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K. Lewers · R. Heinz · H. Beard · L. Marek
B. Matthews

A physical map of a gene-dense region in soybean linkage group A2 near the black seed coat and *Rhg*$_4$ loci

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Abstract Soybean (*Glycine max* L. Merrill) linkage group A2 contains a major resistance gene to the soybean cyst nematode (*Heterodera glycines* Ichinohe) at the *Rhg*$_4$ locus near a gene encoding aspartokinase homoserine dehydrogenase (AK-HSDH) and also near the *I* locus affecting seed coat color. To identify clones related to this region of the genome, we used a PCR assay using primers designed from a gene encoding AK-HSDH to screen approximately 40,000 clones from a bacterial artificial chromosome (BAC) library constructed from genomic DNA of the susceptible cv. Williams 82. The identified BACs were screened with a second PCR assay using primers designed from DNA sequence associated with the *I* locus to confirm the location of the BACs. Only BAC Gm_ISb001_056_G02 (56G2) was positive for both assays. BAC 56G2 contains several genes previously associated with stress or defense response including genes with high sequence similarity to those encoding chalcone synthase, glucosyl-transferase, a heat-shock transcription factor, a membrane-associated salt-inducible protein, adenosyl homocysteinase, a protein kinase, and a G10-like protein. The map contributes to our understanding of the organization of the soybean genome and to the completion of a physical map of the soybean genome. In addition, the genes identified provide landmarks to identify BAC clones near the *Rhg*$_4$ locus in resistant soybean genomic libraries and provide a foundation for comparison of soybean cyst nematode resistant and -susceptible DNA sequences in this region.

Keywords Bacterial artificial chromosome · *Heterodera glycines* · Resistance gene · Soybean · Soybean cyst nematode

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K. Lewers · H. Beard · B. Matthews (✉)
USDA ARS PSI, Soybean Genomics and Improvement Laboratory, Bldg 006 BARC-West, Beltsville, MD 20705, USA
e-mail: matthewb@ba.ars.usda.gov
Tel.: +1-301-504-5730, Fax +1-301-504-5728

R. Heinz
Instituto de Biotecnologia, INTA, CICVyA, Castelar, Buenos Aires, Argentina

L. Marek
Department of Agronomy, Iowa State University, Ames, IA 50011, USA

K. Lewers and R. Heinz are equal co-senior authors

Introduction

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the major pest of soybean (*Glycine max* L. Merrill) and is responsible for the loss of over one billion dollars worth of soybean in the USA each year (Kim et al. 1997). There are multiple genotypes of SCN (Riggs and Schmitt 1988) and several known soybean genes confer resistance to SCN (Riggs and Schmitt 1991). The resistance genes on linkage groups G and A2 of the soybean genetic map are especially important (Webb et al. 1995; Concibido et al. 1996).

On linkage group A2 there is tight linkage of 0.35 cM between resistance against SCN at the *Rhg*$_4$ locus and black seed coat at the *I* locus (Matson and Williams 1965). Several genes encoding chalcone synthase (CHS) isoforms reside near the *I* locus and a deletion in this region causes the production of black pigment in the seed coat (Todd and Vodkin 1996). Restriction fragment length polymorphism (RFLP) molecular marker pBLT65 also maps close to the *I* locus and the *Rhg*$_4$ locus (Weisemann et al. 1992; Webb et al. 1995; Heer et al. 1998). Marker pBLT65 (GenBank accession number AF049706) is a partial cDNA clone from a gene encoding the bifunctional enzyme aspartokinase-homoserine dehydrogenase (AK-HSDH) in soybean cv. Century (Gebhardt et al. 1999). A polymerase chain reaction (PCR) assay for genes encoding AK-HSDH near the *I* and *Rhg*$_4$ loci was described previously (Matthews et al. 1998). This PCR assay detects DNA polymorphisms that can be used to identify genotypes likely to be SCN-resistant (Heer et al. 1998; Matthews et al. 1998).

In this paper, we describe the identification of a bacterial artificial chromosome clone, BAC Gm_ISb001_056_G02 (56G2), that includes the region between genes encoding AK-HSDH and CHS isoforms CHS3 and CHS4 on linkage group A2, and we describe numerous genes that are present on this BAC.

