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A genetic linkage map of azuki bean constructed with molecular and morphological markers using an interspecific population (*Vigna angularis* × *V. nakashimae*)

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Abstract A genetic linkage map of azuki bean (*Vigna angularis*) was constructed with molecular and morphological markers using an F_2 population of an interspecific cross between azuki bean and its wild relative, *V. nakashimae*. In total, 132 markers (108 RAPD, 19 RFLP and five morphological markers) were mapped in 14 linkage groups covering 1250 cM; ten remained unlinked. The clusters of markers showing distorted segregation were found in linkage groups 2, 8 and 12. By comparing the azuki linkage map with those of mungbean and cowpea, using 20 RFLP common markers, some sets of the markers were found to belong to the same linkage groups of the respective maps, indicating that these linkage blocks are conserved among the three *Vigna* species. This map provides a tool for marker-assisted selection and for studies of genome organization in *Vigna* species.

Key words Linkage map · Azuki bean · *Vigna angularis* · Molecular marker · Morphological marker

Introduction

Azuki bean, *Vigna angularis* (Willd.) Ohwi and Ohashi, is one of the most important crops in the subgenus *Ceratotropis*, which includes Asian pulses. This bean is mainly produced in northeast Asian countries, such as Japan, China, Korea and Taiwan (Motomiya and Ito 1972). In Japan, the azuki bean is the second most important pulse after soybean, and is usually used as a material for sweets. In addition, since this bean has purple-red pigment in the seed coat, rice cooked with azuki bean, namely, red rice, has been traditionally prepared on celebration days. The annual production of azuki bean is unstable because of the damage from cold weather and many pests. Therefore, it is necessary to develop high-yield cultivars with cold tolerance and disease resistance. However, genetical research on agronomically important traits of azuki bean has lagged far behind those of other pulses, and only a few studies on morphological characters are available, such as seed and stem color (Matsuura 1933) and pod color and leaf shape (Kakizaki 1923). At the present time, more genetic information is urgently needed to support azuki bean breeding.

The construction of a linkage map is of fundamental importance for the efficient exploration of plant genetic potential. Recently, molecular linkage maps of many crop species have been made and applied to genetic mapping, gene tagging and improved selection in breeding programs. Using RFLP markers, molecular linkage maps have been constructed in several pulse species, including soybean (Shoemaker and Specht 1995), lentil (Simon et al. 1993), mungbean (Menancio-Hautea et al. 1993 a, b), cowpea (Fatokun et al. 1993) and peanut (Halward et al. 1993). Random amplified polymorphic DNA (RAPD) markers also provide genetic information at the DNA level (Williams et al. 1990). As compared to RFLP analysis, RAPD analysis is a technically simple method for constructing genetic maps (Binelli and Bucci 1994; Rowland and Levi 1994) and for use in marker-assisted selection and breeding (Johnson et al. 1995). For azuki bean, however, no genetic map has yet been produced.

In order to construct a linkage map efficiently it is necessary to choose an adequate combination of parents for the mapping population. In particular, high polymorphisms should be detected between the parents, so that they can be crossed to each other. Previously, we

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carried out a RAPD analysis to assess the genetic variation among azuki cultivars (Kaga et al. 1993) and *Ceratotropis* species (Kaga et al. 1996) and found a low level of polymorphism within *V. angularis*. A reproductive isolation barrier was often observed in interspecific crosses between *V. angularis* and other species in the subgenus *Ceratotropis* (Chen et al. 1983; Kaushal and Singh 1988). However, Siriwardhane et al. (1991) reported the successful reciprocal cross of azuki bean with a wild *Ceratotropis* species, *V. nakashimae* (Ohwi.) Ohwi and Ohashi. Based on these facts, we chose *V. angularis* and *V. nakashimae* as parents for a mapping population, and a genetic linkage map of azuki bean was constructed with RFLP, RAPD, and morphological markers using an interspecific population.

Materials and methods

Plant materials

V. angularis cv 'Erimoshouzu' and *V. nakashimae* were used to produce the mapping population. The seeds of *V. nakashimae* were kindly provided by Dr. Y Egawa, National Institute of Agrobiological Resources, Japan. Eighty F_2 individuals from the interspecific cross (*V. angularis* × *V. nakashimae*) were separately grown in pots of 25-cm diameter.

DNA extraction

Total DNA was isolated after the method of Doyle and Doyle (1987) with a slight modification: approximately 200 mg of primary leaves were collected from 1-week-old seedlings, ground in liquid nitrogen, and suspended in 1 ml of 2 × CTAB buffer containing 1% sodium metabisulphite. The appropriate DNA concentration for restriction-enzyme digestion and PCR amplification was determined by visual comparison with lambda DNA of known concentration using the mini-gel method.

