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THESIS

A STUDY ON ESSENTIAL COMPONENTS REQUIRED FOR HYBRID SEED PRODUCTION IN MUNGBEAN

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Mungbean [*Vigna adiate* (L.) Wilczek: Leguminosae] is a short-lived annual legume crop cultivated mainly in Asia for its dry seed as a source of protein and carbohydrate as well as for sprouting as a vegetable. Besides being one of the shortest duration field crops in the world, soil rhizobium bacteria around the mungbean root zone can symbiotically fix N_2 gas from the air and thus makes it among the most popular components in cropping systems. The pure line breeding strategies such as pedigree selection, bulk selection, single seed descent and early generation testing have repeatedly shown a limited success in increasing seed yield in legume crops. One way to break the yield plateau is to produce hybrid seed to utilize heterosis or hybrid vigor inherently available in living organisms. The success in hybrid rice encouraged mungbean breeders to explore for the possibility in developing hybrid cultivars to boost up seed yield. The exploitation of heterosis in hybrid cultivars remains the best approach to maximize yield and yield stability. However, a hybrid seed production is not yet available in mungbean. The objective of this study were (1) to develop new essential characters for supporting hybrid seed production in mungbean by mutation techniques, and (2) to compare levels of heterosis among four F_1 hybrids of mungbeans with different genetic distance.

In the first experiment, new chasmogamous mutants were induced by gamma irradiation at the rate of 100 and 200 Gy. The mutants were identified at a low rate of 0.4-0.7% in the M_2 generation of accession V1197, and observed for their purity by growing in plant-to-row in the M_3 and M_4 generations. A uniform chasmogamous line was hybridized to normal flower lines to study the inheritance of this character. All F_1 plants had normal flowers, while the F_2 plants segregated well with a3:1 ratio of normal : chasmogamous plants. When the F_1 was backcrossed to the chasmogamous parent, the progeny gave a 1:1 ratio of normal to chasmogamous plants. Thus, chasmogamy was controlled by a single recessive gene, *cha*.

In the second experiment, the hybrid combinations based on the genetic distance of parental lines, that were developed by using Sukhothai (SKT) as the female parent and pollinated by male parents of different genetic distance as revealed by SSR markers. They are H192 (close distance), C357 (moderate distance), TC1965 (high distance) and W166 (very high distance). The results revealed that the F_1 from the parents with larger genetic distance showed higher heterosis in yield per plant and number of pods per plant. Thus SSR markers combined with yield components can be used to identify parental lines with high genetic distance for hybrid seed production in mungbean. This approach potentially helps reducing the amount of fieldwork required for evaluation of F_1 hybrids.

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A STUDY ON ESSENTIAL COMPONENTS REQUIRED FOR HYBRID SEED PRODUCTION IN MUNGBEAN

INTRODUCTION

Mungbean (*Vigna radiata* L. Wilczek) is one of the most important legumes in Asian countries and consumed widely throughout the world. Seed yield in mungbean is still low or not improved much owing mainly to the use of cultivars that are exclusively homozygous pure lines. Mungbean is a self-pollinated plant that hybrid seed production requires an intensive hand pollination which is not commercially feasible. The previous exploitation of heterotic for higher yield was largely limited to cross pollinated crops, but evidence is now available to confirm the presence of such effect in self pollinated crops like rice or bean. Yuan (1994) triggered a leap in rice yield by developing hybrid rice varieties which were once considered by scientists as commercially impossible. However, this strategy requires a pollination control system to cross the two fertile grandparent lines to produce the male-sterile female parent line for hybrid seed production. At present, there is no information on this topic in mungbean. Employing model in rice hybrid, this research was investigated on the basic components essential for hybrid seed production beginning with male and female reproductive structures to identify male sterility that would allow higher cross pollination.

Hybrid vigor or heterosis is the superior performance of the heterozygous hybrid progeny over both homozygous parents. Most crops show hybrid vigor but commercial production of hybrids is only feasible if a reliable and cost-effective pollination control system is available, coupled with hybrid vigor in seed yield, to produce hybrid seeds, a pollination control system is required to prevent unwanted self-pollination for large-scale hybrid seed production. This also depends on whether the male-sterile female parent line can be multiplied efficiently and economically.

OBJECTIVES

The objectives of this doctoral thesis were:

1. To develop new essential characters for supporting hybrid seed production in mungbean by mutation techniques
2. To assess the heterotic pattern of F_1 hybrids by comparing levels of heterosis among mungbean hybrids based on the parental distance in morphology and SSR markers

LITERATURE REVIEW

1. Hybrid seed production in legumes

Hybrid seed production in self-pollinated crop had been successful in rice by using CMS system, A-line (female), B-line (maintainer) and R-line (restorer) to obtain F_1 hybrid seeds. In legumes, although self pollination crops, are more difficult to cross than rice due to closure of flowers. Recently, a success case was reported in pigeonpea (*Cajanus cajan* L.) as the world's first pigeonpea hybrid (ICPH 8) released by ICRISAT in 1991 (Saxena *et al.*, 2009). In farmers' fields, this hybrid produced 25-30% more grains than the best pure line variety. ICRISAT identified a CMS line from crossing between the wild relative of pigeonpea (*C. cajanifolius*) and the cultivated variety. Male sterile lines were developed through a series of six backcrosses. In soybean (*Glycine max*), seed production could be done by linking the genetic male sterile with herbicide resistance as marker, thus the female plants can be easily identified from mixed population field. However, commercial production of hybrid soybean is still a major barrier to a full use of hybrid cultivar.