Materials and methods

BAC library screening and restriction fragment profile alignment

BACs that contained inserts near the *Rhg*$_4$ locus were identified by screening a *Glycine max* cv. Williams 82 genomic DNA library of approximately 40000 BACs (Marek and Shoemaker 1997). DNA of BACs archived in microtiter plates served as template for PCR assays. PCR-based screening with primers derived from a portion (GenBank accession number AF049708) of a gene encoding AK-HSDH from soybean cv. Century (primer 548: 5′-GCAGATATCAACAGTTGGGAC-3′ and primer 563: 5′-GGAATGGACAGCTCGTAAAGCC-3′) (Matthews et al. 1998) was carried out as described in Marek and Shoemaker (1997). Nine cv. Williams 82 BACs identified by PCR with primers 548 and 563 were digested to completion with restriction enzymes *Not*I and *Eco*RI and separated on agarose gels to generate restriction fragment profiles.

These BACs were also assayed with primers specific to the *I* locus. Analysis of a series of *I* locus mutants revealed deletions of the promoter of a CHS4 gene (Todd and Vodkin 1996). The CHS4 gene exons and intron are almost identical to those of the CHS5 gene (Akada and Dube 1995). Therefore, a pair of primers for PCR were synthesized specific to the intergenic region between the CHS3 and CHS4 genes just 3′ of the CHS4 gene/*I* locus (849: 5′-ATGTTCCTTATCACCGACCTG-3′ and 850: 5′-GTTGTGCCGATTAACTTAGTCCAG-3′). PCR reactions were as described previously (Matthews et al. 1998) except that the $MgCl_2$ concentration was 3.6 m*M*. The reaction products were separated on 1 g/100 ml agarose/TBE gels.

BAC 56G2 restriction mapping

Restriction fragment organization for BAC 56G2 was determined according to a modified version of Rackwitz et al. (1995). The insert was separated from the vector with *Not*I, gel-purified, and digested with *Apa*I for varying lengths of time (4, 8, 16, and 120 min) at 37°C to produce a series of partial to full digestion products. The DNA fragments were separated by pulse field gel electrophoresis in gels composed of 1 g/100 ml fast lane (FMC BioProducts, Rockland, Me.) agarose in TAE buffer by electrophoresis using a CHEF MAPPER (BioRad Laboratories, Hercules, Calif.) system, and Southern blots were made.

To determine which *Apa*I fragments were terminal, we probed the Southern blots of partial to complete *Apa*I digestions with two oligonucleotides, probe T7 and probe 950, derived from the sequence of the pBeloBAC11 vector. Each terminal probe was designed to detect the vector sequence on each side of the insert and was derived from the sequence of vector between the *Hind*III cloning site and *Not*I sites flanking the *Hind*III cloning site. Probe T7 (AP- GTA ATA CGA CTC ACT ATA GGG C) (Synthetic Genetics, San Diego, Calif.) has alkaline phosphatase (AP) conjugated to the 5′ end. Probe 950 (CAG CTA TGA CCA TGA TTA CGC CAA GC) is an oligonucleotide derived from position 416–441 in the pBeloBAC11.

To determine which *Apa*I fragments contain a gene encoding AK-HSDH and the region between the genes encoding CHS3 and CHS4 (*I* locus), Southern blots of complete digestions with the enzymes *Not*I, *Apa*I, *Bst*XI, *Eco*RI, *Sac*II, *Sma*I, *Xba*I, and *Xmn*I were probed with pBLT65 and the region between genes encoding CHS3 and CHS4. Probes were labeled with alkaline phosphatase using the Genius System (Boehringer Mannheim, Indianapolis, Ind.), and the probe hybridized to the membrane was detected according to manufacturer's instructions.

Subcloning of BAC 56G2

BAC 56G2 was digested to completion with restriction enzymes *Not*I and *Eco*RI. DNA fragments were separated by electrophoresis in a 1 g/100 ml agarose/TAE gel for isolation of most of the fragments. Low-molecular-weight fragments were isolated from a 1.2 g/100 ml agarose/TAE gel after electrophoresis for 10 h at 6 V/cm. Higher molecular-weight fragments were isolated from a 0.75 g/100 ml agarose/TAE gel after electrophoresis for 16 h at 4 V/cm. The *Eco*RI fragments were cloned into the vector pUC18 and electroporated into DH5a *E. coli* cells. The 2-kb *Apa*I fragment of the BAC was cloned in the same manner except that the cloning vector was pCR-Script Amp SK$^+$ and the *Apa*I site was used as the cloning site.

