Development of a molecular marker for a bruchid (*Callosobruchus chinensis* L.) resistance gene in mungbean

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Abstract Bruchid, *Callosobruchus* spp. (Coleoptera: Bruchidae), is a serious pest during storage of seeds of mungbean (*Vigna radiata* (L.) Wilczek) and other *Vigna* species. A source of resistance to this pest has been identified in *Vigna sublobata* (Roxb.) Bairig. accession TC1966. Two hundred recombinant inbred lines at the F_{12} generation have been developed for molecular mapping of bruchid resistance (*Br*) gene in TC1966. Through bulked segregant analysis (BSA), ten randomly amplified polymorphic DNA (RAPD) markers associated with the bruchid resistance gene were successfully identified. A total of four closely linked RAPDs were cloned and transformed into sequence characterized amplified region (SCAR) and cleaved amplified polymorphism (CAP) markers. Seven CAPs developed from the identified RAPD markers showed tighter linkage with the *Br* gene than the original RAPD. Through transformation of RAPDs into CAPs, codominant markers for bruchid resistance were successfully obtained. Homozygous genotypes of these PCR-based markers were estimated to contribute 85% of the variance for seed damage when the insect assay was performed under favorable growth conditions for bruchid.

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Introduction

Bruchid beetle or bean weevil, *Callosobruchus* spp. (Coleoptera: Bruchidae), causes serious damage to several leguminous crops including cultivated *Vigna* species, such as mungbean (*V. radiata* [L.] Wilczek), azuki bean (*V. angularis* [Willd.] Ohwi & Ohasi) and cowpea (*V. unguiculata* [L.] Walp.) during storage (Fernandez and Talekar 1990). Control of this pest by fumigants or other insecticides is not practical because of the primitive nature of seed-storage facilities in most developing countries (where most of munbean and cowpea are produced), the small volume of the seeds and the frequent utilization of the seeds for human consumption (Talekar 1996). Complete resistance against bruchid has been reported in *V. sublobata* (Roxb.) Bairig. (Fujii and Miyazaki 1987; AVRDC 1991), *V. umbellata* (Thunb.) Ohwi & Ohashi, and *V. mungo* (L.) Hepper (Talekar 1996; Tomooka et al. 2000). Resistance could be mediated

through hairiness of the pod or antibiosis (Talekar 1996). TC1966, a strain of *V. sublobata,* showed complete resistance to four species of bruchids, *C. chinensis* (L.), *C. maculatus* (Fabricius), *C. phaseoli* (Gyllenhal) and *Zabrotes subfasciatus* (Boheman) (Fuji et al. 1989). As no cross incompatibility exhibits between mungbean and *V. sublobata*, the resistance factor in TC1966 was regarded as a valuable source for mungbean resistance breeding (Fujii and Miyazaki 1987; Talekar 1988; Tomooka et al. 1992) and might provide useful clues for legume pest management in the future. Inheritance of resistance against azuki bean weevil (*C. chinensis*) in TC1966 was suggested to be under the control of a single, dominant major gene with some modifying factors (Kitamura et al. 1988). Nevertheless, the exact genetic control and mechanism for resistance remained to be explored.

Mapping of the bruchid resistance (*Br*) gene in TC1966 has been conducted by restriction fragment length polymorphism (RFLP) analysis (Young et al. 1992). Two markers that mapped approximately 3.6 and 6.7 centimorgan (cM) from the gene were identified. Unfortunately, these markers were not satisfactory when applied at the advanced mungbean generation (Chen et al. unpublished data). Testing bean bug (*Riptortus clavatus*) within a segregating $BC_{20}F_2$ population, Kaga and Ishimoto (1998) identified eight random amplified polymorphic DNA (RAPD) linked with bean bug resistance. These fragments were subsequently used as RFLP probes and closely linked RFLP markers were reported. RFLP markers are regarded as codominant markers, which facilitate the distinction of homozygous and heterozygous individuals for breeders. Better reproducibility is also found in RFLPs than in RAPDs. However, the RFLP analysis is technically complicated and time consuming and not feasible for high throughput analysis (Powell et al. 1996).

