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Note

Conversion of AFLP Markers Surrounding a QTL, *rhg-t1*, for Soybean Cyst Nematode Resistance into PCR-based Markers

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Soybean cyst nematode (SCN) (*Heterodera glycines*), from the economical view point, is the major pathogen of soybean worldwide. Management practices for SCN have been gradually eroded. Many effective nematicides have either been prohibited or restricted for environmental or toxicity reasons. Thus, the use of resistant cultivars has become a more attractive and more desirable alternative. Several sources of resistance have been reported (Riggs and Schmitt 1988) and several soybean genes confer resistance to SCN (Riggs and Schmitt 1991, Caldwell *et al.* 1960, Concibido *et al.* 2004).

Breeding for soybean cyst nematode resistance may not be routinely performed because screening is time-consuming and requires considerable resources. DNA markers provide an excellent tool for the analysis of the QTLs controlling genetic resistance to SCN races. Molecular markers can facilitate breeding programs via marker-assisted selection (MAS). Concibido *et al*. (1996, 2004) who reported a comparison of SCN-marker assisted selections between SCN greenhouse bioassays in terms of time and cost revealed that MAS is a simple, rapid and inexpensive technique that can be used for analyzing larger numbers of samples. Mudge *et al.* (1997) showed that MAS using SSR markers that flank a major SCN resistance locus, *rhg1*, was 98% accurate in identifying resistant lines.

In Japan, Gedenshirazu, a land race in Akita prefecture, has been used to develop cultivars resistant to SCN race 3. Especially many cultivars carrying the SCN resistance gene derived from Gedenshirazu were released in the Hokkaido and Tohoku areas. For example, the pedigree of Horai, Toyosuzu, Toyokomachi, Toyomusume and Yukihomare developed at the Hokkaido Tokachi Agricultural Experiment Station included Gedenshirazu. A previous study indicated that Gedenshirazu resistance to SCN race 3 might be mainly controlled by two recessive alleles (Shirai *et al.* 1991). We reported that the resistance to SCN race 3 in Toyomusume was also controlled by two loci, *rhg1* and *rhg-t1*, located on the linkage groups G and B1, respectively (Ferdous *et al.* 2006). Moreover, in our experimental material, the effects of these two QTLs and their interaction accounted for approximately 70–80% of the phenotypic variance. The candidate genes for *rhg1* and *Rhg4* which contain a nucleotide-binding site and a leucine-rich repeat domain have already been reported (Hauge *et al*. 2001, Lightfoot and Meksem 2000). However, information about other resistance genes related to SCN race 3 is limited.

A high-density map of the linkage group B1 was constructed, and the *rhg-t1* locus was found to be surrounded by several AFLP markers using recombinant inbred lines of Toyomusume and Tsurukogane (Ferdous *et al.* 2006). It was demonstrated in our previous study that the AFLP marker CAT_CTC1 which is located near GTA_TAC1 was the closest marker to the *rhg-t1* locus. Though AFLP analysis is suitable for linkage mapping, it is unsuitable for large-scale locus-specific uses because of its high cost and complexity. Therefore, it was assumed that converting AFLP markers to PCR-based markers might considerable enhance their usefulness in genetic applications. Thus in the present study, we attempted to convert the surrounding AFLP markers of the *rhg-t1* locus into PCR-based markers for use in selection for nematode resistance in soybean breeding. These markers are generally locus-specific and their amplification is less sensitive to reaction conditions in PCR.

Plant materials

Toyomusume (B, resistant), Tsurukogane (A, susceptible) and RILs derived from these parents were used for the genotyping of newly developed PCR-based markers. Additionally, various resistant and susceptible Japanese cultivars/lines were also employed to analyze the allelic patterns at the

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marker loci surrounding the *rhg-t1*. As sources for resistance, Gedenshirazu, Gedenshirazu No. 1, Toyomusume, Toyokomachi, Toyosuzu, Yukihomare, Oosodenomai, Yukishizuka, Suzuhime and To-8E were used, whereas, Tsurukogane, Hayahikari, Otofukeoosode, Tokachikuro, Chuseihikarigro, Kariyutaka, Suzumaru, Tokachinagaha, Toyohomare, Kitamusume and Tokei 758 were used as susceptible materials.

