รายงานวิจัยฉบับสมบูรณ์

โครงการพันธุศาสตร์และการปรับปรุงพันธุ์พืชไร่ตระกูลถั่วสำหรับประเทศไทย

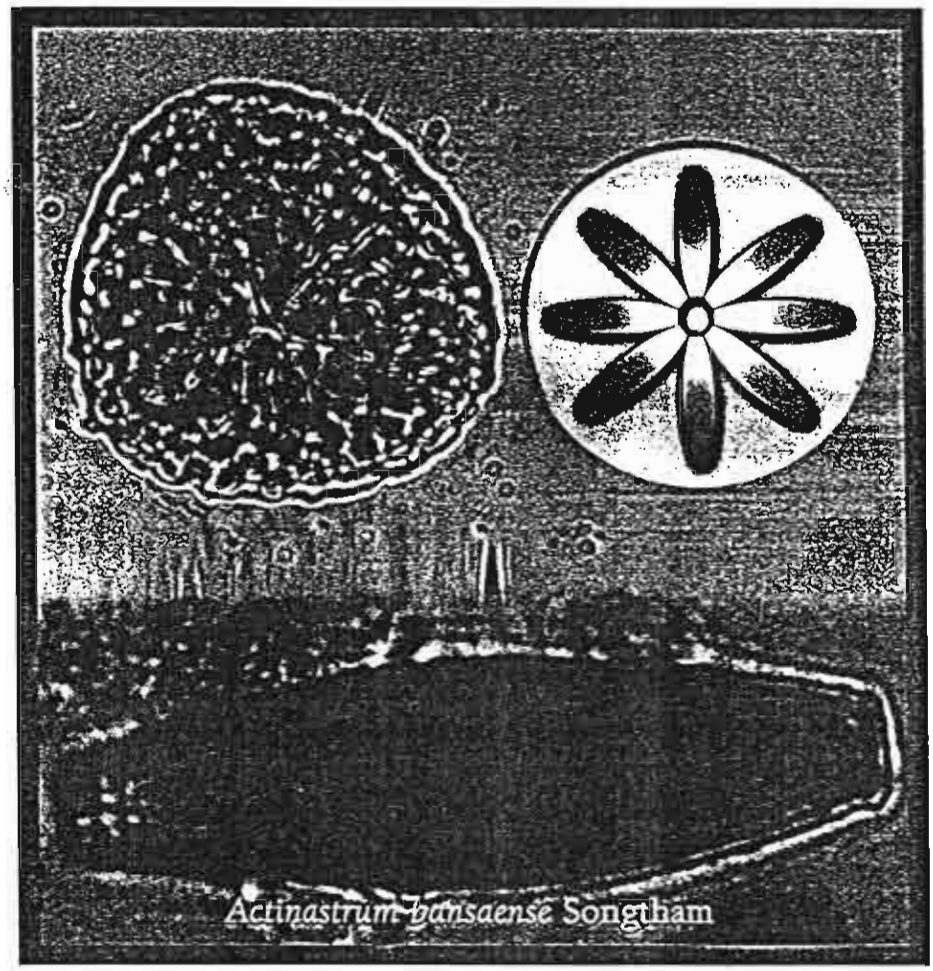
เล่ม 2

โดย ศาสตราจารย์ ดร. พีระศักดิ์ ศรีนิเวศน์ และคณะ

กุมภาพันธ์ 2551.

Science Asia
Journal of the Science Society of Thailand
วารสารของสมาคมวิทยาศาสตร์แห่งประเทศไทย

Vol 30 No 2
June 2004
ISSN 1513-1874
VKSTDB 30(2): 93-209 (2004)



- Bleaching of methylene blue by hydrated titanium dioxide
- Electronic-structure for semiconductor quantum dot
- Ion-pair complexometric determination of CPH
- Convergence analysis of adaptive tabu search
- Mathematical model for pressure controlled ventilation

Science Asia Online http://scienceasia.tiac.or.th

ScienceAsia 30 (2004): 205-209

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

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

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

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

Received 5 Aug 2003
Accepted 25 Feb 2004

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

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

Introduction

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

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

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

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

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

Materials and Methods

Mapping Population

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

Disease Scoring

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

RILs genotyping

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

QTL AnalYsis and Heritability Estimation

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

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

Results

Polymorphism of SSR Markers and Linkage Construction

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

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

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

** significant difference at the probability ≤ .01

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

Heritability Estimation

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

Markers Associated with SDS Sesistance

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

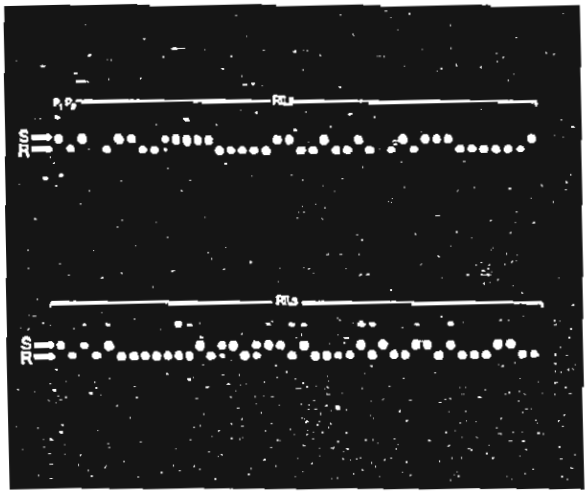


Fig 1. DNA bands obtained from amplification using marker Satt183, separated by 4% metaphore gel and stained by ethidium bromide. Lanes are as follow; lane 1: susceptible parent GC89045-13-1; lane 2: resistant parent GC87012-12-2B-1; the other lanes (above and below) are RILs.

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

Group	Consensus linkage group	SSR markers
1	A1	satt155, satt276, satt454, satt300, satt174, satt200, satt545
2	B1	satt359, sat453
3	B2	satt066, satt304
4	C2	satt100, satt134, satt202, satt277, satt286, satt289, satt307, satt365, satt460
5	D1b+w	sat089, sat135, satt141, satt350, satt546, satt157, satt542, satt549
6	D2	satt301, satt389, satt397, satt514
7	E	sat124, satt263, satt452, satt483, satt598
8	F	satt133, satt114, sct033, satt146, satt269
9	G	sat094, satt012, sat064, satt115, satt138, satt191, satt199, satt288, satt394, satt472, satt517, satt566, satt594
10	H	sat118, satt222, satt469, satt541,satt181, satt302, satt434
11	I	satt062, satt148, satt330, satt292
12	J	satt183, satt456, sct001, sct065, satt285, sct046
13	K	satt167, satt349
14	M	sat121, satt250, satt308, satt323, satt463
15	O	sat173, satt259
Unlinked		satt605, satt509, satt339, sat087, satt113, satt115, satt126, satt129, satt180, satt187, satt192, satt197, satt254, satt257, satt294, satt354, satt422, satt431, satt436, satt488, satt560, satt570, satt571, satt583, satt601

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

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

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

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

The significance of the linked markers was individually analysed by a t-test with unequal variances among the RILs carrying the SSR bands from resistant vs. susceptible parents. All four markers showed significant difference in mean disease score between both groups of RILs (Table 3). The probabilities of t' (significance level at an effective df) for markers Satt183, Satt456, Sct065 and Sct001 were 0.0031, 0.0117, 0.0055 and 0.0073, respectively.

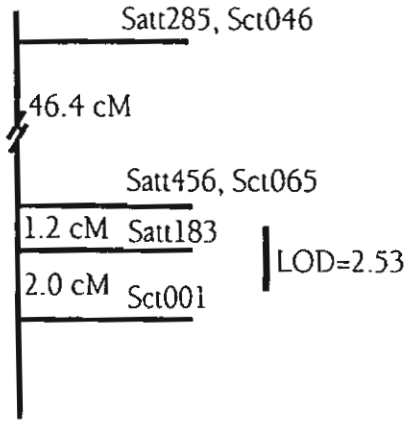


Fig 2. Location of SSR markers on the linkage group J of the soybean genome and the new QTL underlying resistance to SDS.

Discussion

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

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

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

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

Acknowledgements

The authors are grateful to Prof. Dr. Oval Myers Jr., Dr. David A. Lightfoot and the staff members of the Department of Plant, Soil and General Agriculture, Southern Illinois University at Carbondale for laboratory and greenhouse work, the Asian Regional Center of AVRDC at Kasetsart University, Nakhon Pathom, Thailand for seeds and population construction. This research was supported by the Thailand Research Fund, through the KU Legume R&D Project and the Royal Golden Jubilee Ph.D. Scholarship to the first author.

References

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

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Scientia Horticulturae 106 (2005) 137–146
SCIENTIA HORTICULTURAE
www.elsevier.com/locate/scihorti

Genetic diversity in yardlong bean (*Vigna unguiculata* ssp. *sesquipedalis*) and related *Vigna* species using sequence tagged microsatellite site analysis

P. Phansak [a], P.W.J. Taylor [b], O. Mongkolporn [c,*]

[a] *Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[b] *BioMarka, School of Agriculture and Food Systems, Institute of Land and Food Resources, The University of Melbourne, Vic. 3010, Australia*
[c] *Department of Horticulture, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*

Received 15 March 2004; received in revised form 10 March 2005; accepted 17 March 2005

Abstract

A high level of genetic diversity was identified within 15 accessions of yardlong bean from Thailand, Bangladesh, China, Laos, Philippines and Taiwan using STMS analysis based on cowpea *Vigna unguiculata* ssp. *unguiculata* primer-pairs. Accessions clustered into three distinct groups, at a 0.67 coefficient of similarity, with no correlation to geographical origins. Three pairs of accessions appeared to be similar indicating that each pair was of the same accession and most likely originated from the same geographical location. STMS primers designed from the cowpea genome were highly transferable to other *Vigna* species. STMS analysis with these primers also revealed a very high level of variation between six *Vigna* species, which clustered into three groups at a 0.5 coefficient of similarity. Group A represented the African *Vigna* species yardlong bean and cowpea, while groups B (mungbean, blackgram and moth bean) and C (rice bean and adzuki bean) represented the Asian *Vigna* species.

Keywords: Yardlong bean; *Vigna* species; Sequence tagged microsatellite site (STMS); Genetic diversity

* Corresponding author. Tel.: +66 34 282494 8x421; fax: +66 34 282494 8x117.
E-mail address: orarat.m@ku.ac.th (O. Mongkolporn).

0304-4238/$ – see front matter
doi:10.1016/j.scienta.2005.03.010

1. Introduction

The legume genus *Vigna* comprises about 75–80 species originating from regions of Africa, America and Asia. The genus has been subdivided into seven subgenera based on morphology and their centers of origin (Maréchal et al., 1978, 1981). Two of these subgenera contain important grain legumes classified by centers of origin: African *Vigna* species (*Vigna* group) and Asian *Vigna* species (*Ceratotropis* group). Group *Vigna* contains the important species of *V. unguiculate*, which contains the subspecies *unguiculata* (cowpea) and *sesquipedalis* (yardlong bean). Cowpea is believed to have originated in Africa with greatest diversity in western parts of Africa and India, while yardlong bean is found mostly cultivated in southeast Asia (Maréchal et al., 1978; Pandey and Westphal, 1989; Takeya and Tomooka, 1997; Xu and Zong, 1993). Group *Ceratotropis* comprises 16 species, of which 10 are wild species and 6 are cultivated pulses including adzuki bean (*V. angularis*), rice bean (*V. umbellata*), mungbean (*V. radiata*), blackgram (*V. mungo*), bambara groundnut (*V. subterranea*) and moth bean (*V. aconitifolia*).

The Tropical Vegetable Research Center (TVRC), Kasetsart University, Thailand, has a large collection of yardlong bean germplasm from within the country and from other countries within Asia. The germplasm has been morphologically characterized and found to contain variation among accessions. However, morphological studies alone do not provide sufficient information to understand genetic diversity within the species, as well as relatedness to related species. Molecular analysis can provide additional information on genetic diversity that would be useful for breeding programs through selection of diverse parents.

Molecular analysis of germplasm diversity would provide information to plant improvement programs regarding the level of genetic variation within and between species. This information would then be used to select diverse parents of the same species or to identify the most closely related parents for interspecific crossing, to increase heterosis and incorporate desirable genes from more diverse backgrounds into elite germplasm (Henry, 1997).

Several molecular marker techniques have been used to measure genetic diversity of a range of plant species. Random amplified polymorphic DNA (RAPD) was first developed by Williams et al. (1990) and is still among the most widely used because the technique is simple and relatively inexpensive (Harris, 1999). RAPD analysis has proven effective for diversity studies in several legume species, including mungbean (Kaga et al., 1996; Santalla et al., 1998), adzuki bean (Yee et al., 1999), blackgram (Kaga et al., 1996) and cowpea (Mignouna et al., 1998).

Microsatellites, or simple sequence repeats (SSR), are tandem repeats of very short DNA sequences (1–6 bp) (Akkaya et al., 1992). They are abundantly found in eukaryotes (Hamann et al., 1995; Powell et al., 1995; Yu et al., 1999). The variability in microsatellite sequences can be detected by a PCR-based technique, sequence tagged microsatellite site (STMS), which uses primers designed to the flanking sequences to amplify the internal repeat sequences (Beckmann and Soller, 1990). The resulting locus-specific amplification products often exhibit considerable length differences due to variations in the number of repeats (Gupta et al., 1996; Hüttel et al., 1999; Weising et al., 1992). STMS analysis has

been used to study genetic diversity in various legume species such as mungbean (Yu et al., 1999), cowpea (Li et al., 2001), chickpea (Choumane et al., 2000) and field pea (Ford et al., 2002).

The objectives of this study were to determine the level of genetic variation among yardlong bean accessions originating from Thailand, Philippines, China, Bangladesh, Laos and Taiwan and to analyse the genetic relatedness between yardlong bean to six other cultivated *Vigna* species, including mungbean, blackgram, rice bean, adzuki bean, moth bean and cowpea.

2. Materials and methods

2.1. Plant material

A total of 15 accessions of yardlong bean and 6 cultivated *Vigna* species, originating from seven Asian countries, were provided by the TVRC, Kasetsart University, Thailand (Table 1). Total genomic DNA was extracted from three plants per accession using the modified CTAB method of Taylor et al. (1995).

Table 1
Fifteen yardlong bean accessions and six *Vigna* species originating from the TVRC collection at Kasetsart University, Thailand

Common name	Accession	Species	Country
African *Vigna*			
Yardlong bean	VU017	*V. unguiculata* ssp. *sesquipedalis*	Thailand
	VU162		Thailand
	VU157		Thailand
	VU163		Philippines
	VU164		Philippines
	VU169		Philippines
	VU161		China
	VU189		China
	VU196		China
	VU173		Bangladesh
	VU174		Bangladesh
	VU176		Bangladesh
	VU014		Laos
	VU199		Taiwan
	VU200		Taiwan
Cowpea	VS001	*V. unguiculata* ssp. *unguiculate*	Thailand
Asian *Vigna*			
Mungbean	UT1	*V. radiata*	Thailand
Blackgram	PSL2	*V. mungo*	Thailand
Rice bean	Miyazaki	*V. umbellate*	Japan
Adzuki bean	Adzuki	*V. angularis*	Japan
Moth bean	Mothbean	*V. aconotifolia*	Taiwan

2.2. PCR amplification

Twenty-seven pairs of cowpea STMS primers developed by Li et al. (2001) were used in STMS PCR assays. Each 25 μl PCR reaction contained 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 3 mM $MgCl_2$, 0.24 mM of each dNTPs, 0.3 μM of each forward and reverse primer, 50 ng of genomic DNA and 0.5 units of DNA polymerase DyNAzyme™II (Finnzyme, Finland). PCR reactions were performed as follows: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturing at 94 °C for 1 min; annealing temperature at 60–65 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min.

The amplified products were separated on 6% denaturing polyacrylamide gels run in TBE (Tris-base, boric acid, EDTA) buffer. The gels were stained with silver nitrate for 30 min. The Phi174/*Hin*fI DNA ladder (Promega, USA) was used as standard molecular weight marker.

2.3. Genetic diversity analysis

Clearly distinguishable amplified bands were scored as '1' and '0' based on band (allele) presence and absence, respectively. Sizes of amplified bands were estimated using PhotoCaptMW® (Vilber Lourmat, France). Pair-wise similarities were obtained using the simple matching (SM) coefficients (Legendre and Legendre, 1983) within NTSYS-pc Version 2.0 (Rohlf, 1998). The similarity matrix was used in cluster analysis with the unweighted pair-group method with the arithmetic average (UPGMA) to demonstrate genetic relationships among the accessions and species.

Table 2
List of 16 cowpea STMS primers developed by Li et al. (2001) and the number of detected alleles in yardlong bean and related *Vigna* species

Number	STMS primers	Number of alleles						
		Yardlong bean[a]	Cowpea	Mung bean	Black gram	Rice bean	Adzuki bean	Moth bean
1	VM22	1	1	1	1	1	1	1
2	VM26	3	1	0	0	0	0	0
3	VM27	1	1	1	1	1	1	1
4	VM28	1	1	0	0	0	0	0
5	VM30	1	1	0	0	0	0	0
6	VM31	5	1	1	1	1	1	0
7	VM33	1	1	1	1	1	1	0
8	VM34	1	1	0	0	0	0	0
9	VM35	3	1	0	0	0	0	0
10	VM36	1	1	0	0	0	0	0
11	VM37	2	1	1	1	1	1	1
12	VM39	3	1	0	0	0	0	0
13	VM40	1	1	0	0	0	0	0
14	VM68	3	1	1	1	1	1	0
15	VM70	6	1	1	1	1	1	1
16	VM71	1	1	0	0	0	0	0

[a] Representing number of alleles detected by STMS primers in 15 yardlong bean accessions and 1 accession of each of the other *Vigna* species.

Table 3
Similarity matrix of 15 yardlong bean accessions generated from STMS analysis

Accession	VU017	VU157	VU162	VU014	VU161	VU164	VU169	VU163	VU174	VU176	VU173	VU200	VU199	VU196	VU189
VU017	1.00														
VU157	0.67	1.00													
VU162	0.58	0.67	1.00												
VU014	0.50	0.58	0.67	1.00											
VU161	0.67	0.92	0.67	0.58	1.00										
VU164	0.75	0.92	0.67	0.58	0.92	1.00									
VU169	0.50	0.67	0.75	0.58	0.75	0.67	1.00								
VU163	0.75	0.92	0.67	0.58	0.92	1.00	0.67	1.00							
VU174	0.50	0.75	0.67	0.75	0.75	0.75	0.67	0.75	1.00						
VU176	0.50	0.75	0.67	0.58	0.75	0.75	0.83	0.75	0.83	1.00					
VU173	0.50	0.75	0.67	0.75	0.75	0.75	0.67	0.75	1.00	0.83	1.00				
VU200	0.58	0.58	0.50	0.50	0.58	0.58	0.42	0.58	0.58	0.42	0.58	1.00			
VU199	0.75	0.42	0.33	0.33	0.42	0.50	0.33	0.50	0.33	0.33	0.33	0.5	1.00		
VU196	0.58	0.58	0.50	0.50	0.58	0.58	0.42	0.58	0.58	0.42	0.58	1.00	0.5	1.00	
VU189	0.75	0.83	0.67	0.50	0.83	0.83	0.58	0.83	0.67	0.67	0.67	0.75	0.50	0.75	1.00

3. Results

3.1. Genetic diversity of yardlong bean

Of the total 27 STMS primer-pairs screened, 16 STMS primer-pairs successfully amplified a usable DNA marker from all yardlong bean accessions. Of these, seven markers were polymorphic (Table 2). The level of polymorphism detected per primer pair varied from 2 to 6 alleles with an average of 3.4 alleles. The similarity detected among accessions at these loci ranged from 33 to 100% (Table 3). The dendogram demonstrated that three accession clusters existed when the similarity scale was intersected at a value of 0.67 on the 0–1 scale where 1: complete similarity (Fig. 1). Accessions 'VU017' from Thailand and 'VU199' from Taiwan clustered together A, 'VU200' from Taiwan and 'VU196' from China formed another cluster C, and the rest formed cluster B. The STMS profiles for accession pairs 'VU163' and 'VU164' from Philippines, 'VU173' and 'VU174' from Bangladesh, and 'VU200' and 'VU196' from Taiwan and China, were identical to each other.

3.2. Genetic relationship between yardlong bean and related Vigna species

Nine of the 16 STMS primer-pairs assessed were only able to amplify genomic DNA of yardlong bean and cowpea, but not from the other species. However, four STMS primer-

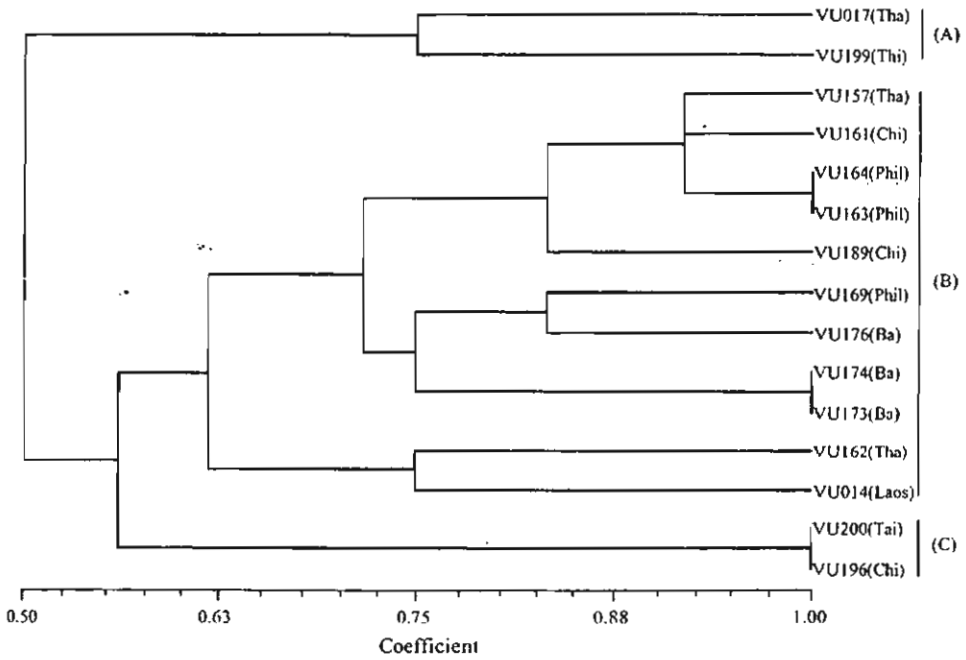


Fig. 1. UPGMA dendrogram of 15 yardlong bean accessions generated from STMS analysis.

Table 4
Similarity matrix of six *Vigna* species generated from STMS analysis

	Yardlong bean	Cowpea	Mungbean	Blackgram	Rice bean	Adzuki bean	Moth bean
Yardlong bean	1.00						
Cowpea	0.93	1.00					
Mungbean	0.00	0.00	1.00				
Blackgram	0.07	0.07	0.87	1.00			
Rice bean	0.00	0.00	0.53	0.53	1.00		
Adzuki bean	0.00	0.00	0.53	0.53	0.67	1.00	
Moth bean	0.00	0.00	0.60	0.67	0.53	0.60	1.00

pairs (VM22, VM27, VM37 and VM70) that amplified clear bands with yardlong bean DNA, could amplify DNA from all *Vigna* species and a further three amplified DNA from all species except for moth bean (Table 2). Where a band was not amplified, the locus was considered to be absent; therefore, the diversity among the *Vigna* species was analysed by incorporating data from all 16 primer pairs with genetic similarity ranging from 0 to 93% (Table 4). The *Vigna* species clustered into three groups (Fig. 2), when the dendrogram was transected at 0.5 on the 0–1 scale. Cluster A consisted of cowpea and yardlong bean accessions representing the African *Vigna*. Clusters B and C contained all other accessions representing the Asian *Vigna*. Cluster B contained mungbean, blackgram and moth bean, and cluster C contained adzuki bean and rice bean.

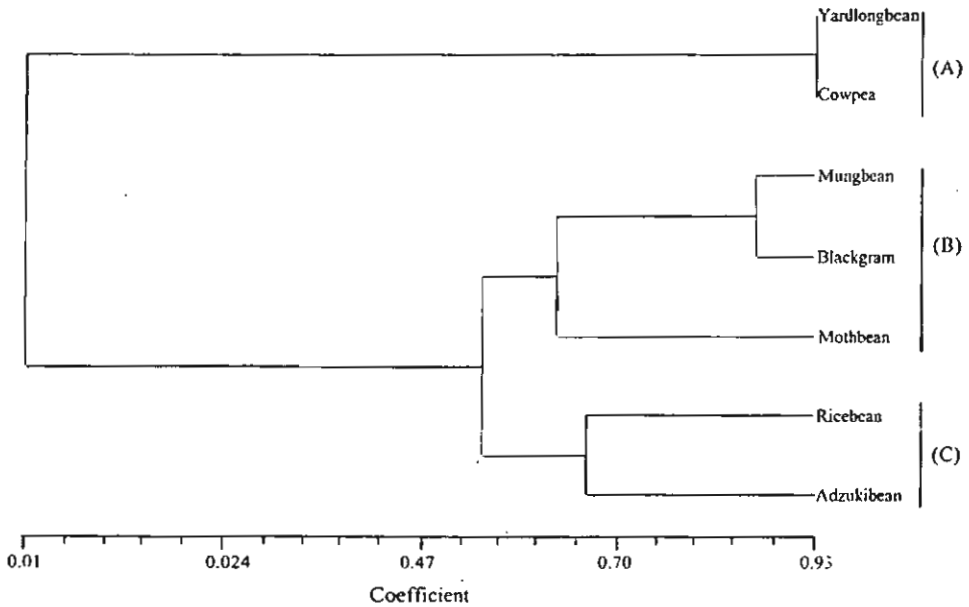


Fig. 2. UPGMA dendrogram of yardlong bean and six related *Vigna* species, generated from STMS analysis.

4. Discussion

4.1. Transferability of cowpea STMS to other Vigna species

The STMS primers used in this study originated from cowpea with 60% being able to amplify genomic DNA from all of the yardlong bean accessions assessed. The high transferability of cowpea STMS primers to amplify yardlong bean DNA was not surprising since these are subspecies of the same species *V. unguiculata*. Nevertheless, 44% of the cowpea STMS primers that amplified yardlong bean DNA were also able to amplify DNA from mungbean, blackgram, rice bean and adzuki bean, while only 25% could amplify moth bean DNA as well. This indicated a level of microsatellite sequence conservation among species. The low level of transferability of cowpea STMS primers to moth bean was reflected in the low level of genetic similarity between moth bean, and cowpea and yardlong bean.

Transferability of STMS primer sequences has recently been shown across other legume species (Choumane et al., 2000; Ford et al., 2002; Pandian et al., 2000). Pandian et al. (2000) reported that 21 of 22 field pea (*Pisum sativum*) STMS primers tested amplified genomic DNA in cultivars of *Vicia*, *Lens* and *Cicer* species, while 11 out of 22 chickpea (*Cicer arietinum*) STMS primers tested produced fragments in cultivars of *Vicia*, *Lens* and *Pisum* species.