Insecticidal factors against bruchids have been reported and reviewed in common bean (*Phaseolus vulgaris* L.) (Fabre et al. 1998; Paes et al. 2000), cowpea (*V. unguiculata* (L.) Walp.) (Macedo et al. 1993) and other legumes (Grossi-de-Sá and Chrispeels 1997; Sales et al. 2000; Carlini and Grossi-de-Sá 2003). The two chemical factors, vignatic acid (Sugawara et al. 1996; Kaga and Ishimoto 1998) and VrCRP (cysteine-rich protein of the plant defensin family) (Chen et al. 2002), have also been isolated from TC1966 and its progenies. Both compounds

were not confirmed as the antibiotic factors directly responsible for bruchid resistance in TC1966.

In addition to bruchid infestation, mungbean yellow mosaic virus (MYMV) is a serious disease in South Asia. To generate PCR-based molecular markers for bruchid and MYMV resistance and to pyramid both traits in mungbean breeding, 200 recombinant inbred lines (RILs) at the F_{12} generation were developed by crossing of MYMV-resistant cultivated mungbean NM92 (AVRDC 2002) with TC1966. Recombinant inbred populations are characteristically a group of segregating pure lines that can be reproduced indefinitely. Thus, this allows repeated phenotyping for different traits and accumulation of useful markers. Here we describe the development of PCR-based markers for bruchid resistance in mungbean by use of a recombinant inbred population. Polymorphism was first explored by the RAPD technique through bulked segregant analysis approach (Michelmore et al. 1991). The associated RAPDs were then cloned and sequenced for further SCAR (sequence characterized amplified region) and CAP (cleaved amplified polymeric region) analyses. In this study, we identified codominant CAP markers for bruchid resistance, which explained up to 85% of the variation for bruchid damage in the tested population.

Materials and methods

Plant materials and DNA extraction

A recombinant inbred (RI) population of 200 lines was generated from an inter-subspecific cross between bruchid susceptible (but MYMV resistant) mungbean variety NM92 and a bruchid resistant subspecies accession TC1966. The RI population was generated as follows: F_1 were selfed for two generations to generate 200 F_3 lines. Two hundred RILs were developed from these F_3 plants by single seed descent for 9 generations to F_{12}. The DNA of these RILs was extracted by a modified CTAB procedure described by Saghai Maroof et al. (1984).

Assay for bruchid resistance

A bruchid (*C. chinensis*) population was maintained on a susceptible mungbean variety VC1973A at the

AVRDC insectary. Forty seeds of each RIL, parents, and resistant and susceptible checks were placed in individual 50 ml Erlenmeyer flasks. Twenty newly emerged bruchid adults were placed into each flask, for mating and laying eggs on the seeds. Seven days after infestation (DAI), all adults were removed and each seed was checked to make sure each had more than two eggs. The flasks were kept in a compartment room of the insectary. The samples were examined at 30 DAI to record the number of bruchid that had emerged and percentage of seed damage. The bruchid assay was repeated three times, in February 2002, May 2003 and July 2005. Mean maximum/minimum temperatures during the bruchid assays were 26.0°/20.5°C, 28.0°/24.5°C and 28.5°/25.0°C for February 2002, May 2003 and July 2005 respectively. In the July 2005 assay, adult emergence was recorded three times a week from 21 DAI to 40 DAI.

Bulked segregant approach and RAPD analysis

After the February 2002 assay, DNA of RILs that showed 0% and 80–100% bruchid infestation were pooled. Two resistant bulks (22 RILs each of 0% damage) and two susceptible bulks (20 RILs of 80–90% damage and 18 RILs of 90–100% damage, respectively) were formed. For the RAPD analysis, 10-mer sets of random primer were purchased from OPERON Inc. (CA, USA) and the University of British Columbia, Nucleic Acid-Protein Service Unit (Canada). Primers were first screened with parental DNA for successful PCR amplification and polymorphic bands. Primers that generated clear and multiple bands were further used to test the bulked samples. The PCR regime used was 94°C for 3 min, followed by 3 cycles of 94°C for 1 min, 40°C 1 min, 72°C 2 min linked to the second program with 40 cycles of 94°C for 30 s, 40°C 30 s, 72°C 1 min and a final extension at 72°C for 10 min. Random primers that showed polymorphism between resistant and susceptible bulks were further analyzed in the 200 RILs.

