually analysed by a t-test with unequal variances among the RILs carrying the SSR bands from resistant vs. susceptible parents. All four markers showed significant difference in mean disease score between both groups of RILs (Table 3). The probabilities of t′ (significance level at an effective df) for markers Satt183, Satt456, Sct065 and Sct001 were 0.0031, 0.0117, 0.0055 and 0.0073, respectively.

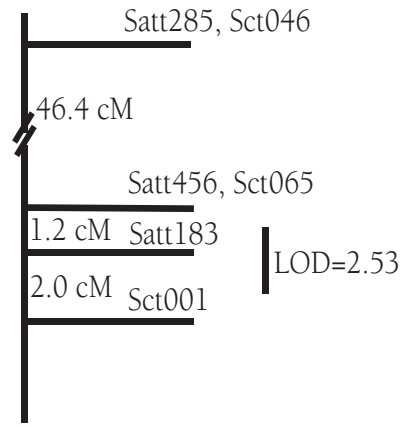

Fig 2. Location of SSR markers on the linkage group J of the soybean genome and the new QTL underlying resistance to SDS.

Discussion

Three major groups of SSR markers, Sat, Satt and Sct, with the respective tandem repeats of AT, ATT and CT, were employed in this study. The percentage of polymorphism of SSR markers surveyed upon the two parents was low. In general, the genetic variation among recommended soybean varieties is so low that polymorphic markers could incorporate only approximately 60% of the whole genetic distance of the soybean genome. A low level of genetic diversity in cultivated soybean has also been reported.[14] The cumulative genetic distance of the soybean genome is 3103.4 cM.[15] Moreover, the genome regions into which SSR markers could not be incorporated included the ones containing QTLs underlying *Fusarium solani* resistance that were previously reported.[8,19]

The putative QTL was located on linkage group J. A cluster of four markers constituted the QTL underlying SDS resistance in this experiment. The probability of the markers in Table 3 had a significant association with the score of leaf scorch. Significant difference between the mean disease score of the RILs carrying SSR marker from the resistant parent against that from the susceptible parent was confirmed by the t-test in Table 3. This implies that the smaller probability of the t-test, the closer the genetic distance from marker to QTL, which may not be quite true as the effective df varied from one comparison another. The df variation was due to occasional difficulty in scoring bands and incomplete PCR reactions for some RILs with certain SSR primers, which resulted in missing data points. If the df is not a consideration, then Satt183 was expected to show a greater probability than both Satt456 and Sct001, as confirmed in Table 3. For each marker, RILs were grouped based on either resistant or susceptible band. The severity scores among the two groups can be compared using a t-test. The mean genotypic scores for the resistant group of RILs were significantly lower than those of the susceptible ones for all markers. This clearly revealed that the resistant lines received a resistant gene from the resistant parent, GC 87018-12-2B-1.

The broad-sense heritability from this population was moderately high (h^2= 0.56), implying that the QTL controlling SDS resistance in this population should be easily transferred to their progenies. New cultivars or

lines can be improved via conventional methods, such as pedigree selection with a possible integration of marker-assisted selection (MAS), if needed. The favorable allele can also be transferred into new improved lines by gene pyramiding using backcross breeding. The linked markers can also be employed to facilitate identification of resistant lines without going through the tedious screening process.

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REFERENCES

1. Hartman GL, Noel GR and Gray LE (1994) Occurrence of soybean sudden death syndrome in East–Central Illinois and associated yield loss. *Plant Dis* **79**, 314 – 8.
2. Ploper LD (1993) Syndrome de la muertesubita : Nueva enfermedad de la soja noroeste Argentino. *Av Agroindustrial* **54**, 5 - 9.
3. Nakajima T, Mitsueda T and Chacha M J (1996) First occurrence of sudden death syndrome of soybean in Brazil. *JARQ* **30**, 31 – 4.
4. Prathuangwong S, Saisangthong S, Fareaudee A and Chutawantana P (1996) The incidence and new occurrence of soybean disease in central area during 1994- 1996. In: *Proceedings of Soybean Research Conference VI,* pp. 242-57. Kasetsart University Pub, Bangkok. (in Thai with English abstract)
5. Stephen PA, Nickell CD and Kolb FL (1993) Genetic analysis of resistance to *Fusarium solani* in soybean. *Crop Sci* **33**, 929 - 30.
6. Njiti VN, Torto GA, Gray LE and Lightfoot DA (1997) An effective greenhouse assay for field resistance to SDS. *Soybean Genetics Newsletter* **24**, 132 - 5.
7. Njiti VN, Shenaut MA, Suttner RJ, Schmidt ME and Gibson PT (1996) Soybean response to sudden death syndrome : Inheritance influenced by cyst nematode resistance in Pyramid x Douglas progenies. *Crop Sci* **36**, 1165 – 70.
8. Hnetkovsky N, Chang SJC, Doubler TW, Gibson PT and Lightfoot DA (1996) Genetic mapping of loci underlying field resistance to soybean sudden death syndrome (SDS). *Crop Sci* **36**, 393 – 400.
9. Karp A and Edwards KJ (1997) DNA markers : a global overview. In : *DNA Markers Protocols, Applications, and Overviews,* pp. 1 - 14. Wiley-VCH, New York.
10. Akkaya MS, Shoemaker RC, Specht JE, Bhagwat AA and Cregan PB (1995) Integration of simple sequence repeat DNA markers into a soybean linkage map. *Crop Sci* 35, 1439 - 45.
11. Walton M (1994) Microsatellite DNA : New tool for plant breeder's box. *Seed World* **132**, 20 - 1.
12. Senior ML, Murphy JP, Goodman MM and Stuber CW (1998) Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. *Crop Sci* **38**, 1088 - 98.
13. Sharon D, Adato A, Mhameed S and Lavi U (1995) DNA fingerprints in plants using simple-sequence repeat and minisatellite probes. *Hort Sci* **30**, 109 - 12.
14. Shoemaker RC and Specht JE (1995) Integration of the soybean molecular and classical genetic linkage groups. *Crop Sci* **35**, 436 - 46.
15. Cregan PB, Jarvik T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, Kaya N, Vantoai TT, Lohnes DG, Chung J and Specht JE (1999) An integrated genetic linkage map of the soybean genome. *Crop Sci* **39**, 1464 - 90.
16. Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, and Newburg L. (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* **1**, 174-81.
17. Steel RGD and Torrie JH (1980) *Principles and Proceedures of Statistics*, 2nd ed., McGraw-Hill, New York.
18. Kearsey MJ and Pooni HS (1996) *The Genetical Analysis of Quantitative Traits*. Chapman & Hall, London.
19. Prabhu RR, Double TW, Chang, SJC and Lightfoot DA (1997) Development and utility of sequence characterized amplified regions (SCARS) from RAPD markers linked to SDS and SCN QTLs. *Soybean Genetics Newsletter* **24**, 128-31.