DNA sequencing and analysis

The ends of the inserts of genomic clones were sequenced, and the sequences were compared to each other and to accessions of the GenBank databases using the BLASTN and BLASTX algorithms (Altschul et al. 1990). Sequence similarity was considered significant at P=e^{-6} for BLASTN and P=e^{-3} for BLASTX.

Mapping of *Eco*RI subclones to *Apa*I fragments

To determine which *Eco*RI subclones have inserts in common with individual *Apa*I fragments, we used the *Apa*I fragments separately as probes on two membranes arrayed with 24 *Eco*RI subclones with unique sequences. Each membrane was inoculated with four replications of the 24 *Eco*RI subclones by using a 96-pin replicating tool dipped in a plate of overnight cultures of the subclones. The inoculated membranes were grown overnight at 37°C, and processed as colony lifts.

To obtain *Apa*I fragment template from BAC 56G2, the DNA insert was excised from the vector with the restriction enzyme *Not*I, then separated from the vector on a 0.5 g/100 ml low-melt agarose gel in TAE buffer electrophoresed at 3.6 V/cm. The insert was digested overnight with *Apa*I. The *Apa*I fragments were separated on a 1 g/100 ml agarose gel in TAE buffer containing ethidium bromide and run at 1.4 V/cm at 4°C, then excised and used as a template in random priming reactions. The 6-kb, 8-kb, 13-kb, 20-kb, 23-kb, and 50-kb *Apa*I probes were used individually and together to hybridize to nylon membranes containing the arrayed *Eco*RI subclones. The 2-kb and 28-kb fragments were not used as probes because they contained BAC vector sequences that were expected to hybridize with the pUC18 vector sequences of the *Eco*RI subclones. An *Eco*RI subclone was mapped to an *Apa*I fragment only if all four replicates of the subclone on the membrane were identified by the *Apa*I fragment.

Results

BAC library screening with PCR

The PCR assay specific to genes encoding AK-HSDH that map near the *I* locus identified nine BACs, although amplification from BAC 6E3 could not be repeated (Fig. 1A). Only one DNA fragment was amplified from each BAC. From BACs 86I18, 57A5, 56G2, and 7B18 a DNA fragment was amplified with electrophoretic mobility similar to that of band 4 (960 bp) from the PCR amplification of genomic DNA of susceptible cultivars

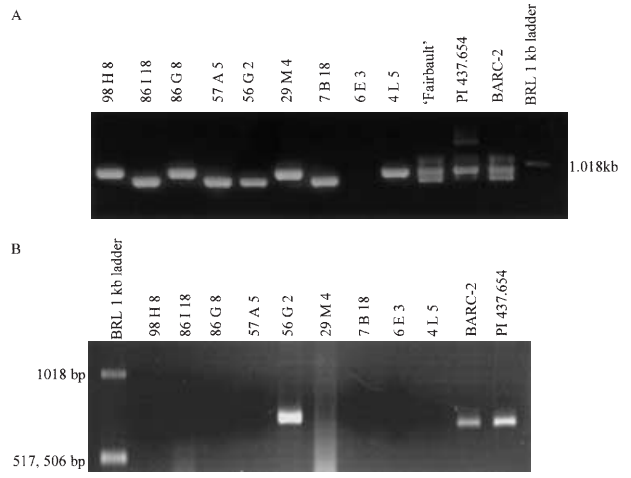

Fig. 1 A PCR products amplified from soybean cv. Williams 82 BAC library clones. Primers 548 and 563 were used in a PCR assay for genes encoding AK-HSDH. This PCR assay detects polymorphisms that map near the *I* locus and the Rhg_4 locus and was used to screen the library and identify clones containing genes encoding AK-HSDH and possibly Rhg_4. PCR products were compared by agarose gel electrophoresis with those from soybean genomic DNA from SCN-susceptible cv. Fairbault and BARC-2 and resistant accession PI 437.654. **B** Soybean cv. Williams 82 BAC library clones identified by the PCR assay using primers 548 and 563 were screened with a second PCR assay using primers 849 and 850 specific to the region between genes encoding CHS3 and CHS4, also known to be near Rhg_4. Only BAC 56G2 was positive with both assays. Soybean genomic DNA from BARC-2 and PI 437.654 also were used as template for comparison of PCR product sizes