RAPD analysis

PCR was performed in a volume of 10 µl consisting of 0.2 ng of genomic DNA, 0.2 µM primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 0.4 mM each of dNTPs and 0.2 units of *Taq* DNA polymerase (TOYOBO, Japan). Decamer oligonucleotide primers were purchased from TOYOBO, Japan, or Operon Technologies, USA. Amplifications were carried out in a BioOven (BioTherm, USA) programmed for 45 cycles of 30 s at 93°C, 1 min at 36°C and 2 min at 72°C, and ending with 1 min at 72°C. Amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

RFLP analysis

Total DNA of azuki bean was digested with *Hin*dIII, electrophoresed (5–10 µg per lane) using 0.8% agarose gel, and transferred to Hybond N+ membrane (Amersham, UK). Mungbean and cowpea genomic DNA probes were kindly provided by Dr N. D. Young, University of Minnesota, USA. Probe labelling, hybridization and band detection were carried out using an ECL direct nucleic acid labelling and detection system (Amersham, UK) according to the manufacturer's instruction.

Genetic analysis of morphological traits

Five morphological traits were analyzed: epicotyl color, seed testa color, hilum shape, pod color and pod shattering. The phenotypes of both parents, the F_1 hybrid and F_2 individuals were evaluated to determine the mode of inheritance.

Linkage analysis

F_2 segregation data for RFLP and RAPD markers, and morphological traits were examined by chi-square tests. Linkage analysis was assessed using MAPMAKER (Lander et al. 1987), rewritten for the Macintosh computer and kindly provided by Dr. S. Tingey, Du Pont Co., USA. In this program, a Twopoint/Group command was used to establish possible linkage groups with a LOD value of 3 and a recombination fraction of 0.25. The order of markers in each group was determined by Multipoint/First order command (LOD = 3, $r = 0.25$). Recombination frequencies were converted into map distances (centiMorgans) using the Kosambi function (Kosambi 1944).

Results

Parental polymorphism survey and F_2 segregation of molecular markers

In order to detect RAPD markers between *V. angularis* and *V. nakashimae*, 200 decamer primers were examined. Among them, 36 primers gave at least three distinct polymorphic fragments (Table 1). They produced a total of 116 RAPDs which were also detected in the amplification products of F_1 plants, indicating their inheritance to the next generation. Concerning RFLP markers, out of 67 mungbean and cowpea genomic probes 42 (62.7%) gave polymorphic patterns between parents in respect of *Hin*dIII digestion. Among them, 20 RFLP markers which gave clear hybridization bands were further used for F_2 segregation analysis.

The segregation of molecular markers (116 RAPD and 20 RFLP markers) was examined with 80 F_2 plants derived from the cross of *V. angularis* × *V. nakashimae*. One-hundred and fifteen RAPDs (99.1%) turned out to be dominant markers while one RAPD (0.9%) and 20 RFLP markers were co-dominant. The segregation of 23 RAPD and three RFLP markers deviated significantly from the expected ratios of 3:1 or 1:2:1 ($P < 0.05$).

Morphological traits

Five morphological traits which showed polymorphisms between parents were examined with F_1 and F_2 plants (Table 2). The F_1 hybrid had the same characteristics as *V. nakashimae*: namely, a purple stem, a black mottle on the seed testa, a dark brown pod, a shattering pod and a concave hilum cushion. F_2 segregation of these characters showed a good fit to a 3:1 ratio, indicating that each of them is controlled by a single dominant gene. Therefore, in this study, tentative dominant genes for these characters from *V. nakashimae* were assumed as follows: *Ps* (purple stem), *Bm* (black mottle on the seed testa), *Dbp* (dark brown pod), *Sp* (shattering pod) and *Chc* (concave hilum cushion). In addition, the intensity of stem color also segregated in F_2 plants giving purple stems and suggesting the existence of a

