

SNP assay to detect the ‘Hyuuga’ red-brown lesion resistance gene for Asian soybean rust

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Abstract Asian soybean rust (ASR), caused by *Phakopsora pachyrhizi* Syd., has the potential to become a serious threat to soybean, *Glycine max* L. Merr., production in the USA. A novel rust resistance gene, *Rpp?*(Hyuuga), from the Japanese soybean cultivar Hyuuga has been identified and mapped to soybean chromosome 6 (*Gm06*). Our objectives were to fine-map the *Rpp?*(Hyuuga) gene and develop a high-throughput single nucleotide polymorphism (SNP) assay to detect this ASR resistance gene. The integration of recombination events from two different soybean populations and the ASR reaction data indicates that the *Rpp?*(Hyuuga) locus is located in a region of approximately 371 kb between STS70887 and STS70923 on chromosome *Gm06*. A set of 32 ancestral genotypes which is predicted to contain 95% of the alleles present in current elite North American breeding populations and the sources of the previously reported ASR resistance genes (*Rpp1*, *Rpp2*, *Rpp3*, *Rpp4*, *Rpp5*, and *rpp5*) were genotyped with five SNP markers. We developed a SimpleProbe assay based on melting curve analysis for SNP06-44058 which is

tightly linked to the *Rpp?*(Hyuuga) gene. This SNP assay can differentiate plants/lines that are homozygous/homozygous or heterozygous/heterozygous for the resistant and susceptible alleles at the *Rpp?*(Hyuuga) locus.

Introduction

Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi* Syd. is a widespread disease of soybean [*Glycine max* (L.) Merr.] and has the potential to cause serious economic losses in soybean production (Sinclair and Hartman 1996; Grau et al. 2004). The pathogen was recently reported for the first time in North America (Schneider et al. 2005). Researchers during the 1970s and 1980s identified four unique dominant ASR resistance genes. PI 200492 (‘Komata’) has a single dominant gene (*Rpp1*) that confers resistance to an Australian Asian rust isolate (McLean and Byth 1980; Bromfield and Hartwig 1980). PI 230970 (‘Ankur’) has *Rpp2* (Singh and Thapliyal 1977), and PI 462312 has the ASR resistance gene *Rpp3* (Hartwig and Bromfield 1983). The cultivar Bing Nan (PI 459025) from China possesses a dominant resistance allele (*Rpp4*) at a locus different from the other three resistance alleles (Hartwig 1986). Recently, a new dominant resistance allele (*Rpp5*) and a recessive resistance allele (*rpp5*) were reported by Garcia et al. (2008). A novel rust resistance gene, *Rpp?*(Hyuuga) from the Japanese cultivar Hyuuga, produces a red-brown (RB) or resistant lesion type (LT) with current rust isolates prevalent in the USA. The resistance gene was identified and mapped to a 3.5 cM region on soybean chromosome *Gm06* [linkage group (LG) C2] (Monteros et al. 2007). The use of native Asian soybean rust resistance genes should be an important management component for the disease, but to date no commercial

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cultivars possessing any ASR resistance genes are currently available in the USA.

Strategically, DNA markers are used to identify genetic factors for traits of interest and to introgress them more efficiently into elite cultivars than can be accomplished using traditional breeding approaches. Single base changes in sequence, called single nucleotide polymorphisms (SNPs), are the most abundant source of variation in plant genomes and include small insertions and deletions (indels) (Zhu et al. 2003). Compared to simple sequence repeats (SSRs), SNPs are more amenable to high-throughput automated genotyping assays that allow samples to be genotyped faster and more economically than with SSRs (Hurley et al. 2004; Ha and Boerma 2008). SNP detection technologies have evolved from expensive, time consuming, and labor intensive processes to some of the most highly automated, efficient, and relatively inexpensive methods of DNA marker detection (Kwok and Chen 2003).

Soybean has a relatively low sequence variation due to historical genetic bottlenecks and narrow genetic base (Hyten et al. 2006; Gizlice et al. 1993; Carter et al. 2004). A combination of soybean ancestors and first progeny that contribute at least 95% of the alleles found in public cultivars in North America from 1947 to 1988 has been identified (Gizlice et al. 1994). Fourteen genotypes estimated to have contributed 80.5% of the allelic diversity present in North American soybean cultivars were used to determine the SNP frequency in coding and non-coding soybean DNA sequence (Gizlice et al. 1994; Zhu et al. 2003). Soybean has, on average, about 1.95 SNPs per 534 bp and over 1,100 single nucleotide polymorphism (SNP) markers across 20 soybean chromosomes have been mapped (Choi et al. 2007). A SNP haplotype refers to a distinct combination of SNPs that are tightly linked in a region of a chromosome or blocks of DNA which tend to be inherited as entire units from a parent to its progeny (Shastry 2004). SNPs that differentiate one haplotype from another are potentially useful as markers linked to QTL or genes and can be used for marker-assisted selection (MAS). Individuals susceptible to a disease and having a shared haplotype can be grouped together, and the haplotype can be used to describe other individuals that will likely be susceptible to a disease. The use of MAS allows selection to be performed in early generations at early stages of plant development and in the absence of the pest or pathogen (Mohan et al. 1997; Ha et al. 2007).