Isolation and cloning of AFLP fragments

The AFLP bands of interest were excised directly from the polyacrylamide gel and DNAs were eluted using a QIAGEN gel extraction kit, according to the manufacturer's instructions. The fragments were re-amplified under the same conditions as those in the AFLP procedure with the selective *Eco*RI and *Mse*I primers, and standard tailing was adopted to ensure that a single deoxyadenosine was added to the 3′-end of the re-amplified fragment. The fragments were ligated into a TA cloning vector (pGEM T easy vector system, Invitrogen). The plasmids were then transformed into the bacterial strain *Escherichia coli* JM109 and cultured. Plasmid DNA was extracted from the positive colonies using a spin miniprep plasmid isolation kit (QIAGEN), according to the manufacturer's instructions. To determine whether the targeted bands were cloned, amplification using the appropriate AFLP primers was performed, and the amplified products were run adjacent to the original AFLP reaction on a polyacrylamide gel. ABI Prism 3100-Avant Genetic Analyzer with the T7 and Sp6 vector primers was then used to sequence the plasmids containing the correct bands.

Converting the AFLP markers to sequence-characterized amplified regions (SCARs)

To develop a SCAR marker from each cloned fragment, one pair of 20–24-mer oligonucleotide primers was designed based on the sequences inside the selective AFLP primer pair and synthesized. Care was taken to avoid the possible formation of dimer or secondary structure in the primers. The primers were tested on the Toyomusume and Tsurukogane parents. SCAR analysis was performed in 10 µl reaction solutions containing 20 ng of genomic DNA, 2.5 mM of each deoxynucleotide (dATP, dTTP, dGTP and dCTP), 10× EX Taq buffer, 0.5 µM of each forward and reverse primer, 0.1 unit of Ex Taq polymerase (Takara). The PCR reaction included 35 cycles at 94°C for 1 min., 58°C for 45 sec. and 72°C for 1 min. and a final extension at 72°C for 10 min. The PCR products were fractionated on a 13% polyacrylamide gel in 1× Tris-Glycine buffer and photographed under UV light after staining with ethidium bromide. Primers that enabled to distinguish the resistant parent from the susceptible one were then analyzed in the recombinant inbred lines of Toyomusume and Tsurukogane.

BAC library screening

The BAC library used in the present study consisted of 53,760 clones of genomic DNA from Misuzudaizu. For the detection of a positive clone, PCR-based screening was performed as described previously (Xia *et al.* 2004).

PCR-SSCP analysis

The PCR products that did not show polymorphism in the SCAR analysis were subjected to PCR-SSCP (Polymerase chain reaction-single strand conformation polymorphism, Orita *et al.* 1989) analysis. One volume of the PCR product was mixed with an equal volume of the denaturing solution (95% (v/v) formamide, 0.05% (w/v) xylene cyanol, 0.05% bromophenol blue, and 20 mM EDTA) and heated at 94°C for 5–10 min. The denatured samples were immediately placed on ice. The samples were loaded a 8% native polyacrylamide gel in TG buffer and run at 150 V or 200 V at 4°C. After electrophoresis, the gel was stained with Vistra Green (Amersham Pharmacia Biotech, UK) and the amplified fragments were detected using Typhoon 9410 (Amersham Biosciences).