4.2. Genetic diversity of yardlong bean

The level of genetic similarity detected among the 15 accessions of yardlong bean, based on allele frequency, varied from 33 to 100% using STMS analysis. The range of variation detected using STMS primers was quite high compared to previous studies that have used STMS to detect variation within species of legumes (Li et al., 2001; Yu et al., 1999). STMS primers are specific in detecting repetitive DNA in the genome. Microsatellite sequences are abundant throughout plant genomes, are hypervariable and are thus more likely to mutate through evolutionary forces (Cregan et al., 1994). STMS analysis separated the yardlong bean accessions into three groups; however, the clustering of accessions was not correlated to geographical origin. Yardlong bean is found widely cultivated in Asia, especially southeast Asia with accessions exhibiting a high level of morphological diversity.

Three pairs of accessions appeared to be identical, which were 'VU163' and 'VU164' from Philippines, 'VU173' and 'VU174' from Bangladesh, and 'VU200' and 'VU196' from Taiwan and China, respectively. Interestingly, accessions 'VU200' and 'VU196' originated from different countries but were identical. The reason for this may be that they were collected from the same geographical location but were given different accession numbers or alternatively, the same accessions were sent to different germplasm collections before being forwarded to the TVRC collection in Thailand. The degree of genetic diversity detected by STMS analysis correlated with the level of morphological diversity for these accessions (TVRC Report, unpublished data). Germplasm passport data such as: stem colour, internode colour, leaf shape, colour of flower keel, colour of flower wings, pod colour and seed coat texture of these accessions showed that there were no differences within each pair of similar accessions.

4.3. Genetic relationship between yardlong bean and related Vigna species

STMS analysis clearly separated *Vigna* species into the African *Vigna* (cowpea and yardlong bean) and Asian *Vigna* (mungbean, blackgram, rice bean, adzuki bean and moth bean) groups. These results correlated with taxonomic relationships in *Vigna* established using RAPD and RFLP analysis (Fatokun et al., 1993; Kaga et al., 1996) where the Asian and African *Vigna* groups were clearly differentiated. Within the Asian *Vigna* group, mungbean and blackgram clustered closely to moth bean; while rice bean and adzuki bean clustered together. The STMS analysis of *Vigna* species was based on only one accession per species. Further analysis using several accessions per species may produce a more accurate identification of the genetic diversity between these species.

Acknowledgments

This project was part of the Legume Cultivar Research and Development Project of the Thailand Research Fund. The authors thank Dr. Hugo Volkaert, Center for Agricultural Biotechnology, Kasetsart University, for his invaluable suggestions regarding data analysis; Dr. Rebecca Ford, BioMarka, the University of Melbourne for comments on the manuscript and the Tropical Vegetable Research Center for supplying the germplasm used in this study and also for the nursery facilities.

References

Akkaya, M.S., Bhagwat, A.A., Cregan, P.B., 1992. Length polymorphisms of simple sequence repeat DNA in soybean. Genetics 132, 1131–1139.

Beckmann, J.S., Soller, M., 1990. Towards a unified approach to genetic mapping of eucaryotes based on sequence tagged microsatellite sites. Biotechnology 8, 930–932.

Choumane, W., Winter, P., Weigand, F., Kahl, G., 2000. Conservation and variability of sequence tagged microsattellite sites (STMS) from chickpea (*Cicer aerietinum* L.) within the genus *Cicer*. Theor. Appl. Genet. 101, 269–278.

Cregan, P.B., Bhagwat, A.A., Akkaya, M.S., Rongwen, J., 1994. Microsatellite fingerprinting and mapping of soybean. Methods Mol. Cell. Biol. 5, 49–61.

Fatokun, C.A., Danesh, D., Young, N.D., Stewart, E.L., 1993. Molecular taxonomic relationships in the genus *Vigna* based on RFLP analysis. Theor. Appl. Genet. 86, 97–104.

Ford, R., Le Roux, K., Itman, K., Brouwer, J.B., Taylor, P.W.J., 2002. Genome-specific sequence tagged microsatellite site (STMS) markers for diversity analysis and genotyping in *Pisum* species. Euphytica 124, 397–405.

Gupta, P.K., Balyan, H.S., Sharma, P.C., Ramesh, B., 1996. Microsatellites in plants: a new class of molecular markers. Curr. Sci. 70, 45–54.

Hamann, A., Zink, D., Nagl, W., 1995. Microsatellite fingerprinting in the genus *Phaseolus*. Genome 38, 507–515.

Harris, S.A., 1999. RAPDs in systematics—a useful methodology. In: Hollingsworth, P.M., Bateman, R.M., Gornall, R.J. (Eds.), Molecular Systematics and Plant Evolution. Taylor & Francis, London, pp. 211–228.

Henry, R., 1997. Practical Applications of Plant Molecular Biology. Chapman & Hall, London.

Hüttel, B., Winter, P., Weising, K., Choumane, W., Weigand, F., Kahl, G., 1999. Sequence tagged microsatellite site markers for chickpea (*Cicer arietinum* L.). Genome 42, 210–217.

Kaga, A., Tomooka, N., Egawa, Y., Hosaka, K., Kamijima, O., 1996. Species relationships in the subgenus *Ceratotropis* (genus *Vigna*) as revealed by RAPD analysis. Euphytica 88, 17–24.

Legendre, P., Legendre, L., 1983. Numerical Ecology, first English ed. Elsevier, Amsterdam.

Li, C.-D., Fatokun, C.A., Ubi, B., Singh, B.B., Scoles, G.J., 2001. Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. Crop Sci. 41, 189–197.

Maréchal, R., Mascherpa, J.M., Stainier, F., 1978. Etude taxonomique d'un groupe complexed' espéces des genres *Phaseolus et Vigna* (Papilionaceae) sur la base de données morpholpgiques et polliniques, traitées par l' analyse informatique. Boissiera 28, 1–273.

Maréchal, R., Mascherpa, J.M., Stainier, F., 1981. Taxonometric study of the *Phaseolus–Vigna* complex and related genera.. In: Polhill, R.M., Raven, P.H. (Eds.). Advances in Legume Systematics. The Royal Botanic Garden, Kew. pp. 329–335.

Mignouna, H.D., Ng, N.Q., Ikea, J., Thottapilly, G., 1998. Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. J. Genet. Breed. 53, 151–159.

Pandey, R.K., Westphal, E., 1989. *Vigna unguiculata* (L.) Walp.. In: Van der Maesen, L.J.G., Somaatmadja, S. (Eds.), Plant Resources of South-East Asia (PROSEA) No. 1: Pulses. Pudoc Wageningen, Wageningen, pp. 77–81.

Pandian, A., Ford, R., Taylor, P.W.J., 2000. Transferability of sequence tagged microsatellite site (STMS) primers across four major pulses. Plant Mol. Biol. Rep. 18, 395a–395h.

Powell, W., Morgante, M., Andre, C., McNicol, J.W., Machray, G.C., Doyle, J.J., Tingey, S.V., Rafalski, J.A., 1995. Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. Curr. Biol. 5, 1023–1029.

Rohlf, F.J., 1998. NTSYS-PC Numerical Taxonomy and Multivariate Analysis System. Version 2.0. Exter Publications, Setauket, NY.

Santalla, M., Power, J.B., Davey, M.R., 1998. Genetic diversity in mungbean germplasm revealed by RAPD markers. Plant Breed. 117, 473–478.

Takeya, M., Tomooka, N., 1997. The Illustrated Legume Genetic Resources Database on The World Wide Web. Miscellaneous Publication of National Institute of Agrobiological Resources, Ibaraki, Japan, No. 11.

Taylor, P.W.J., Fraser, T.A., Ko, H.L., Henry, R.J., 1995. RAPD analysis of sugarcane during tissue culture. In: Terzi, M., Cella, R., Falavigna, A. (Eds.), Current Issues in Plant Molecular and Cellular Biology. Kluwer Academic, Dordrecht, pp. 241–246.

Weising, K., Kaemmer, D., Weigand, F., Epplen, J.T., Kahl, G., 1992. Oligonucleotide fingerprinting reveals various probe-dependent levels of informativeness in chickpea (*Cicer arietinum* L.). Genome 35, 436–442.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18, 6531–6535.

Xu, W.S., Zong, X.X., 1993. Yardlong bean. In: Chomchalow, N., Gowda, C.L.L., Laosuwan, P. (Eds.), Proceedings of the FAO/UNDP Project RAS/89/040 Workshop on Underexploited and Potential Food Legumes in Asia. Chiang Mai, Thailand, pp. 116–119.

Yee, E., Kidwell, K.K., Sills, G.R., Lumpkin, T.A., 1999. Diversity among selected *Vigna angularis* (adzuki) accessions on basis of RAPD and AFLP markers. Crop Sci. 39, 268–275.

Yu, K., Park, S.J., Poysa, V., 1999. Abundance and variation of microsatellite DNA sequences in bean (*Phaseolus* and *Vigna*). Genome 42, 27–34.

Science Asia
Journal of the Science Society of Thailand
วารสารของสมาคมวิทยาศาสตร์แห่งประเทศไทย

Vol 31 No 3
September 2005
ISSN 1513-1874 CODEN: SCIASFZ
31(3): 203-317 (2005)

- Electronic conducting polymer composite films
- Korat clays as raw materials for lightweight aggregates
- Pathogenesis of *Nomuraea rileyi* against *Spodoptera litura*
- Preservation of banana blossom as a dehydrated vegetable
- Ocean wave forecasting in Thailand Gulf during typhoon Linda

ScienceAsia Online http://www.scienceasia.org

RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2005.31.251

ScienceAsia 31 (2005): 251-255

Gamma Radiation Induced Mutations in Mungbean

Chontira Sangsiri,[a] Worawit Sorajjapinun,[b] and Peerasak Srinives[c*]

[a] Center for Agricultural Biotechnology Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
[b] Asian Regional Center AVRDC - the World Vegetable Center, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
[c] Department of Agronomy Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.

* Corresponding author, E-mail: agrpss@yahoo.com

Received 6 Jan 2005
Accepted 25 May 2005

Abstract: Seeds of mungbean varieties KPS 2, VC 6468-11-1B, their F_1 and F_2 were treated with gamma rays (Cs-137 source) at the dose of 500 Gy. The M_1 seeds were sown in the field with the controls (non-irradiated seeds) and bulk-harvested. The M_2 seeds were sown to observe their characters and number of mutants in each population. Among over 430,000 plants observed, irradiated F_1 population gave the highest frequency of mutants at 0.168%, followed by F_2, VC 6468-11-1B, and KPS 2 at 0.165%, 0.152%, and 0.142%, respectively. Mutant characters were grouped as chlorophyll, leaf, flower, and pod mutants. Chlorophyll mutations included albino, coppery leaf, light-green leaf, variegated leaf, waxy leaf, white streak leaf, and xantha leaf. Leaf mutations were lanceolate leaf, narrow-rugose leaf, multiple leaflet, round-cuneate leaf, unifoliate leaf, and wrinkled leaf. The flower mutant was cock's comb raceme while the pod mutant was a lobed one. All mutants were purified for genetic study and possible uses of the traits.

Keywords: *Vigna radiata*, mungbean, gamma rays, mutants.

Introduction

Mungbean (*Vigna radiata* (L.) Wilczek) (2n=2x=22) is a self-pollinated legume originated in South Asia. It is an economically important crop in India, Pakistan, Thailand, Vietnam, Myanmar, and China with the combined planted area of over 5 million ha. The crop is considered rather wild as it still gives low seed yield (<1 t/ha), with uneven maturity. This opens an ample room for mungbean breeders to improve the crop. Besides natural genetic variation available in mungbean germplasm collections, mutation techniques are proven useful in obtaining novel traits and creating genetic variability. Gamma irradiation as a mutagen can induce useful as well as harmful mutation in plants[1,2]. Singh and Sharma[3] isolated a few pentafoliate and tetrafoliate mutants from the gamma rays- and ethyl methanesulphonate (EMS) - treated mungbean. These mutants showed a significant increase in dry matter production, total chlorophyll content and yield, as compared to their parents in M_2 and M_3 generations. Santos[4], and Bahl and Gupta[5] described the mutant characters and their inheritance in mungbean and reported that variegated, multifoliata, xantha, chlorina, albino, unifoliata were each controlled by a recessive gene. Variation in quantitative traits by mutation breeding was also reported by several scientists[6-11]. The major traits were seed yield, seed size, pods per plant, seeds per pod, days to maturity, and plant height. Additionally, Wongpiyasatid *et al*[12] reported an improvement in resistance to powdery mildew, Cercospora leaf spot, and cowpea weevil through gamma radiation induced mutation.

The objective of this study is to induce mutation in four mungbean populations using gamma radiation to determine the mutation frequency, observe the mutant traits and purify them for possible uses.

Materials and Methods

Seeds of the parental lines, 'Kamphaeng Saen 2' (KPS 2) designated as P_1, and VC 6468-11-1B designated as P_2 were obtained from the Asian Region Center of the Asian Vegetable Research and Development Center (ARC-AVRDC), Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand. KPS 2 is a popular Thai mungbean cultivar sown over 150,000 ha annually, owing to its high yielding, shiny seed coat with moderately large seed size (~66 g per 1000 seeds), green hypocotyl, and moderately resistant to powdery mildew and Cercospora leaf spot diseases. VC 6468-11-1B is an elite breeding line with a dull seed coat and a large seed size (~70 g per 1000 seeds), purple hypocotyl, and resistant to both diseases.

Crosses were made using KPS 2 as the female parent. The parents and F_1 seeds were sown in the successive

season. All F_1 seedlings had purple cotyledons confirming that they were derived from crossed seeds, since the purple hypocotyl is dominant to the green one. Another set of F_1 seed was also made in parallel to the production of F_2 seeds. Thus, all four mungbean populations (P_1, P_2, F_1 and F_2) were finally obtained in that same season. The initial M_0 seeds were determined for germination percentage in each population and converted to the seed weight of 156, 187, 159, and 212 g for KPS 2, VC6468-11-1B, F_1 and F_2 respectively. Each amount is equivalent to ~2500 seeds that can readily germinate.

The gamma irradiator used in this study is installed at the Gamma Irradiation Service and Nuclear Technology Research Center (GISC), Kasetsart University, Bangkok. It was manufactured by J.L. Shepherd & Associates, under the Model MARK I-30, Serial No. 1116, loaded with 4500 Curies of Cs-137 having a half-life of 30.12 years. The gamma irradiator was calibrated to irradiate 500 Gy of gamma rays to the seed lots for 82 minutes. The rate of 500 Gy was found to produce much variance while leaving over 60% of the surviving plants[13]. The M_1 seeds were sown in the field surrounded by non-irradiated population as the control. The M_2 seeds were bulk-harvested in each population. There were 7.76, 5.12, 11.02, and 8.72 kg from KPS 2, VC6468-11-1B, F_1, and F_2, respectively. The seeds were drilled in rows, after which the mutants were periodically observed right after germination. In each visit to the field, the mutant plants were marked with bamboo sticks for subsequent observations. Data were recorded on characters and number of the

Table 1. Amount of M_2 mungbean seed sown, number of seedlings germinated, and number of mutants found in the populations of KPS 2, VC6468-11-1B, their F_1 and F_2.

Populations	M_2 seeds sown (kg)	No. of seedlings germinated	Mutant Type					Total	Percent of mutants
			Albino(lethal)	Chlorophyll	Leaf type	Flower	Pod		
KPS	27.76	127,880	113	27	35	0	7	182	0.143
VC6468-11-1B	5.12	81,708	45	26	45	0	8	124	0.152
F_1	11.02	134,607	164	16	35	1	10	226	0.168
F_2	8.72	89,647	105	8	29	0	6	148	0.165
Total	32.62	433,842	427	77	144	1	31	680	0.157

Table 2. Types and number of mutants found in M_2 plants of the four mungbean populations.

Mutant characters	Populations				Total
	KPS 2	VC 6468-11-1B	F_1	F_2	
1. Chlorophyll mutation					
Albino	113	45	164	105	427
Coppery leaf	1	0	0	0	1
Light green leaf	2	2	3	0	7
Variegated leaf	2	3	4	3	12
Waxy leaf	2	6	5	0	13
White streak leaf	1	2	2	3	8
Xantha leaf	19	13	2	2	36
2. Leaflet mutation					
Lanceolate leaflet	2	2	2	0	6
Multiple leaflet	29	37	29	27	122
Narrow-rugose leaflet	2	1	0	0	3
Round-cuneat leaflet	0	0	0	1	1
Unifoliate leaf	2	0	0	1	3
Wrinkled leaf	0	5	4	0	9
3. Flower mutation					
Cock's comb raceme	0	0	1	0	1
4. Pod mutation					
Lobed pod	7	8	10	6	31
Total	182	124	226	148	680

Table 3. Description of the mutant characters found in M_2 plants of the four mungbean populations.

Mutant characters	Character descriptions
1. Chlorophyll mutation	
Albino	Entirely white leaves. Seedlings survived for less than 2 weeks after germination
Coppery leaf	Copper-like color leaflet beginning from flowering till harvesting
Light-green leaf	Lighter green leaves as compared to normal leaves
Variegated leaf	Persistent variegated yellow-green leaves
Waxy leaf	Normal leaf shape with pale waxy leaflet
White streak leaf	White streak from edge to middle vein
Xantha	Orange yellow to light yellowish white, survived for only 2-3 weeks after germination
2. Leaflet mutation	
Lanceolate leaf	Elongated middle leaflet with broader lateral leaflets
Multiple leaf	Compound leaf with 4 - 9 leaflets per leaf
Narrow-rugose leaf	Narrow and elongated leaflet
Round-cuneat leaf	Short petiole, round leaf, did not set pod
Unifoliate leaf	Single leaf, did not set pod
Wrinkled leaf	Leaf has wrinkled character
3. Flower mutation	
Cock's comb raceme	Raceme look like cock's comb, did not set pod
4. Pod mutation	
Lobed pod	Distinct lobes on pod possibly due to semi-sterility

mutants. At maturity, each mutant plant was individually harvested. The remaining plants were bulk-harvested for M_3 seeds and sown for further observation.

Field cultural practices on this experiment were conducted based on standard management for mungbean grown in Thailand. Briefly, the seeds were drilled in rows of 50 cm apart at the rate of 20 seeds per a meter. Weeds were controlled by pre-emergence spraying of Imazathapyr at 250 g(ai)/ha. Late weeds were eradicated by hand weeding twice at 15 and 30 days after sowing. Insects were controlled by spraying with triazophose (Hostathion 40% EC) at the rate of 40 cc per 20 liters of water when the insect population was building up beyond the threshold level. Irrigation water was applied during the cropping season as needed.

Results and Discussion

Since the gamma rate of 500 Gy was almost at Lethal Dose-50 (LD-50) for mungbean[13], the M_1 seed lost its germination up to 20-30% from the effect of irradiation. Some seedlings showed either albino or xantha leaf, and died prematurely. A number of mutant plants were identified in M_2 generation and the mutation percentages in KPS 2, VC6468-11-1B, F_1 and F_2 population were 0.142, 0.152, 0.168, and 0.165, respectively (Table 1). The percentages were much smaller than that reported by Srichot[13] and Thongpimyn[14] who found the mutant rate of up to 1-4% in both qualitative and quantitative traits. In our experiment, no distinct mutant plants were found regarding yield components, possibly due to such a low mutant rate.

The mutants found were mainly of leaf chlorophyll mutation such as albino, coppery leaf, light-green leaf, variegated leaf, waxy leaf, white streak leaf, and xantha leaf. Leaf mutations were lanceolate leaflet, narrow-rugose leaflet, multiple leaflet, round-cuneat leaflet, unifoliate leaf and wrinkled leaf. Flower mutation gave looks like cock's comb with pollen sterility. Similar mutants were also reported by Lamseejan *et al*[15], Santos[4], and Srichot[13]. A lobed pod mutation with fewer seeds per pod was also found. This trait may associate with partial sterility, causing constriction at the point where there was undeveloped seed. The number of mutants found and their descriptions are shown in Table 2 and 3. These mutants were not found in the control populations. Therefore, they were considered the real mutants and not the results of genetic recombination between the parental lines.

Characteristics of leaflet mutants are shown in Fig 1, while those of the other types are given in Fig 2. The unifoliate leaf mutant was also sterile, in agreement with that reported by Santos[4]. The mutant produced numerous flower buds but failed to open. The round-cuneat leaflet mutant produced flowers but its pollen scattered all over the corolla and thus expressed partial sterility. However, coppery leaf, variegated leaf, waxy leaf, white steak leaf, lanceolate leaflet, narrow-rugose leaflet, multiple leaflet, and wrinkled leaf were fertile

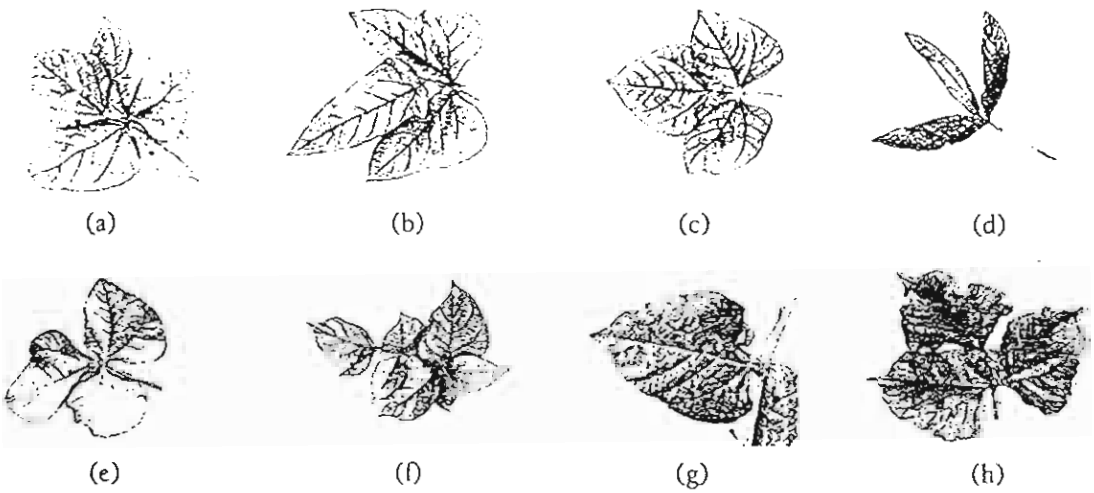


Fig 1. Leaf mutant variation found in the M_2 plants: (a) five multiple leaflet, (b) lanceolate leaf, (c) normal trifoliate leaf, (d) narrow-rugose leaf, (e) round-cuneat leaflet, (f) seven multiple leaf, (g) unifoliate leaf, (h) wrinkled leaf.

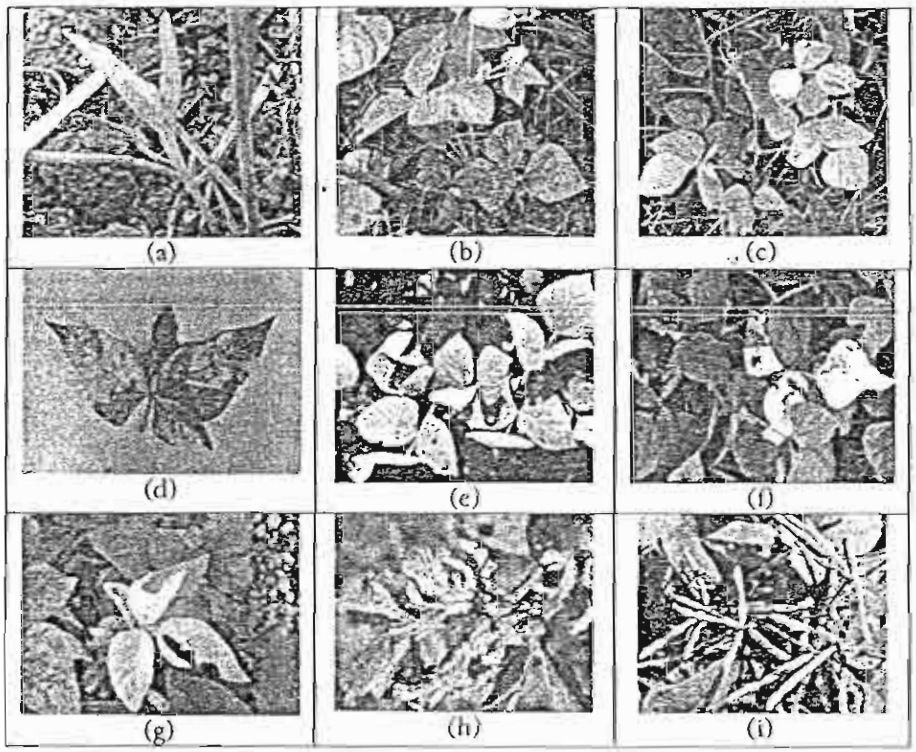


Fig 2. Chlorophyll, flower, and pod mutations found in the M_2 plants: (a) albino, (b) coppery leaf, (c) light-green leaf, (d) variegated leaf, (e) waxy leaf, (f) white streak leaf, (g) xantha leaf, (h) cock's comb raceme, (i) lobed pod due to sparse seed set.

with low yield. The variegated leaf and narrow-rugose leaf mutants produced only few pods while waxy leaf produced pods with lean seeds. These mutants have been reported by a number of scientists,[1,3-6,13,15] but we have found them all in one experiment, possibly due to the high population used (up to 433,842 seedlings).

Although not statistically significant, the rate of mutation was slightly higher in F_1 and F_2 as compared to the parents, since the progenies are more heterozygous than the parents. The heterozygous genotypes have more possible target alleles to mutate than the pure line parent. However, the mutation rate in this experiments is rather low and thus the result needs to be confirmed in more experiments. The mutant

plants were individually harvested for 2 consecutive generations to establish pure mutant lines for further studies. All mutants were bred-true and can be utilized in breeding and genetic study. Some multiple leaflet lines set profuse pods that might be useful as a marker for mungbean yield improvement in the future.

Acknowledgements

This study was supported by Center for Agricultural Biotechnology, Kasetsart University, Asian Regional Center-AVRDC, and the Project on Genetics and Breeding of Field Legumes for Thailand of the Thailand Research Fund.

References

1. Gupta PK (1996) Mutation breeding in mungbean. *In* Asthana AN and Kim DH (Eds.) Recent Advances in Mungbean Research. Indian Society of Pulses Research, Kanpur, India, pp 124-36.
2. Micke A and Donini B (1993) Induced mutations. *In* Hayward MD, Bosemark NO and Romagosa I (Eds.) Plant Breeding Principles and Prospects. Chapman and Hall, London, pp 52-62.
3. Singh VP and Rashmi Sharma (1993) γ-Rays-and EMS-induced leaf mutants in mungbean (*Vigna radiata* (L) Wilczek). *Curr. Sci.*, 65, 636-8.
4. Santos IS (1969) Induce of mutations in mungbean (*Phaseolus aureus* Roxb.) and genetic studies of some of the mutants. Induced mutations in plants. IAEA, Vienna, pp. 169-79.
5. Bahl, JR and Gupta PK (1982) Inheritance of two induced lethal chlorophyll mutations in mungbean. *Curr. Sci.* 53, 147-8.
6. Ekasamtramage Theera (1982) Induce mutations in mungbean by gamma irradiation. M.S. Thesis, Kasetsart University, Bangkok (in Thai with English abstract).
7. Singh VP and Yadav RDS (1982) Gamma induced yellow testa colour mutant of green gram cv T44. *Curr Sci.* 51, 891-2.
8. Srinives P, Hual-alai N, Saengchot S and Ngampongsai S (2000) The use of wild relative and gamma radiation in mungbean and blackgram breeding. Proc 7th MAFF International Workshop on Genetic Resources, 13-15 October 1999. AFFRC and NIAR, Japan.
9. Wongpiyasatid A, Chotechuen S, Hormchan P, Ngampongsai S, Lamseejan S and Pichitporn S (1998) Mutant mungbean lines from radiation and chemical induction. *Kasetsart J. (Nat. Sci)* 32, 203-12.
10. Wongpiyasatid A, Chotechuen S, Hormchan P, Ngampongsai S and Promcham W (2000) Induced mutations in mungbean breeding: regional yield trial of mungbean mutant lines. *Kasetsart J. (Nat. Sci.)* 34, 443-9.
11. Yaqoob M and Rashid A (2001) Induce mutation studies in some mungbean (*Vigna radiata* (L.) Wilczek) cultivars. Online *J. Biol. Sci.* 1, 805-8.
12. Wongpiyasatid A, Chotechuen S, Hormchan P and Srihuttagum M (1999) Evaluation of yield and resistance to powdery mildew, *Cercospora* leaf spot and cowpea weevil in mungbean mutant lines. *Kasetsart J. (Nat. Sci.)* 33, 204-15.
13. Srichot Wanipa (1982) Radiation effects in mungbean. M.S. Thesis, Kasetsart University, Bangkok (in Thai with English abstract).
14. Thongpumyn Bandit (2002) Mungbean improvement by gamma radiation. M.S. Thesis, Suranaree University of Technology, Nakhon Ratchasima (in Thai with English abstract).
15. Lamseejan, S, Samutkupt S and Wongpiyasatid A (1983) Gamma ray induce mutants in mungbean. *J. Agri. Sci.* 16, 446-54 (in Thai with English abstract).