There are many techniques available to genotype SNPs (Kwok and Chen 2003). Homogenous, closed-tube methods for SNP genotyping that do not require a separation step are attractive for their simplicity and containment of amplified products. Most of these methods are based on PCR and use oligonucleotide probes (Liew et al.

2004). High-resolution melting curve analysis without oligonucleotide probes has proven to be a highly sensitive method for mutation discovery and SNP genotyping (Cho et al. 2008). Nucleic acid melting is tracked by monitoring the fluorescence of the samples across a defined temperature range, generating high-resolution melting profiles that can be used to identify the presence of sequence variation within the amplicon (deSilva and Blackett 2007). Wittwer et al. (2003) used high-resolution melting curve analysis of a 110 bp PCR amplicon to genotype the single nucleotide polymorphisms hemoglobin (Hb)C(G16A) and HbS (A17T) near the middle of the amplicon. Another closed-tube method for SNP genotyping is high-resolution melting analysis using an unlabeled probe. In this case, DNA amplified in the presence of the DNA binding saturation dye emits a high level of fluorescence when double stranded but loses fluorescence as the DNA duplex melts apart enabling the detection of sequence variations in the unlabeled probe (Margraf et al. 2008; Zhou et al. 2004).

Unlike high-resolution melting curve analysis, the SimpleProbe system utilizes a single-labeled genotyping probe designed to increase in fluorescence upon target hybridization. SimpleProbes show a decrease in fluorescence as the genotyping probe is melted from its target strand. The genotypes are determined by evaluating the probe-target duplex melting temperature (T_m) of the genotyping probe as it melts from a site of variance producing a characteristic DNA duplex melting profile (Bennett et al. 2003). The T_m difference between SNP alleles is determined by the probe length and the base mismatch (Liew et al. 2007). Base-pair mismatches shift the stability of a duplex by varying amounts depending on the particular mismatch, the mismatch position, and neighboring base pairs (SantaLucia et al. 1996). The SimpleProbe assay was successfully applied in a soybean marker-assisted breeding program for selecting southern root-knot nematode resistance (Ha and Boerma 2008).

The objectives of this research were to fine map the genomic location of the *Rpp?*(Hyuuga) locus and to develop a SNP assay using the SimpleProbe system to detect the *Rpp?*(Hyuuga) ASR resistance gene for use in MAS.

Materials and methods

Plant materials

Two populations were used for fine-mapping the *Rpp?*(Hyuuga) gene in this study. The first population consists of 100 F_6 -derived recombinant inbred lines (RILs) from the cross of 'Dillon' (tan or susceptible lesion

type) \times Hyuuga (RB or resistant lesion type) (Shipe et al. 1997; GRIN 2009). The second population consists of 92 F_6 -derived RILs from the cross of ‘Benning’ (tan or susceptible lesion type) \times Hyuuga (Boerma et al. 1997). DNA was extracted from the RILs in both populations and they were genotyped with SSR markers (Satt460, Satt079, Staga001, and Satt307) on chromosome *Gm06* as described by Monteros et al. (2007). Based on the genotype of four SSR markers and five newly developed SNP markers in this study, six recombinant substitution lines (RSLs) were identified from the Dillon \times Hyuuga population (Fig. 1). Three replications of each RIL and the parents, with four plants per replication, were planted in a randomized complete block design in the greenhouse at the University of Georgia’s Griffin campus on 6, October, 2006, to evaluate their reaction to ASR. Plants were inoculated with the ASR spore suspension on 23, October, 2006, according to the procedure described in Monteros et al. (2007). The *P. pachyrhizi* spores were obtained from ASR-infected soybean plants collected at the University of Georgia’s Attapulgus Research and Education Center, located in the southwest corner of Georgia, near the Georgia–Florida border. Data on lesion type, lesion number, and visible sporulation was obtained on 9, November, 2006.

In the Benning \times Hyuuga population, one plant that was heterozygous for the genomic region between markers Satt460 and Satt307 was initially identified from among the F_7 plants within a single RIL. After self-pollination of this plant, we obtained 52 F_8 seeds and these seeds were planted in the greenhouse. DNA was extracted from individual F_8 plants using a modified CTAB procedure described by Keim et al. (1988) and both SSR and SNP markers located in the Satt460 to Satt307 genomic region were used to find recombinant events. Based on their marker genotypes, four plants out of 52 F_8 plants were placed into four recombinant classes, Class I–IV (Fig. 2),

but they were heterozygous for the genomic region. After self-pollination of the four heterozygous plants we obtained a total of 376 F_9 seeds. DNA was extracted from individual seed chips using the procedure described by Edwards et al. (1991). After genotyping with SNP and SSR markers from F_9 seeds, we identified homozygous seeds: 12 seeds in Class I, 20 in Class II, 20 in Class III, and 2 in Class IV. To evaluate their reaction to ASR, six seeds each from Class I, II, and III, the two seeds from Class IV and the parents, Hyuuga and Benning, were planted in the greenhouse at the University of Georgia’s Griffin campus on 10, July, 2009. Plants were inoculated twice with the ASR spore suspension on 6, August, 2009 and 10, August, 2009. Data on lesion type, lesion number, and visible sporulation were obtained on 24, August, 2009.