2. Hybrid vigor in mungbean

The success in hybrid rice, another self-pollinating crop, helped adding another 20% increment in the average rice yield in China (Yuan, 1987; 1997; 1998; Wu, 2009). This encourages mungbean breeders to explore for the possibility of producing hybrid cultivars to utilize heterosis in mungbean. Chen *et al.* (2003) and Soehendi and Srinives (2005) showed a wide range of heterosis in mungbean yield and yield components. However, a major barrier in hybrid seed production in mungbean is the cleistogamous (close flower) nature of the flowers and thus allows self-fertilization before the flowers are open. This limitation also lowers natural outcrossing by wind and insects. Although, emasculation can be done by hands prior to flowering, it is too laborious, expensive and time consuming. One strategy is to modify morphology of the flowers to encourage higher outcrossing rate. Then, male sterile lines should be developed as the next step to enable commercial hybrid seed production in mungbean.

3. Out-crossing in mungbean:

Studying from rice as the model crop in developing hybrid varieties in self-pollinating species, the anther and stigma of rice naturally extrude out of the flower to allow outcrossing. There have been many reports confirming that outcrossing rates in plants are genetically controlled (Waines and Hegde, 2003; Abdel-Ghani *et al*., 2005; Parzies *et al*., 2008). In tomato, a self-pollinating vegetable, variation in outcrossing rate was found correlated with floral morphology (Georgiady *et al*., 2002). Outcrossing rate was also influenced by environmental conditions such as high rainfall and cool temperature that promoted outcrossing in barley (Abdel-Ghani *et al*., 2004).

To change the mating system from self-pollinating to cross-pollinating, the donors of the traits related to pollination are usually wild varieties or related species. However, traits promoting flower opening has not been reported in mungbean germplasm and wild relatives. The use of mutation technique such as gamma ray is an alternative method to induce new flower morphology in mungbean. Although an optimum dose to induce flower mutation in mungbean has not been identified, yet only a few mutation works in mungbean have touched on flower morphology. Santos (1969) was the first who reported that some M_6 mungbean progenies showed degrees of male sterility and allowed natural outcrossing with other varieties. Yadav and Singh (1987) treated mungbean seeds with 40 kR gamma ray and observed male sterile plants with narrow leaves and compact growth habit. Hual-alai *et al*. (2000) reported that gamma ray at the rate of 100-700 Gy reduced pollen fertility up to 50% from the control. Sangsiri *et al*. (2005) found that gamma ray at a high dose of 500 Gy induced sterile plants with some changes in flower structure. However, the traits were not heritable.

4. Prediction of the performance of hybrids in mungbean

Heterosis is a well-known phenomenon of showing superiority in hybrid progeny compared with their parents (Shull, 1908). There is a long dramatic history of success in hybrid maize, a cross-pollinating crop (Duvick, 1999). In a self-pollinating crop, Yuan (1997) reported a success story in developing hybrid rice, which has recently sustained food security in China and India. The success in hybrid rice encouraged mungbean breeders to explore for the possibility in developing hybrid cultivars to boost up seed yield. The exploitation of heterosis in hybrid cultivars remains the best approach to maximize yield and yield stability.

Genetic difference between the inbred or pure line parents presumably contributes to the genetic basis of heterosis. Farooq and Azam (2002) reviewed the uses of molecular markers in measuring genetic distance and heterosis in plant breeding. Schrag *et al*. (2010) applied joint analyses of hybrids and parental inbred lines for prediction of performance of untested hybrids in maize. In mungbean, Sangiri *et al*. (2007) examined a set of 615 cultivated, wild and weedy mungbeans by using simple sequence repeat (SSR) markers and found that the markers divided the germplasm into distinct sets according to their genetic distances and origins. Their information can be utilized to compare the performance of hybrids derived from the parents with different degrees of genetic distance. The heterotic patterns detected from various parental lines will be useful to the plant breeder to make cross combinations only from promising parents. This approach will help reducing the number of hybrids entering into various yield trials.

Several methods have been developed to predict hybrid performance in maize using genetic markers (Dudley *et al*., 1991; Frisch *et al*., 2010; Maenhout *et al*., 2010; Schrag *et al*., 2010; Steinfath *et al*., 2010). Information from molecular markers was also used to assign new germplasm to heterotic pools in maize (Reif *et al*., 2003). Xu *et al*. (2004) could not predict yield heterosis in maize, although SSR makers showed high polymorphism among the parental inbreds. While Liu and Wu (1998) showed that SSR marker technology could be used to identify heterotic patterns of the parental lines in hybrid rice production. High heterotic effects were obtained from hybrids of genetically diverse parental plants through analysis of RAPD markers (Pirkhezri *et al*., 2010). However, there has been no report so far in using SSR markers to predict hybrid performance in legumes. Recently, SSR markers have been developed to detect genetic diversity in mungbean (Sangiri *et al*., 2007 and Somta *et al*., 2009). The variation in SSR markers together with morphology can be used to form hybrid combinations with distinct heterotic patterns in mungbean. It can be a key to the success in breeding for hybrid mungbeans.

MATERIALS AND METHODS

1. Study on induction of novel characters enabling commercial hybrid seed production in mungbean

1.1. Plant materials and mutation techniques

Five-hundred seeds each of six mungbean pure lines, “Sukhothai” (SKT) from Thailand, JP226609 from East Timor, JP211874 from Myanmar, AusTRCF94436 and AusTRCF94437 from Australia and “V1197” from Pakistan, were treated with gamma ray at the rates of 100 and 200 Gy. The gamma irradiator used in this study is installed at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University (KU), Bangkok. It was manufactured by J.L. Shepherd & Associates, under the Model MARK 1-30, Serial No. 1116, loaded with 4500 Curies of Cs-137 having a half-life of 30.12 years. The treated seeds (M_0) were sown in the field at KU Kamphaeng Saen campus where individual mutant plants were observed for their purity, harvested and grown from M_2 until M_4 generations. In each generation, all mutant plants were observed on flower morphology and individually harvested. A mutant from V1197 was chosen as a promising line and studied on inheritance of the character by crossing with original V1197. The F_1 seeds were sown in the field to backcross to the mutant and individually harvested at maturity. The F_2 seeds from each F_1 plant were sown in rows and number of normal and chasmogamous plants were recorded at flowering stage. The observed number of plants was tested against the expected number by a Chi-square goodness-of-fit test (Mather, 1951).