SABRAO JOURNAL of BREEDING and GENETICS

ISSN 1029-7073

VOL. 37 NO. 2 DECEMBER 2005

CONTENTS

SABRAO
THE SOCIETY FOR THE ADVANCEMENT OF BREEDING RESEARCHES IN ASIA AND OCEANIA

Visit our website at: http://www.irri.org/sabrao

SABRAO Journal
of Breeding and Genetics
37(2) 91-96, 2005

QUANTITATIVE INHERITANCE OF RESISTANCE TO POWDERY MILDEW DISEASE IN MUNGBEAN (*Vigna radiata* (L.) Wilczek)

W. SORAJJAPINUN[1], S. REWTHONGCHUM[1], M. KOIZUMI[1], and P. SRINIVES[2,*]

SUMMARY

Powdery mildew disease caused by the fungus *Erysiphe polygoni* D.C. is devastating to mungbean growing in several countries including Thailand. Inheritance of resistance to powdery mildew was studied using progeny from the cross between moderately resistant KPS 2 (P_1) and resistant VC 6468-11-1A (P_2). Six populations *viz.*, P_1, P_2, F_1, F_2, P_1F_1, and P_2F_1 were sown in a randomized complete block design with six replications at Kasetsart University, Kamphaeng Saen Campus, Thailand to study the genetic control of powdery mildew resistance. Genetic effects were determined with generation mean analysis as proposed by Gamble (1962). The results showed that VC 6468-11-1A had a lower rate of disease development than KPS 2. Mean infection in the F_1 and F_2 populations fell at the midparent level, while P_2F_1 had a similar level of resistance as its recurrent parent. In contrast, backcross to the moderately resistant parent, (P_1F_1) produced progeny as the resistant as the F_1 and F_2. Additive gene action was found to play a major role in controlling powdery mildew resistance in mungbean. Thus, a breeding method employed for self-pollinated species, such as the pedigree, bulk, or single seed descent selection should be effective in selecting for powdery mildew resistant genotypes in the progenies derived from this cross.

Key words: Powdery mildew, *Erysiphe polygoni*, mungbean, *Vigna radiata*, inheritance

Powdery mildew, caused by the fungus *Erysiphe polygoni* D.C., is a major disease found widely in mungbean planting areas. Breeding for resistance is the best way to avoid yield loss due to disease infestation. A promising mungbean line, VC 6468-11-1A, is predominantly resistant, with large seed size, and high yield (Xiao, 2002). Its resistance genes are inherited from the varieties ML-3, ML-5, and ML-6 from India (Fernandez and Shanmugasundaram, 1988).

The powdery mildew disease starts from the lower leaves and spreads upwards under favorable conditions, as in a dry cool season (AVRDC, 1987). Yield losses up of to 20-40% have been reported (Reddy *et al.*, 1994). The damage is more serious when the epidemic starts at the seedling stages. The white mycelia covering the infected plants eventually adversely affect the plants' physiological processes. However, field infection reportedly varied with changing seasons. Humphry *et al.* (2003) found different races of the pathogen prevailing in two seasons in Queensland, Australia. Thus, the same pathogen

[1] The World Vegetable Center-AVRDC - Asian Regional Center (AVRDC-ARC), Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand
[2] Department of Agronomy, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand
* Corresponding author: agrpss@ku.ac.th

races may not attack mungbean grown in different seasons or locations. Therefore, genetics of resistance to powdery mildew in mungbean is rather location/season specific and need to be addressed each time a breeding program against the disease is set up. Horizontal resistance genes should be stacked into novel mungbean lines in order to secure a broader and more reliable disease resistance management program.

Inheritance of resistance to powdery mildew in mungbean has been reported to be controlled by a single dominant gene in the breeding line VC 1560A (AVRDC, 1981) as well as in the breeding line ATF 3640 (Humphry *et al.*, 2003). However, it was reported to be controlled by two dominant genes in the RUM breeding line from India (Reddy *et al.*, 1994) and three genomic regions were also found to harbor Quantitative Trait Loci (QTL) that explained up to 58% of the total variation in disease reaction (Young *et al.*, 1993). Chaitieng *et al.* (2002) reported that the F_2 population derived from the cross between a moderately resistant breeding line, VC 1210A, and a susceptible wild relative (*Vigna radiata* var. *sublobata*, accession TC 1966) showed a continuous distribution and resistance was treated as a quantitative trait. They detected 2 RFLP loci associated with a major QTL conferring up to 65% of the total variation in resistance. Humphry *et al.* (2003) identified RFLP flanking markers closely linked to the QTL conferring resistance to powdery mildew and recommended their application for improving future breeding programs of mungbean.

The application of chemical fungicides is an effective means for controling powdery mildew in mungbean but has adverse effects on health and the environment. Development of resistant varieties is a safe and economic way for disease control. Information on genetic inheritance of disease resistance in mungbean is a prerequisite, especially when the trait is quantitatively inherited. There are several genetic models available for the analysis of gene effects through generation mean analysis (GMA) as proposed by Anderson and Kempthorne (1954), Hayman (1958), and Gamble (1962).

The promising mungbean lines being developed at the Asian Regional Center of the Asian Vegetable Research and Development Center (ARC-AVRDC), Kasetsart University, Thailand are increasingly resistant to powdery mildew. In this experiment, the genetic control of resistance in a cross between a moderately resistant and a highly resistant mungbean was studied using GMA analysis.

MATERIALS AND METHODS

The two parental mungbeans used in this study were the cultivars 'Kamphaeng Saen 2' (KPS 2, P_1) and the breeding line VC 6468-11-1A (P_2). KPS 2 was selected from the cross BPIglab-3//CES44/ML-3///CES1D-21/PHLV18. It is currently the most popular mungbean cultivar grown in Thailand, occupying over 200,000 ha annually. Since its resistance comes only from the cultivar ML-3, it is moderately resistant to powdery mildew in Thailand. VC 6468 is derived from the cross VC 6040 x VC 6209. VC 6040 carries resistance traits from ML-3 and ML-6, while VC 6209 derives its resistance from ML-3 and ML-5. This makes the line highly resistant to the disease. F_1 seeds were obtained using KPS 2 as the female parent. The F_1 plants were backcrossed to P_1 and P_2 to produce P_1F_1 and P_2F_1 progenies, respectively. F_2 seeds were collected from self-pollinated pods on the F_1 plants.

A total of six generations, *viz.* P_1, P_2, F_1, F_2, P_1F_1, and P_2F_1 were sown in a randomized complete block design with six replications at Kasetsart University,

Kamphaeng Saen Campus, Thailand. Each replication was surrounded by preplanted border rows of a highly susceptible variety, CN 60, which served as spreader rows. The treatments were sown 30 days later. Data were recorded on percent disease infection per plant (PIP) and per leaves (PIL) 30 days after sowing, then transformed into the scale of 1-4 and 1-5 (AVRDC, 2004), as shown in Table 1. Genetic effects of the disease rating were determined from the generation means based on the equations derived by Gamble (1962), as follows:

$$m = F_2,$$
$$a = P_1F_1 - P_2F_1,$$
$$d = 1/2P_1 - 1/2P_2 + F_1 - 4F_2 + 2P_1F_1 + 2P_2F_1,$$
$$aa = -4F_2 + 2P_1F_1 + 2P_2F_1,$$
$$ad = -1/2P_1 + 1/2P_2 + P_1F_1 - P_2F_1, \text{ and}$$
$$dd = P_1 + P_2 + 4F_2 - 4P_1F_1 - 4P_2F_1.$$

The six estimated effects represent mean (*m*) effects, additive (*a*) and dominance (*d*) gene effects, and the three types of digenic epistatic effects (*aa, ad,* and *dd*). Significance of each estimate was judged from a t-test against its standard error estimate. Upon significance testing, another analysis was performed including only major effects. Then the deviation of the new estimates from the perfect fit (6 components) estimates were determined from a Chi-square (x^2) test as follows:

$$\chi^2 = \sum_i \frac{(Oi - Ei)^2}{Ei},$$

where *Oi* is the new value of the i^{th} estimate and *Ei* is the perfect fit value of the corresponding estimate. The degree of freedom required for checking the significance of the x^2 is the difference in the number of parameters between the two analyses.

Table 1. Disease reaction scaling based on percentage of powdery mildew infection on plants and leaves of mungbean.

Percent infection per plant (PIP)		Percent infection per leaf (PIL)	
Scale	% infected	Scale	% infected
1	<10	1	< 5
2	11 - 30	2	6-10
3	31-60	3	11-25
4	>60	4	26-50
		5	>50

RESULTS AND DISCUSSION

The results of the present study revealed that both parents, KPS 2 and VC 6468-11-1A, showed variable responses in percent infection per plant (PIP) and infection per leaf (PIL) (Table 2). KPS 2 had PIP and PIL ratings of 2.38 and 2.62, while VC 6468-11-1A had 1.25 and 1.36, respectively. VC 6468-11-1A had lower disease development than KPS 2. The F_1 and F_2 populations showed average disease infection levels close to the mid-parent, at PIP of 1.95 and 1.83, and PIL of 1.88 and 1.98, respectively. Since resistance levels among the parents were not sharply different, disease reactions in the progenies were

Table 2. Average scale of disease infection per plant and per leaf in six mungbean generations.

Generation	Scale of infection per plant (PIP)	Scale of infection per leaf (PIL)
P_1 (KPS 2)	2.38a	2.62a
P_2 (VC 6468-11-1A)	1.25c	1.36d
F_1 (P_1xP2)	1.95b	1.88cd
F_2 (F_1 selfing)	1.83b	1.98bc
P_1F_1 (F_1 x P_1)	2.03b	2.13b
P_2F_1 (F_1 x P_2)	1.25c	1.70d
F-test	**	**
CV%	30.34	33.65

** Significant at .01 level of probability.
Means within a column followed by the same letter are not different at the .05 level by DMRT.

still within the moderately resistant scales. In the backcross to the susceptible parent (P_1F_1), PIP was 2.03, while PIL was 2.13 (Table 2). Backcross progeny of the resistant parent (P_2F_1) gave as low a scaling level as the resistant parent (1.25 and 1.70 for PIP and PIL, respectively). This showed that inheritance of powdery mildew resistance from a moderately resistant x highly resistant cross is governed by quantitative trait loci.

Gene effects governing the powdery mildew resistance were significant only for the additive parameter (Table 3). The non-significant epistatic components were removed from the model and another analysis was done on the other three major parameters: *m*, *a*, and *d* (Table 4). The gene effects upon removal of epistatic components were not significantly different from the perfect fit with six components. The χ^2 values from the estimate of PIP and PIL were 3.86 and 0.21, respectively. Both were less than the $\chi^2_{.05}$ at *df*=3 (7.81). Thus, it can be concluded that the additive gene effect was still the most important component controlling powdery mildew resistance in this mungbean cross.

Table 3. Estimates of the six-parameter gene effects from scaling data of powdery mildew disease from the mungbean cross KPS 2 x VC6468-11-1A.

Gene effects	PIP	PIL
M	1.83± 0.141**	1.98± 0.155**
A	0.53± 0.188**	0.43± 0.204*
D	-0.17± 0.707	-0.37± 0.776
Aa	-0.26± 0.677	-0.27± 0.743
Ad	-0.08± 0.219	-0.19± 0.238
Dd	0.82± 1.027	0.35± 0.117

*,** Significant at .05 and .01 levels of probability, respectively.

AVRDC (1981), Reddy *et al.* (1994), and Humphry *et al.* (2003) reported that major genes control powdery mildew disease resistance in mungbean. On the other hand, Chaitieng *et al.* (2002) found that the F_2 from the cross between a moderately resistant line with a susceptible line showed a continuous trait distribution and resistance can be treated as a quantitative trait. Young *et al.* (1993) found three genomic regions responsible for powdery mildew resistance. A more stable resistance might be established by combining the quantitative genes with the major genes. The quantitative "modifying genes" can add a

Table 4. Estimates of three-parameter gene effects from the scaling data of powdery mildew disease in the mungbean cross KPS 2 x VC6468-11-1A.

Gene effects	PIP	PIL
m	2.12 ± 0.69**	2.26 ± 0.75**
a	0.61 ± 0.11**	0.62 ± 0.15**
d	-0.99 ± 1.64	-0.72 ± 1.79

*,** Significant at and .01 level of probability.

buffer to the resistant cultivar and thus help in prolonging the lifespan of a resistant cultivar.

The above conclusion confirms that the breeding methods employed for self-pollinated species, such as the pedigree, bulk, or single seed descent selection, should be effective in breeding for mungbean genotypes resistant to powdery mildew in this population.

ACKNOWLEDGMENTS

This research was supported by AVRDC-ARC, Kasetsart University, Thailand and the Project on Genetics and Breeding of Field Legumes for Thailand of the Thailand Research Fund.

REFERENCES

Anderson, V.L. and O. Kempthore. 1954. A model for the study of quantitative inheritance. *Genetics* 39:883-898.

AVRDC. 1981. *1979 Progress Report.* Asian Vegetable Research and Development Center, Shanhua, Taiwan. pp. 42-55.

AVRDC. 1987. *1984 Progress Report.* Asian Vegetable Research and Development Center, Shanhua, Taiwan. pp. 196-199.

AVRDC. 2004. *AVRDC Report 2003.* AVRDC- The World Vegetable Center. Shanhua, Taiwan. pp. 146-148.

Chaitieng, B., A. Kaga, O.K. Han, X.W. Wang, S. Wongkaew, P. Laosuwan, N. Tomooka, and D. A. Vaughan. 2002. Mapping a new source of resistance to powdery mildew in mungbean. *Plant Breeding* 121:521-525.

Fernandez, G.C.J., and S. Shanmugasundaram. 1988. The AVRDC mungbean improvement program: The past, present and future. In: *Mungbean, Proc. Second Inter. Symp.* AVRDC. Shanhua, Taiwan. pp. 58-70.

Gamble, E.E. 1962. Gene effects in corn (*Zea mays* L.) I. Separation and relative importance of gene effects for yield. *Can. J. Plant Sci.* 42:339-348.

Hayman B.I. 1958. The separation of epistatic from additive and dominance variation in generation means. *Heredity* 12:371-390.

Humphry, M.E., T. Magner, C.L. McIntyre, E.A.B. Aitken, and C.J. Liu. 2003. Identification of a major locus conferring resistance to powdery mildew (*Vigna radiata* L. Wilczek) by QTL analysis. *Genome* 46:738-744.

Reddy, K.S., S.E. Pawar, and C.R. Bhatia. 1994. Inheritance of powdery mildew (*Erysiphe polygoni* DC.) resistance in mungbean (*Vigna radiata* L Wilczek). *Theor. Appl. Genet.* 88:945-948.

Young, N.D., D. Danesh, D. Menancio-Hautea, and L. Kumar. 1993. Mapping oligogenic resistance to powdery mildew in mungbean with RFLPs. *Theor. Appl. Genet.* 87:243-249.

Xiao T. 2002. Preliminary yield trial of promising mungbean lines. In: *19th ARC Training Report 2001*. Asian Vegetable Research and Development Center- Asian Regional Center, Thailand.

SABRAO JOURNAL of BREEDING and GENETICS

ISSN 1029-7073

VOL. 37 NO. 2 DECEMBER 2005

CONTENTS

SABRAO
THE SOCIETY FOR THE ADVANCEMENT OF BREEDING RESEARCHES IN ASIA AND OCEANIA

Visit our website at: http://www.irri.org/sabrao

SABRAO Journal
of Breeding and Genetics
37(2) 97-105, 2005

SIGNIFICANCE OF HETEROSIS AND HETEROBELTIOSIS IN AN F_1 HYBRID OF MUNGBEAN (*Vigna radiata* (L.) Wilczek) FOR HYBRID SEED PRODUCTION

R. SOEHENDI[1,2] and P. SRINIVES[1,*]

SUMMARY

The increase in mungbean (*Vigna radiata* (L.) Wilczek) production volume comes mainly from the increase in mungbean-cultivated area. A possible breakthrough for this production limitation is to exploit hybrid vigor of the F_1 for possible production of hybrid varieties. The magnitude of hybrid vigor is normally presented in terms of heterosis (H = superiority of the F_1 hybrid over its parental mean) and heterobeltiosis (Hb = superiority of the F_1 hybrid over its better parent). However, the significance of H and Hb in each cross has rarely been tested. We are proposing a simple t-test method for evaluating the significance of H or Hb in each cross using data obtained from individual plants. As an example, heterosis and heterobeltiosis were estimated in four cross combinations involving three diverse mungbean genotypes. The data were collected on plant height, number of leaves/plant, leaf area/plant, number of pods/plant, pod length, number of seeds/pod, 100-seed weight, and yield/plant. All crosses showed significant yield heterosis over the mid-parent and better-parent. Crosses showing heterosis for grain yield also showed heterosis for pod length, number of seeds per pod, and plant height. However, only plant height expressed heterobeltiosis. Superiority over the mid-parent for grain yield ranged from 52.2 to 95.7%, and that over the better parent ranged from 31.8 to 78.5%. The highest heterosis over the mid- and better parent was shown in the cross SMxLM. In self-pollinated crops, hybrid seeds can be produced using a male sterile line as the female parent. The detected yield heterosis must be reasonably high to compensate for the cost of seed production.

Key words: *Vigna radiata*, mungbean, heterosis, heterobeltiosis, t-test

Mungbean (*Vigna radiata* (L.) Wilczek) is an important source of protein in India, Indonesia, Myanmar, Pakistan, Thailand, Vietnam, among other countries. Farmers grow it as a supplemental crop or cash crop. Mungbean is the shortest maturing field crop. However, its yield is low compared to other grain legumes. The annual increase in production comes mainly from the increase in cultivated area. Yield productivity is not easily achieved by the current methods of cultivar improvement and cultural practices. Using hybrid cultivars can improve the yield limitation in pure line cultivars.

Heterosis or hybrid vigor is manifested by F_1 hybrids. Hybrid varieties have contributed greatly worldwide to the production of many crop species, including the most important food crops such as maize and rice. The commercial exploitation of heterosis has

[1] Department of Agronomy, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[2] Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang 65101, Indonesia
* Corresponding author: agrpss@yahoo.com

been one of the driving forces behind the rapid and extensive development of seed production. Heterosis breeding has allowed yield breakthroughs in several crops, including cross-pollinated, often cross-pollinated, and self-pollinated species. The exploitation of heterosis to raise productivity in grain legumes, as in any other crop, depends on three major factors: the magnitude of heterosis; feasibility of large-scale production of hybrid seeds; and type of gene action involved. Heterosis may take the form of an increase in yield, size, number of plant parts, chemical components, and disease resistance. The hybrid is a plant type resulting from the fusion of dissimilar gametes or those having heterozygous gene pairs for a particular character. Heterosis and heterobeltiosis are normally expressed in percentage without testing for significance. If the standard error associated with each generation mean is great, the high heterosis may not be statistically significant, and thus, the data would not convince a seed company to commercially produce the hybrid.

Ahn *et al.* (2004) applied a paired t-test to identify significant differences among 11 *Japonica* rice cultivars and their 44 F_1 hybrids. The test was based on the average heterosis of each trait across all crosses rather than on a per cross basis. Thus the plant breeder cannot utilize the information for selection of a particular hybrid but rather for comparison between traits showing different significant levels. Singh *et al.* (2004) used the pooled error mean square from the ANOVA table to calculate the standard error of difference for testing the significance of heterosis and heterobeltiosis in 45 hybrids derived from crosses of 10 bread wheat cultivars. In their test, it is required that the error variance associated with all crosses is assumed homogeneous so that a pooled error variance can be used for testing the significance of all crosses. This assumption is rather difficult to meet, especially when the plant breeder is working with diverse breeding materials. Our test utilizes variances associated with the particular generation involved in the test to calculate the standard error of estimate of H and Hb. This helps reduce the magnitude of the standard error, making the test more sensitive.

The objective of this study were: 1) to examine a general t-test method for determining the significance of heterosis and heterobeltiosis from each cross combination, using data for yield and major agronomic characters in mungbean; and 2) to determine the prerequisites in commercially producing an F_1 hybrid for mungbean seed production.

MATERIALS AND METHODS

Plant materials

Three mungbean genotypes, namely small-multiple leaflet (SM), large-multiple leaflet (LM), and normal-trifoliate (NT) were crossed during June to August 2002 at Kasetsart University-Kamphaeng Saen Campus (KU-KPS), Nakhon Pathom Province, Thailand. The small-multiple leaflet (SM) was obtained from gamma-irradiated mungbean (Srinives *et al.*, 2000). The large-multiple leaflet (LM) parent is a BC_9 progeny having the cultivar 'Kamphaeng Saen 1' as the recurrent parent and the large-multiple leaflet mutant (V5926) from the Asian Vegetable Research and Development Center (AVRDC), Taiwan as donor parent (Kowsurat *et al.*, 1999). The normal-trifoliate, VC6468-11-1B is an advanced breeding line carrying powdery mildew resistance genes. It was derived from crossing VC 6040A and VC 6209-1 at the Asian Regional Center of the AVRDC. To minimize the environmental effect under the growing condition, the parents and F_1s were sown with two plants per pot in 10-inch pots filled with mixed potted soil. The pots were placed in an open area receiving full sunshine. Each genotype consisted of 20 plants. All the optimum recommended practices for mungbean growth were applied (Park, 1978). At

50 days after sowing, the leaf area was measured using the leaf area meter model LI-3100 (Licor, Inc., Lincoln, Nebraska, USA). At harvest, ten random plants were measured for plant height (cm), number of leaves per plant, leaf area per plant (cm^2), number of pods per plant, pod length (cm), number of seeds per pod, 100-seed weight (g), and yield per plant (g).

Significance testing of heterosis

For each F_1 cross, percent heterosis (%H) and heterobeltiosis (%Hb) for a particular trait were calculated as follows:

$$\%H = \left(\bar{F}_1 - \overline{MP}\right) \times 100 / \overline{MP} \text{ , and}$$

$$\%Hb = \left(\bar{F}_1 - \bar{P}_i\right) \times 100 / \bar{P}_i,$$

Where:

$\bar{F}_1$ = mean observation of the F_1 progenies from the total of n_1 plants,

$\overline{MP}$ = mean observation of both parents from $n_2 + n_3$ plants, and

$\bar{P}_i$ = mean observation of the i^{th} parent from n_2 plants for P_1, and n_3 plants for P_2.

Significance of H and Hb were determined by a t-test as follows:

$$\text{t-test for H} = \frac{\bar{F}_1 - \overline{MP}}{S_H}, \text{ and}$$

$$\text{t-test for Hb} = \frac{\bar{F}_1 - \bar{P}_i}{S_{Hb}},$$

where S_H and S_{Hb} are the standard error of estimates of H and Hb which can be derived as shown in the attached note.

The degree of freedom (*df*) for each test was obtained by summing up the *df* of each generation involved in the estimate. Thus, the *df* for testing H is $(n_1-1)+(n_2-1)+(n_3-1)$, and the *df* for testing Hb is $(n_1-1)+(n_i-1)$, i = 2 or 3, depending on whether the high parent is P_1 or P_2.

RESULTS AND DISCUSSION

All the characters observed from the cross SMxLM were similar to those from its reciprocal cross (LMxSM) and the data from both sets could be combined to gain degrees of freedom for the t-test. It was our intention to separately test the significance of each cross.

Seed yield per plant in the normal-trifoliate (NT), small-multiple leaflet (SM), and large-multiple leaflet (LM) were 12.47, 10.56, and 8.70 g/plant, respectively. The F_1 of SMxLM gave the highest seed yield (18.85 g) among the progenies (Table 1). All four crosses showed significant heterosis over the mid- and better parent for seed yield. Superiority over the mid-parent ranged from 52.2 to 95.7% (Table 2), while those over the better parent ranged from 31.8 to 78.5% (Table 3). The highest heterosis, both over mid- and better parents, was found in SMxLM.

Table 1. Yield, yield components, and agronomic characters of three mungbean lines and their F_1s grown at Kasetsart University, Kamphaeng Saen Campus, Thailand, Late Rainy Season, 2002.

Mungbean genotypes	Seed yield/ plant (g)	No. of pods/plant	No. of seeds/pod	100-seed weight (g)
		Value ± SE		
SM	10.56 ± 0.45	43.0 ± 1.7	9.6 ± 0.3	2.57 ± 0.07
LM	8.70 ± 0.51	13.3 ± 0.9	10.9 ± 0.4	6.05 ± 0.13
NT	12.47 ± 0.98	17.9 ± 0.9	11.5 ± 0.5	5.99 ± 0.10
SM X LM F_1	18.85 ± 1.02	28.2 ± 1.7	11.6 ± 0.3	5.80 ± 0.26
LM X SM F_1	17.90 ± 0.80	27.1 ± 1.2	11.5 ± 0.3	5.77 ± 0.26
SM X NT F_1	17.55 ± 0.81	31.0 ± 1.2	11.8 ± 0.3	4.79 ± 0.27
LM X NT F_1	16.43 ± 1.20	20.0 ± 1.2	12.2 ± 0.4	6.70 ± 0.21
Mungbean genotypes	**Pod length (cm)**	**Plant height (cm)**	**No. leaves per plant**	**Leaf area/ plant (cm^2)**
		Value ± SE		
SM	4.65 ± 0.13	47 ± 1.1	66.0 ± 5.9	1054 ± 127.0
LM	8.75 ± 0.20	49 ± 0.4	7.8 ± 0.7	971 ± 169.1
NT	9.70 ± 0.28	42 ± 0.4	12.2 ± 0.9	1153 ± 145.2
SM X LM F_1	8.20 ± 0.21	55 ± 0.8	16.8 ± 3.0	1884 ± 242.5
LM X SM F_1	8.25 ± 0.21	55 ± 0.8	16.4 ± 2.4	1800 ± 246.0
SM X NT F_1	8.95 ± 0.14	53 ± 1.2	15.6 ± 2.2	1824 ± 277.4
LM X NT F_1	10.00 ± 0.26	51 ± 0.6	13.0 ± 1.3	1751 ± 189.8

Even though NT had the highest seed yield of the three parents, its hybrids had lower heterosis and heterobeltiosis values than those from the other parents. Crosses showing heterosis for seed yield also gave heterosis for pod length, number of seeds per pod, plant height, and leaf area per plant. Chopra (1994) reported a high degree of heterosis for seed yield and its components in almost all grain legumes, which are essentially self-pollinated.