Twenty-five seeds of the 32 soybean ancestors and first progeny that contribute at least 95% of the alleles found in public cultivars in North America from 1947 to 1988 (Gizlice et al. 1994) and previously reported sources of ASR resistance genes, PI 200492 (*Rpp1*); PI 230970 (*Rpp2*); PI 462312 (*Rpp3*); PI 459025B (*Rpp4*); PI 200526 (*Rpp5*); PI 200487 (*Rpp5*); PI 471904 (*Rpp5*); PI 200456 (*rpp5*); and PI 506764 (*Rpp?*(Hyuuga)), obtained from the USDA Soybean Germplasm Collection maintained at the University of Illinois (Urbana, IL, USA) were planted in the greenhouse. Trifoliolate leaves from all plants were harvested and bulked, and processed for DNA extraction as previously described by Monteros et al. (2007).

SNP identification

An amplicon resequencing approach was used to identify SNPs associated with *Rpp?*(Hyuuga). Chromosome *Gm06* from 43 to 45 Mbp templates were obtained from the soybean genome sequence database (<http://www.phytozome.net/soybean>), and a total of 50 primers were designed from

Fig. 1 Graphical genotypes of representative RILs from Dillon \times Hyuuga in the *Rpp?*(Hyuuga) region on chromosome *Gm06*. *LT* indicates the type of lesion, *Tan* is the susceptible reaction and *RB* is the resistant reaction

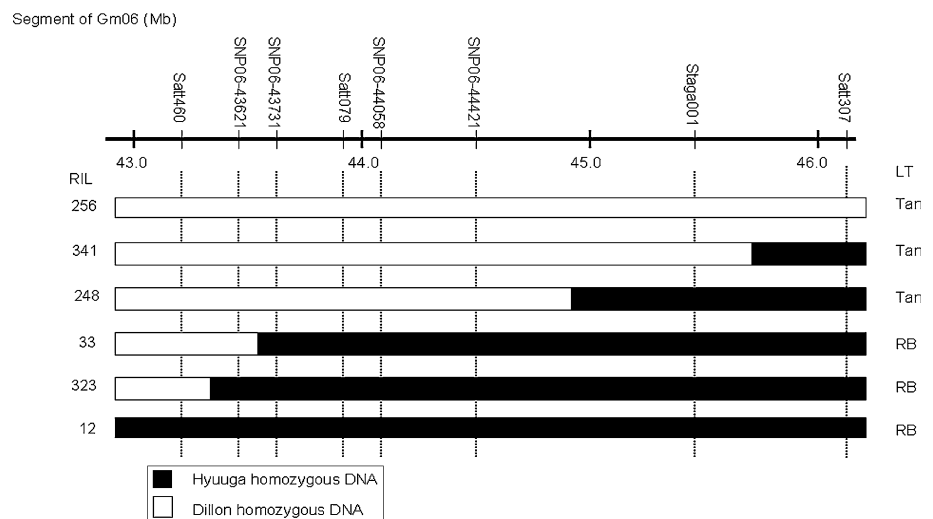
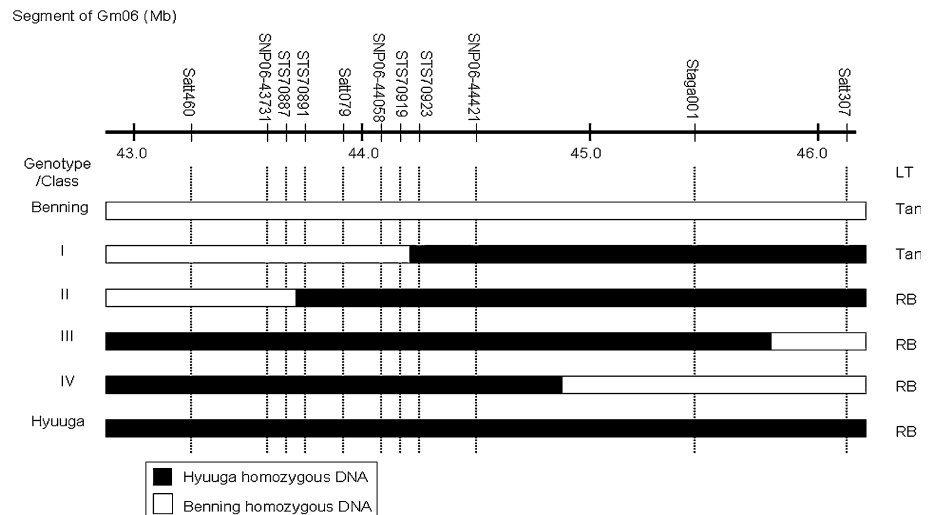


Fig. 2 Graphical genotypes of representative RSLs from Benning \times Hyuuga in the *Rpp?*(Hyuuga) region on chromosome *Gm06*. *LT* indicates the type of lesion, *Tan* is the susceptible reaction and *RB* is the resistant reaction. STS70887, STS70891, STS70919, and STS70923 previously identified by Hyten et al. (2009) were located at nucleotide 43746435, 43786392, 44086038, and 44118117 on *Gm06*, respectively



this genomic region with the LightCycler Probe Design software (Roche Applied Science, Indianapolis, IN, USA).