1.2 Field planting

Field cultural practices in this experiment were conducted based on the standard management method for mungbean grown in Thailand. Briefly, the seeds were sown at spacing of 50 cm between rows and 12.5 cm between plants within row. Urea fertilizer was basally applied at 312.5 kg/ha. Hand-weeding was done twice at

20 and 40 days after sowing. Insecticides were sprayed at seedling, flowering and pod filling stages to control insect pests. Irrigation water was applied during the cropping as needed.

1.3 Determination of outcrossing rates

To study outcrossing, three pure lines, *viz*. KPS1 (green hypocotyl and normal flower), V1197 (purple hypocotyl and normal flower) and chasmogamous mutant line (purple hypocotyl and open flower) were sown in each block of 2x5 m^2. At maturity, seeds from each line were harvested and sown in the following season to observe for outcrossed plants. Hypocotyl color and chasmogamous flower were used as markers to detect the outcrossing rate.

2. Comparison of hybrid vigor based on parental distance in SSR markers and agronomic traits in mungbean (*Vigna radiata* (L.) Wilczek)

2.1. Plant materials

Five parental mungbean lines were chosen based on genetic distance by SSR markers from the previous study on genetic diversity of mungbean germplasm (Sangiri *et al*., 2007). Three of them are cultivated mungbean accessions (*V. radiata* var. *radiata*), *viz*. C357, H192 and Sukhothai (SKT), while two of them are wild accessions (*V. radiata* var. *sublobata*), *viz*. TC1965 and W166. The mungbean accessions were chosen based on their genetic distance as revealed by morphology and SSR markers (Sangiri *et al*., 2007). All mungbean accessions were obtained from Genebank, National Institute of Agrobiological Science, Japan. The experiment was conducted on January 2009 to December 2010 at the field of Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand. The field is located at latitude 14° 1' 48.54" N, longitude 99° 57' 51.67" E with the elevation of 10 m above sea level. In the first year, the F_1 hybrid seeds were produced by hand-pollination, using Thai indigenous variety, Sukhothai as the female parent and crossed with male parent with different degrees of genetic distance, i.e. H192 (close distance), C357 (moderate distance), TC1965 (high distance) and W166 (very high distance). Four F_1 hybrid combinations were produced, viz. SKTxC357, SKTxH192, SKTxTC1965 and SKTxW166. All hybrids and their parents were arranged in a randomized complete block (RCB) design with two replications. Each F_1 hybrid and its parents were sown in a plot of 5-meter rows, 2 rows per plot, with an equal spacing of 50 cm between plants and between rows. This planting density is lower than that recommended for a production field in order to reduce competition between vigorous F_1 plants in the field. The field management followed normal agricultural practices for mungbean (Park, 1978). Insecticides (cypermethrin, abamectin, chlorfluazuron and omethoate) were alternately sprayed at the manufacturers' recommended rates. Weeds were controlled by using plastic mulching and hand-weeding. True hybrid plants were confirmed by comparing morphological characters, days to first flowering and SSR markers with the parents. At maturity, individual plants were hand-harvested to observe for number of branches per plant, clusters per plant, pods per cluster, pods per plant and seeds per pod. Pod length (cm), 100-seed weight (g) and number of seeds per plant were also observed. Data were analyzed based on individual plants as well as plot means.

2.2. DNA extraction and SSR analysis

Total genomic DNA from each of the parental lines and F_1 hybrids were isolated from young leaves using the method modified from Dellaporta *et al.* (1983). Each polymerase chain reaction (PCR) was carried out in a total volume of 10 µl containing 2 ng of DNA, 1X Taq buffer, 2 mM MgCl2, 0.2 mM dNTPs, 1 unit Taq DNA polymerase (Fermentas) and 5 pmol of each forward and reverse SSR primers. The PCR was performed under the following conditions: 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min in a GeneAmp® PCR System 9700 (Applied Biosystems). The PCR products were run on 4.5% denaturing polyacrylamide gel and visualized by a silver staining solution.

Nineteen SSR primers as reported by Sangiri *et al*. (2007) were used to detect polymorphism between the parental lines and their progenies. They are CEDG013, CEDG015, CEDC050, CEDG056, CEDG075, CEDG087, CEDG088, CEDG100, CEDG108, CEDG139, CEDG149, CEDG150, CEDG174, CEDG191, CEDG247, CEDG264, CEDG269, CEDG304 and CEDG305.

2.3. Statistical analysis

The data were analyzed base on their sources of variation (ANOVA) using the R program version 2.10 for Windows (Venables *et al*., 2009). When an F-test was declared significant, the treatment means were compared by Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Heterosis and heterobeltiosis of each agronomic trait were calculated in percentage as followed:

$$\% \text{ Heterosis (H)} = [(F_1\text{-mid parent})/\text{mid parent}]\text{x}100$$

$$\% \text{ Heterobeltiosis (Hb)} = [(F_1\text{-high parent})/\text{high parent}]\text{x}100$$

Significance of H and Hb were tested against their standard errors following the method advocated by Soehendi and Srinives (2005).

Agronomic data were standardized to the mean of zero and variance of one before being used in similarity analyses by Euclidean distance and clustered by an Unweighted Pair Group Method with Arithmatic Mean (UPGMA) (Sneath and Sokal, 1973). A cluster analysis was performed based on similarity between two samples, and a distance can be represented by difference between transformed values of the samples.