The highest number of pods per plant (43.0) was found in the parent SM and in the F_1 of SMxNT (31.0). The heterosis was not significant over either mid- or better parent, except only in the cross LMxNT (28.2%). All crosses having SM as a parent showed negative heterobeltiosis for number of pods per plant (Table 3) indicating that SM had a very high number of pods.

Number of seeds per pod in the hybrids ranged from 11.5 to 12.2 (Table 1). SM had the lowest number of seeds (9.6) compared to LM and NT (10.9 and 11.5, respectively). All four crosses showed significant heterosis ranging from 8.9 to 13.2% (Table 2), but heterobeltiosis was not significant, ranging from 2.6 to 6.4 % (Table 3).

One hundred seed weight of SM was the lowest (2.57 g) compared to the parents LM and NT (6.05 and 5.99 g, respectively). The hybrid LMxNT showed the largest seed size and had significant heterosis and heterobeltiosis.

Tabel 2. Significance test of heterosis over the mid-parent (MP) for yield, yield components, and agronomic characters of four F_1 mungbeans grown at Kasetsart University, Kamphaeng Saen Campus, Thailand, Late Rainy Season, 2002.

Cross combination	Seed yield /plant (g)		No. of pods/plant		No. of seeds /pod		100-seed weight (g)	
	value ± SE	% H	value ± SE	% H	value ± SE	% H	value ± SE	% H
SM X LM F_1	9.22 ± 1.07 **	95.7	0.05 ± 1.96 ns	0.2	1.35 ± 0.42 **	13.2	1.49 ± 0.27 **	34.6
LM X SM F_1	8.27 ± 0.87 **	85.9	-1.05 ± -0.04 ns	-0.1	1.25 ± 0.39 **	12.2	1.46 ± 0.27 **	33.9
SM X NT F_1	6.04 ± 0.98 **	52.5	0.55 ± 1.54 ns	0.1	1.25 ± 0.39 **	11.9	0.51 ± 0.28 ns	11.9
LM X NT F_1	5.84 ± 1.32 **	55.2	4.40 ± 1.34 **	28.2	1.00 ± 0.47 **	8.9	0.68 ± 0.23 *	11.3

Cross combination	Pod length (cm)		Plant height (cm)		No. of leaves /plant		Leaf area /plant (cm^2)	
	value ± SE	% H	value ± SE	% H	value ± SE	% H	value ± SE	% H
SM X LM F_1	1.50 ± 0.25 **	22.4	7.15 ± 0.96 **	14.9	-20.1 ± 3.75 **	-54.5	871.7 ±215.5 **	86.0
LM X SM F_1	1.55 ± 0.25 **	23.1	7.05 ± 0.99 **	14.7	-20.5 ± 3.83 **	-55.6	787.2 ±267.8 **	77.7
SM X NT F_1	1.78 ± 0.21 **	24.7	8.50 ± 1.30 **	19.1	-23.5 ± 3.68 **	-60.1	720.9 ±293.6 **	65.3
LM X NT F_1	0.78 ± 0.31 **	8.4	5.55 ± 0.71 **	12.2	3.0 ± 1.43 *	30.0	689.3 ±220.1 **	64.9

*Significant at $P \leq 0.05$; ** Significant at $P \leq 0.01$; ns = Non-significant.

Table 3. Significance test of heterobeltiosis for yield, yield components, and agronomic characters of four F_1 mungbeans grown at Kasetsart University, Kamphaeng Saen Campus, Thailand, Late Rainy Season, 2002.

Cross combination	Seed yield /plant (g)		No. of pods/plant		No. of seeds /pod		100-seed weight (g)	
	value ± SE	%Hb	value ± SE	% Hb	value ± SE	% Hb	value ± SE	% Hb
SM X LM F_1	8.29 ± 1.11 **	78.5	-14.8 ± 2.41 **	-34.4	0.7 ± 0.49 ns	6.4	-0.25 ± 0.28 ns	-4.13
LM X SM F_1	7.33 ± 0.92 **	69.4	-15.9 ± 2.09 **	-36.9	0.6 ± 0.46 ns	5.5	-0.28 ± 0.29 ns	-4.62
SM X NT F_1	5.08 ± 1.27 **	40.7	-12.0 ± 2.08 **	-27.9	0.3 ± 0.56 ns	2.6	-1.20 ± 0.29 **	-20.03
LM X NT F_1	3.96 ± 1.55 **	31.8	2.1 ± 1.49 ns	11.7	0.7 ± 0.62 ns	6.1	0.65 ± 0.25 *	10.74

Cross combination	Pod length (cm)		Plant height (cm)		No. of leaves /plant		Leaf area /plant (cm^2)	
	value ± SE	%Hb	value ± SE	% Hb	value ± SE	% Hb	value ± SE	% Hb
SM X LM F_1	-0.55 ± 0.29 ns	-6.3	6.2 ± 0.87 **	12.7	-49.2 ± 6.32 **	-74.6	830.3 ± 226.7 **	78.8
LM X SM F_1	-0.50 ± 0.29 ns	-5.7	6.1 ± 0.91 **	12.5	-49.6 ± 6.37 **	-75.2	745.9 ± 276.9 *	70.8
SM X NT F_1	-0.75 ± 0.31 *	-7.7	6.0 ± 1.58 **	12.8	-50.4 ± 6.27 **	-76.4	671.3 ± 313.1 *	58.2
LM X NT F_1	0.30 ± 0.38 ns	3.1	2.1 ± 0.76 *	4.3	0.8 ± 1.59 ns	6.6	598.4 ± 239.0 *	51.9

*significant at $P \leq 0.05$; ** significant at $P \leq 0.01$; ns = non-significant.

SM had the shortest pod and thus the hybrid SMxNT expressed a negative heterobeltiosis of 7.7% (Table 3). The hybrid LMxNT had the longest pods (10.0 cm). All cross combinations showed significant heterosis ranging from 8.4 to 24.7% (Table 2).

Plant height showed significant heterosis and heterobeltiosis in all cross combinations. The hybrid SMxLM and its reciprocal gave taller plants than the other crosses (Table 1). The range of significant heterosis and heterobeltiosis varied from 12.2 to 19.1% (Table 2) and from 4.3 to 12.8%, repectively (Table 3). The hybrid SMxNT gave the highest value both for heterosis and heterobeltiosis.

The small-multiple leaflets (SM) had the highest average number of leaves (66.0), as compared to LM and NT which had 7.8 and 12.2 leaves, respectively. The F_1 derived from SM showed negative values for both heterosis and heterobeltiosis. The F_1 of LMxNT showed significant heterosis (30%; Table 2), but not heterobeltiosis (6.6%; Table 3).

For leaf area per plant, all crosses showed significant heterosis and heterobeltiosis. Heterosis values ranged from 64.9 to 86%, while heterobeltiosis values ranged from 51.9 to 78.8%. The highest value was found in the cross SMxLM (Table 1).

Our findings agreed well with a number of previous studies. A high degree of heterosis was reported in the F_1s of various grain legumes, such as pigeon pea, pea, and lentil (Singh *et al.*, 1975). In mungbean, Khattak *et al.* (2000) found heterosis over the mid-parent for pod clusters on the main stem in the cross VC 3902A x ML-5. Chen *et al.* (2003) reported that a Korean mungbean variety (K7) gave F_1 progenies with significant heterobeltiosis for seed yield in many crosses. In these studies, the magnitudes of heterosis were dependent upon the genotype of the parents.

In self-pollinated plant species, it is rather easy to produce hybrid seed if male sterile lines are available and can be used as the female parent. Cross and Schulz (1997) discussed a development in chemical induction of male sterility. There are at least four classes of chemical agents, *viz.* plant-growth regulators and substances that disrupt floral development, metabolic inhibitors, inhibitors of microspore development, and inhibitors of pollen fertility. Since the hybrid seeds must be harvested from the female parent only, the magnitude of heterosis should be sufficiently high to compensate for the cost of producing open-pollinated seeds in self-pollinating crops. Our significant test show that heterosis obtained from two diverse mungbeans was sizable and worth exploring further.

Hybrid rice breeding has been very successful in China since the 1970s. With the development of photo-thermo-sensitive genic male sterile (P/TGMS) or environment-sensitive genic male sterile (EGMS) lines, a two-line breeding system has been developed as a simplified alternative to the traditional three-line breeding that requires a male-sterile line, a sterility maintainer line, and a fertility restorer (Yuan, 1992). The two-line breeding system is much simplified since an EGMS line can serve as a sterile line under one environmental condition and can propagate itself under different environments. The ability to maintain sterility makes EGMS lines practicable as a female to cross with other lines. In recent years, a number of two-line hybrids have been commercialized in China, and several other Asian countries have established hybrid breeding programs using EGMS lines (Lu *et al.*, 1994; Li and Yuan, 2000).

With the success in the use of hybrid rice varieties, the possibility of using hybrid mungbean should be explored. The high heterosis identified in this study and by Chen *et al.* (2003) are encouraging. However, a large-scale production of hybrid seed is possible only when a male sterility system is available, coupled with the availability of insect pollen

vectors. Generally, legume pollen is heavier than that of cereals and thus could not be effectively transferred by wind. These are interesting topics for mungbean breeders to investigate in the future. A male sterile line, if available, should open up a large dimension of yield improvement through the use of hybrid seed in mungbean.

CONCLUSIONS

The yield superiority of the F_1 hybrid over the mid- and better parents is evident in mungbean. A simple t-statistical test was developed to help support evidence of heterosis and heterobeltiosis. The test is sufficiently sensitive to be able to conclude that mid-parent and better parent heterosis occurred in varying degrees for seed yield and its components. The highest mid- and better parent heterosis for seed yield were 95.7% and 78.5%, respectively, found in the cross SMxLM. Commercialization of hybrid cultivars in mungbean can be justified by the significant heterosis for yield. Future research should be directed to making feasible, among others, the reduction of manpower required for hybrid seed production.

Note on derivation method for S_H and S_{Hb}.

$$H = \bar{F}_1 - \frac{(\bar{P}_1 + \bar{P}_2)}{2},$$

$$= \bar{F}_1 - \frac{\bar{P}_1}{2} - \frac{\bar{P}_2}{2}.$$

Using the property of expectation (Steel and Torrie, 1980; Chapter 5, topic 5.10) then,

$$\text{Variance of H} = \text{Var}\left(\bar{F}_1 - \frac{\bar{P}_1}{2} - \frac{\bar{P}_2}{2}\right),$$

$$= V\bar{F}_1 + \frac{V\bar{P}_1}{4} + \frac{V\bar{P}_2}{4},$$

(assuming no covariation between generations).

$$= \frac{VF_1}{n_1} + \frac{VP_1}{4n_2} + \frac{VP_2}{4n_3},$$

$$= \frac{SSF_1}{n_1(n_1 - 1)} + \frac{SSP_1}{4n_2(n_2 - 1)} + \frac{SSP_2}{4n_3(n_3 - 1)}.$$

Where $V\bar{F}_1$, $V\bar{P}_1$, and $V\bar{P}_2$ are the variances of the mean of each generation; and VF_1, VP_1, VP_2, SSF_1, SSP_1 and SSP_2 are variances and sums of squares of the specified generations, respectively.

Then, the standard error of estimate of H (or S_H) = $\sqrt{\text{variance of H}}$.

In the same manner, variance of Hb can be obtained from

$$\text{Variance of Hb} = \text{Var}(\bar{F}_1 - \bar{P}_i),$$

$$= \frac{VF_1}{n_1} + \frac{VP_i}{n_i} = \frac{SSF_1}{n_1(n_1 - 1)} + \frac{SSP_i}{n_i(n_i - 1)},$$

and $S_{Hb} = \sqrt{\text{variance of Hb}}$.

ACKNOWLEDGEMENTS

We would like to thank the Participatory Development of Agricultural Technology Project (PAATP)- Indonesia for the scholarship of the senior author to pursue a Ph.D. degree at Kasetsart University. A part of the research was also supported by the Project on Genetics and Breeding of Field Legumes for Thailand funded through Kasetsart University by the Thailand Research Fund.

REFERENCES

Ahn, S.N., E.G. Jeong, and J.D. Yea. 2004. Effect of low temperature on heterosis for traits related to cold tolerance in *Japonica* rice. *Korean J. Breed.* 36:140-145.

Chen X., W. Sorajjapinun, S. Reiwthongchum, and P. Srinives. 2003. Identification of parental mungbean lines for production of hybrid varieties. *Chiang Mai University J.* 2(2):97-105.

Chopra, V.L. 1994. *Plant Breeding Theory and Practice*. Oxford & IBH Publishing Co. PVT. LTD, New Delhi, India. 471p.

Cross, J.W., and P.J. Schulz. 1997. Chemical induction of male sterility. In: K.R. Shivanna and V.K. Sawhney, eds, *Pollen Biotechnology for Crop Production and Improvement*. Cambridge University Press, Cambridge, UK. pp. 218-236.

Khattak, G.S.S., M.A. Haq, M. Ashraf, and G.R. Tahir. 2000. Heterosis for some morphological traits in mungbean (*Vigna radiata* (L.) Wilczek). *Kasetsart J. (Nat. Sci.)* 34(4):439-442.

Kowsurat, S., P. Srinives, P. Kasemsap, and S. Lamseejan. 1999. Effects of the multiple leaflet gene on agronomical and physiological characters of mungbean (*Vigna radiata*). *J. Agric. Sci. Cambridge* 133:321-324.

Li, J., and L. Yuan. 2000. Hybrid rice: genetics, breeding, and seed production. *Plant Breed. Rev.* 17:15-158.

Lu, X., Z. Zhang, S.S. Virmani, and K. Maruyama. 1994. Current status of the two-line method of hybrid rice breeding. *Chinese J. Rice Sci.* 8:48-54.

Park, H.G. 1978. *Suggested Cultural Practices for Mungbean*. International Cooperator's Guide. Asian Vegetable Research and Development Center. Shanhua, Taiwan. 2p.

Singh, H., S.N. Sharma, R.N. Sain, and E.V.D. Sastry. 2004. Heterosis studies for yield and its components in bread wheat under normal- and late-sowing conditions. *SABRAO J. Breed. Gen.* 36:1-11.

Singh, T.P., K.B. Singh, and R.S. Malhotra. 1975. Heterosis and combining ability in lentil. *Indian J. Agric. Sci.* 45(6):259-263.

Srinives, P., N. Hual-alai, S. Saengchot, and S. Ngampongsai. 2000. The use of wild relatives and gamma radiation in mungbean and blackgram breeding. In: *Proc. Seventh MAFF Int. Workshop on Genetic Resources Part 1. Wild Legumes. October 21-25, 1999, Tsukuba, Japan*. National Institute of Agrobiological Resources, Tsukuba, Japan. pp.205-218.

Steel, R.G.D. and J.H. Torrie. 1980. *Principles and Proceedures of Statistics: A Biometrical Approach (2nd ed.)*. Mc Graw-Hill, Inc., New York, USA. 633p.

Yuan, L.P. 1992. The strategy of breeding rice PGMS and TGMS lines. *Hybrid Rice* 1:1-4.

Korean J. Genetics 27 (4) : 351-359 (December 2005)

Identification of SSR Markers Associated with N_2-Fixation Components in Soybean [*Glycine max* (L.) Merr.]

Patcharin Tanya[1], Peerasak Srinives[1*], Teerayuth Toojinda[2], Apichart Vanavichit[1] and Suk-Ha Lee[3*]

[1]Department of Agronomy, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand [2]DNA Technology Laboratory, National Center for Genetic Engineering and Biotechnology, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand, [3]Department of Plant Science, College of Agriculture and Life Science, Seoul National University, San 56-1, Sillim-dong, Kwanak-gu, Seoul 151-742, Korea

Received May 6, 2005; accepted December 2, 2005

ABSTRACT

Regulation of nodulation and nitrogen fixation in legume plants relies mainly on the interaction between the host plant and the symbiotic bacteria. The purpose of this study was to identify quantitative trait loci (QTLs) for nodulation and nitrogen fixation characters in a soybean [*Glycine max* (L.) Merr.] population. Simple sequence repeat (SSR) markers were utilized to identify QTLs for the number of nodules per plant, nodule fresh and dry weight per plant, plant dry weight, and acetylene reduction activity (ARA) in 136 F_2-derived recombinant inbred lines (RILs) from the cross 'SJ2' x 'Suwon 157'. A genetic linkage map was constructed using 85 simple sequence repeat (SSR) markers onto 20 linkage groups covering 1094 cM. Using single-factor analysis of variance (SF-ANOVA) and multiple regression analysis, five QTLs were associated with the number of nodules per plant. For the QTLs linked to linkage group (LG) O, Satt529 on LG J, Satt440 on LG I, and Satt157 on LG D1b+W the positive alleles were derived from 'Suwon 157', whereas at the QTL linked to Satt385 on LG A1 the positive allele was from 'SJ2'. The QTLs conditioning the nodule fresh weight were linked to Sat_108 and Sat_274 in LG O, and Sct_001 in LG J, with the positive alleles derived from 'Suwon 157', whereas the positive allele of Satt545 on LG A1 was derived from 'SJ2'. Sat_274 and Sat_108 on LG O, and Satt260 on LG K were associated with QTL conditioning nodule dry weight, with the positive allele of 'Suwon 157'. There were four QTLs associated with plant dry weight, viz. Sat_274 and Sat_038 on LG O, Sct_001 on LG J, and Satt440 on LG I, with all the positive alleles from 'Suwon 157'. Finally, Sat_274 on LG O and Satt157 on LG D1b+W were linked to QTLs conditioning ARA, with the positive alleles were

*To whom correspondence should be addressed.
E-mail: agripss@gmail.com, sukhalee@snu.ac.kr. This research was supported by the Royal Golden Jubilee Ph.D. Program and the Project on Genetics and Breeding of Field Legumes for Thailand, of the Thailand Research Fund, the Soil Microbiology Group of the Department of Agriculture, Kasetsart University, Thailand. This research was also supported in part by a grant (code no. CG3121) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program, which is funded by the Ministry of Science and Technology (MOST) of the Republic of Korea.

from 'Suwon 157'. These data demonstrated that effective marker-assisted selection may be feasible for improving nodulation and nitrogen fixation in soybean. The existence of a major QTL on LG O conditioning greater than 10% of the phenotypic variation in nodulation and nitrogen fixation characters provides an opportunity to select progeny lines in a segregating population.

Key words: soybean, quantitative trait loci, SSR marker, N_2-fixation.

INTRODUCTION

Nodulation of soybean [*Glycine max* (L.) Merr.] is a developmentally complex process requiring interaction between *Bradyrhizobium* and the plant host, which is regulated by both genotypic and environmental factors (Gresshoff, 1990). A number of plant alleles controlling nodulation have been identified. The rj_1 allele was found to be associated with restriction of nodulation in a broad range of *Bradyrhizobium* strains (Caldwell, 1966), whereas the dominant allele Rj_2 conditioned an ineffective nodulation in strain USDA7, 14 and 122 (Devine et al., 1991). The dominant allele Rj_3 conditioned an ineffective nodulation inoculated with USDA33 (Vest, 1970). Rj_4 was a dominant allele controlling an ineffective nodulation upon inoculation with USDA61 (Vest and Caldwell, 1972). The two recessive genes rj_5 and rj_6, are found to be responsible for non-nodulating 'NN5', a mutagenized from 'Williams'. Pracht et al. (1993) confirmed that nod139 induced from 'Bragg', carrying the non-nodulating gene allelic to NN5. Hypernodulation in soybean mutants was controlled by rj_7 (Vuong et al., 1996; Vuong and Harper, 2000) and rj_8 (Vuong et al., 1996), whereas supernodulation was controlled by *nts* (Kolchinsky et al., 1997).

The potential of N_2 fixation can be observed by plant characters, viz. number of nodules per plant, fresh nodule weight per plant, dry nodule weight per plant, dry plant weight, and ARA (Pazdernik et al., 1996; King and Purcell, 2001). Nitrogen fixation activity increases sharply after flowering stage and gradually decreases after green pod stage. Although the ARA method is a direct measurement of the activity, it is sometimes not sufficiently sensitive for determination of nitrogen fixation (Patterson and LaRue, 1983). In order to evaluate fixation efficiency of a soybean-*Bradyrhizobium* interaction, all of the above-mentioned components should be determined. Then, a plant breeder could compile all the fixation components into soybean breeding lines by accumulating the favorable markers linked to these components.

With the advent of DNA marker technology, QTLs can be detected and located in the plant genome (Tanksley et al., 1989; Van et al., 2004). Desirable gene associated with agronomic traits can be selected via its linkage to easily detectable markers (Yu et al., 2005). SSR marker has several advantages in genetic mapping studies because it is co-dominant, high in heterozygosity, rapid, reliable, abundance, and highly polymorphic (Akkaya et al., 1992). In addition, the SSR labeled M13 (-21) primer is more specific in amplifying the repeated DNA sequence and can be widely used in a multiplexed manner, although the marker alleles per primer are reduced in expense of the specificity (Schuelke, 2000).

In this study, SSR mapping was utilized to identify QTLs associated with N_2-fixation components in the F_2-derived RILs created from a cross between 'SJ2' and 'Suwon157'. Number of nodules per plant, fresh nodule weight per plant, dry nodule weight per plant, dry plant weight, and ARA were included as N_2-fixation components.

MATERIALS AND METHODS

Mapping Population

One hundred and thirty-six RILs derived from a cross between a Thai cultivar 'SJ2' and a Korean cultivar 'Suwon 157' were used. The RILs were developed at Kamphaeng Saen Campus of Kasetsart University, Thailand by single seed descent method originating from a different F_2 plant during December 2000 - December 2003. Finally, 136 F_6 lines were obtained and treated as the RILs. The two cultivars were chosen as parental lines in this study because of their polymorphism in N_2-fixation components (Tanya et al., 2005).

Table 1. Mean squares and expected mean squares (EMS) from the analysis of variance for N_2-fixation components.

Source of variation	df	No. of nodules/plant	Nodule fresh wt/plant (g)	Nodule dry wt/plant (g)	Plant dry weight (g)	ARA (μmole C_2H_4/pl/hr)	EMS
Between RILs	135	587**	0.339**	0.017**	0.74**	45.5**	$\sigma^2 + 2\sigma^2_R$
Error	136	73	0.056	0.005	0.15	15.0	σ^2
Heritability (h^2)		0.78	0.72	0.55	0.66	0.50	

Genotypic Data

Genomic DNA was extracted from 3 leaflets at R_3 stage using the protocol modified from Lambrides et al. (2000). The extraction buffer consisted of 1 mM Tris-HCl pH 8.0, 5 mM EDTA, 5 mM NaCl, 20% sodium dodecyl sulfate (SDS) and sodium bisulfide was used After genomic DNA was extracted with 5 M KOAc and resuspended in TB buffer, DNA concentration was measured using F-4500 Fluorescence Spectrophotometer (Model F-4500, Hitachi, Ibaragi, Japan). A total of 195 SSR and 7 SSR labeled M13 (-21) primers were used for this study. The PCR reaction for SSR followed the suggestion by Diwan and Cregan (1997), while the reaction for SSR labeled with M13 (-21) followed Schuelke (2000). Initially, the forward primers were labeled with fluorescent color tags (Applied Biosystems, Foster City, CA, USA), viz. blue (6-FAM), yellow (NED), and green (HEX). The SSR reaction contained the genomic DNA (10 ng/μl), 10x Buffer (w/$MgCl_2$), 2.5 mM of each dNTP, 2 unit Taq DNA polymerase, and 5 μM Primer Mix. Each PCR cycle consisted of 25 sec of denaturation at 94°C, 25 sec of annealing at 46°C, and 25 sec of extension at 68°C on PCT-225™ Thermal Controller (MJ research, Watertown, MA, USA). The PCR process was repeated for 32 cycles. The reaction mixture for SSR labeled M13 (-21) primers contained genomic DNA (10 ng/μl), 10x Buffer (w/$MgCl_2$), 2.5 mM of each dNTP, 2 unit Taq DNA polymerase, 5 μM forward primer with M13 tail, 10 μM reverse primer, and fluorescent labeled with M13 (-21). Two PCR steps were run; the first step required 30 sec of denaturation at 95°C, 45 sec of annealing at 54°C, and 45 sec of extension at 72°C for 30 cycles, the second step required 30 sec of denaturation at 95°C, 45 sec of annealing at 53°C, and 45 sec of extension at 72°C for 10 cycles. The PCR product quality was checked by 3% agarose gel before mixing into a set of 6 primers. The 2 μl PCR mixture was taken into a new well with 2 μl mixture of a standard size cocktail consisted of 200 μl formamide deionized, 100 μl loading buffer, 40 μl Genscan 500 (500XL). The final mixture was loaded in ABI Prism® 377 sequencer (Applied Biosystems, Foster City, CA, USA). The GeneScan® Analysis software, version 2.1.1 (Applied Biosystems, Foster City, CA, USA) and the Genotyper® software, version 2.0 (Applied Biosystems, Foster City, CA, USA) were used to analyze the allele size and gel image.

Phenotypic data analysis

The RILs and parental soybeans were inoculated with *B. japonicum* strain DASA 01026 and planted in a completely randomized design with 2 replications. The method modified from Somasegaran and Hoben (1985) was used to measure five phenotypic traits, viz. number of nodules per plant, nodule fresh weight per plant, nodule dry weight per plant, plant dry weight, and ARA. Phenotypic data were collected from two plants per each replicate. The phenotypic assay for N_2-fixation components were conducted at the Laboratory of the Soil Microbiology Group, Soil Science Division, Department of Agriculture, Bangkok in Thailand during March to November 2003.

Statistical analysis

The difference between RILs in each component trait was determined by an analysis of variance (ANOVA) using SAS program (SAS, 1999-2000). The expected mean square (EMS) components (Table 1) were used to estimate the heritability of each trait from the formula $h^2 = \sigma_R^2/(\sigma_R^2 + \sigma^2/r)$, where σ_R^2 and σ^2 are the variance components associated with RILs and error; r is number of replications (= 2 in this case).

Construction of linkage map and QTL analysis

In each trait, a single factor analysis of variance (SF-ANOVA) was used to evaluate the association between the genotypic and phenotypic data. The significant markers from SF-ANOVA were assigned into

Table 2. SSR markers linked to QTLs associated with the number of nodules per plant.