All PCR primers were used to amplify genomic DNA of Dillon and Hyuuga. The PCR products were analyzed on a 1.5% agarose gel stained with ethidium bromide. A total of 29 out of 50 primer pairs produced a single amplicon from genomic DNA. These PCR products were directly sequenced using one of the PCR primers with a BigDye Terminator v3.1 Cycle Sequencing Kit (PE-ABI, Foster City, CA, USA). These amplicons were analyzed on an ABI 3730 automated sequencer (PE-ABI, Foster City, CA, USA). The sequence data were analyzed with SequencherTM 4.2 (GeneCodes Corp., Ann Arbor, MI, USA). Only 12 out of 29 amplicons (41%) contained SNPs or InDels between Dillon and Hyuuga (Table 1). An additional 23 STS markers located between Satt460 and Sat_263 from the study of Hyten et al. (2009) were also evaluated by sequencing as previously described.

SNP genotyping using high-resolution melting analysis with unlabeled probe

The SNP markers identified above were used to genotype the Dillon \times Hyuuga RILs, the 32 ancestral genotypes, Dillon, and the nine plant introductions with previously reported ASR resistance genes (Table 4). High-resolution melting analysis with unlabeled oligonucleotide probes targeting SNP sites and a saturation DNA dye was applied in this study. Five SNPs were successfully genotyped using unlabeled probes (Table 2).

All PCR reactions were performed in 384-well plates with a total volume of 3 μ L per well and conducted as asymmetry. The PCR reaction mixture consisted of 20–30 ng of genomic DNA, 3 mM MgCl₂, 1 μ M of excess primer, 0.2 μ M of limiting primer, 0.5 \times of LightCycler

480 Genotyping Master mix (Roche Diagnostics, Indianapolis, IN, USA), and 0.6 \times LCGreen Plus (Idaho Technology, Salt Lake City, UT, USA). PCR was performed in the LightCycler[®] 480 (Roche Applied Science, Indianapolis, IN, USA) for 50 cycles with 10 s denaturation at 95°C, 15 s of annealing at 58°C, and 20 s extension at 72°C. After amplification, 1.0 μ M of unlabeled oligonucleotide probe was added and a final melting cycle was performed by raising the temperature to 95°C for 2 min, lowering the temperature to 40°C for 5 min, and increasing the temperature to 90°C with continuous fluorescent acquisition followed by a cool down to 40°C.

The fluorescence signal (*F*) was plotted in real time against temperature (*T*) to produce melting curves for each sample. Melting curves were then converted to negative derivative curves of fluorescence with respect to temperature ($-dF/dT$) by the LightCycler[®] Data Analysis software (Roche Diagnostics, Indianapolis, IN, USA). The same software was used to group similar melting curves and automatically call genotypes based on melting standards for known genotypes in the experiment or software-defined melting standards.

Design of a simple probe to detect *Rpp?*(Hyuuga)

A SNP site from SNP06_44058 was detected at position 44058927 on chromosome *Gm06*, in which the ASR-susceptible lines had the “G” allele and the ASR-resistant lines had the “T” allele. For melting curve analysis, a 180 bp fragment was amplified using the forward 5'-GGT TTT CAG ACA AGA TCA AAT CG-3' and reverse primers 5'-CCA TTC ATA GTG AAC TCA AC-3'. The SimpleProbe 5'-CTG AAG GGT **XI** CAA AGT **GCC** AGA TC-3' was internally labeled with fluorescein and blocked with phosphate at the 3' end (TIB MOLBIOL, Adelphia,

Table 1 Primer combinations for detection of single nucleotide polymorphism (SNP) using the Williams 82 soybean genomic sequences (<http://www.phytozome.net/soybean>) on chromosome Gm06 in the region of the *Rpp2*(Hyyuga) gene