CAP analysis

RAPD fragments highly correlated with bruchid resistance were then cloned into the pDrive cloning vector using QIAGEN PCR Cloning kit (QIAGEN Inc.). The inserts of each clone were sequenced using an ABI373 DNA Sequencer. Primer sets were

designed based on both ends of the obtained sequences. PCR was carried out in a volume of 25 μl containing 50 ng of total genomic DNA, 5 pmoles of each primer, 0.6 U *Taq* DNA polymerase, 0.1 mM of each dNTP, 3 mM MgCl$_2$, and 1× PCR buffer. PCR was processed with the following program: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 1 min, 55°C (depend on pretest results with gradient thermocycler) 1 min, 72°C 2 min and a final extension at 72°C for 10 min. The amplified products from the two parental DNAs were digested with different restriction enzymes to determine polymorphism within the insert. The identified CAPs were also tested for all 200 RILs.

Sequence analysis and marker linkage analysis

Sequence homologies of cloned RAPDs and SCARs were analyzed and compared at NCBI (http://www.ncbi.nlm.nih.gov/BLAST), using both blastn and blastx procedures. Associations between markers and traits were determined by QGENE program using single point regression analysis (Nelson 1997). Based on this analysis, coefficients of statistics (R^2, SE, genotype mean, etc.) were reported for all significant markers. Only marker-phenotype associations with a probability threshold of $P < 0.001$ were considered. Linkage analysis was performed using the software Mapmaker v. 3.0b (Lander et al. 1987) at a log likelihood 5.0 with a maximum Haldane distance of 50 cM. To verify the order of markers obtained by three-point analysis, the Ripple command was used at the window-size of 5 and log likelihood threshold of 6.0.

Results

Bruchid reaction among 200 RILs

Genetically, F$_{12}$ RILs are deemed to be a set of highly homogeneous pure lines. However, the results of bruchid bioassays over 3 years were not consistent (Table 1). The results of bruchid assays in May 2003 and July 2005 were comparable, where as the February 2002 assay's results deviated from the aforementioned two assays. Lower temperatures may not be favorable for growth of bruchid thus less damages was observed in the February 2002 assay.

Table 1 Bruchid-infesting distribution of 200 RILs in the three bruchid assays conducted in Feb 2002, May 2003 and July 2005

Damage (%)	No. of RILs		
	Feb 2002	May 2003	July 2005
0	44	57	48
10	12	1	16
20	7	1	6
30	14	3	3
40	19	2	6
50	13	1	2
60	15	4	3
70	18	4	0
80	20	5	1
90	20	2	2
100	18	120	113

Interestingly, however, about one fourth of the total RILs consistently expressed complete resistance (0% damage) in all three assays (Table 1). More than half of the 200 RILs expressed complete susceptibility (100% damage) in May 2003 and July 2005, but only 18 lines showed similar susceptibility in the February 2002 assay. Damage differences between the 2002/2003 and 2005 assays could be attributed to temperature effect on infestation ability of bruchid. Low temperatures in the February 2002 assay could be a negative factor for larva emergence and growth hence resulting in a longer period for adult emergence (i.e., >30 DAI) and therefore less seed damage was recorded. Given the unstable factors frequently encountered for insect bioassays, marker-assisted selection, which is environment-independent, could circumvent this hurdle.

Bulked segregant and RAPD analyses

More than 600 primers were pre-tested for effective RAPD amplification in tested materials. In total, 360 primers were selected and RAPD tested with four bulked samples and two parents. Ten out of 360 primers produced polymorphic bands ranging from 700 to 1800 bp. Some primers (e.g., OPV02) produced unique bands in the two resistant bulks and resistant parent, whereas some primers (e.g., UBC223) produced a unique band in the two susceptible bulks and the susceptible parent, in