Development of PCR-based markers

Each PCR-based SCAR marker was developed by ensuring that the PCR band pattern was consistent with that in the corresponding AFLP analysis. An example of developed SCAR marker is shown in Fig. 1. Of the ten AFLP markers, eight (TGG_GCA4, CAT_CTC1, CAA_TAT, GGG_CTA2, TCA_GTT1, TAG_TAG1, GGC_ATG2 and ACT_TCA2) were successfully converted to SCAR markers designated as SCAR2, SCAR4, SCAR6, SCAR7, SCAR11, SCAR19, SCAR21 and SCAR24, respectively. All the markers, except for SCAR7 were codominant in the RIL population. These codominant markers enabled to distinguish readily and accurately homozygous resistant, homozygous susceptible and heterozygote individuals by electrophoresis. No polymorphism could be detected between the resistant and susceptible parents when the SCAR primers (SCAR 8 and SCAR 9) were derived from the AFLP markers GTT_CAG1 and GTA_TAC1. In both cases, the BAC library was screened to extend sequence information flanking the marker fragments. Primers designed from the internal sequence of the regions of the AFLP markers GTT_CAG1 and GTA_TAC1 were used in the PCR assays to isolate the BAC clones associated with these specific sequences. In the current study, a three-dimensional pooling strategy combined with HEGS (High efficiency genome scanning system, Kawasaki and Murakami 2000) was applied, which remarkably facilitated PCR-based library screening. A PCR signal was considered to be positive only if it was present in all the three-pool types. After DNA isolation and sequencing, only one clone was found to be identical with each AFLP fragment (GTT_CAG1 and GTA_TAC1). After obtaining the extension sequences of the AFLP fragments, although new primer pairs were designed, there was no polymorphism between Toyomusume and Tsurukogane. Thereafter, the PCR-SSCP technique was applied to detect polymorphisms between the parents, and the developed new markers were designated as PCR-SSCP8 and PCR-SSCP9. A BAC-end marker,