| STS ID | SNPs/Indel ^a | Upper position on Gm06 | Lower position on Gm06 | Forward primer | Reverse primer | PCR length |
|-------------|-------------------------|------------------------|------------------------|-----------------------------------|-------------------------------------|------------|
| Satt460 | | 43291288 | 43291438 | | | |
| STS06_43617 | 0/1 | 43617983 | 43618457 | ACC AAG AAC TTT AAC AAG ATT TCG | TGG GAT AGG CAA TAA GAT AGA CCA TA | 475 |
| STS06_43621 | 1/0 | 43621002 | 43621457 | TTA TGG TTA ACT TGG TGG GAG | CAG ATC TTC GAC CCG GA | 456 |
| STS06_43731 | 10/1 | 43731429 | 43731946 | GTA GAA CTT ATG GGC CGT | CAT TGC CAC ATC ATA TAA TCG C | 518 |
| STS06_43831 | 3/1 | 43831609 | 43832161 | GTC GGT AGT AAA GAA AGA TG | TGA AAT ATG TTA TTG GGT AGA C | 553 |
| STS06_43930 | 2/2 | 43930463 | 43931013 | TTT AAA CAG TTT GTA TCA AGT CA | TCC TCA ATA CAG CTC TGG | 551 |
| Satt079 | | 43950925 | 43951071 | | | |
| STS06_43958 | 18/1 | 43958861 | 43959374 | GCA TGT ACG TTC TTA TTT GTG G | CTC ATT AGT AAG TAA TCA TGG ATT AGC | 514 |
| STS06_43983 | 3/1 | 43983099 | 43983608 | TTC TGC CAT TTC TTC GG | TGT ACT TAC TGT CCT TTC AC | 510 |
| STS06_44058 | 1/0 | 44058853 | 44059359 | GAC AAG ATC AAA TCG TTG GAG | CAG TTT ATT TAC CTG CTG GTT | 507 |
| Sat_263 | | 44188149 | 44188270 | | | |
| STS06_44257 | 26/0 | 44257478 | 44257987 | TCT CAT CTA GGG TGT TGG C | ATT AGC ATC AGA GTT GAA CGA | 510 |
| STS06_44421 | 5/1 | 44421514 | 44422064 | CAT GTA TAG AAG TTG GTT GAG T | GTG CAA CAA TAC CCG GAG | 551 |
| STS06_44422 | 7/0 | 44422369 | 44422877 | TGC CCA TCA ACA GCA TA | TGC ATG ACA CAA AGG TA | 509 |
| STS06_44947 | 1/0 | 44947224 | 44947726 | ATA AGC CTG TTC ATT TAA TTT CTC C | GTC ACT AAC CAA ATC CAG GTC | 503 |
| Staga001 | | 45427178 | 45427426 | | | |
| Satt307 | | 46286824 | 46286980 | | | |

^a Indicates the number of SNPs/indels identified between Dillon versus Hyyuga

Table 2 List of primers and concentrations used in high-resolution melting analysis with unlabeled probes

| Primer name | | Primer sequence (5'-3') | PCR (μ M) | SNP Position on Gm06 (Dillon/Hyuuga) |
|-------------|-------|--|----------------|--------------------------------------|
| SNP06-43621 | F | CTG GGC TGA AGA AAC CAA C | 0.2 | |
| | R | GCC ATT CTT CTT ACC ATG CT | 1.0 | |
| | Probe | TGA GAG TGG TAC TAT CAC AGG ACA <u>AAA</u> ATA | 1.0 | 43621233 (T/A) |
| SNP06-43731 | F | GTA GAA CTT ATG GGC CGT | 0.2 | |
| | R | ATC TTC TCC TTG AAG TGT CAT C | 1.0 | |
| | Probe | ATC AAT <u>GGC</u> AGC GGA ATG GAG | 1.0 | 43731481 (T/C) |
| SNP06-43983 | F | TTC TGC CAT TTC TTC GG | 0.2 | |
| | R | GAC TGC CTA GAT TTC AAC CT | 1.0 | |
| | Probe | TGC AGC CAG TTC <u>ATC</u> AAT CCT CTC | 1.0 | 43983266 (C/T) |
| SNP06-44058 | F | GAC GAT TCA AAT CTC TTC C | 1.0 | |
| | R | CCA TTC ATA GTG AAC TCA AC | 0.2 | |
| | Probe | ATC TGG <u>CCC</u> TTT GAC CCT | 1.0 | 44058927 (G/T) |
| SNP06-44421 | F | CCA CTT AAT GAG GTA ACG GT | 0.2 | |
| | R | TGA CTT CAG TAA TTC AAA TGT TGG G | 1.0 | |
| | Probe | GGC ACA ACC <u>ACA</u> TCC ATC TCA C | 1.0 | 44421992 (C/T) |

NJ, USA). The probe was designed to match the resistant allele.

PCR reactions were performed in 384-well plates with a total volume of 3 μ L per well in a LightCycler 480 (Roche Applied Science, Indianapolis, IN, USA), following the standard protocol for asymmetrical PCR. The PCR reaction mixture consisted of 20–30 ng of genomic DNA, 0.5 μ M of forward primer, 1.0 μ M of reverse primer, 0.2 μ M of SimpleProbe, 3.0 mM MgCl₂, and 0.5 \times of LightCycler 480 Genotyping Master mix.

After an initial denaturation of 5 min at 95°C, 50 PCR cycles were performed with 10 s of denaturation at 95°C, 15 s of annealing at 55°C, and 20 s extension at 72°C. A final melting cycle was performed by raising the temperature to 95°C for 2 min, lowering the temperature to 40°C for 2 min, and increasing the temperature to 90°C, at this point using continuous fluorescent acquisition, and followed by a final cool down to 40°C. The data from the Light Cycler[®] 480 was analyzed as described earlier.