Molecular data were obtained by scoring amplicons of PCR product as present (1) or absent (0) and entered in a form of binary data matrix. Then, Dice coefficient of similarity was calculated and a dendrogram on genetic similarity was constructed. The computer package NTSYS-PC Version 2.2 (Rohlf, 1998) was used for molecular cluster analysis.

Regression analysis was used to explain a relationship between genetic distance based on SSR and agronomic traits with the heterosis and heterobeltiosis values. Scatter plots were constructed to visually identify the relationship between the variables, then the regression equations were estimated. Coefficient of determination (R^2) was used to test the accuracy of a regression model. R^2 of closer to 1.00 revealed more reliability of the regression equation in predicting a dependent variable (i.e. heterosis and heterobeltiosis in this case).

RESULTS AND DISCUSSION

Results

1. Study on induction of novel characters enabling commercial hybrid seed production in mungbean

1.1. Isolation of the chasmogamous mutant line

Effect of gamma radiation could not be detected on the M_1 plants. When bulked M_2 seeds were harvested and sown, the individual mutants could then be identified, harvested and purified. The flower mutants were obtained from both gamma doses of 100 and 200 Gy only in accession V1197 (Table 1). The mutants showed disorder or misshapen flowers, with a wide range of flower morphology (Figure 1). They showed various forms of open flowers such as multiple corollas with exerted stigma, tri-stigmas, corolla with only standard but no wings and keel, double stigmas and double wings, flowers without keel, and open keel with exerted stigma. The gamma ray seemed to be specific to certain mungbean accessions in giving these types of mutant. However, most misshaped flower mutants were not solid mutants, i.e. not all flowers in a plant were misshapen. Finally, a corolla mutant line without wings and keel was purified and found breed-true from M_2 to M_4 (Figure 1c and 2b).

1.2. Outcrossing in the chasmogamous mutant

The mixed plant types in each pure line were identified from hypocotyl color, flower form and plant uniformity. The chasmogamous mutant showed an increase in outcrossing rate up to 9.6% compared to non-detectable rate in the pure lines KPS1 and V1197 (Table 2).

Table 1 Effect of gamma radiation on six mungbean accessions observed in M_2 generation.

Accessions	Gamma rates	No of plant survivors	No of chasmogamous plants	% Mutation
JP226609	100	950	0	0
	200	830	0	0
JP211874	100	1,090	0	0
	200	980	0	0
AusTRCF94436	100	2,080	0	0
	200	1,720	0	0
AusTRCF94437	100	1,880	0	0
	200	1,650	0	0
SKT	100	1,120	0	0
	200	800	0	0
V1197	100	2,400	10	0.42
	200	2,880	20*	0.69
	Total	18,380	30	0.163

*Only two plants from 200 Gy which have all flower showed the chasmogamous, another lines have chasmogamous on some of the flower in that plant.

Table 2 Percentage of outcrossing in the chasmogamous mutant, its parental variety (V1197) and standard variety (KPS1).

Lines	% outcrossing	Main characters of the original lines
KPS1	0	Determinate growth, large plant canopy, green hypocotyl and normal flowers
V1197	0	Indeterminate growth, small plant canopy, purple hypocotyl and normal flowers
Chasmogamous mutant	9.6	Indeterminate growth, small plant canopy, purple hypocotyl and open flowers

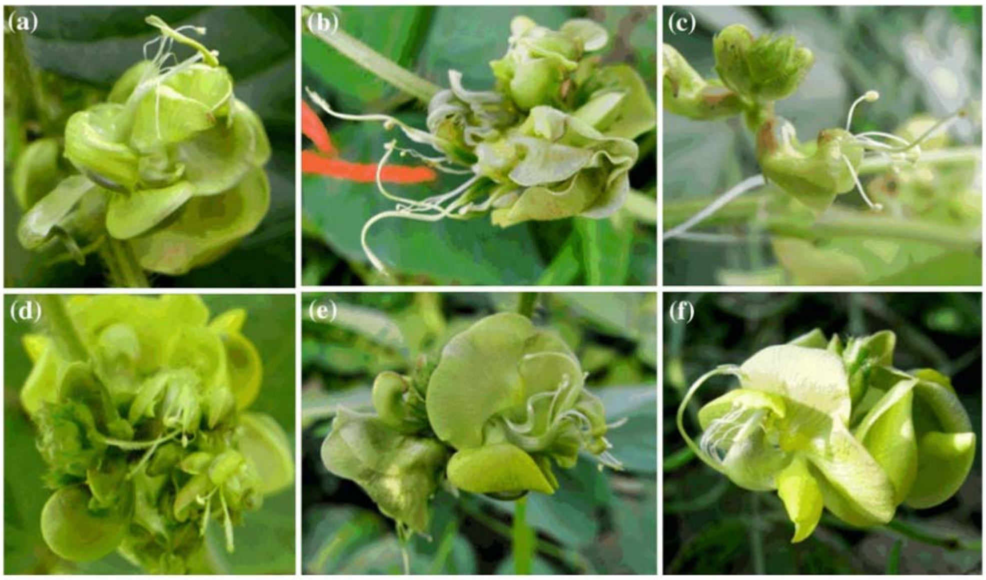

Figure 1 Flowers of mutants obtained from V1197 mungbean showing various forms of open flowers: (a) multiple corollas with exerted stigma, (b) tri-stigmas, (c) corolla with only standard with no wings and keel, (d) double stigmas and double wings, (e) flower without keel, and (f) open keel with exerted stigma.