Table 1 Nucleotide sequences of the primers used in this study

Primer code[a]	Sequence	Primer code[a]	Sequence
SDA08	5′-TGGACACTGA-3′	OPD02	5′-GGACCCAACC-3′
SDA09	5′-TGGCCACTGA-3′	OPD05	5′-TGAGCGGACA-3′
SDA11	5′-TGCTCACTGA-3′	OPD08	5′-GTGTGCCCCA-3′
SDA27	5′-TGGTCACTGC-3′	OPD09	5′-CTCTGGAGAC-3′
OPA07	5′-GAAACGGGTG-3′	OPD11	5′-AGCGCCATTG-3′
OPA08	5′-GTGACGTAGG-3′	OPD15	5′-CATCCGAGCT-3′
OPA17	5′-GACCGCTTGT-3′	OPD16	5′-AGGGCGTAAG-3′
OPB07	5′-GACCGCTTGT-3′	OPD20	5′-ACCCGGTCAC-3′
OPB08	5′-GTCCACACAG-3′	OPE01	5′-CCCAAGGTCC-3′
OPC01	5′-TTCGAGCCAG-3′	OPE02	5′-GGTGCGGGAA-3′
OPC02	5′-GTGAGGCGTC-3′	OPE03	5′-CCAGATGCAC-3′
OPC05	5′-GATGACCGCC-3′	OPE06	5′-AAGACCCCTC-3′
OPC06	5′-GAACGGACTC-3′	OPE09	5′-CTTCACCCGA-3′
OPC08	5′-TGGACCGGTG-3′	OPE17	5′-CTACTGCCGT-3′
OPC10	5′-TGTCTGGGTG-3′	OPF07	5′-CCGATATCCC-3′
OPC13	5′-AAGCCTCGTC-3′	OPG10	5′-AGGGCCGTCT-3′
OPC19	5′-GTTGCCAGCC-3′	OPH04	5′-GGAAGTCGCC-3′
OPD01	5′-ACCGCGAAGG-3′	OPH17	5′-CACTCTCCTC-3′

[a] OP: Primers purchased from Operon Technologies, USA. SD: Commercially synthesized primers from TOYOBO, Japan

Table 2 Morphological characters of *V. angularis*, *V. nakashimae* and F_1 hybrid, and their segregation in F_2 population

Character	*V. angularis*	*V. nakashimae* F_1 hybrid		F_2 segregation		
				Phenotype	No. of plants	$\chi^2(3:1)$
Epicotyl color	Green	Purple	Purple	Purple:green	61:19	0.07
Purple epicotyl	–	Intense	Intense	Intense:weak	43:18	0.66
Black mottle on seed testa	Absent	Present	Present	Present:absent	63:17	0.60
Pod color	Straw	Dark brown	Dark brown	Dark brown:straw	55:22	0.52
Pod shattering	Non-shattering	Shattering	Shattering	Shattering:non shattering	58:17	0.22
Hilum cushion	Smooth	Concave	Concave	Concave:smooth	62:18	0.27

hypostatic gene for the intensity of pigmentation. Among 61 F_2 plants with purple stems, 43 and 18 showed intense and weak purple colors, respectively. Since this segregation agreed with a 3:1 ratio ($\chi^2 = 0.66$), a hypostatic gene, *Isc* (intense stem color), was also added to the tentative dominant genes from *V. nakashimae*.

Linkage analysis

Linkage analysis was carried out with 116 RAPD and 20 RFLP markers and six tentative phenotypic genes, using MAPMAKER. In total, 14 linkage groups containing at least three markers were constructed, and ten markers (7.6%) remained unlinked (Fig. 1). The sizes of the largest and smallest linkage groups were 196.2 and 18.8 cM respectively. The total map size was 1250 cM and the average distance between markers was 10.6 cM. Clusters of markers showing distorted segregation in the F_2 population were located in linkage groups, 2, 8 and 12.

Discussion

Linkage map of azuki bean

The first genetic linkage map of azuki bean has been constructed using an F_2 population derived from an interspecific cross of *V. angularis* and *V. nakashimae*. In total, 132 markers (108 RAPD, 19 RFLP and five morphological markers) were included in 14 linkage groups covering 1250 cM, while ten remained unlinked. Since the basic chromosome number of azuki bean is 11, a highly saturated molecular map should consist of the same number of linkage groups. In this study, the azuki linkage map was mainly constructed with RAPD markers, because the genomic and cDNA probes from azuki bean were not available. Previously, many researchers reported that RAPD markers often arise from the repeated sequences in the genome (Devos and Gale 1992; Williams et al. 1993). Therefore in future work,