Results

The Dillon \times Hyuuga RILs and the RSLs developed from Benning \times Hyuuga were genotyped with SSR and SNP markers to fine-map the *Rpp?*(Hyuuga) gene. The *Rpp?*(Hyuuga) locus was previously mapped on chromosome *Gm06* (LG-C2) in the 3.5 cM interval between the SSR markers Satt460 and Satt307 (Monteros et al. 2007). Two additional SSR markers, Satt079 and Staga001, were mapped between Satt460 and Satt307. Resequencing of the amplicons containing the SNP site was used based on the

availability of the soybean whole genome shotgun assembly (<http://www.phytozome.net/soybean>). Sequence information from the four SSR markers was used in BLAST searches against the soybean genomic sequence database. The SSR markers Satt460, Satt079, Staga001, and Satt307 were physically placed at approximately 43291288, 43950925, 45427178, and 46286824 on chromosome *Gm06*, respectively (Table 1). Of the 50 PCR primers designed to amplify 2,135 bp of sequence located in the Satt460-Staga001 interval from Dillon and Hyuuga, only 29 of those primers produced a single band on agarose gels which was then used for sequencing. Twelve out of 29 PCR fragments contained sequence variation between Dillon and Hyuuga and produced a sequence tagged site (STS) for fine-mapping of *Rpp?*(Hyuuga). A total of 77 SNPs and 8 InDels were identified in these 12 STS. We used high-resolution melting analysis with unlabeled probe to genotype the STS. One SNP for each of the 12 STS was selected and used to design single oligonucleotide probes to anneal with the DNA from either Dillon or Hyuuga. Five probes showed clearly different melting curves between Dillon and Hyuuga (Table 2).

These five SNP markers and four SSR markers covering 3 Mbp on chromosome *Gm06* were used to screen RILs from Dillon \times Hyuuga. A total of six RILs, four of which contain different recombinant events in the target region, were identified and inoculated with ASR (Fig. 1). The RIL 256, 341, and 248 were susceptible to ASR, while RILs 33, 323, and 12 were resistant to ASR based on lesion type. RIL 248 is homozygous for the Dillon genome in the interval between Satt460 and SNP06-44421 and has a tan or susceptible ARS phenotype. RIL 33 is homozygous for

the Dillon allele at SNP06-43621 but homozygous for Hyuuga allele at the SNP06-43731, and has a RB or resistant phenotype. The integration of recombination events and the ASR reaction data indicate that the *Rpp?*(Hyuuga) locus is located between SNP06-43621 and Staga001 on chromosome *Gm06*.

Additional recombinant events between SNP06-43621 and Staga001 were identified in the Benning × Hyuuga RSLs. After identification of the four initial recombinant classes, more STS markers identified by Hyten et al. (2009) were used to further refine the genomic location of recombination events. Plants in Class I contain a recombinant event between STS70919 and STS70923 and have a tan or susceptible ARS phenotype, while Class II plants contain a recombinant event between STS70887 and STS70891 and have a RB or resistant phenotype. These data suggested that *Rpp?*(Hyuuga) is located in the region of approximately 371 kb between STS70887 at nucleotide 43746435 and STS70923 at nucleotide 44118117. Seven genes showing a similarity with NBS-LRR disease resistance proteins were annotated in this 371 kb sequence from the Williams 82 genomic sequence (Soybean Genome Project, DoE Joint Genome Institute) (Table 3).

The SNP genotypes for the 32 ancestral lines, Dillon, Hyuuga, and the other eight previously reported sources of ASR resistance genes at five SNP markers are shown in Table 4. All 32 ancestral soybean accessions and Dillon possess a G allele at nucleotide 44058927 (SNP06-44058) on chromosome *Gm06*, while Hyuuga contains a T allele. ‘Arksoy’, ‘Ralsoy’, and ‘Mejiro’ showed the same SNP genotypes as Hyuuga at the other four markers, SNP06-43621, SNP06-43731, SNP06-43983, and SNP06-44421. Hyuuga, PI 462312 (*Rpp3*), PI 200487 (*Rpp5*), and PI 471904 (*Rpp5*) showed the same SNP genotypes for all five SNPs. The SNP marker SNP06-44058 can be used to distinguish the *Rpp?*(Hyuuga) allele from the *rpp?*(Hyuuga) allele in these 32 ancestral genotypes and the previously reported sources of ASR resistance other than PI 462312, PI 200487, and PI 471904. We developed melting curve analysis with SimpleProbe chemistry for use in MAS for

Rpp?(Hyuuga). The plants containing the Hyuuga allele yielded melting peaks at 60°C, and the plants containing the Dillon allele yielded melting peaks at 54°C (data not shown). The probe annealed with the Hyuuga sequence and resulted in a 6°C higher T_m of the probe with the target sequence compared with the T_m of the probe with the mismatch in the Dillon sequence. The heterozygous samples generated both peaks. This assay was further used to genotype the two parents and 100 RILs from the cross of Dillon × Hyuuga. Linkage analysis from the 100 RILs from the cross of Dillon × Hyuuga mapped the SNP marker to the interval between Satt079 and Staga001 (data not shown). The location of the SNP marker from the genetic mapping analysis is in agreement with its physical location on chromosome *Gm06*.