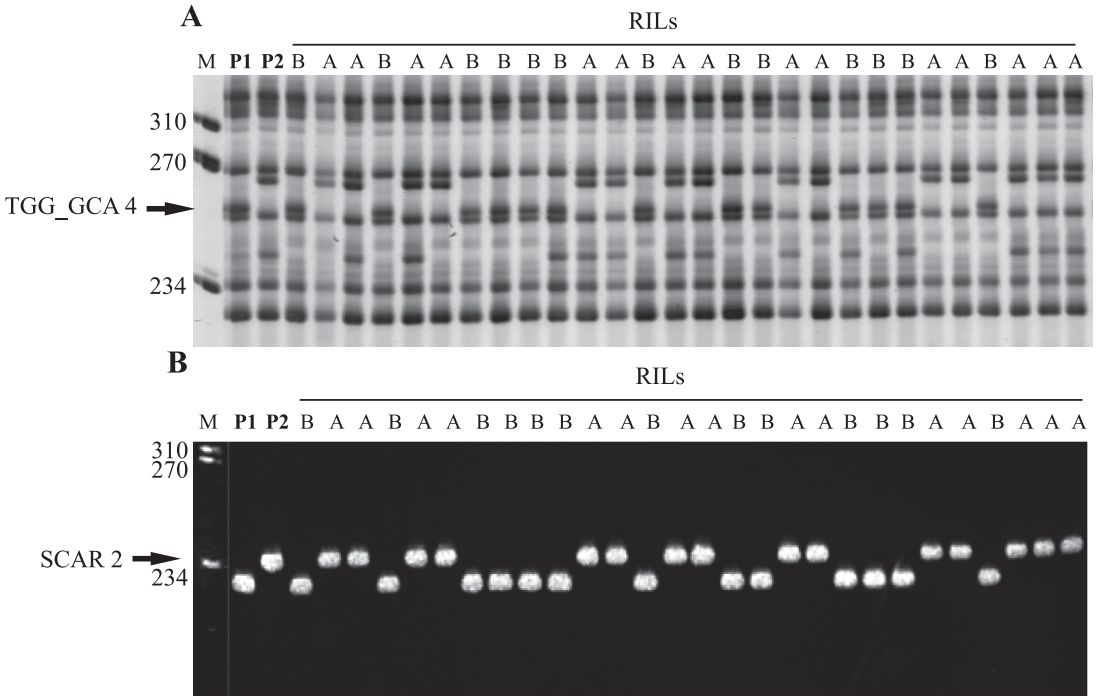

Fig. 1. Fragment patterns of a developed SCAR marker. A: Segregation of the AFLP marker TGG_GCA 4. B: Segregation of the SCAR2 marker developed from the AFLP marker TGG_GCA 4. P1: resistant parent (B), Toyomusume; P2: susceptible parent (A), Tsurukogane.

BAC136H11RV, was also developed from the AFLP fragment GTT_CAG1 using the BAC-end sequence. The developed PCR-based markers with sequence-specific primers were listed in Table 1.

Validity of the developed PCR-based markers

To confirm the validity of the developed PCR-based markers (7 SCAR markers, 2 PCR-SSCP markers and one BAC-end primer), 19 cultivars/lines of soybean were used (Table 2). Unfortunately, the genotype data of the SCAR4 marker linked to *rhg-t1* were the same in all the cultivars except for Tsurukogane. This polymorphism may be specific between Tsurukogane and other cultivars, and may not apply to other populations. All the resistant cultivars derived from Gedenshirazu No.1 displayed the same genotype at five SCAR marker loci around the *rhg-t1* locus (SCAR4, PCR-SSCP9, SCAR6, 2 and 24), indicating that the linkage block including *rhg-t1* and these markers may have been inherited in each cultivar. On the other hand, the resistant cultivars derived from PI84751 showed the same genotype as that of the cultivars derived from Gedenshirazu No.1 only for the PCR-SSCP9 and SCAR24 markers. We were unable to examine the allelic identity between the SCN resistance gene derived from Gedenshirazu and that from PI84751. The phenotype of each cultivar could not be determined by using only the genotype data of a single QTL, because of the interaction between *rhg-t1* and other loci.

Even though DNA-based MAS has been applied in plant breeding for more than a decade, still some molecular markers may not be consistent in different populations. The reasons why some of the linked markers may not be generally useful or exhibit a restricted utility are as follows: i) the linkage intensity may display wide variations across different genetic backgrounds or the developed markers are not closely linked to the target genes, ii) the marker is line-specific and does not change depending on the alleles of the target gene. Markers tagging QTLs differ in efficiency in various breeding populations, and may not be effective in some populations. For instances, all the cultivars derived from Gedenshirazu showed a resistant genotype at the SCAR6 marker locus, whereas two cultivars derived from PI84751 showed a susceptible genotype. Therefore, indirect selection of this marker could be used for the Gedenshirazu gene pool with few exceptions. However, as indicated in Table 2 and Figure 2, the PCR-SSCP9 marker appeared to be more useful for distinguishing resistant from susceptible cultivars, except for Kariyutaka, Toyohomare and Tokei758.

The practical application of the developed markers in the breeding process requires a pre-screening of all the available elite lines for polymorphism at the corresponding marker locus. In the absence of polymorphism, available information about fragment sequence can be used for fragment extension strategies to screen for line-specific polymorphism. Therefore, time-consuming and comprehensive search of new AFLP markers and their conversion into PCR-based markers would not be necessary.

The co-dominant PCR-based markers SCAR6 and PCR-SSCP9 in the present study can be used for initial