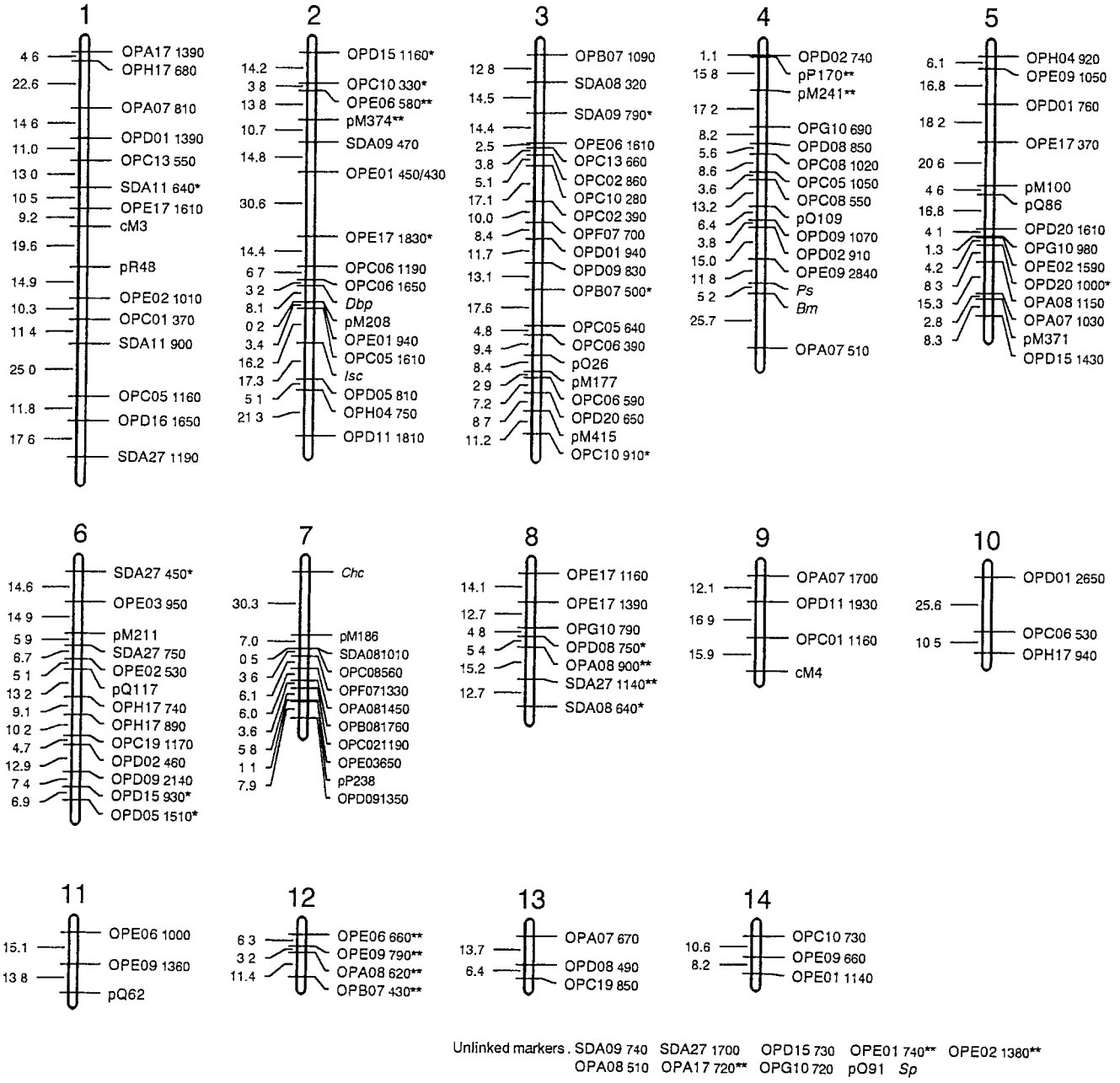

Fig. 1 A linkage map of azuki bean constructed with a *V. angularis* × *V. nakashimae* F_2 mapping population. Fourteen linkage groups are numbered in order of the size. Map distances and the marker names are shown on the left and right sides of the linkage groups, reespectively. *cM pM pO pP pQ* and *pR* indicate mungbean and cowpea RFLP markers provided from Dr. N. D. Young. RAPD markers are presented with the primer codes listed in Table 1 and the approximate molecular sizes. Markers showing significant deviations from the expected segregation ratios at 0.05 and 0.01 level are indicated with * and **, respectively

more RFLP markers are needed to be added to this linkage map in order to fill the gaps, to integrate some linkage groups, and to cover the entire genome.