Discussion

The susceptibility of currently available elite soybean cultivars to ASR and the presence of ASR in most soybean-growing regions in the world make the development of ASR-resistant soybean cultivars an important breeding objective. The detection of additional SNP and SSR markers in the 3.5 cM interval on chromosome *Gm06* where *Rpp?*(Hyuuga) was previously mapped allowed us to increase the resolution in mapping this resistance gene. A similar approach of combining the genotypes of RSLs with their phenotypic response to defoliating insects was used by Zhu et al. (2006) to fine map a major insect resistance QTL on chromosome *Gm07* (LG-M). A higher density of markers in this region of chromosome *Gm06* reduces the likelihood of recombination between markers and the target loci during the breeding process and should increase the feasibility of fine mapping and map-based cloning of this ASR resistance gene.

In this study, an increase in the marker density allowed us to identify RSLs in the region of the *Rpp?*(Hyuuga) locus. The integration of the SNP genotypes from selected RSLs and the ASR reaction data allowed us to narrow

Table 3 Candidate resistance genes showing a similarity with NBS-LRR family in the *Rpp?*(Hyuuga) region on soybean chromosome *Gm06*

| Name | Genomic size (bp) | Amino acids (aa) | Genomic span | Putative conserved domain ^a |
|---------------|-------------------|------------------|-----------------------------------|--|
| Glyma06g40680 | 2,421 | 328 | Gm06:43840929..43843349 (–strand) | None |
| Glyma06g40690 | 4,348 | 1124 | Gm06:43857506..43861853 (–strand) | TIR-NBS-LRR |
| Glyma06g40710 | 4,847 | 1100 | Gm06:43881927..43886773 (–strand) | TIR-NBS-LRR |
| Glyma06g40740 | 4,520 | 1203 | Gm06:43913641..43918160 (–strand) | TIR-NSB-LRR |
| Glyma06g40780 | 4,346 | 1066 | Gm06:43967782..43972127 (–strand) | TIR-NBS-LRR |
| Glyma06g40820 | 3,446 | 672 | Gm06:44001679..44005124 (+strand) | TIR-NBS |
| Glyma06g40830 | 10,289 | 572 | Gm06:44015320..44025608 (+strand) | LRR |

^a Putative conserved domain in a protein sequence was identified by searching NCBI’s conserved domain database

Table 4 The SNP haplotypes of ancestral soybean germplasm and the original sources of other known ASR resistance genes near the *Rpp?*(Hyyuga) gene on chromosome *Gm06*

| PI number | Cultivar name | SNP06-43621 | SNP06-43731 | SNP06-43983 | SNP06-44058 | SNP06-44421 |
|------------|----------------------|-------------|-------------|-------------|-------------|-------------|
| PI 548298 | A.K. (Harrow) | A | T | C | G | T |
| PI 548438 | Arksoy | A | C | T | G | T |
| PI 548302 | Bansei | T | T | C | G | C |
| PI 548311 | Capital | T | T | C | G | C |
| PI 548445 | CNS | T | T | C | G | C |
| PI 548318 | Dunfield | T | T | C | G | C |
| PI 548325 | Flambeau | T | T | C | G | C |
| PI 548456 | Haberlandt | T | T | T | G | T |
| PI 548348 | Illini | A | T | C | G | T |
| PI 548461 | Improved Pelican | T | C | T | G | C |
| PI 548657 | Jackson | T | T | C | G | C |
| PI 548352 | Jogun | T | T | C | G | C |
| PI 548356 | Kanro | T | T | C | G | C |
| PI 548360 | Korean | A | T | C | G | C |
| PI 548362 | Lincoln | T | T | C | G | C |
| PI 548379 | Mandarin(Ottawa) | T | T | C | G | C |
| PI 548382 | Manitoba Brown | T | T | C | G | C |
| PI 548391 | Mukden | T | C | C | G | C |
| PI 548477 | Ogden | T | T | C | G | C |
| PI 548402 | Peking | A | T | C | G | T |
| PI 548603 | Perry | T | T | C | G | C |
| PI 548484 | Ralsoy | A | C | T | G | T |
| PI 548406 | Richland | T | T | C | G | C |
| PI 548485 | Roanoke | T | T | C | G | C |
| PI 548488 | S-100 | A | T | C | G | T |
| FC 33243 | Anderson | T | T | C | G | C |
| PI 80837 | Mejiro | A | C | T | G | T |
| PI 180501 | Strain No. 18 | T | T | C | G | C |
| PI 438471 | Fiskeby III | T | T | C | G | T |
| PI 438477 | Fiskeby 840-7-3 | T | T | C | G | T |
| FC 31745 | | T | T | C | G | T |
| PI 240664 | Bilomi No. 3 | T | T | C | G | C |
| PI 592756 | Dillon | T | T | C | G | C |
| PI 506764 | <i>Rpp?</i> (Hyyuga) | A | C | T | T | T |
| PI 200492 | (<i>Rpp1</i>) | T | T | C | G | T |
| PI 230970 | (<i>Rpp2</i>) | T | T | C | G | T |
| PI 462312 | (<i>Rpp3</i>) | A | C | T | T | T |
| PI 459025A | (<i>Rpp4</i>) | T | T | C | G | C |
| PI 200487 | (<i>Rpp5</i>) | A | C | T | T | T |
| PI 200526 | (<i>Rpp5</i>) | T | T | C | G | C |
| PI 471904 | (<i>Rpp5</i>) | A | C | T | T | T |
| PI 200456 | (<i>rpp5</i>) | T | T | C | G | T |