Table 1. List of developed PCR-based markers with sequence-specific primers

AFLP marker	Converted marker	Marker type	Marker behaviour	Distance from CAT_CTC1[1)]	Primer sequence (5′-3′)	Fragment size (App.)	
						Toyomusume	Tsurukogane
GTT_CAG 1	a) PCR-SSCP 8	PCR-SSCP	Codominant	−6.6 cM	Forward, CCAACTTAGATGTACAGTAGC Reverse, TTCCTGCTCATTACCCCATC	ND	ND (900 bp)[4)]
	b) BAC 136H11RV[2)]	BAC end primer	Codominant	ND[3)]	Forward, GGGGTCACAAACTACCAGCA Reverse, AAGCCCCAAAGCTGATTTTT	ND	ND (510 bp)[4)]
GGG_CTA 2	SCAR 7	SCAR	Dominant	−2.1 cM	Forward, AGACCCATAAGCTTATGTAATG Reverse, ATCGCCCTGAGTCTCAAAGC	no product	77 bp
CAT_CTC 1	SCAR 4	SCAR	Codominant	+0.0 cM	Forward, ATAACTTGATATCTTGGTCAAG Reverse, AATTTCGCAGTTATCAGATAC	207 bp	250 bp
GTA_TAC 1	PCR-SSCP 9	PCR-SSCP	Codominant	+1.5 cM	Forward, ACCTAAACTCGATGGGTATG Reverse, ATTTTGTTATTCTCTCTGTGTAC	ND	ND (430 bp)[4)]
CAA_TAT	SCAR 6	SCAR	Codominant	+3.2 cM	Forward, TCGAGGACTTCAATGTTCATGG Reverse, TAGTAAATTGTATCCTAGTACC	540 bp	570 bp
TGG_ GCA 4	SCAR 2	SCAR	Codominant	+6.9 cM	Forward, ACACCAGCAATGAGATTCAAGAG Reverse, AGATGATGGAGGTGTTTGAGC	220 bp	245 bp
ACT_TCA 2	SCAR 24	SCAR	Codominant	+23.3 cM	Forward, GTGGGACTCCGATTCAAA ATATG Reverse, GAGAGTTTACATCACGGGATTTC	220 bp	210 bp
TAG_TAG 1	SCAR 19	SCAR	Codominant	+39.4 cM	Forward, TGGCACTCTCCGGTTTACTG Reverse, ATCCCTAGCAGAGGTAAACTC	620 bp	610 bp
TCA_GTT 1	SCAR 11	SCAR	Codominant	+43.4 cM	Forward, AGGTGGAAGCTAATTCATCTAG Reverse, AAGACACTCTAATATGGCCA	700 bp	720 bp
GGC_ATG 2	SCAR 21	SCAR	Codominant	+50.5 cM	Forward, GCAGTACACTCCACCTTATTTG Reverse, GTACGTTAGGTTTCGGTCAC	510 bp	494 bp

[1)] '+' indicates the upper direction and '−' indicates the lower direction of the CAT_CTC1 marker.
[2)] BAC-end primer derived from the GTT_CAG1 marker
[3)] Not determined
[4)] Size indicated in parentheses is determined in Misuzudaizu sequences.

Table 2. Genotypes of a set of resistant / susceptible cultivars at the PCR-based marker loci