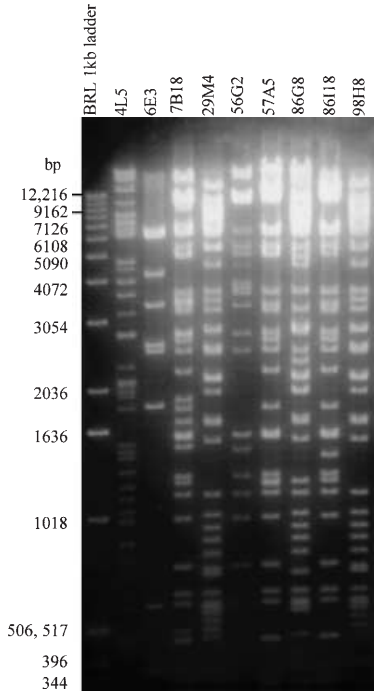

Fig. 2 Restriction fragment comparison of soybean cv. Williams 82 BACs identified by a PCR assay, using primers 548 and 563, that detects polymorphisms mapping near the *I* locus and the Rhg_4 locus. BACs were digested with *Not*I to isolate the inserts, the inserts were digested with *Eco*RI, and the restriction fragments were separated by agarose gel electrophoresis for restriction fragment pattern comparison. The sizes of the vector fragments generated by digestion with *Eco*RI were 0.5 kb and 6.3 kb

(Matthews et al. 1998). Sequencing of DNA from band 4 indicated it was most similar to pBLT65 (GenBank accession number AF049707), the partial cDNA clone from cv. Century. *Eco*RI fragment profile analysis (Fig. 2) of these BACs revealed that three of the four BACs (57A5, 86I18, and 7B18) had many *Eco*RI fragments in common and that the fourth BAC in this group, BAC 56G2, was the least similar to the other three. These four BACs are of interest because band 4 has been genetically linked with the Rhg_4 locus on linkage group A2 in several populations as an RFLP probe (Webb et al. 1995) and as a PCR marker (Heer et al. 1998; Matthews et al. 1998; Meksem et al. 2000).

From BACs 86G8, 98H8, 29M4, and 4L5, a DNA fragment was amplified with electrophoretic mobility similar to that of band 3 (996 bp) from the PCR amplification of genomic DNA from either resistant or susceptible cultivars (Matthews et al. 1998). The sequence of this band indicated that it is most similar to GenBank accession number AF049708, the cv. Century genomic clone from which the primers 548 and 563 were derived. *Eco*RI fragment profile analysis (Fig. 2) of these four BACs revealed that BACs 86G8, 98H8, and 29M4 were very similar and that BAC 4L5 was least similar to the other three BACs. Because the 996 bp fragment is not polymorphic between resistant and susceptible parents, it has never been used for genetic mapping or genetically linked to the Rhg_4 locus.

The nine BACs were assayed by PCR using the primers specific to the region 3′ of the CHS4 gene/*I* locus (Fig. 1B). A product was amplified only from BAC Gm_ISb001_056_G02 (56G2). Because BAC 56G2 was positive for this assay, and because the AK-HSDH fragment produced by PCR from this BAC was the same size as that used to map to linkage group A2 and the Rhg_4 locus, BAC 56G2 was most likely to represent part of linkage group A2 and was further analyzed.