addition, others (e.g., OPW02) produced polymorphisms between resistant and susceptible bulks but not between resistant and susceptible parents (Fig. 1).
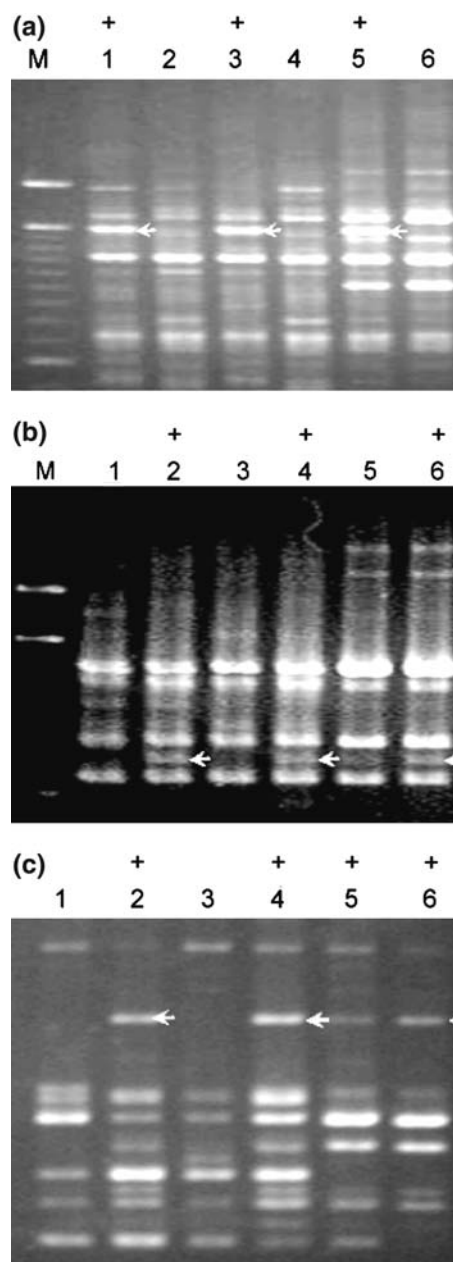
Fig. 1 Bulked segregant analysis for bruchid resistance using random decamers. OPV02 (**a**), UBC223 (**b**) and OPW02 (**c**). M: molecular weight marker; Lane 1: resistant bulk I; Lane 2: susceptible bulk I; Lane3: resistant bulk II; Lane 4: susceptible bulk II; Lane 5: resistant parent TC1966; Lane 6: susceptible parent NM92. Arrow indicates polymorphism between resistant and susceptible bulks

Ten polymorphic primers (i.e., UBC66, UBC168, UBC223, UBC313, UBC353, OPM04, OPU11, OPV02, OPW02 and OPW13) were further tested on a total of 200 RILs. Single marker regression analysis was processed using genotype and phenotype data to investigate their association using the QGene program. Four RAPDs with significant correlations were produced by OPW02, UBC223, OPU11 and OPV02 primers. The phenotypic correlation (R^2 value) explained by the markers were 0.72 for OPW02a4, 0.6 for OPU11a4, 0.55 for UBC223a7 and 0.5 for OPV02a3 when using data collected in 2005.

Development for SCAR and CAP markers

OPW02a4, UBC223a7, OPU11a4 and OPV02a3, were successfully cloned and sequenced. Sets of primers were designed and tested for SCAR analysis. Both OPU11a4for/rev and OPV02a3for/rev primers produced dominant bands for the resistant parent. These two SCARs were successfully reproduced in segregating population. Both UBC223a7 and OPW02a4 for/rev primers produced monomorphic bands between the two parents.

Further digestion with various restriction enzymes revealed that the UBC223a7 fragment generated a polymorphic band in the resistant parent when digested with *Hae*III enzyme, however this polymorphism was irreproducible within the segregating population. In addition to the original 1.5 kb RAPD region, OPW02a4 for/rev primers also produced other bands located between 0.25 kb and 1.7 kb in size. This implied that multiple regions were tagged by these two primers. Although no clear polymorphism was observed in the SCAR profile, there was higher band intensity around the 1.5 kb region for the resistant parent than for susceptible parent. Further digestion of the OPW02a4 for/rev SCAR reaction with different restriction enzymes revealed many cleaved amplified polymorphisms (CAP) between resistant and susceptible parents, i.e., *Hae*III (7 CAPs, W02s1-W02s7), *Msp*I (3 CAPs, W02s8-W02s10) and *Hin*fI (2 CAPs, W02s11-W02s12). With *Msp*I digestion, two monomorphic bands were shown for both parents, and three polymorphic bands (i.e., 1.7, 0.6 and 0.48 kb) observed in the resistant parent. The 1.7 kb band was not related with the bruchid reaction as determined by genetic progeny testing. Among