Bold indicates the same SNP genotype as Hyyuga

down the location of the *Rpp?*(Hyyuga) locus to a 371 kb segment on chromosome *Gm06*. Based on the gene annotation in the Soybean Genome Project, seven candidate

genes containing TIR-NBS-LRR groups were identified in the sequence between STS70887 and STS70923 on chromosome *Gm06* (Table 3). These results should increase the

feasibility of cloning the *Rpp?*(Hyuuga) gene following a similar approach used recently by Meyer et al. (2009) to identify the *Rpp4* candidate gene from the resistant genotype PI459025B. They found that this candidate gene is a member of the CC-NBS-LRR family of disease resistance genes. This report suggests that other ASR genes may also belong to the NBS-LRR family of disease resistance genes.

Five SNP markers produce a distinct haplotype which can be used to distinguish Hyuuga from other reported sources of ARS resistance genes including *Rpp1*, *Rpp2*, *Rpp4*, *Rpp5* from PI 200526, and *rpp5*, as well as from the soybean ancestors evaluated which contribute a high percent of the genetic variation to elite soybean breeding populations in the USA (Table 4). Both the *Rpp1* and the *Rpp4* loci have been mapped to chromosome *Gm18* (LG-G), and the *Rpp2* locus has been mapped to chromosome *Gm16* (LG-J) using SSR markers (Hyten et al. 2007; Silva et al. 2008) indicating that *Rpp?*(Hyuuga) is different from *Rpp1*, *Rpp2*, and *Rpp4*. In our study the sources of *Rpp3* (PI 462312) and *Rpp5* (PI 200487 and PI 471904) had the same genotype at the five SNP markers as Hyuuga. Hyten et al. (2009) used a $F_{2:3}$ population from the cross of Williams 82 \times PI 462312 to map the *Rpp3* gene to a similar region on chromosome *Gm06* as *Rpp?*(Hyuuga). They also found that Hyuuga (PI 506764) and PI 463312 had the same SNP genotypes at 273 of 275 SNPs in this region. However, when PI 462312 and Hyuuga were simultaneously evaluated using Brazilian isolates of ASR, PI 462312 showed a susceptible tan lesion type while Hyuuga produced the resistant RB lesion type (Garcia et al. 2008). Also, *Rpp3* was defeated by the *P. pachyrhizi* MT isolate just 2 years after ASR was first detected in Brazil (Pierozzi et al. 2008). Current efforts include screening Hyuuga and PI 462312 with multiple ASR isolates in Bio-Safety Level 3 containment facilities at Ft. Detrick to identify an isolate of *P. pachyrhizi* that differentiates *Rpp?*(Hyuuga) from *Rpp3* (Reid Fredrick, Fort Detrick MD, personal communication).

Although *Rpp?*(Hyuuga) was identified in a Dillon \times Hyuuga population, SSR markers near this ASR resistance loci correctly predicted the ASR-resistant reaction in progeny from a Benning \times Hyuuga population (Monteros et al. 2007) suggesting that this resistance gene loci can be effective in different genetic backgrounds. The development of a high-throughput SNP-based marker enables breeders in a wide range of soybean breeding programs to use MAS to select for the *Rpp?*(Hyuuga) ASR resistance gene. The application of the melting curve assay for high-throughput and rapid SNP genotyping in soybean was described by Ha and Boerma (2008). One limitation of using linked markers in MAS is that often a given marker is not polymorphic across all populations (Xu and Crouch 2008). Having a rapid SNP assay that differentiates the *Rpp?*(Hyuuga) resistance allele from the *rpp?*(Hyuuga)

allele in most elite US breeding populations greatly enhances its utility and efficiency for MAS.

Our newly developed SimpleProbe assay can detect alternate alleles at the *Rpp?*(Hyuuga) locus and will allow breeders to effectively select ASR-resistant progeny in early segregating generations and facilitate breeding efforts to pyramid *Rpp?*(Hyuuga) with other ASR resistance genes. These strategies should accelerate the development of ARS-resistant soybean cultivars adapted to the various production regions of the USA.

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