BAC 56G2 restriction mapping

Complete digestion of the BAC with *Apa*I revealed at least eight fragments varying from 2 kb to 50 kb in length (Fig. 3A). A series of partial restriction digests with *Apa*I indicated the order of fragments (Rackwitz et al. 1995) (an example is shown in Fig. 4A). The 22-base oligonucleotide probe T7 derived from the sequence of one end of the BAC vector hybridized to the 28-kb *Apa*I fragment, placing this fragment at one end of the BAC 56G2 insert (Fig. 4B). The 26-base oligonucleotide probe 950 derived from the sequence of the other end of the BAC vector hybridized to the 2-kb *Apa*I fragment of the BAC 56G2 insert, placing the 2-kb fragment at the other end of the BAC56G2 insert. The location of the gene encoding AK-HSDH on the 50-kb *Apa*I fragment of BAC 56G2 was de-

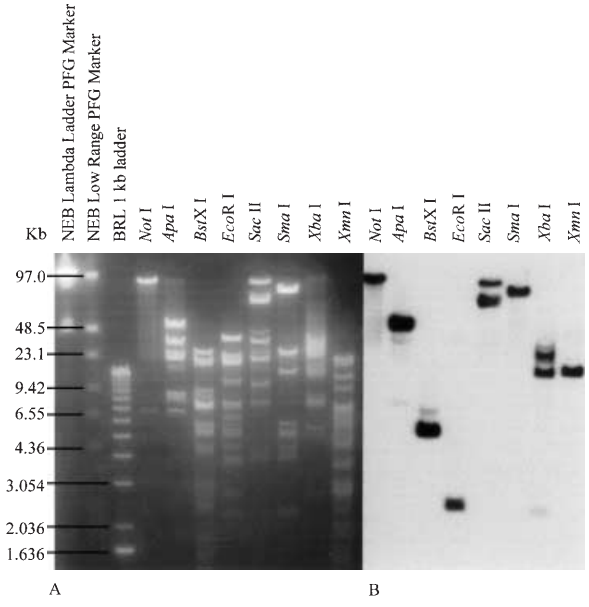

Fig. 3 A Soybean cv. Williams 82 BAC 56G2 was digested with eight restriction enzymes, and the fragments were separated by pulse field gel electrophoresis. **B** A Southern blot of the gel was hybridized with molecular marker pBLT65, GenBank accession number AF049706, a partial cDNA clone from a gene encoding AK-HSDH in soybean cv. Century, indicating that this portion of a gene encoding AK-HSDH is located in the 50-kb *Apa*I fragment

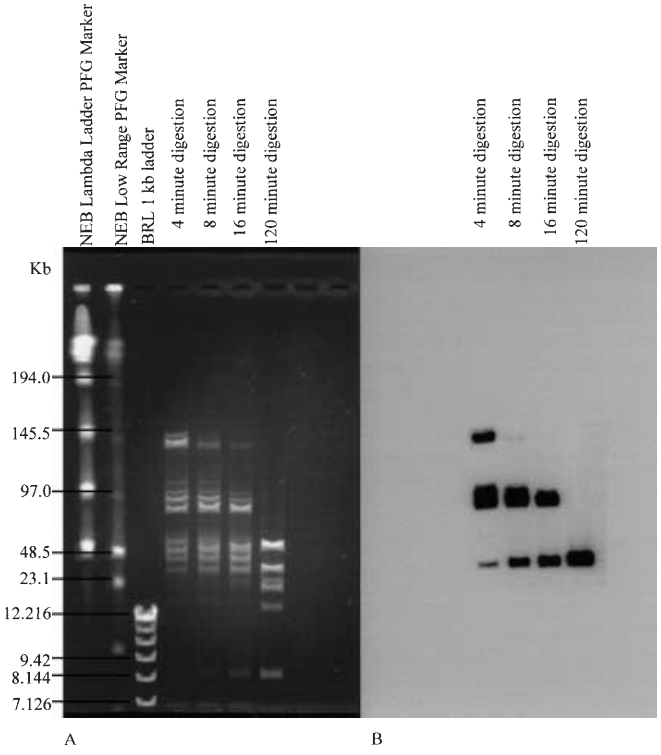

Fig. 4 A Partial *Apa*I digests of soybean cv.Williams 82 BAC 56G2 insert were separated by pulse field gel electrophoresis for size comparison with three DNA mass ladders and to generate an *Apa*I restriction map of the insert. The 6-kb and 2-kb fragments are not visible in this figure. **B** A Southern blot was made of the gel and hybridized with probes designed from the vector. This was done to determine which *Apa*I fragments were terminal. In this case, the T7 end was used as probe, and it was determined that the 28-kb *Apa*I fragment was one of the terminal fragments

termined by hybridizing pBLT65, a partial cDNA clone from a gene encoding AK-HSDH, to a Southern blot containing the *Apa*I digestion fragments of BAC 56G2 (Fig. 3B). The probe corresponding to the region between genes encoding CHS3 and CHS4, associated with the *I* locus, hybridized with the 20-kb *Apa*I fragment.