these CAPs, three of them revealed allelic polymorphism between R and S parents. Digestion with *Hae*III enzyme produced two bands at about 600 and 900 bp for resistant parent and one bands at 1100 bp for susceptible parent. Testing among 200 RILs revealed that each RIL either produced two bands from resistant parent or one band from susceptible parent (Fig. 2). These bands can be used as codominant marker when making selection in the breeding program. According to the aforementioned results, it is suggested that OPW02a4 is the most informative RAPD for *Br* gene of TC1966. The complete sequence of OPW02a4 fragment and related RAPD and CAP primers are shown in Fig. 3.

Linkage and sequence analysis

Results of QGene analysis with the three bruchid assays revealed seven most closely linked CAPs and four RAPDs (Table 2). Among the four RAPDs, segregation of OPW02a4 (126:74) and OPV02a3 (116:84) skewed to the susceptible parent NM92. The segregation of UBC 223a7 (100:96) and OPU11a4 (104:94) did not deviate statistically from the expected ratio of 1:1 ($P > 0.7$). CAPs derived from OPW02a4 also skewed to the susceptible parent.

The R^2 value for each marker varied among three assays. The assay in the February 2002 assay had least association while the July 2005 assay showed the highest association for all listed markers. Nevertheless, these markers consistently showed high correlation with bruchid resistance comparing with
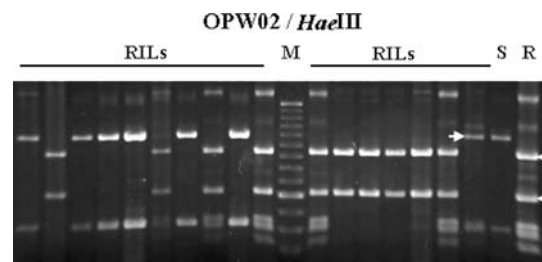
Fig. 2 CAP analysis for bruchid resistance gene using OPW02a4 for/rev primers followed by *Hae*III enzyme digestion. Three bands (two from R parent and one from S parent) revealed allelic polymorphism between two parents when tested across 200 RILs. S: susceptible parent NM92; R: resistant parent TC1966; M: molecular weight marker. Arrows at left and right sides indicate polymorphic bands for susceptible and resistant parents respectively

Fig. 3 Sequence of the
cloned OPW02a4 fragment.
Bold letters are RAPD
primers, underlined letters
indicate forward and
reverse primers used in
CAP analysis