The clusters of markers showing distorted segregation were found in linkage groups 2, 8 and 12. Such clusters have been commonly observed in the linkage maps derived from interspecific populations (Bernatzky 1986; Foolad et al. 1995). Zamir and Tadmor (1986) reported that abnormal segregation may result from the expression of linked lethal genes either at the gametic or zygotic stages of plant development. In the interspecific population of *Prunus*, the parental and reproductive differences were also thought to cause abnormal segregation (Foolad et al. 1995). In the present study, relatively weak F_2 plants which did not produce seeds could not be included in the F_2 mapping population. Therefore, the distorted segregation observed might result from growth-related factors, such as seed production.

Mapping of the genes for morphological traits

Using the interspecific population of *V. angularis* and *V. nakashimae*, six morphological traits were analyzed and

five tentative genes were mapped on the azuki linkage map. There are no studies available on pod-shattering behavior and the hilum cushion, while the mechanisms of stem-, seed- and pod-color expression were studied by Matsuura (1933). He analyzed these characters using azuki cultivars and identified two genes for stem color, eight for seed testa color and two for pod color. Stem color is explained by the control of the gene *P* (pigmentation of anthocyanin in the stem) and the hypostatic gene *I* (intensity of pigmentation on the stem). He also designated the genes *M* and *B2* for black mottle on the seed testa and the black pod, respectively. He further confirmed the linkage relationships between *I* and *B2*, and between *P* and *M*. In the present study, all four characters segregated in the F_2 interspecific population and their tentative genes were mapped. Similar linkage relationships to those mentioned above were also observed; namely, *Isc* (intense stem color) and *Dbp* (dark brown pod) in linkage group 2, and *Ps* (purple stem) and *Bm* (black mottle on seed testa) in linkage group 4. Since these dominant genes were from *V. nakashimae*, allelism tests are required to confirm that the genes of these common characters are located at the same locus.

Comparison of linkage maps between azuki bean and other *Vigna* species

Comparative linkage maps constructed with heterologous probes have been used to investigate the chromosomal evolution of the entire genome between related species, such as tomato and pepper (Tanksley et al. 1988), tomato and potato (Bonierbale et al. 1988) and pea and lentil (Weeden et al. 1992). In the present study, 19 out of 20 RFLP markers from mungbean and cowpea genomic probes could be mapped on the linkage map of azuki bean. Table 3 shows the comparison of linkage groups between the three *Vigna* species, azuki bean, mungbean and cowpea. Although their linkage maps are not saturated with a number of markers, some sets of the markers were found to belong to the same linkage groups of the respective maps. This suggests that some linkage blocks are conserved among these *Vigna* species. In addition, one of the RFLP markers, pM241, was found to be located in the cluster showing distorted segregation in the azuki linkage map as well as in the mungbean linkage map (Menancio-Hautea et al. 1993b), indicating that the distorted segregation in this cluster might be caused by a similar mechanism in the two *Vigna* species.

Further study for azuki bean breeding

The development of a genetic linkage map in azuki bean will greatly enhance the ability of breeders to monitor the introgression of desirable traits from wild species into the azuki cultivar. In the present study, we have made a preliminary analysis of dominant morphological characters from *V. nakashimae*. In addition this wild species has a resistance gene to blown stem rot which is one of the most serious diseases for azuki bean. Once this gene is tagged and mapped with molecular markers, marker-assisted selection will be applied for the improvement of azuki cultivars.

The red-seed color of azuki bean is also one of the important characters for azuki breeding. In the present study we made a cross between an azuki cultivar and *V. nakashimae* having a green seed color. However, we could not clarify the mechanism of red seed color expression because of its complicated segregation and the small number of F_2 plants analyzed. According to Matsuura (1933), the ground color of the seed testa was explained by a seven-gene model with both inhibitor and complementary genes. Therefore, for this character, which is under multiple gene control, we need to analyze the plants of advanced generations, such as recombinant inbred lines.

Table 3 Comparison of linkage groups of 20 RFLP markers among three *Vigna* species, azuki bean, mungbean and cowpea

Probe	Linkage group		
	Azuki bean	Mungbean[a]	Cowpea[b]
cM3	1	1	6
pR48	1	6	–
pM374	2	5	–
pM208	2	7	4
pO26	3	3	1
pM177	3	3	–
pM415	3	3	–
pP170	4	–	3
pM241	4	2	3
pO109	4	13	–
pM100	5	8	–
pQ86	5	8	–
pM371	5	1	–
pM211	6	10	8
pQ117	6	4	–
pM186	7	–	4
pP238	7	1	1
cM4	9	7	4
pQ62	11	2	–
pO91	(unlinked)	5	–

– not examined
[a] Mapped by Menancio-Hautea et al. (1993a)
[b] Mapped by Fatokun et al. (1993)

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