Markers	LG	SF-ANOVA[a]		Allelic means		SLG-Regr[b]		MLG-Regr[c]	
		P	R^2 (%)	Suwon 157	SJ2	*P*	R^2 (%)	*P*	R^2 (%)
Satt385	A1	0.0186	4.10	46	53	0.0186	4.10	0.0373	2.98
Satt180	C1	0.0126	4.65	45	52	0.0148	4.51	-	-
Satt294	C1	0.0196	4.15	45	52	-	-	-	-
Satt041	D1b+W	0.0462	3.02	52	46	-	-	-	-
Satt157	D1b+W	0.0204	4.01	52	45	0.0210	4.09	0.0489	2.75
Satt314	H	0.0391	3.41	44	50	0.0391	3.41	-	-
Satt440	I	0.0075	5.49	53	45	0.0075	5.49	0.0279	3.54
Satt529	J	0.0341	3.57	52	45	0.0321	3.68	0.0030	6.84
Sct_001	J	0.0274	3.77	52	45	-	-	-	-
Satt388	L	0.0175	4.77	52	45	-	-	-	-
Sat_038	O	<0.0001	18.51	55	40	<0.0001	21.98	<0.0001	17.04
Sat_108	O	<0.0001	16.25	55	41	-	-	-	-
Sat_109	O	<0.0001	14.87	55	42	-	-	-	-
Sat_190	O	0.0008	8.15	53	43	-	-	-	-
Sat_274	O	0.0001	10.36	55	44	-	-	-	-
Satt123	O	0.0381	3.42	52	46	-	-	-	-
Satt153	O	<0.0001	15.64	55	41	-	-	-	-
Satt331	O	0.0008	8.38	54	44	-	-	-	-
Satt581	O	<0.0001	11.77	55	43	-	-	-	-
									33.15

[a]SF-ANOVA: single factor analysis of variance.
[b]SLG-Regr: multiple regression with markers on each linkage group.
[c]MLG-Regr: multiple regression with all significant markers from the SLG-Regr model.

each linkage group. Then, a multiple regression analysis was conducted by including all the significant markers on that linkage group in the model (SLG-Regr) to detect unique QTLs. Then, all the significant markers from the SLG-Regr analysis and unlinked single markers identified from SF-ANOVA were combined in a multiple linkage group regression model (MLG-Regr) to identify the markers linking to the trait. The regression analysis was forward and stepwise selection of the significant markers at $P < 0.05$. The percent of phenotypic variation explained by the markers was estimated from the coefficient of determination (R^2) from MLG-Regr using SAS program (SAS, 1999-2000). The MAPMAKER program version 3.0 (Lincoln et al., 1993) was used to construct linkage maps from the genotypic data. Markers were assigned to linkage groups using LOD threshold of 3.0 and maximum distance of 50 cM. Map distance was estimated using Kosambi mapping function.

RESULTS

A total of 202 SSR markers were used to construct a genetic linkage map. The map covered about 1094 cM with 78 SSR and 7 SSR labeled M13 (-21) primers. The average distance between two adjacent marker loci was about 12.9 cM.

Significant differences among RILs were observed in the nodulation and nitrogen fixation characters including nodule dry and fresh weight and ARA (Table 1). The heritability estimates were moderate in the ARA to high in the number of nodules per plant, implying that these traits can be effectively improved through selection.

The SF-ANOVA identified nineteen markers as potentially associated with QTLs for the number of nodules per plant at $P < 0.05$ (Table 2). Individually, these markers accounted for 3.02 to 18.51 % of the phenotypic variation. Nine markers were assigned on LG O, two markers each on LG C1, D1b+W, and J, while the rest four markers on LG A1, H, I, and L were

Table 3. SSR markers linked to QTLs controlling the nodule fresh weight per plant.

Markers	LG	SF-ANOVA[a]		Allelic means Nodule fresh wt/plant (g)		SLG-Regr[b]		MLG-Regr[c]	
		P	R^2 (%)	Suwon 157	SJ2	*P*	R^2 (%)	*P*	R^2 (%)
Satt385	A1	0.0171	4.20	0.920	1.089	-	-	-	-
Satt545	A1	0.0184	4.27	0.920	1.092	0.0184	4.27	0.0075	4.24
Satt380	J	0.0388	3.48	1.082	0.924	-	-	-	-
Satt414	J	0.0211	4.46	1.091	0.910	-	-	-	-
Satt529	J	0.0172	4.49	1.088	0.909	-	-	-	-
Sct_001	J	0.0090	5.26	1.092	0.900	0.0263	4.29	0.0296	2.69
Satt388	L	0.0219	4.45	1.068	0.893	-	-	-	-
Sat_038	O	<0.0001	16.39	1.137	0.802	-	-	-	-
Sat_108	O	<0.0001	13.43	1.137	0.831	0.0054	5.28	0.0018	6.15
Sat_109	O	<0.0001	12.74	1.139	0.843	-	-	-	-
Sat_190	O	0.0019	7.00	1.096	0.879	-	-	-	-
Sat_274	O	<0.0001	19.65	1.194	0.828	<0.0001	19.33	<0.0001	20.83
Satt123	O	0.0064	5.84	1.102	0.903	-	-	-	-
Satt153	O	<0.0001	14.73	1.117	0.816	-	-	-	-
Satt331	O	<0.0001	12.70	1.152	0.854	-	-	-	-
Satt581	O	<0.0001	17.84	1.185	0.833	-	-	-	-
									33.91

[a]SF-ANOVA: single factor analysis of variance.
[b]SLG-Regr: multiple regression with markers on each linkage group.
[c]MLG-Regr: multiple regression with all significant markers from the SLG-Regr model.

not linked with the other markers. SLG-Regr for the two markers on LG C1, the two markers on LGD1b+W, the two markers on LG J, and the nine markers on LG O retained Satt180 on LG C1, Satt157 on LG D1b+W, Satt529 on LG J, and Sat_038 on LG O, indicating the existence of a single QTL for the number of nodules on each linkage group. The MLG-Regr analysis with the seven independent markers retained five of these seven SSR markers. For the QTL linked to Satt385 on LG A1 the positive allele was derived from 'Suwon 157', whereas at the other four QTLs the positive alleles were from 'SJ2'. MLG-Regr accounted for 33.15% of the phenotypic variation in the number of nodules.

Sixteen markers were associated with the nodule fresh weight per plant at $P < 0.05$ using SF-ANOVA (Table 3). Each marker accounted for 3.48 to 19.65% of the total variation of this trait. QTLs on LG J, L, and O increased nodule fresh weight by 'Suwon 157' alleles, whereas 'SJ2' provided the positive alleles at QTL on LG A1. Satt545 on LG A1, Sct_001 on LG J, and Sat_108 and Sat_274 on LG O were detected by SLG-Regr analysis. It is interesting to note that all of these four markers identified using SLG-Regr analysis were also retained even after MLG-Regr analysis. This indicated that two independent QTLs on LG O were associated with nodule fresh weight. Sct_001 on LG J, Sat_108, and Sat_274 on LG O were found to be associated with the positive alleles from 'Suwon 157', whereas 'SJ2' provided the positive alleles at QTL on LG A1. The amount of phenotypic variation explained by these four QTLs was 33.91%.

Based on SF-ANOVA, twelve markers were detected as potentially linked to QTLs for the nodule dry weight per plant at $P < 0.05$ (Table 4). Individually, these markers showed variation from 1.67 to 13.27% among RILs. All markers indicated that all 'Suwon 157' alleles increased dry nodule weight. SLG-Regr analysis for the four markers retained Satt414 on LG J, Satt260 on LG K and two markers (Sat_108 and Sat_274) on LG O. This analysis indicated that Sat_108 and Sat_274 have detected unique QTLs. MLG-Regr analysis with four markers identified by SLG-Regr analysis confirmed that QTLs conditioning the nodule dry weight per plant were linked to markers Satt260 on LG K and Sat_108 and Sat_274 on LG O. At

Table 4. SSR Markers linked to QTLs associated with the nodule dry weight per plant.

Markers	LG	SF-ANOVA[a]		Allelic means		SLG-Regr[b]		MLG-Regr[c]	
		Nodule dry wt/plant(g)							
			R^2				R^2		R^2
		P	(%)	Suwon 157	SJ2	*P*	(%)	*P*	(%)
Satt414	J	0.0254	4.20	0.236	0.198	0.0214	4.48	-	-
Sct_001	J	0.0409	3.25	0.236	0.202	-	-	-	-
Satt260	K	0.0279	3.64	0.234	0.199	0.0279	3.64	0.0446	2.60
Sat_038	O	<0.0001	10.72	0.244	0.183	-	-	-	-
Sat_108	O	<0.0001	10.97	0.247	0.186	0.0125	4.66	0.0005	8.38
Sat_109	O	0.0001	1.67	0.228	0.216	-	-	-	-
Sat_190	O	0.0034	6.21	0.240	0.194	-	-	-	-
Sat_274	O	<0.0001	13.27	0.255	0.187	0.0001	12.18	<0.0001	17.76
Satt123	O	0.0376	3.44	0.237	0.203	-	-	-	-
Satt153	O	0.0004	9.24	0.239	0.186	-	-	-	-
Satt331	O	0.0006	8.71	0.248	0.193	-	-	-	-
Satt581	O	<0.0001	13.65	0.256	0.187	-	-	-	-
									28.74

[a]SF-ANOVA: single factor analysis of variance.

[b]SLG-Regr: multiple regression with markers on each linkage group.

[c]MLG-Regr: multiple regression with all significant markers from the SLG-Regr model.

Table 5. SSR markers linked to QTLs associated with the plant dry weight.

Markers	LG	SF-ANOVA[a]		Allelic means		SLG-Regr[b]		MLG-Regr[c]	
		Plant dry wt (g)							
			R^2				R^2		R^2
		P	(%)	Suwon 157	SJ2	*P*	(%)	*P*	(%)
Satt440	I	0.0293	3.68	2.07	1.84	0.0293	3.68	0.0069	4.80
Satt215	J	0.0205	4.43	2.09	1.83	-	-	-	-
Satt380	J	0.0058	6.12	2.11	1.80	-	-	-	-
Satt414	J	0.0019	7.98	2.13	1.77	-	-	-	-
Satt529	J	0.0016	7.73	2.12	1.77	-	-	-	-
Satt596	J	0.0053	6.39	2.11	1.79	-	-	-	-
Sct_001	J	0.0006	8.79	2.13	1.76	0.0023	8.30	0.0029	6.18
Satt388	L	0.0339	3.82	2.05	1.81	-	-	-	-
Sat_038	O	<0.0001	12.69	2.13	1.69	<0.0001	13.82	0.0320	2.91
Sat_108	O	<0.0001	11.76	2.14	1.72				
Sat_109	O	<0.0001	11.76	1.88	1.81	-	-	-	-
Sat_190	O	0.0026	6.55	2.09	1.78	-	-	-	-
Sat_274	O	<0.0001	14.75	2.20	1.73	0.0190	4.06	<0.0001	14.17
Satt123	O	0.0376	3.44	2.06	1.84	-	-	-	-
Satt153	O	<0.0001	11.49	2.11	1.71	-	-	-	-
Satt331	O	0.0005	8.99	2.15	1.78	-	-	-	-
Satt581	O	<0.0001	14.02	2.20	1.74	-	-	-	-
									28.06

[a]SF-ANOVA: single factor analysis of variance.

[b]SLG-Regr: multiple regression with markers on each linkage group.

[c]MLG-Regr: multiple regression with all significant markers from the SLG-Regr model.

Table 6. SSR markers linked to QTLs associated with acetylene reduction activity.

Markers	LG	SF-ANOVA[a]		Allelic means (ARA μmole C_2H_4/pl/hr)		SLG-Regr[b]		MLG-Regr[c]	
		P	R^2 (%)	Suwon 157	SJ2	*P*	R^2 (%)	*P*	R^2 (%)
Satt041	D1b+W	0.0324	3.50	10.58	8.68	-	-	-	-
Satt157	D1b+W	0.0147	4.46	10.61	8.49	0.0125	4.81	0.0021	6.18
Satt388	L	0.0239	4.32	10.50	8.48	-	-	-	-
Sat_038	O	0.0023	3.76	10.83	8.23	-	-	-	-
Sat_108	O	0.0436	3.03	10.25	8.77	-	-	-	-
Sat_109	O	0.0449	3.06	11.07	7.96	-	-	-	-
Sat_274	O	<0.0001	11.92	11.24	8.16	<.0001	12.36	<0.0001	12.34
Satt153	O	0.0135	4.57	10.30	8.47	-	-	-	-
Satt331	O	0.0012	7.91	11.02	8.21	-	-	-	-
Satt581	O	0.0002	10.17	11.25	8.21	-	-	-	-
									18.52

[a]SF-ANOVA: single factor analysis of variance.
[b]SLG-Regr: multiple regression with markers on each linkage group.
[c]MLG-Regr: multiple regression with all significant markers from the SLG-Regr model.

these three marker loci, the 'Suwon 157' allele increased nodule dry weight per plant. The multiple regression analysis accounted for 28.74% of the variation in nodule dry weight per plant among progeny.

The initial SF-ANOVA of plant dry weight detected seventeen SSR markers associated with QTLs for dry plant weight (Table 5). Each of these markers is located on LG I, LG J, LG L, and LG O. Individually, these markers accounted for 3.44 to 14.75% of the variation in plant dry weight. At all QTLs, the 'Suwon 157' allele increased plant dry weight. Both SLG-Regr and MLG-Regr analyses identified four significant markers, viz. Satt440 on LG I, Sct_001 on LG J, Sat_038 and Sat_274 on LG O. The multiple regression analysis accounted for 28.06% of the phenotypic variation.

Ten markers were identified by SF-ANOVA as being potentially associated with QTLs conditioning ARA at $P < 0.05$ (Table 6). Each marker accounted for 3.03 to 11.92% of the phenotypic variation in ARA among progeny. At all marker loci, the 'Suwon 157' alleles increased ARA. Combined MLG-Regr with SLG-Regr analyses revealed that only two markers, Satt157 on LG D1b+W and Sat_274 on LG O were associated with ARA. These two markers linked to QTLs conditioning ARA explained 18.52% of the total variation for ARA.

DISCUSSION

When compared with the heritability, the combined R^2 explaining the QTL in each fixation component may not be high. The MLG-Regr analysis accounted for less than 33.91% of the phenotypic variation in nodule fresh weight (Table 3). For the ARA, only 18.52% of the phenotypic variation was explained (Table 6). However, the heritability estimates varied from 50 to 78% depending on the phenotypic traits (Table 1). This would suggest that the other QTLs remain undetected, the markers were not linked to the true QTLs, the heritability estimate was poor, or that the epistatic interaction effects remain undetected. This may be partially due to the incomplete genomic coverage using only 202 SSR markers. More SSR markers should be used in combination with other type of markers to fill up the gaps in each linkage map and to identify more markers tightly linked to QTLs for each N_2-fixation component.

Our experiment identified different markers from the earlier works, possibly due to different parents used in developing the mapping population and phenotyping. Of specific interest in this study was a major QTL associated with the N_2-fixation components on LG O.

The minor ones were located on LG A1, D1b+W, I, J and K. Cregan et al. (1999) reported that Rj_1 allele for conditioning nodulation was located on LG D1b+W, while Rj_2 allele for conditioning ineffective nodulation was found on LG J in three mapping populations, viz. 59 F_2 plants from *G. max* x *G. soja*, 240 RILs from Minsoy x Noir, and 57 F_2 plants from Clark x Harosoy. The genes Rj_2 for ineffective nodulation, *Rmd* for powdery mildew resistance and *Rps*2 for phytophthora root and stem rot resistance were located on the classical linkage group 19 in the BARC-4 x Clark63 population (Devine et al., 1991). A study from the progenies derived from two isolines of a soybean cultivar 'Williams' showed that LG J was related to classical linkage group 19 with certain common loci such as Rj_2, *Rmd*, and Rps_2 (Polzin et al., 1994). A gene for supernodulation (*nts*) was linked to the RFLP anchor markers on LG H (Kolchinsky et al., 1997).

The results from the present study indicated that marker-aided selection may be effectively feasible for enhancing nodulation and nitrogen fixation in soybean. The existence of a major QTL on LG O conditioning more than 10% of the phenotypic variation in nodulation and nitrogen-fixing ability provides an opportunity to select lines in a segregating population. However, further confirmation will be needed for the markers detecting variation.

REFERENCES

Akkaya MS, Bhagwat AA and Cregan PB (1992) Length polymorphisms of simple sequence repeat DNA in soybean. Genetics 132: 1131-1139.

Caldwell BE (1966) Inheritance of a strain-specific ineffective nodulation in soybeans. Crop Sci 6: 427-428.

Cregan PB, Jarvik T, Bush AL, Shoemaker RC, Lark KG, Kahler AL, Kaya N, VanToai TT, Lohnes DG, Chung J and Specht JE (1999) An integrated genetic linkage map of the soybean genome. Crop Sci 39: 1464-1490.

Devine TE, Kilen TC and O'Neill JJ (1991) Genetic linkage of phytophthora resistance gene Rps_2 and the nodulation response gene Rj_2 in soybean. Crop Sci 31: 713-715.

Diwan N and Cregan PB (1997) Automated sizing of fluorescent-labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean. Theor Appl Genet 95: 723-733.

Gresshoff PM (1990) The importance of biological nitrogen fixation to new crop development. In: Janick J and Simon JE (eds.) Advances in New Crops. Timber Press, pp. 113-119, Portland, OR.

King CA and Purcell LC (2001) Soybean nodule size and relationship to nitrogen fixation response to water deficit. Crop Sci 41: 1099-1107.

Kolchinsky A, Landau-Ellis D and Gresshoff PM (1997) Map order and linkage distances of molecular markers close to the supernodulation (*nts-1*) locus of soybean. Mol Gen Genet 254: 29-36.

Lambrides CJ, Lawn RJ, Godwin ID, Manners J and Imrie BC (2000) Two genetic linkage maps of mungbean using RFLP and RAPD markers. Aust J Agric Res 51: 415-425.

Lincoln SL, Dary MJ and Lander ES (1993) Constructing genetic linkage maps with MAPMAKER/EXP version 3.0, a tutorial and reference manual. Available Source: http://linkage.rockefeller.edu/soft/mapmaker/index.html, December 3, 2005.

Patterson TG and LaRue TA (1983) Nitrogen fixation by soybeans: seasonal and cultivar effects, and comparison of estimates. Crop Sci 23: 488-492.

Pazdernik DL, Graham PH, Vance CP, and Orf JH (1996) Host genetic variation in the early nodulation and dinitrogen fixation of soybean. Crop Sci 36: 1102-1107.

Polzin KM, Lohnes DG, Nickell CD and Shoemaker RC (1994) Integration of Rps_2, *Rmd*, and Rj_2 into linkage group J of the soybean molecular map. J Heredity 85(4): 300-303.

Pracht JE, Nickell CD and Haper JE (1993) Genes controlling nodulation in soybean: Rj_5 and Rj_6. Crop Sci 33: 711-713.

SAS institute, Inc. (1999-2000) SAS/STAT User's Guide, 8.01st ed. SAS institute, Inc., Cary, NC, USA.

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18: 233-234.

Somasegaran P and Hoben HJ (1985) Methods in Legume-*Rhizobium* Technology. USAID, Hawaii.

Tanksley SD, Young ND, Paterson AH and Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7: 257-264.

Tanya P, Srinives P, Toojinda T, Vanavichit A, Nuntakij A, Kotepong S and Lee SH (2005) Evaluation of N_2-fixation traits in Thai and Korean soybean cultivars. (submitted to ScienceAsia)

Van K, Ha BK, Kim MY, Moon JK, Paek NC, Heu S

and Lee SH (2004) SSR mapping of genes conditioning soybean resistance to six isolates of *Xanthomonas axonopodis* pv. *Glycines*. Korean J Genetics 26 : 47-54.

Vest G (1970) *Rj*$_3$-A gene conditioning ineffective nodulation in soybean. Crop Sci 10: 34-35.

Vest G and Caldwell BE (1972) *Rj*$_4$-A gene conditioning ineffective nodulation in soybean. Crop Sci 12: 692-693.

Vuong TD and Harper JE (2000) Inheritance and allelism analysis of hypernodulation genes in the NOD3-7 and NOD2-4 soybean mutants. Crop Sci 40: 700-703.

Vuong TD, Nickell CD and Harper JE (1996) Genetic and allelism analyses of hypernodulation soybean mutants from two backgrounds. Crop Sci 36: 1153-1158.

Yu S, Moon JK, Hwang RY, Kim YH, Kim HM and Jeong SC (2005) Genetic and sequence analysis of the RAPD fingerprint containing a *Rsv3*-linked marker rapidly identified by using RILs in soybean. Korean J. Genetics 27: 127-132.

Plant Breeding 125, 77—84 (2006)

Development of an interspecific *Vigna* linkage map between *Vigna umbellata* (Thunb.) Ohwi & Ohashi and *V. nakashimae* (Ohwi) Ohwi & Ohashi and its use in analysis of bruchid resistance and comparative genomics

P. Somta[1], A. Kaga[2], N. Tomooka[2], K. Kashiwaba[2], T. Isemura[2], B. Chaitieng[2], P. Srinives[1] and D. A. Vaughan[2,3]

[1]Department of Agronomy, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand; [2]National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba 305-8602, Japan; [3]Corresponding author. E-mail: duncan@affrc.go.jp

With 4 figures and 1 table

Received November 18, 2004/Accepted February 14, 2005
Communicated by M. Yano

Abstract

To facilitate transfer of bruchid resistance to azuki bean (*Vigna angularis*) from its relatives an interspecific mapping population was made between rice bean, *V. umbellata*, and the related wild species *V. nakashimae*. The *V. umbellata* parent is completely resistant and *V. nakashimae* is completely susceptible to the bruchid beetle pests, azuki bean weevil (*Callosobruchus chinensis*) and cowpea weevil (*C. maculatus*). There is very low cross compatibility between *V. umbellata* and azuki bean. Therefore, *V. nakashimae*, that crosses with both *V. umbellata* and *V. angularis* without the need for embryo rescue, is used as a bridging species. A genetic linkage map was constructed based on an interspecific F_2 mapping population between *V. umbellata* and *V. nakashimae* consisting of 74 plants. A total of 175 DNA marker loci (74 RFLPs and 101 SSRs) were mapped on to 11 linkage groups spanning a total length of 652 cM. Segregation distortion was observed but only three markers were not linked to any linkage group due to severe segregation distortion. This interspecific genome map was compared with the genome map of azuki bean. Of 121 common markers on the two maps, 114 (94.2%) were located on the same linkage groups in both maps. The marker order was highly conserved between the two genome maps. Fifty F_2 plants that produced sufficient seeds were used for quantitative trait locus (QTL) analysis and locating gene(s) for *C. chinensis* and *C. maculatus* resistance in *V. umbellata*. The resistance reaction of these F_2 plants differed between *C. chinensis* and *C. maculatus*. Both resistances were quantitatively inherited with no F_2 plants completely susceptible to *C. chinensis* or *C. maculatus*. One putative QTL for resistance to each of these bruchid species was located on different linkage groups. Other putative QTLs associated with resistance to both *C. chinensis* and *C. maculatus* were localized on the same linkage group 1. Linked markers associated with the bruchid-resistant QTL will facilitate their transfer to azuki bean breeding lines.

Key words: *Vigna umbellata* — *Callosobruchus chinensis* — *C. maculatus* — comparative mapping — quantitative trait loci

Vigna species are an important source of protein for people, particularly in tropical Africa and Asia and several *Vigna* species have been domesticated in Asia. Among them the cultigens, mungbean [*V. radiata* (L.) Wilczek], black gram [*V. mungo* (L.) Hepper] and azuki bean [*V. angularis* (Willd.) Ohwi & Ohashi] are economically most important. Rice bean [*V. umbellata* (Thunb.) Ohwi & Ohashi] is occasionally cultivated in different parts of South-east and East Asia. It grows prolifically and is used for its green pods, seeds or for forage.

A major production constraint of the Asian *Vigna* is susceptibility to bruchid beetles (*Coleoptera*; *Bruchidae*) that eat seeds of legumes in storage. The most important of the bruchid beetles are azuki bean weevils (*Callosobruchus chinensis* L.) and cowpea weevils (*C. maculatus* F.) (Southgate 1979). Bruchid beetles first infest *Vigna* species in the field where the adult bruchid lays eggs on young pods, hatched larvae bore through the pod wall and feed while remaining concealed within seeds (Southgate 1979). When the crop is harvested and stored the bruchid continues feeding and eventually comes out as an adult and causes secondary infestation, which can result in total destruction of a seed lot within a period of 3–4 months (Banto and Sanchez 1972). Hence a major objective of *Vigna* breeding programmes is to produce bruchid-resistant varieties. The most studied source of bruchid resistance is an accession of wild mungbean, TC1966 [*V. radiata* var. *sublobata* (Roxb.) Verdc.], which has complete resistance to azuki bean weevils and cowpea weevils (Fujii et al. 1989). The resistance in TC1966 is controlled by a single dominant gene (*Br*) (Kitamura et al. 1988), which has been mapped with molecular markers (Young et al. 1992, Kaga and Ishimoto 1998) and successfully used in a breeding programme (Tomooka et al. 1992). Wild black gram (*V. mungo* var. *silvestris* Lukoki, Maréchal & Otoul) is also reported to be immune to *C. chinensis* and *C. maculatus* (Fujii et al. 1989, Dongre et al. 1996).

In order to develop azuki bean cultivars with resistance to both bruchids, the germplasm collection of azuki bean has been evaluated but no good sources of resistance have been found and most accessions are completely susceptible (Tomooka et al. 2000, Kashiwaba et al. 2003, N. Tomooka, unpublished data). Bruchid-resistant sources in *V. radiata* var. *sublobata* and *V. mungo* var. *silvestris* mentioned above are not cross compatible with azuki bean and so new suitable sources of bruchid-resistant germplasm are needed for the development of azuki cultivars with resistance to these weevils. Evaluation of a *Vigna* species core collection revealed new resistance sources with a high level of bruchid resistance (Tomooka et al. 2000). Among the resistant sources, cultivated rice bean (*V. umbellata*) is considered to be potentially the most useful because many accessions show complete resistance to the bruchids and it is a cultivated species (Kashiwaba et al.

2003). The chemicals responsible for bruchid resistance in *V. umbellata* (cv. Menaga) have recently been identified (US Patent 6,770,630B2). These chemicals are all derivatives of the flavanoid naringenin, two of the derivatives being isomers of the other two. Transfer of bruchid resistance from *V. umbellata* to susceptible azuki bean is difficult due to cross incompatibility. As an alternative approach to gene transfer the use of a bridging species has been proposed (Tomooka et al. 2000). Species of the *V. minima* complex, *V. minima* (Roxb.) Ohwi & Ohashi, *V. riukiuensis* (Ohwi) Ohwi & Ohashi and *V. nakashimae* (Ohwi) Ohwi & Ohashi, have been suggested as the most suitable bridging species between *V. umbellata* and *V. angularis* (Vaughan et al. 2005).

Restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs or microsatellites) are widely employed to construct linkage maps and mapping of agronomically important traits in many crop plants because they are highly informative and reproducible. In *Vigna* species, SSR markers have been developed in cowpeas [*V. unguiculata* (L.) Walpers] (Li et al. 2001), mungbean (Kumar et al. 2002) and azuki bean (Wang et al. 2004b). In *Vigna* crops, genetic linkage maps have been constructed for mungbean, cowpea and azuki bean and used to map genes/quantitative trait loci (QTLs) for seed weight in mungbean and cowpea, bruchid resistance and powdery mildew resistance in mungbean (see Kaga et al. 2005 for review).

The study reported here analysed an interspecific cross between rice bean (*V. umbellata*) showing complete resistance and *V. nakashimae* showing complete susceptibility to bruchids. The resulting F_2 population was used to construct a genetic linkage map and address the following objectives:

1 Analyse the inheritance of the bruchid resistance in a cross between *V. umbellata* and *V. nakashimae*;
2 Locate the bruchid-resistance gene(s) on the genome map;
3 Compare the genome map developed with that of azuki bean.