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   1  ACCCCGCCAA AGGAGTCGAG TGAAACTCTT GGAGGATCCT GTTGAATGGA GAAATCATGG
  61  AACATGGAAG GGTCCTGCAA AATATTTCCC TTAAGATCCA GGAAAGCAGT ATCAAAAGCC
 121  AAAGTGTGTT TAAGATCTTG GAGAAAGTCC ATGTGAGAGA CCGAAATGAG GAAAAATGAG
 181  TCTGCATTAG GTGAAACACG GGAAAGTGCA TCAGAACTGG TGTTTGTTTT TCCTGGTTTA
 241  TAATGTATCT CAAAGTCGAA TCCCAATAAT TTGGCTACAT AAGACTGTTG GTCAAGAGTT
 301  AAGATAGTTT AAGATAACAA TTCCTTGATG GCTTGTTGAT CAATGTAGAT GATGAAGTGG
 361  CAACCGAGCA AGTATTGGCG CCATTTCTGA ATAGCAGATG TGATAGCACA AAGTTCCCTC
 421  ATGTACGTTG ATGAATGTCT CAATTTAGGG CAAAATTGCT TACTGAAAAA AGCAATTGGG
 481  TGTTCTTCCT GTGATAAAAC AACCCCTATA CCAAAACCAG AGACATCTGT CTGAATAGTG
 541  AAAACTAAAT CAAACCGTGG CAGAGCCAAT ACTAGAGCAG TAGTAACCAC AGTTTTAATG
 601  TTCTCAAAAG TCGTAGTAGC TGAAGTAGAC CATATAAGGT TATCCTTCTT AAGTAAATTT
 661  GTTAAGGCAG AAGCAATAGA GGCATACCCC TTTACAAAAT CGGCGGTAAA AACCTGTGAG
 721  TCCAAGAAAA CCCCGTAGGG ACTTCAAAGT GGTTGGTAGT GGCTAGCTAA CGATATCTTG
 781  GACCTTTGAA GGATCTGGTG TGACACCCTA TGACGAGATT ACATGACCCA AGTATTGAAT
 841  CGATTGCTTC CCTATGAAAC ACTTGGAACC CTTGAGACGA AAACTGTTTG TGAGGAGCAA
 901  TTTGAAACCG GTTTCCAAGT GAGAGATGTG GTCCTCCAAG GTGGTGCTGT AGGCAAGGAT
 961  ATCATAAAAA AATAATAATA AAACGACGTA AATGAGGGCG GAACAAGTCA TTAATGTTAG
1021  CTTGAAAGGT GGATGGTGCA TTGCACAATC CGAAAGGCAT GACCTTGAAC TCGAAGTGGC
1081  CGTTGTGGGT GCGGAACACC GTTTTAGAGA AATCAGTAGG CAACATTAGG ATTTGGTGAA
1141  AACCTGAGAA GAGGTCGAGT TTCGAGAACC ACGTAGCATT ACCAAGTTCA TCCAAAAGAT
1201  CATCGACCGT AGGAATTGAA AATTTATCTT TAACGGTAAC GACATTGAGG GCACGATAAT
1261  CGATGCAAAA TCGCCATGTA CTGCCTTTCT TCTTGACAAG AAGAACAAGG GAAGAATAAG
1321  GGTTGGTGCT TGGCGTAATG AACCCCGAGT CCAACATCTT TTGAACCTGG ACCTTAATTT
1381  CTTGCTTCTG AAAATGGGGG TATCGGTAAG GACGCACTGA AATGGGAGCA ATGTTGGTTA
1441  AAAGGTTGAT TGAATGATCG GTGCAACGAG ATAGAGGAAG GGTTGTTGGC GGGGT
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other markers in the three assays. For OPW02a4
locus, mean bruchid damage was 90% for individuals
that were homozygous for NM92 allele and 9% for
individuals that were homozygous for TC1966 allele
(Table 2). Six CAPs, such as W02s02, W02s03,
W02s04, W02s09, W02s10 and W02s11, derived
from RAPD OPW02a4 are more closely linked to *Br*
gene (R^2 = 0.85) compared to the original RAPD
(Table 2). Genetic mapping of RAPDs, SCARs and
CAPs with MAPMAKER/EXP 3.0b also showed the
same result (Fig. 4). The SCAR markers of OPU11a4,
OPV02a3 and UBC223a7 were not able to generate
more informative markers for this experiment. With
low stringency PCR conditions, RAPD was not
always reproducible to amplify all RILs. However,
using a more stringent temperature regime for SCAR
analysis, all samples were successfully amplified and
the corresponding CAPs were identified.

As differences in SCAR intensity and CAPs were
observed by OPW02a4for/rev primers, sequences of
these SCAR products derived from resistant and
susceptible lines might provide more inside informa-
tion. To improve resolution, the SCAR products were
electrophoresised on 1.3% agarose gel for longer
period. The result indicated only one 1.45 kb band for
the susceptible parent NM92. However, two bands
(about 1.45 and 1.6 kb) were generated specifically
for resistant parent TC1966 and the tested resistant
lines. SCAR sequence of this band (1470 bp) of
NM92 is the same as the sequence of the cloned
RAPD (Fig. 3). Further comparison of these
sequences suggested high homology of the 1470 bp
band between resistant parent and resistant RIL
(98%), the homology between resistant RIL and
susceptible parent is 93% (data not shown). The
CAPs were basically derived from this 1470 bp

Table 2 Names and orders of CAPs and RAPDs that showed close linkage in the three bruchid assays