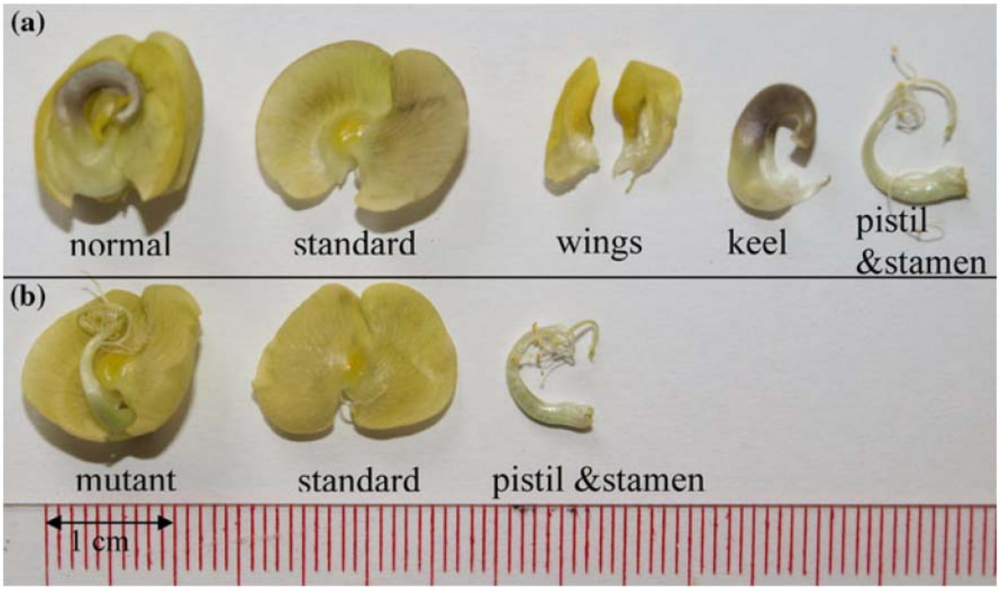

Figure 2 Parts of flowers of normal V1197 mungbean (a), and the chasmogamous mutant (b).

Table 3 Description of the chasmogamous mutant line and its parent, V1197.

Traits*	Chasmogamous	V1179
Branches/plant	3.3±0.11	2.5±0.19
Pods/plant	9.0± 0.96	18.7± 1.67
Seeds/pod	8.6± 0.38	11.5± 0.41
Pod length (cm)	5.9± 0.14	7.0± 0.32
100-seed weight (g)	3.3± 0.06	2.7± 0.187
Yield/plant (g)	2.2± 0.26	5.3± 0.51

*All traits showed highly significant different between the mutant and the parental line as tested by group comparison t-test at $P \leq 0.001$

1.3 Inheritance of chasmogamous mutant

The chasmogamous mutant expresses different yield components and agronomic characters from its original V1197 line (Table 3). The mutant has more branches and higher 100 seed weight, but less of pods per plant, seeds per pod, and yield per plant. The change in flower morphology not only has a direct effect on pollination, but also on the yield-related traits. When the chasmogamous mutant was crossed with the normal parent, all the F_1 plants had normal flowers (Table 4). The number of F_2 plants across 4 families were 104 normal flowered plants and 25 chasmogamous plants, which agreed well with a 3:1 ratio at df = 1. The observed ratio in each F_2 family (each at df = 1) also agreed with 3:1. All four families were homogeneous in segregation pattern (df = 3). When the normal flower F_1 was backcrossed by the chasmogamous parent, a ratio of 1 normal plants: 1 chasmogamous plants was obtained. The chasmogamous mutant found in this study was controlled by a single gene designated herewith as *Cha* for the normal flower, which is completely dominant to the chasmogamous *cha*.

Table 4 Number of plants segregating for normal and chasmogamous flowers in various generations of mungbean.

Generations	Number of plants			Expected ratio	Chi-square values[a]	Prob.
	Total	Normal	Chasmogamous			
Normal flower, P_1	10	10	-	-	-	
Chasmogamous, P_2	10	-	10	-	-	
F_1 (P_1xP_2)	10	10	-	-	-	
F_2	129	104	25	3:1	2.17	0.140
Family 1	20	17	3	3:1	1.07	0.300
Family 2	47	40	7	3:1	2.56	0.109
Family 3	29	19	10	3:1	1.71	0.190
Family 4	33	28	5	3:1	1.47	0.225
Heterogeneity among families					4.55[b]	0.207
BC_1F_1 (F_1xP_2)	17	6	11	1:1	1.47	0.225

[a] Chi-square value at (P=0.05, df=1) = 3.84

[b] Chi-square value at (P=0.05, df=3) =7.81

2. Comparison of hybrid vigor based on parental distance in SSR markers and agronomic traits in mungbean

2.1 Cluster analysis based on molecular data and agronomic traits

The results showed that SSR markers divided the parental lines into four groups with different degrees of genetic distance from SKT, where H192, C357, TC1965 and W166 showed close, moderate, high and very high distance, respectively. A dendrogram was constructed to show the relationship between the mungbean accessions and their F_1 progenies (Figure 3). In this study, SKT was used as the common female parent and as the reference genotype to measure genetic distance among the parents and their hybrids and then group them. The entries can be divided in to three groups. The first group consists of the cultivated mungbeans with closer genetic distances, *viz*. SKT, C357 and H192 as well as their F_1s, i.e. SKTxH192 and SKTxC357. The second group includes the wild mungbean from Australia, TC1965 which is high genetic distance from SKT. Their F_1 hybrid, SKTxTC1965 also falls into this group. While the third group which shows the highest genetic distance from SKT consists of W166, a wild mungbean from Myanmar and the F_1, SKTxW166. When agronomic traits were considered, the dendrogram shows three distinct groups. The first group comprises the cultivars SKT, C357 and H192 with their hybrids (Figure 4). The second group consists of both hybrids between the cultivated and wild accessions, SKTxTC1965 and SKTxW166, which show blending of characters among their parents. While the third group comprises the two wild parents, TC1965 and W166, which show the highest morphological distance from SKT. A heterotic pattern can be established from the above information into two groups. The first pattern is recognized as the hybrids between the cultivars, SKTxC357 and SKTxH192. While the second pattern comprises the hybrids of the cultivated and wild mungbeans, SKTxTC1965 and SKTxW166.