Mapping the *Eco*RI subclones to the *Apa*I fragments of BAC 56G2

*Apa*I fragments were hybridized to the *Eco*RI subclone array (Fig. 5) to determine which *Eco*RI subclones contained inserts in common with each *Apa*I fragment. This allowed us to associate the *Apa*I restriction map and the *Eco*RI subclones (Fig. 6). Several *Eco*RI subclones of the BAC had sequence similarity to genes previously associated with stress and defense responses (Table 1). When an individual *Apa*I fragment was used to probe the array of *Eco*RI subclones, it hybridized with a small population of the clones. Most of the *Eco*RI subclones that hybridized with only one *Apa*I fragment hybridized with the 50-kb fragment; subclone 915 hybridized only with the 6-kb fragment. Other *Eco*RI subclones hybridized with multiple *Apa*I fragments, suggesting that there are duplicated regions in BAC 56G2. Duplication of regions of *Apa*I fragments was indicated by the hybridization of subclones 924, 999, 1006, and 1010 – and only these subclones – with both the 13-kb and 20-kb fragments. Additional duplication was indicated by hybridization of

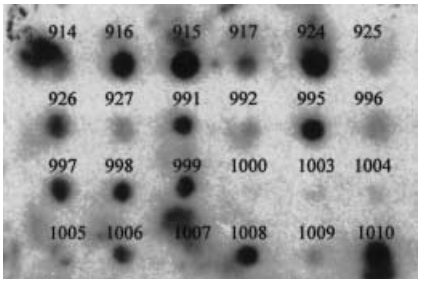

Fig. 5 Soybean cv. Williams 82 BAC 56G2 *Eco*RI sub-clones were hybridized to a membrane and probed with the BAC 56G2 *Apa*I fragments to order the BAC 56G2 *Eco*RI sub-clones on the *Apa*I restriction map. In this demonstration, six *Apa*I fragments (50-kb, 13-kb, 6-kb, 8-kb, 23-kb, and 20-kb) were used as template in the random priming reaction to make a single probe. One replication of the array of *Eco*RI subclones is depicted

subclone 999 with the 6-kb, 8-kb, 13-kb, 20-kb and 23-kb fragments. One end of this 16-kb subclone had very high sequence identity with CHS, and repeated attempts at additional sequencing using primers derived from the sequenced end of subclone 999 indicated multiple priming sites on the subclone. It is very likely that