Marker	RSq	AA[a]	SE[b]	N[c]	aa[a]	SE[b]	N[c]
February 2002							
w02s3	0.414	62.5	2.45	118	18.2	2.80	82
w02s4	0.414	62.5	2.45	118	18.2	2.80	82
w02s10	0.412	62.5	2.45	118	18.3	2.80	82
w02s11	0.397	62.4	2.47	117	19.0	2.87	83
w02s2	0.395	61.6	2.45	121	18.0	2.89	79
w02s9	0.371	60.9	2.52	122	18.6	2.92	78
w02a4	0.300	58.6	2.62	126	20.2	3.12	74
u223a7	0.299	62.7	2.63	100	25.6	3.13	96
w02s6	0.258	60.4	2.61	107	25.9	3.30	93
u11a4	0.222	59.5	2.77	104	27.6	3.27	94
v02a3	0.178	56.5	2.85	116	27.6	3.39	84
May 2003							
w02s2	0.661	95.2	1.39	121	20.6	4.20	79
w02s4	0.642	95.7	1.30	118	22.6	4.27	82
w02s3	0.642	95.7	1.30	118	22.6	4.27	82
w02s10	0.606	94.9	1.53	118	23.8	4.36	82
w02s11	0.599	95.0	1.54	117	24.5	4.36	83
w02s9	0.573	92.9	1.92	122	23.2	4.42	78
w02a4	0.573	91.8	1.97	126	21.4	4.53	74
w02s6	0.484	94.8	1.55	107	32.3	4.60	93
u223a7	0.463	95.9	1.55	100	35.0	4.53	96
u11a4	0.403	92.9	2.05	104	36.0	4.69	94
v02a3	0.333	87.8	2.77	116	35.3	4.88	84
July 2005							
w02s3	0.852	95.8	1.44	118	9.2	2.28	82
w02s4	0.852	95.8	1.44	118	9.2	2.28	82
w02s2	0.841	94.5	1.60	121	8.0	2.23	79
w02s10	0.836	95.4	158	118	10.0	2.31	82
w02s11	0.822	95.5	1.59	117	10. 7	2.47	83
w02s9	0.774	92.7	1.96	122	9. 6	2.56	78
w02a4	0.724	90.4	2.24	126	9.1	2.66	74
w02s6	0.628	94.4	1.71	107	21.1	3.83	93
u11a4	0.604	94.2	2.00	104	22.2	3.78	94
u223a7	0.551	93.7	1.87	100	25.1	4.09	96
v02a3	0.504	88.1	2.78	116	21.8	3.93	84

Data showed results of single marker regression analysis by QGene program
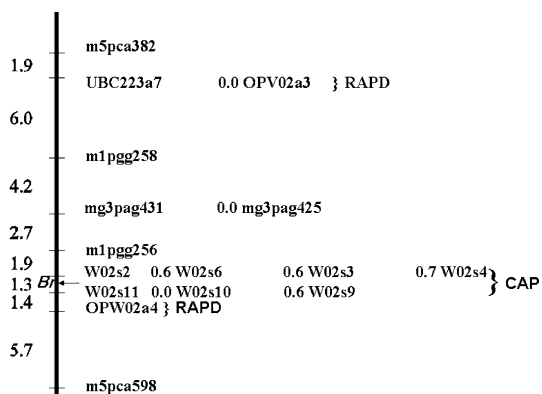
[a] AA Mean bruchid damage % for genotype homozygous to NM92; aa Mean bruchid damage % for genotype homozygous to TC1966

[b] Standard error

[c] No. of recombinant inbred lines

region. Sequence of this fragment did not show high homology with any reported gene on NCBI web site. However, comparison of the translated query with the protein database (blastx) suggested high correlation with RNA-directed DNA polymerase and gypsy/Ty-3 retroelement. Sequence alignment of the 1.6 kb