2.2. Performance of the hybrids and heterosis

F_1 hybrids in this experiment showed significant heterosis over mid- and better-parents in almost all characters (Table 5). The cultivated parents and hybrids were earlier in flowering (33-38 days after planting) as compared to wild accessions (53-54 days after planting). Most hybrids had higher yield per plant than their parental lines. The highest seed yield was obtained from the crosses between cultivar and wild accessions SKTxTC1965 and SKTxW166, yielding 169.2 and 144.9 grams per plant, respectively. While the F_1s from the cultivated accessions, SKTxH192 and SKTxC357 yielded at 130.7 and 103.3 grams per plant, respectively. Number of pods per plant showed high heterosis in the same pattern of crosses from which SKTxTC1965 and SKTxW166 gave 1148.2 and 547.2 pods per plant, giving heterobeltiosis at 1007.08% and 427.57%, respectively. The other yield-related traits, *viz*. 100-seed weight, number of seeds per pod, and pod length tended to express near to either one parent or mid-parent. Thus the useful heterosis in this study came from higher number of pods and seed yield per plant.

Among the cultivars crossed, SKT is more similar to H192 than C357 in agronomic traits, especially days to first flowering, number of branches, number of clusters and seed yield. Their similarity was also confirmed by SSR clustering. The heterosis percentage in seed yield per plant varied from 51.65% and 64.01% in the cultivar crosses SKTxH192 and SKTxC357 to 211.01% and 248.69% for cultivar x wild crosses SKTxW166 and SKTxTC1965, respectively (Table 5). Likewise, the heterobeltiosis in seed yield per plant from the crosses between SKT with the cultivars C357 and H192 gave yield advantage of only 12.69% to 42.56% over the better parents, while the crosses with wild mungbeans, TC1965 and W166 showed 58.06% to 84.58% over the better parent, respectively. The hybrids showed less number of seeds per pod and 100-seed weight, but more pods per plant. Thus number of pods per plant is the major trait contributing to hybrid vigor in seed yield per plant in this study. Means and heterosis of the other agronomic traits revealed that the hybrids were early in flowering, higher in number of branches and clusters per plant, and shorter in pod length. Although number of clusters per plant was not much different between the parents and the hybrids, number of pods per cluster of the hybrids showed high vigor over their parents.

F_1 crosses obtained from the parents with wider relationship (i.e. between cultivars and wild mungbeans) generally gave higher H and Hb than those obtained from the cultivar crosses. Ranges of hybrid vigor were also higher in the wider crosses as compared to the narrower crosses. H and Hb of the cultivar crosses varied from 96.17% in H of number of pods per plant to -38.64% in Hb of 100 seed weight of SKTxH192. While crosses between the cultivar and wild accessions gave the lowest value of Hb at -65.27% for 100-seed weight and the highest value of H at 1141.30% for number of pods per plant (Table 5). A negative heterosis was obtained for seed size in all crosses when compared to the better parent.

2.3. Relationship between genetic distance and hybrid performance:

Relationship between H, Hb and the observed agronomic traits of the four F_1 hybrids were presented in Table 6. H of yield per plant showed positive correlation with number of clusters and number of pods per plant, but showed negative correlation with days to first flowering at the correlation coefficients (r) of 0.996, 0.991 and -0.999 (df = 2), respectively. Thus number of clusters and pods per plant are considered major contributors to heterosis in seed yield of hybrid mungbean in this study. In contrast, H of 100-seed weight, another yield component, is negatively correlated with H of seed yield per plant (r = -0.968) and number of clusters per plant (r = -0.986). Small seed size and high pod number are unique characters of wild mungbean and strongly inherited to their hybrid progenies. For phenotypic correlation, number of pods per plant was negatively correlated with number of seeds per pod (r = -0.974), while pod length is positively correlated with 100 seed weight (r = 0.950).

Heterotic patterns of the four F_1 hybrids were also presented by graph (Figure 5), showing the relationship between genetic distance based on both SSR and phenotype with the H and Hb values. The genetic distance and hybrid yield performance as measured from H and Hb showed R^2 of 0.78 to 0.99 and could be classified into two groups (Figure 5). The first group comprises SKTxW166 and SKTxTC1965 at the left end of regression line, and the other group comprises SKTxC357 and SKTxH192 at the right end of the regression line. Grouping by SSR markers (Figure 5a and 5c) agreed well with grouping by agronomic traits (Figure 5b and 5d).