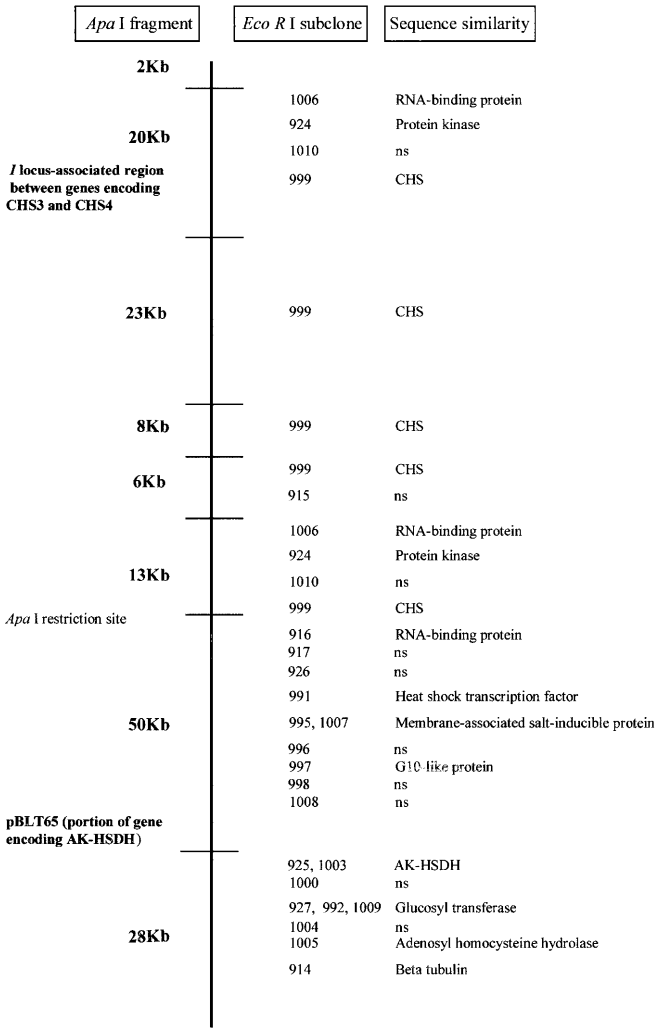

Fig. 6 *Apa*I restriction map of soybean cv. Williams 82 BAC 56G2. The location of a gene encoding AK-HSDH is indicated on the 50-kb fragment, and the location of the region between genes encoding CHS3 and CHS4 is indicated on the 20-kb fragment. The locations of *Eco*RI subclones on *Apa*I fragments also are indicated. *Eco*RI subclones not located to the 20-kb, 23-kb, 8-kb, 6-kb, 13-kb, or 50-kb fragments were assumed to be on the 28-kb fragment by default. *Eco*RI subclones with significant (P=e^{-3}) sequence similarity (BLASTX) to GenBank accessions are identified. *ns*=No significant similarity with any GenBank accession

subclone 999 contains multiple CHS genes. This is not surprising, because CHS gene family members have been reported in clusters of CHS1, CHS3, and CHS4, plus CHS3 and CHS5 (Akada and Dube 1995). It is possible that the association of subclone 999 with several *Apa*I fragments is a result of a large region of CHS duplications. Another possible gene family is indicated in this part of the genome by subclones 916 and 1006, which have sequence similarity to RNA-binding proteins and hybridized with the 50-kb fragment and with the 13-kb and 20-kb fragments, respectively. Subclones 914, 925, 927, 992, 1000, 1003, 1004, 1005, and 1009 were assumed to be part of the 28-kb fragment because they did not hybridize with any *Apa*I fragment used as a probe and because sequence comparison between these subclones and the complete sequence of the 2-kb *Apa*I fragment indicated no significant similarity. The DNA sequence of the 2-kb *Apa*I fragment was not significantly similar to any accession in GenBank.

Discussion

Our data suggest that we have isolated a region of the susceptible genome that may contain the Rhg_4 locus. BAC 56G2 is part of linkage group A2 because simple sequence repeat markers (SSRs Sat_157 and Sat_162) developed from BAC 56G2 mapped to linkage group A2 and cosegregated with the *I* locus in a population of 240 recombinant inbred lines (RILs)(Cregan et al. 1999). However, the possibility exists that BAC 56G2 represents a physically and genetically linked duplication of the true *I* locus region. In that case, there would be at least two very similar regions immediately adjacent to each other on linkage group A2. This would pose an obstacle to a map-based cloning approach of Rhg_4 because one of the assumptions made when utilizing genetic mapping techniques is no longer valid. That assumption is that a single polymorphic marker (such as a DNA fragment derived from restriction digest or PCR) represents only one locus. This assumption is usually checked with the Chi-square goodness-of-fit test before calculating recombination frequency. However, with physically close duplication, the data would pass the goodness-of-fit test. The segregation ratios at codominant loci in tandem duplicated regions will be skewed slightly toward the heterozygote class, but not so much that they would fail a Chi-square goodness-of-fit test, because the tandem duplicated regions are so physically close to each other. It is interesting to note that many researchers have observed that RIL populations have more heterozygous individuals than predicted at most loci; this phenomenon has not been an obstacle to mapping, because heterozygous individuals are ignored in RIL mapping populations by recording a "–" score for heterozygous individuals. Although tandem duplicated regions do not interfere with applications like marker-assisted selection, the marker data coming from them are misleading if used in high-resolution mapping efforts to pin-point the Rhg_4 or other genes in similar regions.