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1.9 ─┬─   m5pca382
     │
     ├─   UBC223a7        0.0 OPV02a3    } RAPD
6.0  │
     │
     ├─   m1pgg258
4.2  │
     │
     ├─   mg3pag431       0.0 mg3pag425
2.7  │
1.9  ├─   m1pgg256
1.3  ├─ Br◄ W02s2  0.6 W02s6     0.6 W02s3      0.7 W02s4 }
1.4  ├─   W02s11 0.0 W02s10    0.6 W02s9                  } CAP
     │    OPW02a4 } RAPD
5.7  │
     │
     ┴─   m5pca598
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Fig. 4 Linkage mapping for RAPD and CAP markers with the *Br* gene. The linked markers included 3 RAPDs (UBC223a7, OPV02a3 and OPW02a4), 7 CAPs derived from OPW02a4 (W02s2, W02s3, W02s4, W02s6, W02s9, W02s10 and W02s11) and 6 Amplified fragment length polymorphism (AFLP) markers (m5pca382, m1pgg258, mg3pag431, mg3pag425, m1pgg256 and m5pca598)

fragment by blastn suggested high homology with chloroplast complete genome of soybean and other legumes. Alignment by blastx correlated this 1.6 kb fragment to NADH dehydrogenase subunit. Whether these regions play a role in plant defense to insects remains further study.

Discussion

Each RIL material used in this study, after selfing for 12 generation, were uniform in terms of agronomic phenotype in the field. However, their reactions to bruchid infestation obtained in three assays were temperature-dependent. In general, temperatures of 25–30°C are regarded as suitable for growth of bruchid pest (Dongre et al. 1996; Lambrides and Imrie 2000; Jackai and Asante 2003). During the February 2002 assay, mean max/min temperature (26.0°/20.5°C) fell below the range of favorable growth conditions for bruchid, consequently lowered seed damage. Correlation analysis confirmed that the same set of markers was linked to resistance for all three assays. However, these markers expressed their highest R^2 values and genetic effects in the July 2005 assays followed by the May 2003 assay then the February 2002 assay. In the July 2005 assay, mean bruchid damage for mungbean lines carrying homozygous susceptible and resistant alleles were 95% and

9%, respectively (Table 2), while those of the February 2002 assay were 62% and 18%, respectively. Even under environmental conditions unfavorable for bruchid growth and development, the results suggested that these markers were closely linked with the major gene responsible for bruchid resistance. Since 28–30°C is commonly used for bruchid assay, it is reasonable that the developed markers show the highest correlation with data collected in the July 2005 assay when the max/min temperature was 28.5°/25.0°C. The resistance of TC1966 was reported to be under the control of a single dominant allele together with modifier factors (Kitamura et al. 1988). Theoretically, one half of the materials are expected to carry the homozygous R allele, however, only about one fourth expressed stable resistance to the pest insect. The current assay with 200 RILs suggests that expression of bruchid resistance gene could be modified by some environmental factors. Sequences of the polymorphic markers were associated with putative plant disease resistance polyprotein and a retrotransposon protein by blastx. The occurrence of susceptible and resistant progenies from parents whose genotypes were estimated as homozygous R/R and homozygous r/r has been observed occasionally in our previous populations derived from different susceptible parents (VC3890A and VC1973A, our laboratory data) as well as reported by Kitamura et al. (1988). The presence of this retroelement-related gene may partially explain the reason behind high SNP frequency within the marker region and might be a potential reason for variations in bruchid resistance. It is not clear whether retro-element-related genes or NADH dehydrogenase subunit in the resistant line play a role on insect resistance or plant defense mechanism. This remains to be further explored.

In this study, bulked segregant analysis was successfully employed to identify molecular markers linked to a major gene for bruchid resistance. Several of the RAPDs, though not closely linked to *Br*, were allocated to the same linkage group with markers associated with the *Br* gene. This confirms the effectiveness of the bulked segregant approach to identify polymorphism associated with the trait of interested, especially when this is governed by the simple Mendelian inheritance. Conversion of RAPD markers into SCAR or CAP markers has been reported and applied to improve the low reproduc-

ibility of RAPD (Paran and Michelmore 1993). Our study also suggests the possibility of exploring codominant markers through transfer of RAPD into CAP. Besides *C. chinensis*, TC1966 is also known to be resistant to other bruchids, e.g., *C. maculates*, *C. phaseolus, C. analis* and *Z. subfasciatus* (Fuji et al. 1989). It would be interesting to investigate the correlation of the newly developed markers with the resistance to other *Callosobruchus* spp. using the obtained RILs. This will also help clarify whether single or more chemical compounds are responsible for resistance to different storage pests in TC1966.

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