Materials and Methods

Plant materials: Bruchid-resistant cultivated rice bean (*Vigna umbellata*) accession JP100304 was used as the female parent in a cross with the bruchid-susceptible, wild species, *V. nakashimae* accession JP107879, produced at the National Institute of Agrobiological Sciences, Tsukuba, Japan. The *V. umbellata* accession was a landrace from Nepal and the *V. nakashimae* accession originated from a wild population collected in Nagasaki prefecture, Japan. Both accessions came from the National Institute of Agrobiological Sciences Genebank, Tsukuba, Japan. The wild species *V. nakashimae* is a bridging species between rice bean and azuki bean and was the male parent in this cross. An F_1 plant (*V. umbellata* × *V. nakashimae*) was grown in a greenhouse. Among 131 F_2 seeds planted 74 F_2 individuals grew normally.

Bioassay of bruchid resistance: *Callosobruchus chinensis* (azuki bean weevils) and *C. maculatus* (cowpea weevils) were reared on mungbean seeds in an incubator at 30°C and 70% relative humidity and used to evaluate resistance in the parents and F_3 seeds of F_2 individuals that produced sufficient seeds for the assay. Between 15 and 20 F_3 seeds from individual F_2 plants and 20 seeds from parents were placed on 9 cm diameter Petri dishes with the hilum oriented down on some sticky tape. Twenty (10 males and 10 females) newly emerged bruchid adults were introduced into each Petri dish for 24 h. The infested seeds were maintained in incubators at 30°C and 70% relative humidity. Twenty days after insect introduction, the number of damaged seeds was recorded daily and the damaged seeds promptly removed from the Petri dish. A damaged (susceptible) seed was considered to be one from which an insect had emerged. As the generation time for bruchids is about 30 days under these experimental conditions the evaluation was continued until 50 days after insect introduction. Data recording was then terminated to avoid counting second-generation infestation. Seeds that showed no surface damage were further examined for internal damage by X-ray analysis (model TV-PBO-C; SOFTEX, Tokyo, Japan).

DNA extraction: Genomic DNA was extracted from leaves of 74 individual F_2 plants and parents by the method of Draper and Scott (1988). DNA concentration was adjusted to 200 ng/µl for RFLP analysis and to 1 ng/µl for SSR analysis by comparing with known concentrations of standard λDNA on a 1.5% agarose gel.

RFLP analysis: Genomic DNA from parents was digested with six restriction enzymes (*Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Xba*I) and was electrophoresed on 1% agarose gels, followed by DNA blotting on to Hybond N+ membrane (Amersham, Little Chalfont, UK). The blots were hybridized with mungbean, cowpea, common bean and soybean probes to determine RFLP between parents. DNA from F_2 plants was digested with enzymes that yielded clear polymorphisms between the parents, subjected to electrophoresis and blotted on to membranes. Probe labelling, hybridization and detection were carried out using an electrochemiluminescence direct nucleic acid labelling system according to the manufacturer's instructions (Amersham).

SSR analysis: The SSR markers mapped on the azuki bean linkage map (Han et al. 2005) were selected and used to detect polymorphism between the two parents following the methods described by Wang et al. (2004b) with slight modifications. In brief, 10 µl of PCR reaction mixture contained 1 ng of genomic DNA, 1 U KOD-plus- DNA polymerase (TOYOBO, Tokyo, Japan), 1x PCR buffer for KOD-plus-, 0.2 mM dNTPs, 1 mM $MgSO_4$ and 5 pmol of the forward and reverse primers. The 5'-end of the reverse primer was fluorescent labelled with one of the four following fluorescent dyes, 5-Fam, VIC, NED and PET (Applied Biosystems, Foster City, CA, USA). After checking for polymorphism between parents, three or four differentially coloured primers were mixed into single PCR reaction mixtures and amplified at the same time. Fluorescent signal strengths of each amplified fragment were adjusted to the same level by adding non-fluorescent labelled primer pairs instead of labelled primers. Such multiplex sets were used to genotype F_2 individuals of the mapping population. PCR reactions were performed with a GeneAmp PCR System 9700 (Applied Biosystems) programmed as follows: 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 60°C for 15 s and 68°C for 15 s. Following amplification, 2 µl of PCR product was mixed with 13 µl of Hi-Di formamide containing 0.17 µl GeneScan 500 LIZ size standard (Applied Biosystems). The multiplex PCR products separated into four colours on an ABI Prism 3100 Genetic Analyzer and the genotypes were determined with GENEMAPPER ver. 3.0 (Applied Biosystems).

Linkage map construction and QTL mapping The genetic map was constructed using JoinMap ver. 3.0 (Van Ooijen and Voorrips 2001). A segregation ratio for each marker in the F_2 population was tested for the goodness-of-fit to a 1 : 2 : 1 or 3 : 1 ratio using chi-square test at 5, 1 and 0.1% significance levels. According to Pham et al. (1990), the type of gametic or zygotic selection for co-dominant loci is determined by the chi-square test at the 5% significance level. Markers were assigned to linkage groups based on pairwise recombination frequencies and LOD values. An LOD value of 3 or greater was used to create linkage groups and the recombination value was converted into a map distance (cM) using the Kosambi mapping function (Kosambi 1944). Double crossovers between adjacent loci due to miss-scored individuals were confirmed manually. Linkage groups were named following azuki bean linkage groups (Han et al. 2005).

A subset of molecular markers spaced across the genetic linkage map with no missing genotype data was selected and used for composite interval mapping (Zeng 1994) (CIM; ZmapQtl Model 6). QTL analysis was carried out using WinQTL Cartographer (Wang et al. 2004a). The

markers used as cofactors for CIM were selected by the forward stepwise regression (F method in SRmapQtl). The number of background markers 'window size' was set to 5 and 10 respectively. Arcsine-transformed data on percentage of cumulative number of damaged F_3 seeds for each day from 20 to 50 days was used for QTL analysis. The presence of a putative QTL was declared whenever the LOD score exceeded the threshold of 2.5. To determine the effect of seed size, which might affect bruchid resistance, QTLs for 100 seed weight (SW) were analysed and compared to those for bruchid resistance.

Results

Bruchid resistance

Resistance to *C. chinensis* and *C. maculatus* was tested in the parents *V. umbellata* and *V. nakashimae*. The average number of eggs laid per seed (E/S) by *C. chinensis* was 2.4 for *V. umbellata* and 3.1 for *V. nakashimae*. For *C. maculatus*, the bruchids laid 2.8 and 3.1 E/S on *V. umbellata* and *V. nakashimae* respectively. *Vigna umbellata* was completely resistant to the two bruchid species, neither *C. chinensis* nor *C. maculatus* emerged from seeds of this species. Whereas *V. nakashimae* was completely susceptible to both *C. chinensis* and *C. maculatus*. All seeds of *V. nakashimae* were damaged within 32 days by *C. chinensis* and within 33 days by *C. maculatus*, after insect introduction.

Evaluation of three F_1 seeds showed resistance to both bruchid species. Among 131 F_2 seeds planted, 57 (43.5%) showing abnormalities were excluded from further study: 20 died at the vegetative stage, six failed to flower, eight dropped all their pods and 23 did not produce normal seeds. Finally, 74 F_2 individuals were used to develop the genetic linkage map. A further 24 F_2 individuals were excluded from the bruchid evaluation test because, although plants grew normally, the number of F_3 seeds was insufficient. Thus F_3 seeds from 50 F_2 individuals were used for the bruchid resistance test. Fifteen to twenty F_3 seeds were randomly evaluated. In F_2 lines (F_3 seeds) the average E/S values for *C. chinensis* and *C. maculatus* were 3.9 ± 0.16 and 4.3 ± 0.17 respectively. In the bruchid resistance test of 50 F_2 lines, *C. chinensis* emerged from 32 lines and *C. maculatus* emerged from 13 lines during evaluation. However, none of these lines was as susceptible to *C. chinensis* or *C. maculatus* as their susceptible parent, *V. nakashimae*.

Variation in percentage of damaged F_3 seeds (PDS) in the parents and F_2 population caused by *C. chinensis* at 32 and 39 days and by *C. maculatus* at 33 and 44 days are shown (Figs 1a,b and 2a,b respectively). These two dates correspond to the day on which each bruchid species (a) completely damaged seeds of susceptible parents and (b) stopped emerging from the seeds. The experiment was terminated at 50 days when no bruchids had emerged for more than 1 week. The frequency distribution of PDS by *C. chinensis* at 32 days and *C. maculatus* at 33 days both showed a skewed distribution towards the resistant parent, *V. umbellata* (Figs 1a and 2a respectively). The results suggest that dominant genes control resistance to bruchid beetles. The population average of PDS was 8.5 + 1.9 for *C. chinensis* and 0.90 + 0.3 for *C. maculatus*. By these dates the susceptible parent, *V. nakashimae*, was completely damaged. Emergence of adult bruchids from F_3 seeds continued until 39 days for *C. chinensis* and until 44 days for *C. maculatus*. At the end of evaluation, the average PDS in incompletely susceptible F_2 lines increased to 14.1 + 2.4% and 2.3 + 0.8% in *C. chinensis* and *C. maculatus* respectively. There was a significant positive correlation between the PDS of *C. chinensis* and *C. maculatus* resistance ($r = 0.36$, $P = 0.012$).

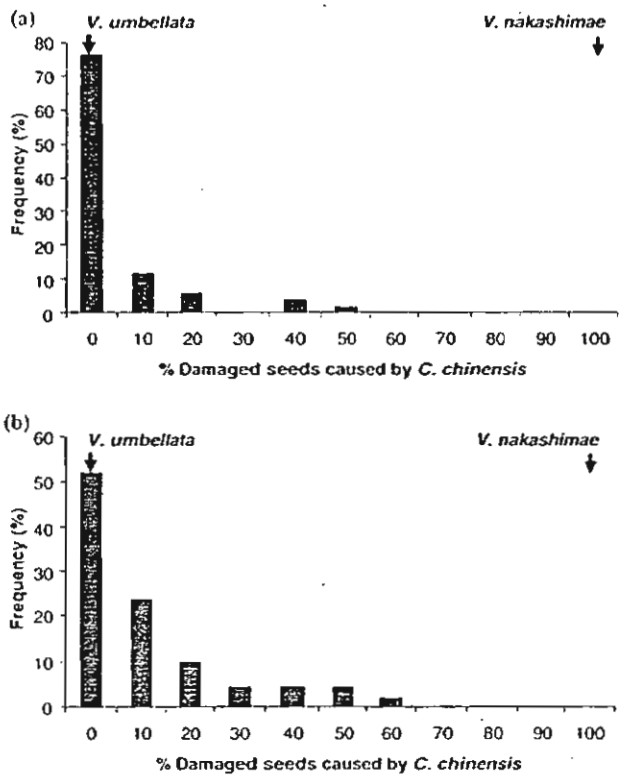


Fig. 1: Frequency distribution of percentage damaged seeds (PDS) caused by *Callosobruchus chinensis* in an F_2 population (based on F_3 seeds) derived from a cross between *Vigna umbellata* and *V. nakashimae* at (a) 32 days and (b) 39 days

Delayed bruchid development was observed in the F_2 population. The average developmental period of *C. chinensis* among susceptible F_2 lines was 32.2 days (range 26–39 days), 5 days longer than the 27 days recorded for *V. nakashimae*. In the case of *C. maculatus*, the average developmental period among susceptible F_2 lines was 34.4 days (range 27–44 days), 6 days longer than the 28 days recorded for *V. nakashimae*.

X-ray analysis of undamaged seeds in the F_2 population revealed that, for a few seeds, the internal parts were in fact damaged. When undamaged seeds were cut open, most bruchids had died at the first instar larval stage (62.9% F_2 individuals for both *C. chinensis* and *C. maculatus*) but some had died during the second instar larva or later stages, pupa or adult. In seeds of *V. umbellata*, bruchids always died during the first larval stage.

The resistant parent, *V. umbellata*, has an SW of 3.9 g and *V. nakashimae* has an SW of 1.8 g. Average SW in the F_2 population was 3.2 ± 0.1 g (range 1.4–5.5). Based on the F_2 population used for bruchid evaluation, the correlations between SW and PDS were −0.19 ($P = 0.186$) for *C. chinensis* and −0.13 ($P = 0.106$) for *C. maculatus* indicating that seed weight had no influence on bruchid resistance.

Interspecific linkage map of *V. umbellata* × *V. nakashimae*

Simple sequence repeat markers and RFLP probes were screened to detect DNA polymorphisms between *V. umbellata* and *V. nakashimae*. Of the 200 SSR markers tested, 132 (66%) amplified DNA fragments in both parents and 122 (61%)

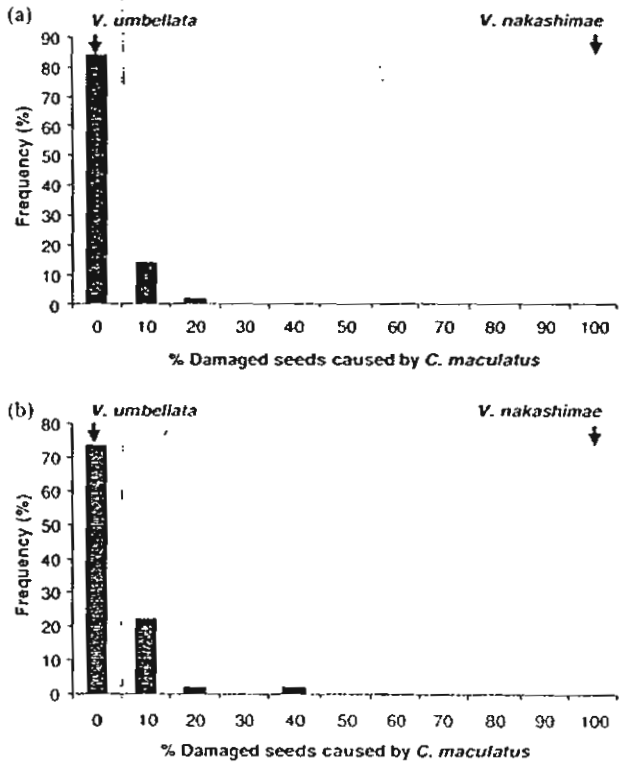


Fig. 2: Frequency distribution of percentage damaged seeds (PDS) caused by *Callosobruchus maculatus* in an F_2 population (based on F_3 seeds) derived from a cross between *Vigna umbellata* and *V. nakashimae* at (a) 33 days and (b) 44 days

markers revealed clear polymorphism. Of the 122 SSR markers 97 were successfully used for mapping in the F_2 population. Of the 185 mungbean, cowpea, soybean and common bean probes surveyed, 152 (82.1%) yielded clear polymorphisms between *V. umbellata* and *V. nakashimae* with at least one of the six restriction enzymes. From these, 152 RFLP probes 68 (26 mungbean, 7 cowpea, 22 common bean and 13 soybean) were used for mapping in the F_2 population.

A molecular-marker based linkage map of the interspecific *V. umbellata* × *V. nakashimae* F_2 population was constructed with 74 RFLP and 101 SSR loci (Fig. 3). The map spanned 652 cM with an average number of 15.9 marker loci per linkage group and an average distance between adjacent markers of 3.7 cM. The largest and smallest linkage groups were 95 cM (26 loci) and 33 cM (9 loci) respectively. SSR markers were distributed throughout the 11 linkage groups. Five RFLP probes (Bng022, Bng081, Bng107, mgM415 and sgA077) and three SSR markers (CEDG024, CEDG094 and CEDG105) had multi-loci with two or three loci segregating independently (suffix lower-case letter in Fig. 3). Five RFLP and 10 SSR loci appear to be dominant as no heterozygous individuals were found (italics in Fig. 3). Among all the loci analysed, 26% (28 SSR and 14 RFLP loci) deviated significantly ($P < 0.05$) from the expected ratios of 3 : 1 or 1 : 2 : 1. Such distorted loci were found on all linkage groups except for linkage group 9. Three SSR loci (CEDG096, CEDG105c and CEDG063) were not located on the linkage map and showed significant distortion towards the heterozygous state ($P < 0.001$). The majority of these distorted loci were clustered on linkage groups 2, 6 and 11. In particular, 10 of 11 loci on linkage group 11, and seven of nine loci on linkage group 6 exhibited distorted segregation under gametophytic selection, in favour of *V. umbellata* alleles, whereas seven loci on linkage group 2 showed distorted segregation under zygotic selection.

Quantitative trait loci for bruchid resistance

Composite interval mapping was performed in two ways. The first approach was to detect QTL location controlling bruchid resistance on the day when seeds of the susceptible parent were completely damaged by bruchids (Figs 1a and 2a). This resulted in finding significant genome regions on linkage group 7 associated with *C. chinensis* resistance and on linkage group 4 for *C. maculatus* resistance (Table 1). A putative QTL for *C. chinensis* resistance located on linkage group 7 at marker interval CEDG064-CEDG111 explained 53.4% of the variation. This QTL is named *Brc*3.1, where *Brc* stands for Bruchid resistance for *C. chinensis*, 3 for the population number and 1 the QTL number. This mapping population was designated as '3', populations '1' and '2' are BC_1F_1 and F_2 populations between *V. nepalensis* Tateishi and Maxted and *V. angularis* (Han et al. 2005, T. Isemura, unpublished data). The putative QTL for *C. maculatus* resistance detected on linkage group 4 accounted for 60.1% of the phenotypic variation and is named *Brm*3.1. *Vigna umbellata* allele at *Brc*3.1 and *Brm*3.1; both had a negative dominant effect, with reduced seed damage by both bruchids.

The second approach was to use data on the day bruchids stopped emerging from seeds of the F_2 population (Figs 1b and 2b). This revealed two putative QTLs located on linkage groups 1 and 7 for resistance to *C. chinensis* and two putative QTLs mapped to linkage groups 1 and 4 for resistance to *C. maculatus* (Table 1). QTL (*Brc*3.1) at CEDG064-CEDG143 on linkage group 1 explained 25.6% of the phenotypic variation. The other QTL, *Brc*3.2, mapped on linkage group 1 between Bng162 and mgM381 accounted for 18.5% of the phenotypic variation. For *C. maculatus* two putative QTLs on linkage groups 1 and 4 were detected. *Brm*3.2 was identified on linkage group 1 between CEDG019 and mgM381 and *Brm*3.1 on linkage group 4 between CEDG154 and CEDG232 and accounted for 18.9 and 37.3% of the phenotypic variation respectively. These QTLs reduced the percentage of damaged seeds in both an additive and a dominant manner.

The QTL analysis for seed weight was also conducted. A single chromosome region located on linkage group 10 between sgA077b and Bng119 was identified associated with seed weight (LOD score = 5.3). *Sdwt*3.1 explained 23.7% of phenotypic variation of seed weight with the effect of the *V. umbellata* allele increasing seed weight (additive effect = 6.8, dominant effect = 3.1).

Discussion

Linkage map and comparison to other linkage maps

Genetic maps are a basic tool in molecular genetic studies and crop improvement. In the genus *Vigna*, 10 genetic linkage maps have so far been developed, mainly using RFLP and/or random amplified polymorphic DNA markers (Kaga et al. 2005). In the present study the molecular linkage map was constructed based on SSR markers, the most useful molecular marker systems in plant breeding. The F_2 population was

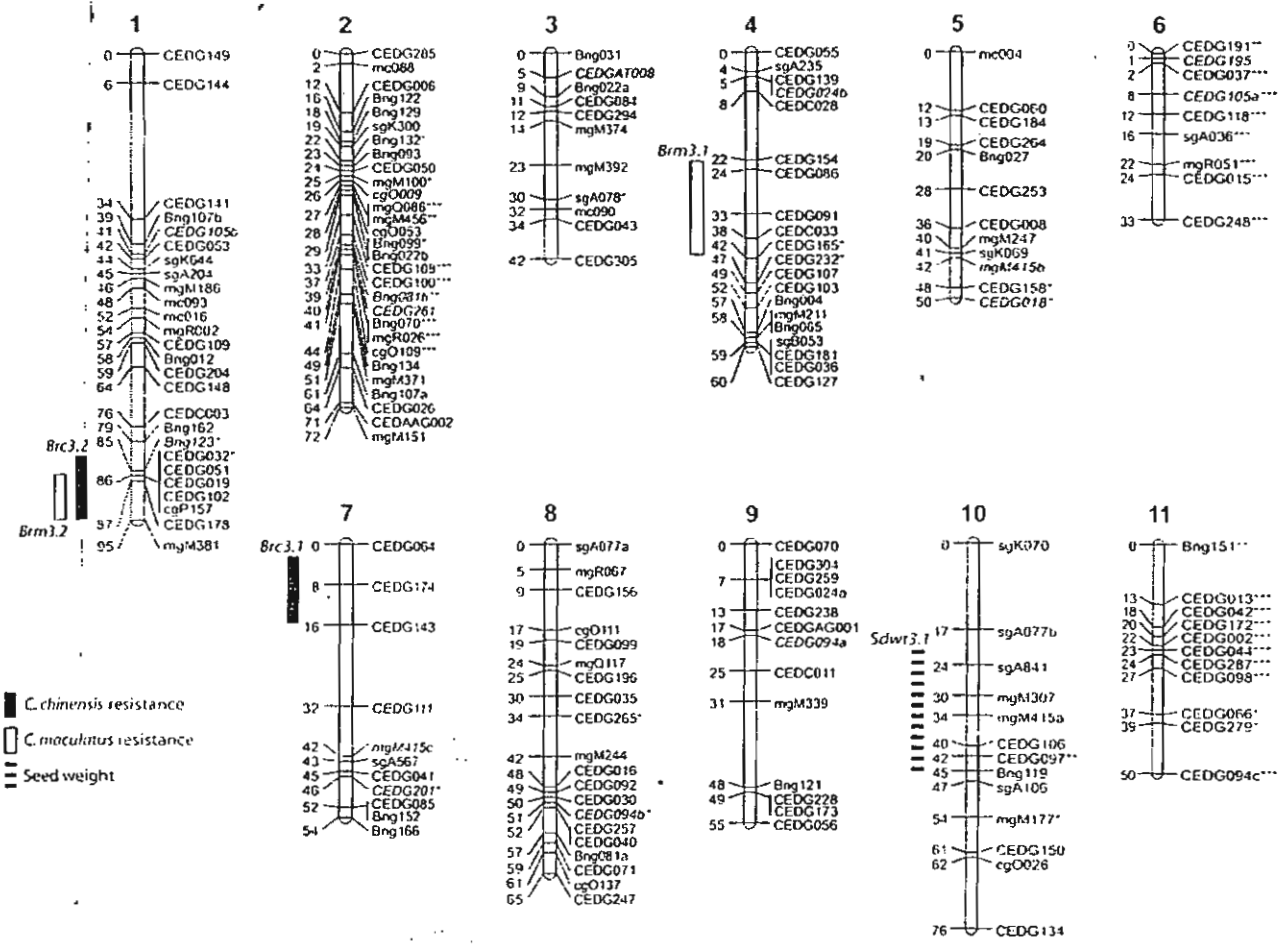


Fig. 3: A genetic linkage map of rice bean (*Vigna umbellata*) and *V. nakashimae* constructed from an F_2 population with RFLP and SSR markers. Map distances and marker names are shown on the left and right sides of the linkage groups respectively. Distances are given in Kosambi centimorgans. Prefixes mgM, mgQ, mgR, and mc indicate mungbean probes; cgO and cgP label cowpea probes; Bng identifies common bean probes; sgA, sgB and sgK represent soybean probes. Lower-case letters are suffixed to markers with multiple loci. SSR markers are presented with primer codes. Italicized markers show dominant loci. Markers showing significant deviation from the expected segregation ratios at 0.05, 0.01, and 0.001 levels are indicated with *, **, and *** respectively. Bars on the left side of the linkage groups indicate the LOD1 support intervals of putative QTLs

Table 1: Results of QTL analysis for *Callosobruchus chinensis* resistance and *C. maculatus* resistance in F_2 population (based on F_3 seeds) derived from *Vigna umbellata* × *V. nakashimae* by composite interval mapping (CIM)[1]

Bruchid species	Evaluation (days)	QTL name	Linkage group	LOD1 support for marker interval	Position (cM)	LOD score	Effect of *V. umbellata* allele on PDS: Additive (a) %	Effect of *V. umbellata* allele on PDS: Dominant (d) %	% PVE
C. chinensis	32	*Brc*3.1	7	CEDG064–CEDG111	4.0	8.0	−4.8	−3.2	53.4
	39	*Brc*3.1	7	CEDG064–CEDG143	4.0	4.0	−3.6	−2.9	25.6
		*Brc*3.2	1	Bng162–mgM381	90.6	2.6	−2.5	−1.2	18.5
C. maculatus	33	*Brm*3.1	4	CEDG154–CEDG232	40.2	11.1	−0.4	−0.4	60.1
	44	*Brm*3.1	4	CEDG154–CEDG232	27.4	5.0	−0.6	−0.4	37.3
		*Brm*3.2	1	CEDG019–mgM381	88.6	2.9	−0.4	−0.5	18.9

[1]CIM was performed using bruchid assay data obtained on the day when seeds of the susceptible parent were completely damaged by bruchids (32 and 33 days for *C. chinensis* and *C. maculatus*, respectively) and the day bruchids stopped emerging from the seeds of the F_2 population (39 and 44 days for *C. chinensis* and *C. maculatus*, respectively).

derived from an interspecific cross between *V. umbellata* and *V. nakashimae*. The map consisted of 74 RFLP and 101 SSR loci, and 11 linkage groups were formed corresponding to the haploid chromosome number of *V. umbellata* and *V. nakashimae*.