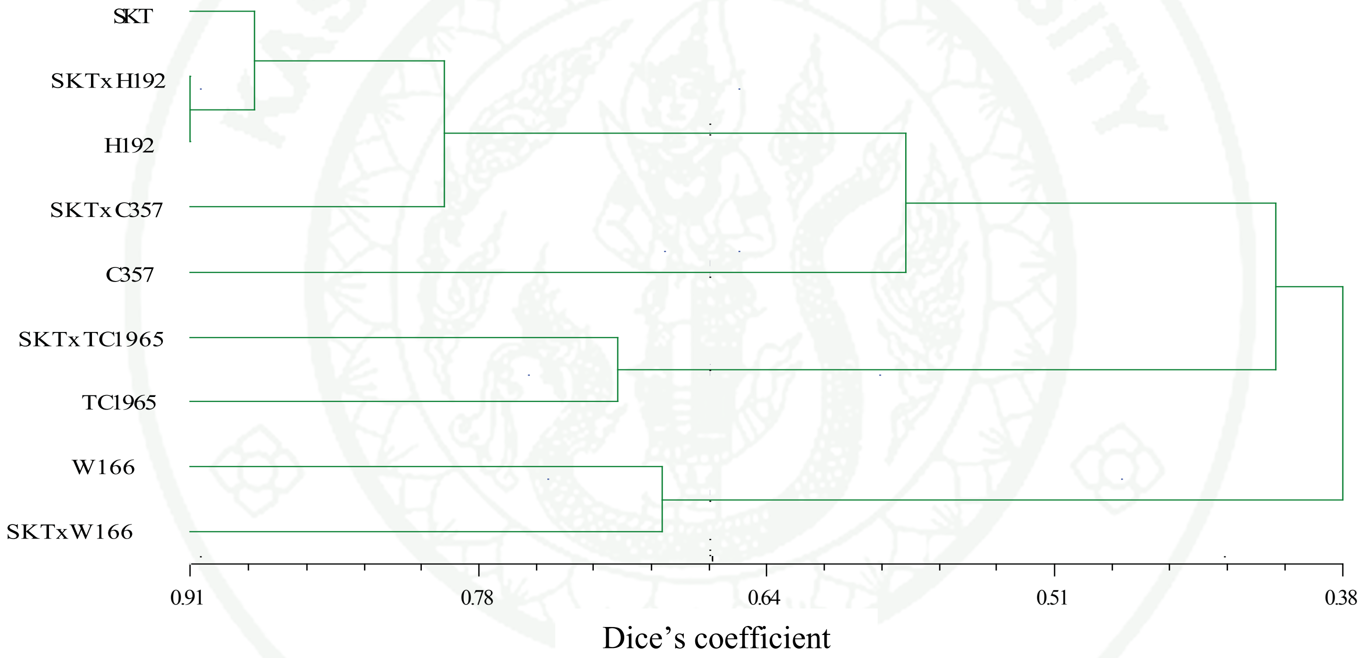

Figure 3 A dendrogram showing relationship between five mungbean accessions and their F_1 progenies based on unweighted pair group method with arithmatic mean (UPGMA) of Dice-similarity coefficients of their SSR markers.

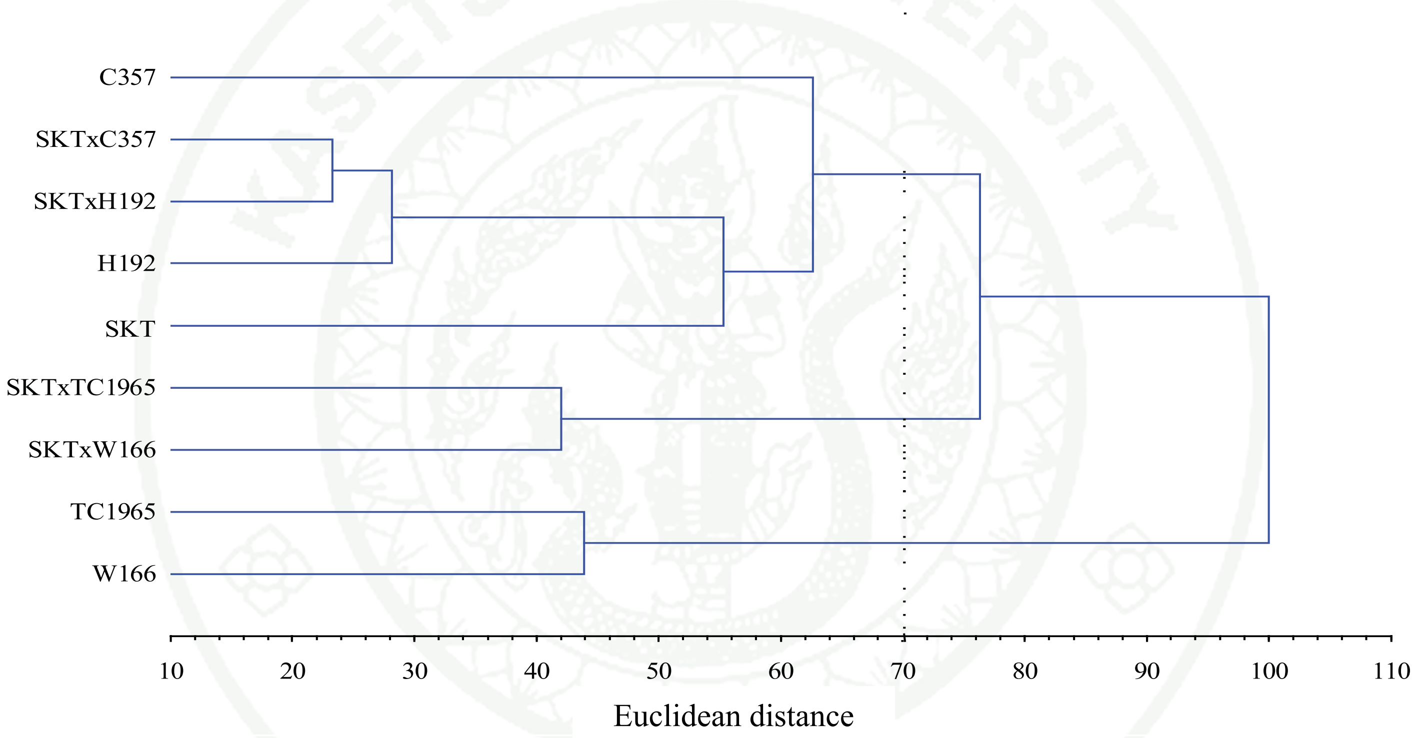

Figure 4 A dendrogram showing relationship between fives mungbean accessions and their F_1 progenies based on unweighted pair group method with arithmatic mean (UPGMA) of Euclidean distances of their agronomic traits.