In addition, high levels of duplication are proposed to lead to genomic instability and are considered mechanisms of regional genome evolution (Michelmore and Meyers 1998). Analysis of BAC 56G2 provided evidence of gene duplication on at least two levels in this region. Regional duplication was evidenced by four *Eco*RI subclones hybridizing to two different *Apa*I fragments separated by approximately 37 kb. Gene duplication was evidenced by the duplication of genes encoding CHS isoforms covering approximately 70 kb and by the duplication of two different RNA-binding proteins on separate *Apa*I fragments. The duplications observed in this region may contribute to the high rate of deletion at the *I* locus (Todd and Vodkin 1996).

Table 1 Genes identified by one-pass sequencing of *Eco*RI subclones of soybean cv. Williams 82 BAC 56G2

Local clone and sequence numbers	GenBank Accession	Tentative gene name[a]	*P* value[b]	Amino acid identity
1009 p10e 31–21 r	AZ313417	Anthocyanin 5-*O*-glucosyl-transferase	1.7e^{-59}	106/176(60%) *Petunia×hybrida*
999 p13e 36–2 r	AZ313412	Chalcone synthase	2.0e^{-33}	74/91(81%) *Glycine max*
1007 p1e 35–1 f	AZ313416	Membrane-associated salt-inducible-like protein	2.7e^{-27}	71/203(34%) *Arabidopsis thaliana*
995 p1e 35 r	AZ313410	Membrane-associated salt-inducible-like protein	1.8e^{-29}	69/203(33%) *Arabidopsis thaliana*
991 p6e 28 f	AZ313408	Heat-shock transcription factor-like protein	5.3e^{-14}	32/47(68%) *Arabidopsis thaliana*
992 p8e 9 f	AZ313409	Glucosyl-transferase	1.0e^{-53}	74/119(62%) *Petunia×hybrida*
1003 pe38–2 r pe38–2 r	AZ313413	Aspartokinase-homoserine dehydrogenase	BLASTN[c] 1e^{-166}	405/434(93%) Glycine max
1005 pe40–3	AZ313414	Adenosyl-homocysteinase	2.7e^{-08}	25/30(83%) *Nicotiana tabacum*
1006 pe41–5 r	AZ313415	Putative RNA-binding protein	2.1e^{-06}	27/76(35%) *Arabidopsis thaliana*
914 puce10 f	AZ313403	Beta-tubulin	8.7e^{-11}	32/40(80%) *Glycine max*
916 puce11 r	AZ313404	Putative RNA-binding protein	1.0e^{-06}	28/84(33%) *Arabidopsis thaliana*
927 puce18 r	AZ313407	Glucosyltransferase	9.9e^{-34}	69/107(64%) *Petunia×hybrida*
924 puce1 f	AZ313405	Putative protein kinase	5.6e^{-11}	22/33(66%) *Arabidopsis thaliana*
925 puce2 f	AZ313406	Aspartokinase-homoserine dehydrogenase	BLASTN[c] 1e^{-106}	263/284(92%) *Glycine max*
997 p7e29–2 r	AZ313411	G10-like protein	2e^{-28}	52/103(50%) *Arabidopsis thaliana*

[a] Amino acid sequence to which the query sequence showed the highest similarity obtained by comparison of each DNA sequence translated in all reading frames against an amino acid sequence database, using the BLASTX program based on the BLAST algorithm

[b] Probability of chance occurrence of a high-scoring segment pair (HSP)

[c] No significant similarity to any amino acid sequence was identified with a comparison of translated DNA sequences, but a significant DNA sequence similarity was identified upon comparison with a DNA sequence database using the BLASTN program based on the BLAST algorithm

Due to gene duplication in the region, it is unlikely that high-resolution mapping alone will identify the *Rhg$_4$* locus. In addition, BAC 56G2 is densely populated with genes previously associated with stress or defense responses, so there is a low probability that *Rhg$_4$* would be identified solely by sequence similarity. Therefore, carefully designed complementation tests using DNA from a resistant genome will be required. Simple sequence repeat markers derived from BAC 56G2 cosegregated with the *I* locus in a population of 240 RILs, indicating that BAC 56G2 represents a physical portion of the genome mapping to linkage group A2, including the *I* and *Rhg$_4$* loci. These and other markers derived from BAC 56G2 can be used to screen a BAC library made from a resistant genotype in order to identify a homologous region for use in the complementation tests required to identify *Rhg$_4$*.

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