Comparative mapping has demonstrated conservation of gene and marker order across genomes for *Vigna* species (Kaga et al. 2005) and also between *Vigna* and related legume taxa (Boutin et al. 1995). The interspecific *Vigna* map constructed in this study and a genetic map of azuki

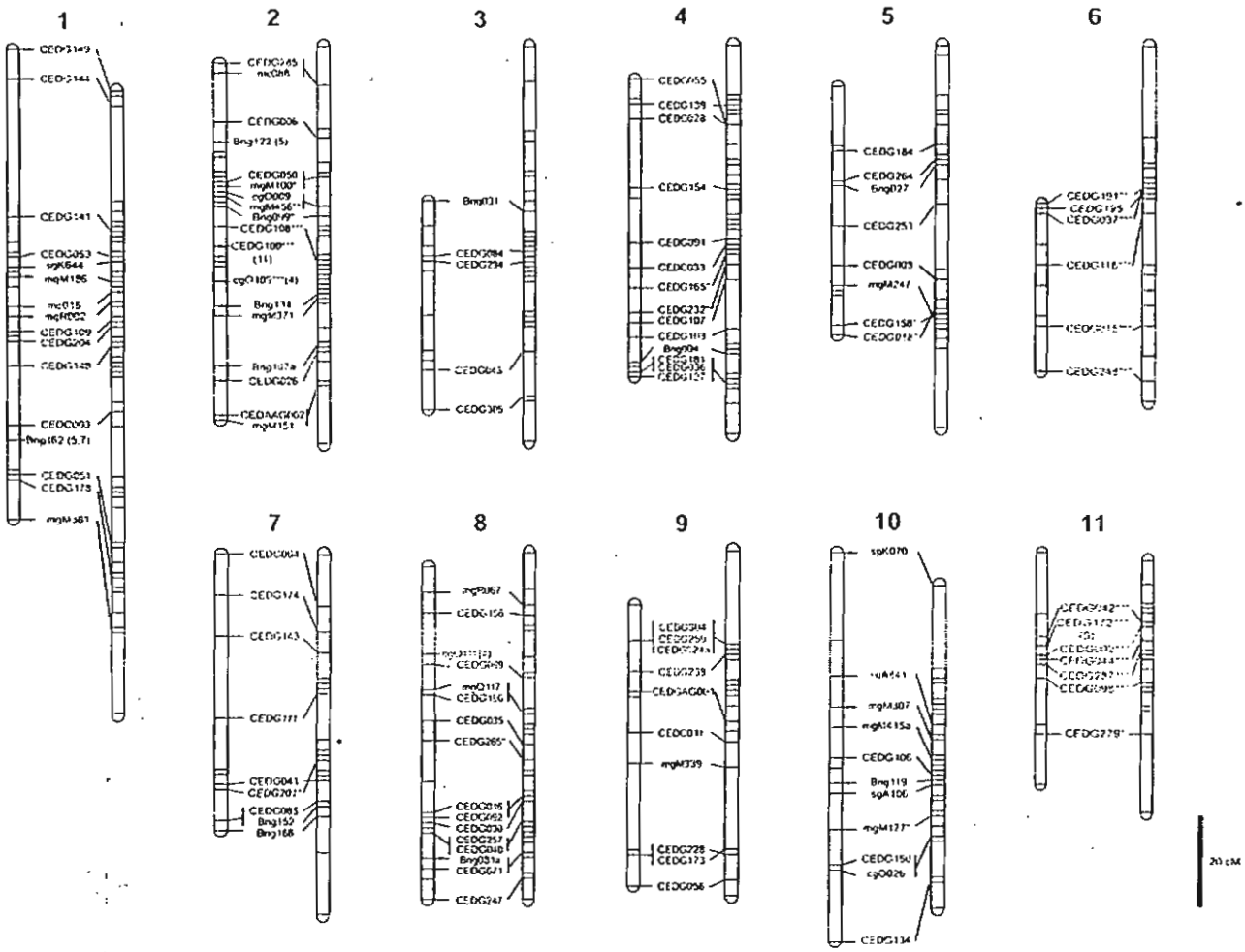


Fig. 4: A comparative linkage map of an interspecific *Vigna umbellata* × *V. nakashimae* map and azuki bean (*V. angularis*) based on common RFLP and SSR markers. Linkage groups are named according to linkage groups of rice bean (1–11), which are aligned on the left. Lines connect common markers between linkage groups. Markers followed by a number in parenthesis indicate locations of the loci on the other linkage groups

bean constructed from a BC_1 population (*V. nepalensis* × *V. angularis*) × *V. angularis* (Han et al. 2005) were compared to examine conserved linkage blocks and marker order between the two maps (Fig. 4). A total of 114 (94.2%) SSR loci were successfully located on the same linkage groups and in a similar order to the linkage map of azuki bean. The SSR primer sequences reveal a well-conserved genome structure among *Vigna* species and these sequences could aid in the study of genomes of other *Vigna* species.

However, a few discrepancies in genetic distance, location on linkage group and order between marker loci were observed (Fig. 4). Some markers mapping to the same position on the azuki bean map were found to be separated in the present map (e.g. linkage group 8; mgQ117 and CEDG196). The CEDG100 locus, which was mapped to linkage group 2 of the *V. umbellata* × *V. nakashimae* map, was located on linkage group 11 of the azuki bean map. Another example comes from the markers on the distal end of linkage group 5, mgM247, CEDG158 and CEDG018 in the *V. umbellata* × *V. nakashimae* map that were in the reverse order in the azuki bean map. Such differences between the two maps can be explained by chromosomal rearrangements and/or translocations during the evolutionary divergence of these species. In addition, distorted segregation in the present study was observed at 24% of loci in contrast to 3.5% of loci showing distorted segregation in the azuki bean map. These results support previous results that *V. angularis* and *V. nepalensis* are more closely related than *V. nakashimae* and *V. umbellata* within section *Angulares* of the subgenus *Ceratotropis* (Tomooka et al. 2002).

Segregation distortion of markers has been noted in previous genetic mapping studies of *Vigna* species such as mungbean, cowpea and azuki bean, with the level of distortion ranging from 12 to 30.8% (Kaga et al. 2005). Several RFLP markers facilitated comparison of the distorted loci between previously reported maps and the present map. Most of these distorted loci are specific to linkage groups 2, 6 and 11 in the present map and they all differ from the markers showing distorted segregation on the interspecific genetic maps of *V. umbellata* × *V. angularis* (Kaga et al. 2000) and *V. angularis* × *V. nakashimae* (Kaga et al. 1996).

Significant differences in the transmission of alleles were found on linkage groups 2, 6 and 11. Among these linkage groups, groups 6 and 11 have a higher than expected number of *V. umbellata* alleles. Preferential transmission of *V. umbellata*

alleles has also been reported in crosses between *V. umbellata* and *V. angularis* (Kaga et al. 2000). Further studies of *V. umbellata* to elucidate the reason for this are needed.

Cross-compatibility relationships in *Vigna* are complex and whether they are used as the male or female parent does affect the success in developing hybrids between species (Tomooka et al. 2002). Ease of hybridization among *V. angularis*, *V. nakashimae* and *V. umbellata* differs. Crosses between *V. angularis* and *V. umbellata* require embryo rescue and there is a high level of abnormality in segregating populations (Kaga et al. 2000). The level of abnormal segregation is high in the cross between *V. umbellata* and *V. nakashimae* but hybrids do not require embryo rescue. Distorted segregation can be attributed to abnormalities resulting from interspecific hybridization.

Bruchid resistance in rice bean

Two putative QTLs, located on two linkage groups (linkage groups 1 and 7) were found to be associated with *C. chinensis* resistance and two QTLs on two putative linkage groups (linkage groups 1 and 4) were identified for *C. maculatus* resistance. QTLs for *C. chinensis* (*Brc*3.1) and *C. maculatus* (*Brm*3.1) resistance are of interest because they explained much of the phenotypic variation (25.6–53.4 and 37.3–60.1% respectively). In addition, the QTLs *Brc*3.1 and *Brm*3.1 were consistently identified from the day seeds of the susceptible parent, *V. nakashimae*, were completely damaged to the end of evaluation.

Insecticidal chemicals in seeds, hardness of cotyledon and/or seed coat and/or inferior nutritional value of seeds (Tomooka et al. 2000) have been suggested as the causes of bruchid resistance. Recently, flavonoids with the basic structure of naringenin have been isolated from *V. umbellata* (cv. Menaga) and these were found to be responsible for bruchid resistance (US patent 6,770,630B2). One naringenin derivative causes resistance to both *C. chinensis* and *C. maculatus* and another naringenin derivative causes resistance only to *C. chinensis*. This supports the results presented here that two QTLs mapped to similar locations are responsible for resistance to the different bruchid species and other QTLs affect *Callosobruchus* species differently.

Seed size is considered to be a factor associated with bruchid resistance (i.e. small seeds have higher resistance than larger seeds) (Southgate 1979). The seed weight of the parents used here differs 2.2-fold, with the *V. umbellata* parent having an SW of 3.9 g compared with *V. nakashimae* with an SW of 1.8 g. However, no relationship between the percentage of damaged seeds and small seed size was observed in cross progeny. The fact that the locations of the putative QTLs (*Brc*3.1 and *Brm*3.1) for bruchid resistance differ from the QTL for seed size (*Sdwt*3.1) is in agreement with the results showing the percentage of damaged seeds is not correlated with seed weight.

Previous mapping studies for bruchid resistance based on an intraspecific cross between mungbean (*V. radiata*) and its presumed wild progenitor (*V. radiata* var. *sublobata*) have revealed a single major gene for resistance to *C. chinensis* (Young et al. 1992, Kaga and Ishimoto 1998). A major QTL underlying bruchid resistance to *C. chinensis* in *V. umbellata* has been found in an interspecific mapping population of *V. umbellata* × *V. angularis* (Kaga 1996). The position of the QTL is close to the pR26 (=mgR026) locus where *C. chinensis* resistance in wild mungbean is located. However, in the present study, no QTL for bruchid resistance was detected on linkage group 2 where the pR26 (mgR026) locus is located. The variety of *V. umbellata* used in the two experiments was different. *Vigna umbellata* used in this study was a landrace from Nepal, whereas, in the previous study the origin of the *V. umbellata* accession was Japan and the bruchid resistance was incomplete (Kaga et al. 1996). Intraspecific variation in the level of resistance to bruchid beetles has been observed among *V. umbellata* accessions (Tomooka et al. 2000). These data suggest there is variation in bruchid resistance genes in *V. umbellata*.

The *V. umbellata* × *V. nakashimae* map constructed in this study has proved to be useful in partly elucidating the inheritance of bruchid resistance in rice bean. That no F_2 plant revealed a phenotype as susceptible to bruchids as the susceptible parent and the frequency distribution of resistance skewed towards the resistant parent in the F_2 population indicates that many dominant genes are involved in resistance to both bruchids. Cytoplasmic effects can also explain the skewed distribution for bruchid resistance. It was not possible to analyse the reciprocal cross in this experiment (*V. nakashimae* × *V. umbellata*) because although F_1 seeds were obtained they did not germinate (Tomooka et al. 2003). Cytoplasmic effects are not considered to be a major factor in bruchid resistance because backcross progeny of hybrids between *V. umbellata* and *V. nakashimae* using a bruchid-susceptible azuki bean (*V. angularis*) as the female parent show resistance to both bruchid species (Tomooka et al. 2003). The skewed distribution of the resistance may be explained by the many abnormal F_2 plants and the resulting small F_2 population with many markers skewed towards *V. umbellata*. As no susceptible line was found, genetic variance was smaller than expected, hence the likelihood that there remain several undetected QTLs for bruchid resistance.

By using the bridging species *V. nakashimae*, the resistance of *V. umbellata* is transferred to an azuki bean bruchid-susceptible cultivar to develop bruchid-resistant lines (Tomooka et al. 2003). Every seed from each backcross between *V. angularis* × (*V. umbellata* × *V. nakashimae*) and recurrent male parent *V. angularis* (to date backcrossed three times) has been sequentially evaluated for resistance to the two bruchid species. In the preliminary evaluation, some lines developed have resistance to both bruchid species (Tomooka et al. 2003). As these backcross lines show segregation, the application of molecular markers linked to putative QTLs for bruchid resistance found in this study will greatly enhance bruchid resistance breeding in azuki bean. A search for other undetected bruchid resistance QTL in *V. umbellata* using backcross lines is also planned.

Acknowledgements

This study was conducted when the first author (PS) was a recipient of the Royal Golden Jubilee PhD Program award from the Thailand Research Fund. The authors thank T. Nobori, S. Hirashima, T. Oomizu, T. Taguchi and H. Tomiyama from the National Institute of Agrobiological Sciences for technical support. The *Vigna* (mungbean and cowpea), common bean, and soybean probes were provided by N.D. Young, University of Minnesota, St Paul, USA, J. Tohme, Centro Internationale por Agricultura Tropicale (CIAT), Cali, Colombia, and R.C. Shoemaker, Iowa State University, Ames, USA respectively.

References

Banto, S. M., and F. F. Sanchez, 1972: The biology and chemical control of *Callosobruchus chinensis* (Linn) (Coleoptera: Bruchidae). Philipp. Ent. 2, 167—182.

Boutin, S. R., N. D. Young, T. C. Olson, Z. H. Yu, R. C. Shoemaker, and C. E. Vellejos, 1995: Genome conservation among three legume genera detected with DNA markers. Genome 38, 928—937.

Dongre, T. K., S. E. Pawar, R. G. Thakare, and M. R. Harwalker, 1996: Identification of resistance sources to cowpea weevil in *Vigna* sp. and its inheritance of their resistance in black gram. J. Stored Prod. Res. 32, 201—204.

Draper, J., and R. Scott, 1988: The isolation of plant nucleic acids. In: J. Draper, R. Scott, P. Armitage, and R. Walden (eds), Plant Genetic Transformation and Gene Expression, 212—214. Blackwell Scientific Publishing, London.

Fujii, K., M. Ishimoto, and K. Kitamura, 1989: Patterns of resistance to bean weevils (Bruchidae) in *Vigna radiata-mungo-sublobata* complex inform the breeding of new resistant varieties. Appl. Entomol. Zool. 24, 126—132.

Han, O. K., A. Kaga, T. Isemura, N. Tomooka, and D. A. Vaughan, 2005: A genetic linkage map for azuki bean (*Vigna angularis*). Theor. Appl. Genet. (in press).

Kaga, A., 1996: Construction and Application of Linkage Maps for Azuki Bean (*V. angularis*). PhD Thesis, Kobe University, Japan.

Kaga, A., and M. Ishimoto, 1998: Genetic localization of a bruchid resistance gene and its relationship to insecticidal cyclopeptide alkaloids, the vignatic acids, in mungbean (*Vigna radiata* L. Wilczek). Mol. Gen. Genet. 258, 378—384.

Kaga, A., M. Ohnishi, T. Ishii, and O. Kamijima, 1996: A genetic linkage map of azuki bean constructed with molecular and morphological markers using an interspecific population (*Vigna angularis* × *V. nakashimae*). Theor. Appl. Genet. 93, 658—663.

Kaga, A., T. Ishii, K. Tsukimoto, E. Tokoro, and O. Kamijima, 2000: Comparative molecular mapping in *Ceratotropis* species using an interspecific cross between *V. umbellata* and *V. angularis*. Theor. Appl. Genet. 100, 207—213.

Kaga, A., D. A. Vaughan, and N. Tomooka, 2005: Molecular markers in *Vigna* improvement: understanding and using gene pools. In: H. Lorz, and G. Wanzel (eds), Biotechnology in Agriculture and Forestry, Vol. 55 Molecular Marker Systems, 171—187. Springer-Verlag, Berlin.

Kashiwaba, K., N. Tomooka, A. Kaga, O. K. Han, and D. A. Vaughan, 2003: Characterization of resistance to three bruchid species (*Callosobruchus* spp., Coleoptera, Bruchidae) in cultivated rice bean (*Vigna umbellata*). J. Econ. Entomol. 96, 207—213.

Kitamura, K., M. Ishimoto, and M. Sawa, 1988: Inheritance of resistance to infestation with azuki bean weevil in *Vigna sublobata* and successful incorporation to *V. radiata*. Jpn. J. Breed. 38, 459—464.

Kosambi, D. D., 1944: The estimation of map distances from recombination values. Ann. Eugen. 12, 172—175.

Kumar, S. V., S. G. Tan, S. C. Quah, and K. Yusoff, 2002: Isolation of microsatellite markers in mungbean, *Vigna radiata*. Mol. Ecol. Notes 2, 96—98.

Li, C.-D., C. A. Fatokun, B. Ubi, B. B. Singh, and G. J. Scoles, 2001: Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microsatellite markers. Crop Sci. 41, 189—197.

Pham, J. L., J. C. Glaszmann, R. Sano, P. Barbier, A. Ghesquire, and G. Second, 1990: Isozyme markers in rice: genetic analysis and linkage relationships. Genome 33, 348—359.

Southgate, B. J., 1979: Biology of the *Bruchidae*. Annu. Rev. Entomol. 24, 449—473.

Tomooka, N., C. Lairungruang, P. Nakeeraks, Y. Egawa, and C. Thavarasook, 1992: Development of bruchid-resistant mungbean using wild mungbean germplasm in Thailand. Plant Breeding 109, 60—66.

Tomooka, N., K. Kashiwaba, D. A. Vaughan, M. Ishimoto, and Y. Egawa, 2000: The effectiveness of evaluating wild species: searching for sources of resistance to bruchid beetles in the genus *Vigna* subgenus *Ceratotropis*. Euphytica 115, 27—41.

Tomooka, N., D. A. Vaughan, H. Moss, and N. Maxted, 2002: The Asian *Vigna*: Genus *Vigna* Subgenus *Ceratotropis* Genetic Resources. Kluwer Academic, Dordrecht, The Netherlands.

Tomooka, N., Y. Egawa, K. Kashiwaba, A. Kaga, T. Isemura, and D. A. Vaughan, 2003: Incorporation of bruchid resistance factors from rice bean (*Vigna umbellata*) to azuki bean (*V. angularis*). Jpn. J. Trop. Agric. 47, 75—76 (in Japanese).

Van Ooijen, J. W., and R. E. Voorrips, 2001: JoinMap® 3.0, Software for the Calculation of Genetic Linkage Maps. Plant Research International, Wageningen, The Netherlands.

Vaughan, D. A., N. Tomooka, and A. Kaga, 2005: Azuki bean. In: R. J. Singh, and P. P. Jauhar (eds), Genetic Resources, Chromosome Engineering, and Crop Improvement. Grain Legumes, 341—352. CRC Press, Boca Raton, FL.

Wang, S., C. J. Basten, P. Gaffney, and Z. B. Zeng, 2004a: Window QTL Cartographer 2.0 User Manual. North Carolina State University, Raleigh, NC.

Wang, X. W., A. Kaga, N. Tomooka, and D. A. Vaughan, 2004b: The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi]. Theor. Appl. Genet. 109, 352—360.

Young, N. D., L. Kumar, D. Menancio-Hautea, D. Danesh, N. S. Talekar, S. Shanmugasundarum, and D. H. Kim, 1992: RFLP mapping of a major bruchid resistance gene in mungbean (*Vigna radiata* L. Wilczek). Theor. Appl. Genet. 84, 839—844.

Zeng, Z.-B., 1994: Precision mapping of quantitative trait loci. Genetics 136, 1457—1466.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Stored Products Research 42 (2006) 313–327
Journal of STORED PRODUCTS RESEARCH
www.elsevier.com/locate/jspr

Characterization of *Callosobruchus chinensis* (L.) resistance in *Vigna umbellata* (Thunb.) Ohwi & Ohashi

P. Somta[a], N.S. Talekar[b], P. Srinives[a,*]

[a]*Department of Agronomy, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand*
[b]*Asian Vegetable Research and Development Center, P.O. Box 42, Shanhua, Tainan 741, Taiwan*

Accepted 13 May 2005

Abstract

Resistance to azuki bean weevil, *Callosobruchus chinensis,* was studied in a series of field and laboratory experiments in two accessions of rice bean (*Vigna umbellata* (Thunb.) Ohwi & Ohashi), one accession of black gram (*V. mungo* (L.) Hepper), and one accession of mungbean, (*V. radiata* (L.) Wilczek). Weevil damage to immature pods of the rice bean accessions, 'Menaga' and 'Miyazaki', was significantly less than to the susceptible mungbean, VC1973A. In mature pods, the pest damage to the pod wall of Menaga was significantly higher than to VC1973A, whereas the damage to Miyazaki was similar to VC1973A. Seeds within the pods of both rice bean accessions were resistant no matter when the pods were harvested. When the insects were exposed directly on dry seeds, both rice bean accessions and a black gram accession VM2164 were resistant to them. In artificial seeds made by mixing flour of the individual resistant *Vigna* accessions with VC1973A and subsequently exposed to bruchid oviposition, the higher the quantity of resistant *Vigna* flour the lower the number of bruchids that emerged from such seeds. No bruchids emerged from artificial seeds containing crude starch fraction from the three resistant *Vigna* accessions when such seeds were exposed to bruchid infestation, whereas many insects emerged from the seeds containing starch of VC1973A or flour of VC1973A alone. In artificial seeds made by mixing crude protein fractions of the three resistant *Vigna* accessions with flour of VC1973A, as the concentration of protein increased the number of *C. chinensis* adults that emerged decreased. Fractionation of crude proteins into acetone-precipitable proteins and peptide and amino acid portions resulted in the loss of antibiosis effect. Artificial seeds made from purified starch-polysaccharides fraction, however,

*Corresponding author. Tel./fax: +66 3428 1267.
E-mail address: agrpss@ku.ac.th (P. Srinives).

doi:10.1016/j.jspr.2005.05.003

exhibited antibiosis effects if prepared from the rice bean seed of Menaga and Miyazaki but not if made from the black gram seed, VM2164.

Keywords: Azuki bean weevil; Host-plant resistance; Rice bean; *Callosobruchus chinensis*; *Vigna* spp.

1. Introduction

Mungbean, *Vigna radiata* (L.) Wilczek, is an important legume produced mainly in South and Southeast Asia. This crop is a major source of dietary protein for poor people in the region and an important nitrogen-fixing legume in tropical cropping systems. Poor genetic background and damage by insect pests and diseases limit the seed yield of this crop. Among several insect pests that attack this crop, two beetle species, azuki bean weevil, *Callosobruchus chinensis* (L.), and cowpea weevil, *C. maculatus* (F.), are the most destructive. The initial infestation takes place in the field, where the adult bruchid lays eggs on green pods and the larvae bore through the pod cover and feed concealed within developing seeds (Southgate, 1979). When such seeds are harvested and stored, the insect continues feeding, eventually emerges as an adult, and causes secondary infestation which, at times, results in total destruction within 3–4 months (Banto and Sanchez, 1972). Since mungbean is generally grown by small land-holders, poor storage facilities are prevalent in such households, and the seeds are frequently used for family consumption, the application of fumigants or insecticides is not suitable (Talekar and Lin, 1981). Efforts have been made to identify sources of resistance and breed a bruchid-resistant mungbean. Among 525 accessions screened for *C. chinensis* resistance at the Asian Vegetable Research and Development Center (AVRDC), two black gram, *V. mungo* (L.) Hepper, accessions, VM2011 and VM2164 were resistant to *C. chinensis* (Talekar and Lin, 1981). The VM2011 exhibited less damage to pods when the insect infestation occurred in the field, while the VM2164 had highly resistant seeds in laboratory tests. Transfer of insect resistance genes, however, from VM2164 into mungbean cultivars was not successful (Fernandez and Talekar, 1990). Two mungbean accessions, V2709 and V2802, were moderately resistant to the bruchid in seeds (Talekar and Lin, 1992). One accession of mungbean, TC1966, *V. radiata* var. *sublobata*, was resistant to *C. chinensis* (Fujii and Miyazaki, 1987). Crosses between TC1966 and other mungbeans were made successfully (Tomooka et al., 1992), but since TC1966 is a wild legume and not consumed by humans anywhere the possibility of some undesirable traits present in the seeds of the progeny of some of the crosses could not be ruled out (Watanasit and Pichitporn, 1996). A feeding test was conducted by Miura et al. (1996) using $BC_{14}F_4$ seed of the cultivar 'Osaka Ryokutou' with TC1966 as the donor of the gene conferring the resistance. They found that the glutamic-oxalacetic transaminase activity was higher and the total cholesterol concentration was lower in female mice fed with the resistant line compared with the control mice. Sugawara et al. (1996) isolated the chemicals responsible for azuki bean weevil resistance in TC1996 and named the mixture vignatic acid. Its major component is cyclopeptide alkaloids. This makes the use of TC1966 questionable for breeding mungbean, which is widely consumed as fresh sprout, resistant to seed-feeding pests such as *C. chinensis*.

Recently, Tomooka et al. (2000) found two cultivated varieties of rice bean, *V. umbellata* (Thunb.) Ohwi & Ohashi, a food legume, to be completely resistant to *C. chinensis* and

C. maculatus. Both accessions, 'Menaga' and 'Miyazaki', are land races of cultivated rice bean from Japan. Kashiwaba et al. (2003) prepared artificial seeds from various proportions of rice bean (resistant) and azuki bean (susceptible) flour and fed these to *C. chinensis*, *C. maculatus* and Graham bean weevil (*C. analis* (F.)). They reported that the number of bruchid adults emerging decreased, and the larval development period (days) was extended, when artificial seed with an increasing proportion of rice bean flour were used. They concluded that a chemical component(s) contained in the cotyledon of rice bean has an inhibitory effect on growth of these bruchid species. They did not attempt to fractionate and characterize the compounds responsible for the resistance. In this study, we partially characterize the mechanism of resistance of these varieties as well as that of VM2164 to *C. chinensis*.

2. Materials and methods

Most of the experiments were conducted at the Asian Vegetable Research and Development Center, Shanhua, Taiwan. The rest were conducted at Kasetsart University, Kamphaeng Saen, Thailand.

2.1. Study with pods

Seeds of Menaga, Miyazaki, VM2164, and one susceptible mungbean breeding line (VC1973A) were planted in individual 3 m long and 2.25 m wide plots, in a randomized complete block design with four replications. The crop was grown by normal cultural practices as recommended by Park (1978). When the earliest maturing accession started forming pods, we released 1000–2000 newly hatched laboratory-reared adults of *C. chinensis* in the field once every week until most pods of the latest maturing accessions were ready for harvest (Talekar and Lin, 1981). All pods at pod-filling (yellow pod) stage and at mature dry pod (black pod) stage were picked weekly from each plot. Pods at the same stage in each replication collected through the course of experiment were combined into one observation. Each observation comprised a few hundred to several thousand pods. The collected pods were placed in nylon net bags and maintained in a greenhouse for further drying before extracting the dry seeds. Once every week, adults that had emerged from the pods were counted and discarded promptly and the number of damaged pods and seeds were recorded. This procedure was continued for up to 6 weeks after final harvest. The accumulated number of damaged pods and seeds among the four accessions were averaged across four replications and compared.

2.2. Study with whole seeds

Seeds of each accession were placed in a water-saturated chamber for 48 h to equalize their moisture content. Ten grams of seed were poured into each of four 125 mL flasks and 40 newly emerged (0–3-day-old) adults of *C. chinensis* were introduced over the seeds in each flask. Bruchid adults were allowed to lay eggs and the feeding damage by larvae emerging from eggs was recognized by the presence of holes in seeds. Percentages of damaged seeds among the four accessions were compared.

2.3. Study with artificial seeds

Dry de-hulled cotyledons of VC1973A, Menaga, Miyazaki, and VM2164 were ground into very fine flour. Flour of VC1973A was used as the base for preparation of artificial seeds consisting of an increasing proportion of Menaga, Miyazaki, or VM2164 in a VC1973A base to study possible antibiotic factors present in the seed (Talekar and Lin, 1992). Preparation of artificial seeds followed the method developed by Shade et al. (1986). The moisture content of artificial seeds was adjusted to 12–15% in the moisture chamber. For each proportion of resistant material, eight artificial seeds, each weighing about 1 g, were made and confined in an 125 mL Erlenmeyer flask as one experimental unit. Forty newly emerged adults of *C. chinensis* were released into individual flasks for oviposition for 4 days. All studies were conducted at 28.5±1 °C with four replicates. Seeds were observed for 50 days and the number of first-generation adults emerging from the seeds was recorded. Using this generalized procedure, various components of seeds of Menaga, Miyazaki, and VM2164 were tested for their antibiotic effect on growth and development of *C. chinensis*.

2.3.1. Antibiosis study

In the first experiment, fine powder of the three resistant accessions was mixed with fine powder of VC1973A at the rates of 25%, 50%, and 75% and made into artificial seeds. Seeds were also made from the fine powder of the individual accessions alone. These seeds were bioassayed with *C. chinensis* as described above.

2.3.2. Preparation of artificial seeds with crude protein and crude starch

In the second experiment, 10 g of flour of each accession was individually suspended and mixed with distilled water. These suspensions were shaken intermittently and held for 2 h before centrifuging at 15 000 rpm, for 15 min at 4 °C. Centrifugation separated the material into liquid phase (mostly protein) and solid phase (mainly starch). The solid fraction was dried at 60 °C for 8 h. The liquid fraction was freeze-dried. To test for possible antibiosis against *C. chinensis* in starch, the solid fraction of each individual accession equivalent to 20% and 25% of original seed was mixed with flour of VC1973A and made into artificial seeds. To determine the equivalent weight, the total weight of the starch fraction was set to 100% and the required equivalent weight (20% or 25% in this case) was calculated accordingly. To test for antibiosis in protein, the solid from the freeze-dried fraction equivalent to 5%, 10%, 20%, and 30% w/w of original seed of each accession was individually added to flour of VC1973A and made into artificial seeds. Artificial seeds made from flour of VC1973A alone were included in the two tests as a susceptible control. All seeds were bioassayed with *C. chinensis* as described above.