Cultivar name	Resistant/ Susceptible	Origin of resistance	Genotype data of PCR-based markers (Distance from CAT_CTC1)[1)]									
			PCR-SSCP8 (−6.6 cM)	BAC136H11 RV (ND[2)])	SCAR4 (+0.0 cM)	PCR-SSCP9 (+1.5 cM)	SCAR6 (+3.2 cM)	SCAR2 (+6.9 cM)	SCAR24 (+23.3 cM)	SCAR19 (+39.4 cM)	SCAR11 (+43.4 cM)	SCAR21 (+50.5 cM)
Gedenshirazu	Resistant		A	A	B	B	B	A	B	B	B	B
Gedenshirazu No. 1	Resistant	Gedenshirazu	B	B	B	B	B	B	B	–	B	A
Oosodenomai	Resistant	Gedenshirazu	B	B	B	B	B	B	B	A	B	A
Yukihomare	Resistant	Gedenshirazu	B	B	B	B	B	B	B	B	B	B
Toyomusume	Resistant	Gedenshirazu	B	B	B	B	B	B	B	B	B	B
Toyokomachi	Resistant	Gedenshirazu	A	A	B	B	B	B	B	B	B	B
Toyosuzu	Resistant	Gedenshirazu	B	B	B	B	B	B	B	B	B	B
Yukishizuka	Resistant	PI84751	C	B	B	B	A	A	B	A	B	A
Suzuhime	Resistant	PI84751	C	B	B	B	A	A	B	A	B	B
To-8E	Resistant	PI84751	B	B	B	B	B	B	B	B	B	B
Hayahikari	Susceptible		C	C	B	A	A	B	B	A	A	A
Otofukeoosode	Susceptible		B	B	B	A	A	B	A	A	A	A
Tokachikuro	Susceptible		B	B	B	A	A	B	A	A	B	A
Chuseihikarigro	Susceptible		B	B	B	A	A	B	A	A	B	A
Kariyutaka	Susceptible		B	B	B	B	B	B	B	A	A	A
Suzumaru	Susceptible		B	B	B	A	A	A	B	A	B	A
Tokachinagaha	Susceptible		–	A	B	A	A	A	B	A	B	B
Toyohomare	Susceptible		B	B	B	B	B	B	B	A	A	A
Kitamusume	Susceptible		C	A	B	A	A	B	B	A	A	A
Tokei 758	Susceptible		A	A	B	B	A	A	B	A	B	A
Tsurukogane	Susceptible		A	A	A	A	A	A	A	A	A	A

A, susceptibility allele; B, resistance allele; C, different allele from resistant and susceptible parents; –, no product
[1)] '+' indicates the upper direction and '−' indicates the lower direction of the CAT_CTC1 marker.
[2)] Not determined

screening of SCN resistance sources. Since the number of cultivars used in the present study was limited, further testing of a large number of cultivars should be performed to reconfirm the validity of these designed markers. Since one QTL can only partially explain SCN resistance in any breeding populations, combining two or more QTL markers in MAS is important to obtain an optimum response.

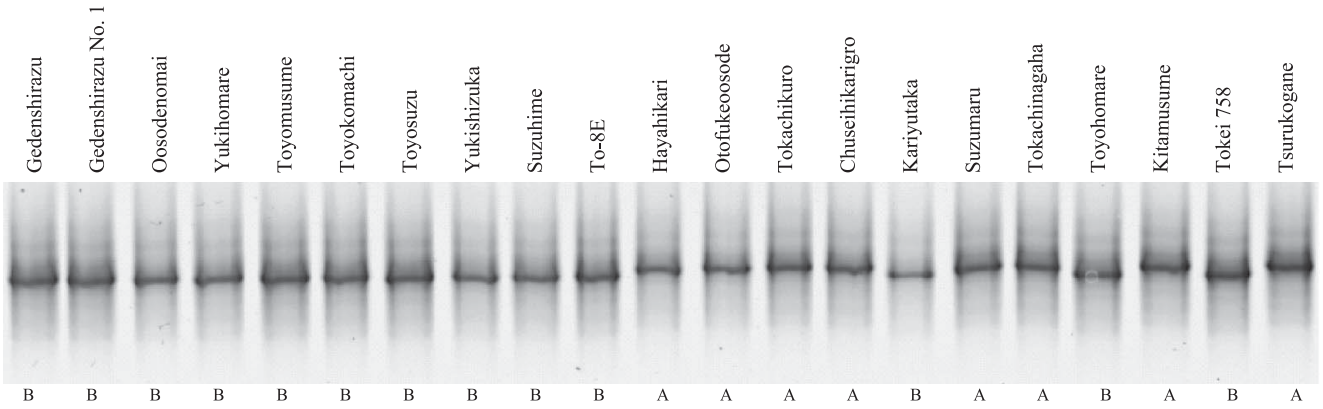

Fig. 2. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis with PCR-SSC9 marker using different cultivars and lines. Primers were designed from the AFLP marker GTA_TAC 1. A, susceptibility allele; B, resistance allele.

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