2.3.3. Preparation of artificial seeds with precipitable protein, peptide and amino-acid fractions

In the third experiment, the protein fraction of each accession was extracted as described above. Cold acetone was then added to this liquid fraction gradually until there was no more precipitation (Englard and Seifter, 1990). The protein–acetone mixtures were allowed to stand for 3 h to let the proteins settle at the bottom of the centrifuge tubes. The tubes were then centrifuged at 15 000 rpm for 15 min at 4 °C. The precipitable proteins at the bottom of the tubes were air dried at room temperature. The liquid fraction was concentrated by a flash evaporator until all the

acetone had evaporated and was then freeze-dried. Precipitable protein equivalent to 25% of the original seed of each accession was individually added to flour of VC1937A and made into artificial seeds. The freeze-dried fraction at a concentration of 2%, 5%, 7%, or 10% equivalents, of original seed was then added to flour of VC1937A and made into artificial seeds. Artificial seeds made from susceptible flour alone were included. All seeds were bioassayed with *C. chinensis* as described above.

2.3.4. Preparation of artificial seeds with solvent extracts of peptide and free amino acid fraction

In the fourth experiment, a freeze-dried fraction of each accession equivalent to 10 g of original seed was subjected to sequential extraction with hexane, dichloromethane, and methanol. Extraction with hexane and dichloromethane was done in a Soxhlet extraction apparatus for 8 h at the rate of 5–6 solvent exchanges per hour. For methanol, the freeze-dried fraction and solvent were shaken vigorously for 8 h. All solvent extracts were concentrated in a flash evaporator until all solvent had been evaporated. The dried residues of each extract as well as solid residues after solvent extraction in quantities equivalent to 30% of those in the original seed were incorporated into flour of VC1973A and prepared into artificial seeds. Artificial seeds having 30% flour of Menaga, Miyazaki, and VM2164 were included as well as the seeds made solely from VC1973A.

2.3.5. Preparation of artificial seeds with purified starch fraction

In the fifth experiment, the water-insoluble fractions comprising mainly starch and other carbohydrates as described in the second experiment were suspended three times with 0.1 M NaOH. After each suspension, the mixture was allowed to stand for 2 h at 4 °C and centrifuged at 15 000 rpm for 15 min at 4 °C. The liquid phase (starch-associated proteins) was collected. The solid phase was subsequently suspended once again in 0.1 M NaOH, and centrifuged at 15 000 rpm for 15 min at 4 °C. All liquid phases from the extraction were combined and dialyzed using dialysis membrane against distilled water for 24 h at 4 °C, to remove NaOH. The dialyzed liquid phases were freeze-dried. The solid phases (purified polysaccharides) were dried at 60 °C for 8 h. The polysaccharides as well as solids from the freeze-dried fractions equivalent to 25% of initial seed compositions were mixed with flour of VC1973A and made into artificial seeds. Artificial seeds made from susceptible flour alone were included as a control.

2.3.6. Preparation of artificial seeds with steam distilled chemicals

In the sixth experiment, 200 g flour of each accession was suspended in water and subjected to steam distillation. The distillate liquid equivalent to 25% of the initial seed was added to flour of VC1973A and made into artificial seeds. The seeds made from flour of VC1973A alone were included as a control. All seeds were bioassayed with *C. chinensis* as described above.

2.3.7. Antibiosis in post-germination cotyledons

In the seventh experiment, seeds of all accessions were germinated in sand previously baked at 100 °C for 24 h. Three-day-old post-germination cotyledons were collected and washed with water. The cotyledons were then dried at 60 °C for 16 h and ground into fine powder. These powders, 25% of the mixture, were mixed with flour of VC1973A (75%) and made into artificial seeds. Seeds made from flour of VC1973A alone were included as a susceptible control. All seeds were bioassayed with *C. chinensis*.

2.4. Statistical analysis

The SAS program was used for analysis of variance (ANOVA) in a completely randomized design (CRD). If significant differences were indicated, means were separated using least-significant difference (LSD) tests with a $P \leqslant 0.5$ level of significance (SAS Institute, 2001).

3. Results and discussion

3.1. Study with pods

The results of the damage caused by *C. chinensis* to immature and mature seeds while within pods are shown in Table 1. Since VM2164 did not flower even after harvest of pods of all other accessions, we did not include it in the data analysis. In Menaga and Miyazaki, the insect damage to seeds, whether the pods were harvested when immature or fully mature, was significantly less than in the susceptible control VC1973A. Pod pericarp characters such as gum production, smooth surface, thin pericarp, indehiscence and flaking surface can influence bruchid damage to seeds (Janzen, 1969). Highly pubescent pods also tend to be less damaged by bruchids (Talekar and Lin, 1981). Because the morphology of the pods of the three accessions was not much different except that the surfaces of Menaga and Miyazaki were slightly glabrous and smoother than that of VC1973A, it is unlikely that the morphology of the two rice bean accessions contributed to the pod resistance of these accessions in pods. When we observed the damage to immature pods, the pod wall of Menaga was resistant but Miyazaki was considerably more damaged and appeared to be as susceptible as VC1973A. At maturity, the pod wall of Menaga was more damaged than those of VC1973A and Miyazaki (Table 1). In both cases, insect holes were visible only over the seed locules. Hence the bruchids appeared to prefer the seeds inside the pods, rather than the pod itself. Pod pericarp did not seem to play any role in the resistance of either rice bean accession to *C. chinensis*. The seeds of Menaga and Miyazaki were about two-thirds the size of those of VC1973A. This fact alone, however, cannot account for the drastic

Table 1
Infestation on immature and mature seeds within pods of various *Vigna* accessions by *Callosobruchus chinensis*[a]

Accession	Damaged seeds (%) (mean ± SE)	Damaged pods (%) (mean ± SE)
Immature stage		
VC1973A	19.42 ± 1.37a	33.52 ± 0.80a
Menaga	1.71 ± 0.25b	5.19 ± 0.50c
Miyazaki	3.26 ± 0.49b	22.48 ± 1.26b
Mature stage		
VC1973A	18.52 ± 2.55a	15.38 ± 3.65b
Menaga	3.76 ± 0.98b	23.21 ± 0.58a
Miyazaki	4.18 ± 0.59b	9.34 ± 1.21b

[a]Means followed by the same letter in each column and for each stage of maturity are not significantly different at $P < 0.5$ by LSD test.

reduction in the damage. We suspect that biochemical differences in the seeds contributed to the differences in the varieties to bruchid attack.

3.2. Study with whole seeds

Callosobruchus chinensis laid large numbers of eggs on dry seeds of all accessions (Table 2). The number of eggs laid on seeds of all four accessions was, however, not significantly different. No adults emerged from seeds of Menaga, Miyazaki, and VM2164, but numerous insects emerged from seeds of VC1973A (Table 2). Menaga, Miyazaki, and VM2164, therefore, appear to be completely resistant to *C. chinensis*. Resistance of VM2164 has already been demonstrated (Talekar and Lin, 1992). Certain factors such as seed hardness, small seed size, absence of nutritional factors, and presence of toxic substances, may affect bruchid damage to legume seeds (Southgate, 1979). Although, in our study, seed size of Menaga, Miyazaki, and VM2164 was smaller than VC1973A (100-seed weights being 4.28, 4.25, 3.69, and 6.73 g, respectively), it did not correlate with bruchid resistance in seeds. Tomooka et al. (2000) demonstrated the absence of a relationship between the level of resistance of some *Vigna* seeds to *C. chinensis* and *C. maculatus* and seed size. Our results implied that antibiosis factors present in the seed coat or seed cotyledon might be responsible for resistance to *C. chinensis*. At times we found dead larvae and pupae in cotyledons or near the embryonic axis of all resistant accessions. A similar observation was made earlier by Ramzan et al. (1990) who found dead *C. maculatus* larvae in cotyledons of rice bean. However, Tomooka et al. (2000) reported no damage caused by *C. chinensis* and *C. maculatus* beyond the seed coat of Menaga and Miyazaki. Biotypes of bruchids and intraspecific variation of plant accessions may result in differences in the level of resistance (Tomooka et al., 2000). Our results hinted at the possibility of antibiosis factors in the cotyledon being responsible for resistance of these rice bean accessions to *C. chinensis*. Hence we conducted a series of resistance-testing experiments with various components of seeds of these *Vigna* species accessions.

3.3. Study with seed components

3.3.1. Antibiosis

When artificial seeds, made by mixing increasing proportions of flour from three resistant accessions with VC1973A, were offered to *C. chinensis* for oviposition, insects laid more than seven eggs per seed. The differences in the constituents of these seeds did not affect the number of

Table 2
Infestation on dry seeds of various *Vigna* accessions by *Callosobruchus chinensis*[a]

Accession	No. of eggs laid per 10 g seed[b] (mean ± SE)	No. of adults emerged (mean ± SE)
VC1973A	524.00 ± 56.25	245.05 ± 21.48a
Menaga	452.00 ± 35.68	0.00 ± 0.00b
Miyazaki	498.00 ± 22.13	0.00 ± 0.00b
VM2614	534.00 ± 27.19	0.00 ± 0.00b

[a]Means followed by the same letter in each column are not significantly different at $P < 0.5$ by LSD test.
[b]No significant difference.

eggs laid. Development and emergence of bruchid adults, however, was strongly affected by the presence of different proportions of resistant seed flour in VC1973A flour. No insects emerged from any combination, except for one combination each of Menaga and VM2164 with VC1973A, from which a mean of less than one adult emerged per seed. More than three adults emerged, however, from each seed of VC1973A (Table 3). The results indicated the possibility of strong antibiosis factors present in the cotyledons of Menaga, Miyazaki, and VM2164, which may be responsible for their resistance to *C. chinensis*.

3.3.2. Crude starch and crude protein

The numbers of *C. chinensis* adults that emerged from artificial seeds containing 25% of the water-insoluble crude starch-polysaccharide fraction from Menaga, Miyazaki, and VM2164 were significantly less than those that emerged from seeds containing the same component of VC1973A or the control diet of VC1973A alone (Table 4). In fact, no adults emerged when flour of Menaga or Miyazaki was used. A similar but much less pronounced antibiosis was observed in a test with 20% crude starch conducted in Thailand. The difference between the two experiments might be due to a difference in the concentration of crude starch used and a possible biotype difference in *C. chinensis* between two locations. Credland (1990) reported the occurrence of genetically different biotypes in bruchids which differ in their host range. Our observation agrees with this report in that we found greater damage of *C. chinensis* to rice bean in Thailand than in Taiwan (unpublished data). Moreover, the number of eggs laid on artificial seeds by Thai bruchids was much higher than those laid by Taiwan bruchids, averaging 39.09 and 11.43 per seed, respectively. This combined difference may explain the discrepancy between the results of the two experiments. A polysaccharide from seeds of common bean, *Phaseolus vulgaris* (L.), was toxic to *C. chinensis* as

Table 3
Preference for oviposition and emergence of adults of *Callosobruchus chinensis* from artificial seeds made from various combinations of resistant and susceptible *Vigna* accessions[a]

Seed combination	No. of eggs per 8-seed replicate (mean ± SE)	No. of adults emerged per 8-seed replicate (mean ± SE)
Menaga 25% + VC1973A 75%	60.75 ± 14.64ab	1.75 ± 1.11b
Menaga 50% + VC1973A 50%	53.00 ± 15.91ab	0.00b
Menaga 75% + VC1973A 25%	77.50 ± 10.94a	0.00b
Menaga 100%	69.50 ± 7.35ab	0.00b
Miyazaki 25% + VC1973A 75%	67.00 ± 14.51ab	0.00b
Miyazaki 50% + VC1973A 50%	51.25 ± 9.04ab	0.00b
Miyazaki 75% + VC1973A 25%	63.00 ± 10.34ab	0.00b
Miyazaki 100%	44.50 ± 7.27b	0.00b
VM2164 25% + VC1973A 75%	71.75 ± 12.60ab	0.00b
VM2164 50% + VC1973A 50%	64.25 ± 8.58ab	0.00b
VM2164 75% + VC1973A 25%	64.25 ± 13.93ab	0.50b
VM2164 100%	65.00 ± 8.24ab	0.00b
VC1973A 100%	58.75 ± 5.88ab	29.25 + 4.03a

[a]Means followed by the same letter in each column are not significantly different at $P \leqslant 0.5$ by LSD test.

Table 4
Effect of addition of crude starch-polysaccharide fraction of various *Vigna* accessions with seed powder of VC1973A on infestation of *Callosobruchus chinensis*[a]

Treatment	No. of adults emerged per 8-seed replicate (mean ± SE)	
	20% crude starch[b]	25% crude starch[c]
VC1973A cotyledon powder	54.75 ± 8.42a	25.25 ± 2.49a
VC1973A + 20% VC1973A starch-polysaccharide	55.00 ± 10.84a	29.75 ± 6.79a
VC1973A + 20% Menaga starch-polysaccharide	20.50 ± 7.67b	0.00 ± 0.00b
VC1973A + 20% Miyazaki starch-polysaccharide	40.50 ± 8.60a	0.00 ± 0.00b
VC1973A + 20% VM2164 starch-polysaccharide	31.00 ± 4.16ab	0.75 ± 0.49b

[a]Means followed by the same letter in each column are not significantly different at $P < 0.5$ by LSD test.
[b]Data collected from the experiment conducted in Thailand.
[c]Data collected from the experiment conducted in Taiwan.

evidenced by increasing larval mortality, reduced growth and development, and fewer eggs laid by the females (Applebaum et al., 1970; Applebaum and Guez, 1972). Similarly, purified carbohydrates from *P. vulgaris* adversely affected another bruchid, *Acanthoscelides obtectus* (Say) in the same manner as *C. chinensis* (Gatehouse et al., 1987). Recently, a polysaccharide, galactorhamnan, isolated from seeds of jack bean, *Canavalia ensiformis* (L.) DC, was shown to be antibiotic to *C. maculatus* and reduce emergence (Oliveira et al., 2001). It is possible that similar factors may be involved in the antibiotic effects of these *Vigna* accessions to *C. chinensis*. Silva et al. (1999) determined changes in the levels of carbohydrases, aminopeptidases, and acid phosphatase in the larval midgut cells and in the luminal contents of *C. maculatus* and *Zabrotes subfasciatus* (Boheman) fed different diets. They found a change in α-amylase in the midgut lumen of the bruchid larvae fed with legume seeds with different starch granules. They speculated that starch may be a barrier to predation by bruchid beetles.

When water-soluble crude protein fractions of resistant accessions were added to the flour of VC1973A, the number of adults that emerged decreased as the concentration of water-soluble crude protein of Menaga, Miyazaki, or VM2164 added to flour of VC1973A increased beyond 10% (Fig. 1). In the case of VC1973A, addition of its own crude protein at any concentration did not affect insect development and emergence of *C. chinensis* adults; there was no difference from the seeds made from VC1973A only. This implied that some factors in the crude protein fraction of seeds of Menaga, Miyazaki, and VM2164 adversely affected development of *C. chinensis*. Insect development and emergence were not affected for up to 10% of crude protein fraction mixtures with VC1973A, but were curtailed at 20% and higher.

3.3.3. Precipitable protein and peptide-amino acids

Fractionation of crude protein into cold-acetone-precipitable proteins and peptides and free amino acids, reduced the antibiotic effects of the crude protein fraction. There was no significant difference in the number of *C. chinensis* adults emerged among the seeds made by adding acetone-precipitable protein of various accessions and artificial seeds made purely from VC1973A flour (Fig. 2). When, after removal of acetone-precipitable protein, the artificial seeds constituted by

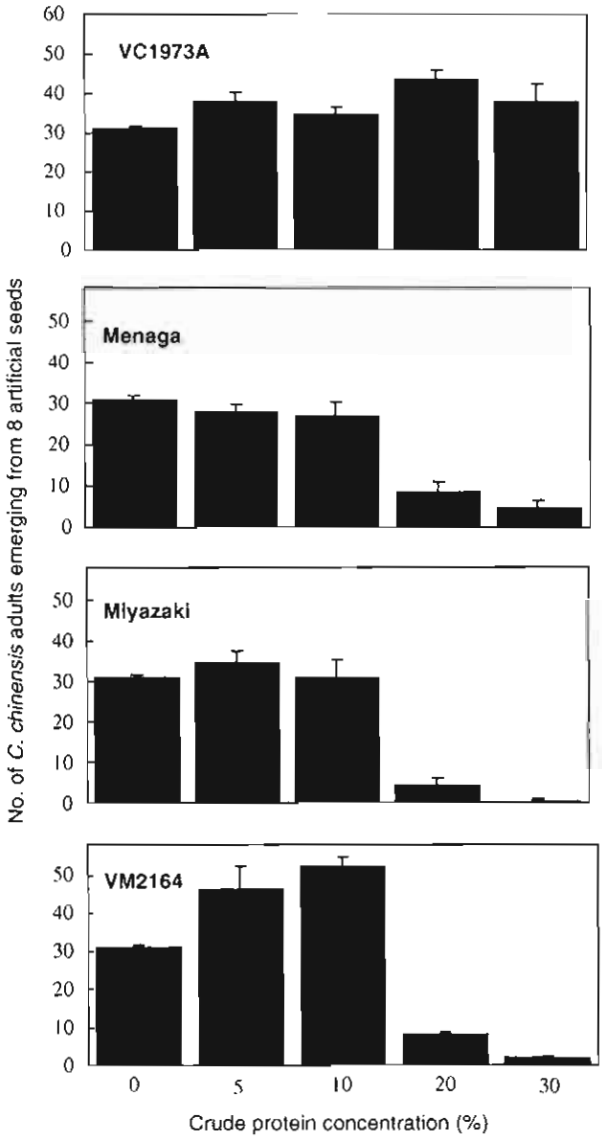


Fig. 1. Emergence of *Callosobruchus chinensis* adults from artificial seeds made by adding various concentrations of crude protein from seeds of susceptible (VC1973A) and resistant (Menaga, Miyazaki, and VM2164) *Vigna* accessions into *Vigna radiata* VC1973A. The vertical line above each solid bar indicates SE of number of the adults emerging from eight artificial seeds.

addition of 2%, 5%, 7%, and 10% water-soluble fraction were exposed to bruchid adults for oviposition, there was a decrease in the number of adults which emerged from the seeds but the change was not specific to the insect resistant accessions (data not shown). The decrease was found in all four accessions, indicating thereby that the resistance of these three *Vigna* accessions may not be due to any antibiotic factors present in this fraction. The crude starch-polysaccharide fraction appears to be more important.

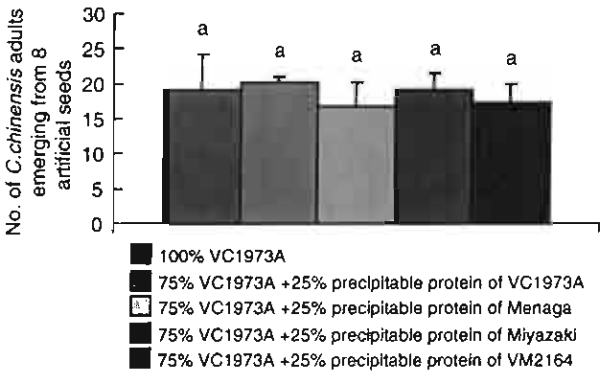


Fig. 2. Emergence of *Callosobruchus chinensis* adults from artificial seeds containing acetone-precipitable proteins equivalent to 25% of original seed content of susceptible and resistant *Vigna* accessions. The vertical line above each solid bar indicates SE of number of the adults emerging from eight artificial seeds. There are no significant differences among the treatments and control.

3.3.4. Solvent extracts of peptides and free amino acids

Extraction with polar and non-polar solvents of peptide and free amino-acid fractions, and addition of such solvent extracts and residual material after solvent extraction to VC1973A did not affect growth and development of *C. chinensis* when artificial seeds made from the mixtures as well as VC1973A alone were exposed to bruchid infestation (data not shown). These results implied that the resistance of *Vigna* accessions is not associated with peptides or free amino acid fractions.

3.3.5. Purified starch-polysaccharide and associated protein

The numbers of *C. chinensis* adults which emerged from artificial seeds, containing 25% original seed equivalent purified starch-polysaccharides from Menaga and Miyazaki were significantly less than those that emerged from seeds containing purified starch-polysaccharides of VC1973A and seeds made from flour of VC1973A alone. Use of this fraction of VM2164, however, did not reduce bruchid damage as compared to addition of the same fractions of VC1973A (Fig. 3). Addition of starch-polysaccharide-associated proteins extracted in 0.1 M NaOH from Menaga, Miyazaki, and VM2164 did not significantly reduce *C. chinensis* emergence, compared with similar proteins from VC1973A (Table 5). These results are in line with our previous findings that proteinaceous materials are not involved in the resistance of Menaga, Miyazaki, and VM2164 to *C. chinensis*, whereas carbohydrates are involved. Carbohydrates are indispensable for supporting optimum growth, development, and reproduction of seed-feeding insects. Some carbohydrates, however, might adversely affect nutrition because they either act as feeding deterrents or are incompletely hydrolyzed and absorbed, whereas others are readily absorbed but not metabolized at high concentrations and may even inhibit enzymatic reactions including those involved in glycolysis and gluconeogenesis (Gatehouse et al., 1987). Carbohydrate binding proteins, lectins (phytohaemagglutins), have been considered to be very toxic to *C. maculatus* (Janzen et al., 1976; Pusztai and Watt, 1974), since they cause disruption of epithelial cells which interferes with nutrient uptake and absorption (Gatehouse et al., 1989). It is possible that carbohydrate-protein combinations may be involved in the antibiotic effects of these rice

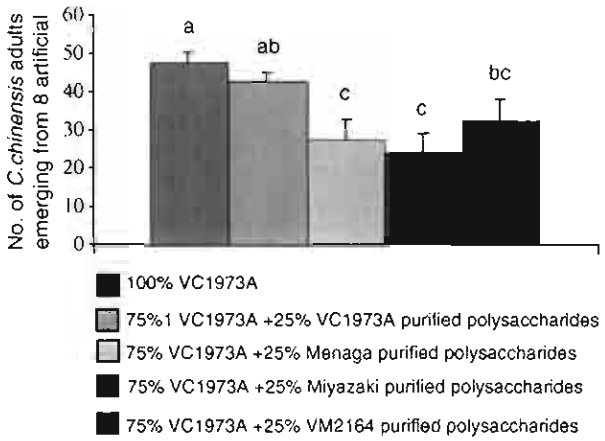


Fig. 3. Emergence of *Callosobruchus chinensis* adults from artificial seeds containing 75% seed powder of *Vigna radiata* VC1973A plus 25% purified polysaccharides of various *Vigna* accessions. Bars indicated by the same letter are not significantly different at $P \leqslant 0.5$ by LSD test. The vertical line above each solid bar indicates SE of number of the adults emerging from eight artificial seeds.

Table 5
Effect of addition of starch-associated proteins of various *Vigna* accessions to seed powder of VC1973A on infestation by *Callosobruchus chinensis*[a]

Treatment[b]	No. of adults emerged per 8-seed replicate (mean ± SE)
VC1973A powder	49.00 ± 5.21a
VC1973A + 25% VC1973A starch-associated proteins	38.00 ± 5.95ab
VC1973A + 25% Menaga starch-associated proteins	29.75 ± 3.61b
VC1973A + 25% Miyazaki starch-associated proteins	29.75 ± 6.55b
VC1973A + 25% VM2164 starch-associated proteins	39.25 ± 4.75ab

[a]Means followed by the same letter are not significantly different at $P < 0.5$ by LSD test.
[b]Starch-associated proteins extracted from 25% equivalent seed powder of various accessions.

bean accessions to *C. chinensis*. These factors, however, do not seem to be involved in the resistance of VM2164 to bruchids. Detrimental effects of some legumes against certain insect species may result from a combination of chemicals rather than from a single component able to cause resistance by itself (Oliveira et al., 1999). The synergy of purified starch-polysaccharides and associated proteins in rice bean may confer complete resistance to *C. chinensis* similar to the proteins and a polysaccharide shown to be toxic to *C. maculatus* in jack bean (Oliveira et al., 1999, 2001).

3.3.6. Steam-distilled chemicals

Chemicals extracted by steam distillation from seed flour of resistant and susceptible accessions had no effect on the development and emergence of adults of *C. chinensis*. There was no

significant difference in the number of bruchid adults emerging from the seeds containing such chemicals and artificial seeds made from VC1973A flour only (data not shown). A number of insects have been reportedly affected by plant volatiles that are specific to their host plant (Visser, 1986). Our results, however, revealed no indication of either adverse or beneficial effects of volatile chemicals from Menaga, Miyazaki, and VM2164 towards *C. chinensis* development or emergence.

3.3.7. Post-germination cotyledons

When artificial seeds containing 25% powder of post-germination cotyledons of resistant and susceptible accessions were offered to *C. chinensis*, no insects emerged (data not shown). Since most of the seed storage chemicals are precursors for new chemical synthesis in germination and seedling development, this could lead to loss or gain of toxicity or antibiosis to bruchids in cotyledons after germination.

Bruchid-resistant gene(s) in rice bean has been successfully incorporated into a bruchid-susceptible *Vigna* species, azuki bean (*V. angularis*), via a bridging species (N. Tomooka pers. commun.). Although rice bean is cross incompatible with mungbean, transferring of bruchid-resistant gene(s) may be achieved by using a bridging species or by genetic engineering methods.

The present study revealed that resistance to azuki bean weevil both in pods (field environment) and seeds (storage environment) of rice bean is due to the existence of antibiosis factors in seeds. The resistance seems to be complex because two or more chemical factors may be involved. Identification of the resistant factor(s) in rice bean and its mode of action will be helpful for breeding programs incorporating the resistance into the other *Vigna* species without the need for extensive bioassay tests in each generation.

Acknowledgements

This research is a part of the thesis of the first author, who is a Ph.D. Fellow of the Royal Golden Jubilee (RGJ) Project of the Thailand Research Fund (TRF). Parts of the research were supported by (1) the Project on Genetics and Breeding of Field Legumes for Thailand which was also funded by the TRF, and (2) AVRDC—the World Vegetable Center in Taiwan. The authors are grateful to Dr Duncan A. Vaughan and Dr Norihiko Tomooka of the National Institute of Agrobiological Resources, Japan, for providing the bruchid-resistant germplasm.

References

Applebaum, S.W., Guez, M., 1972. Comparative resistance of *Phaseolus vulgaris* beans to *Callosobruchus chinensis* and *Acanthoscelides obtectus* (Col. Bruchidae): the differential digestion of soluble heteropolysaccharide. Entomologia Experimentalis et Applicata 25, 64–74.

Applebaum, S.W., Tadmor, U., Podoler, H., 1970. The effect of starch and of a heteropolysaccharide fraction from *Phaseolus vulgaris* on development and fecundity of *Callosobruchus maculatus*. Entomologia Experimentalis et Applicata 13, 61–70.

Banto, S.M., Sanchez, F.F., 1972. The biology and chemical control of *Callosobruchus chinensis* (Linn.) (Coleoptera: Bruchidae). Philippine Entomologist 2, 167–182.