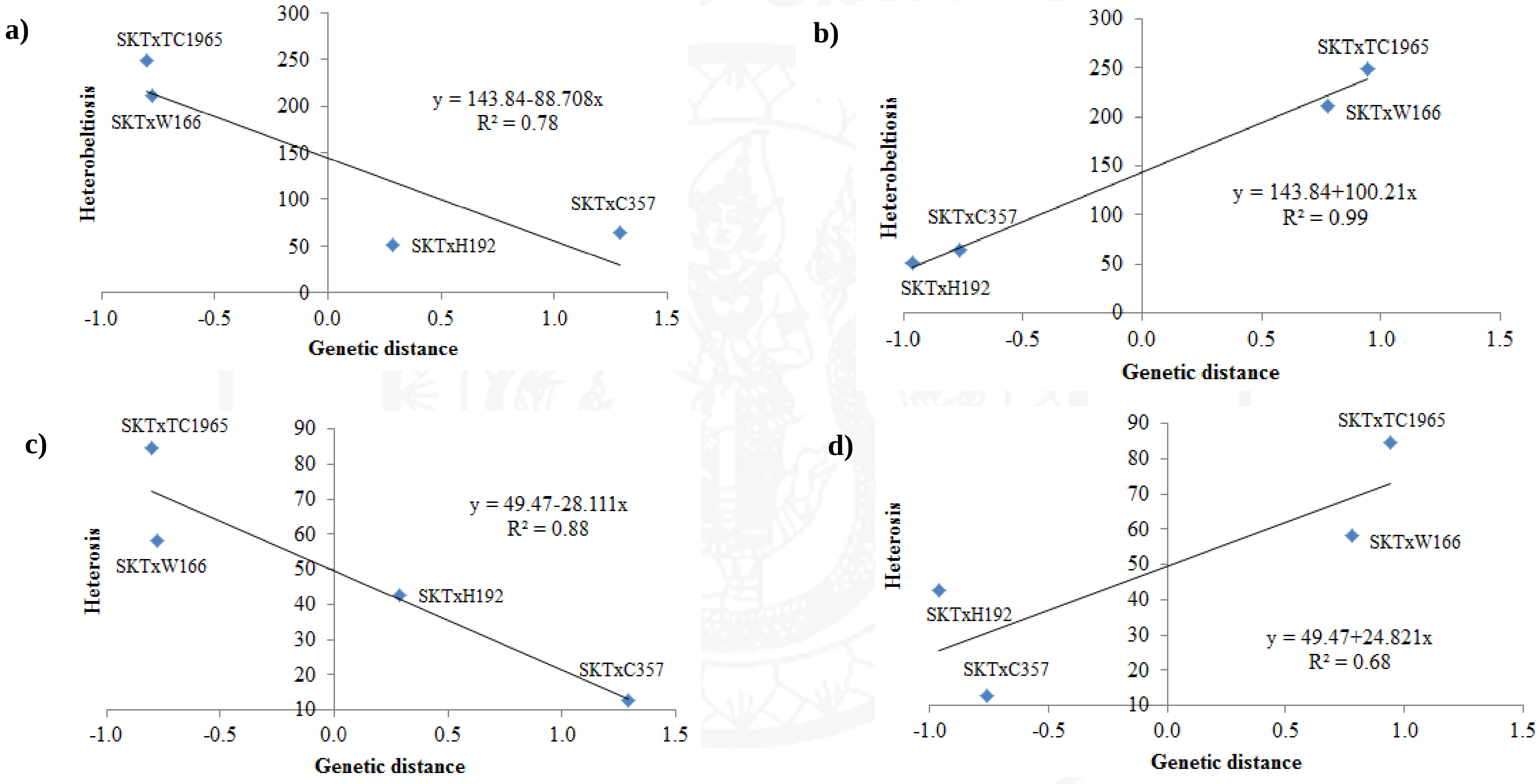

Figure 5 Relationship between genetic distance base on SSR (a,c) and phenotype (b,d) with the heterobeltiosis and heterosis in yield per plant of four F_1 hybrid mungbeans.

Table 5 Mean comparison and significant test on the percentages of heterosis (H) over mid-parent and heterobeltiosis (Hb) over high-parent in yield, yield components and agronomic traits of four F_1 mungbeans and their parental lines.

Entry	Days first flowering	No. branches per plant	No. clusters per plant	No. pods per cluster	Pod length (cm)	No. pods per plant	No. seeds per pod	100-seed weight (g)	Yield per plant (g)
SKT	38.4	4.7b	26.3	3.78b	14.9a	103.7b	15.3a	8.3a	91.7b
H192	36.2	5.2ab	30.2	9.86a	7.2c	302.4a	11.2c	3.6c	80.7b
SKT x H192 (F_1)	37.0	5.8a	30.6	9.38a	9.5b	283.8a	13.3b	5.1b	130.7a
H	-0.75ns	16.55**	8.19ns	32.54**	-13.89**	39.76**	0.30ns	-14.65**	51.65**
Hb	-3.54ns	11.11ns	1.18ns	-4.78	-36.01**	-6.16ns	-13.28**	-38.64**	42.56**
SKT	38.4	4.7a	26.3	3.78b	14.9a	103.7b	15.3a	8.3a	91.7a
C357	33.6	3.0b	27.8	3.80b	6.7c	105.4b	9.8c	4.2c	34.3b
SKT x C357 (F_1)	35.0	4.8a	28.7	7.23a	9.2b	205.1a	12.5b	5.7b	103.3a
H	-2.72ns	23.87**	6.01ns	90.77**	-14.68**	96.17**	-0.92ns	-9.36**	64.01**
Hb	-8.75*	1.35ns	9.06ns	90.26**	-38.21**	94.60**	-18.65**	-31.6**	12.69ns
SKT	38.4b	4.7c	26.3b	3.78a	14.9a	103.7b	15.3a	8.3a	91.7b
TC1965	53.6a	7.4a	129.9a	0.65c	4.4c	81.3b	9.4b	0.9c	5.4c
SKT x TC1965 (F_1)	35.9b	6.1b	136.0a	8.59a	6.0b	1148.2a	7.5c	2.8b	169.2a
H	-21.9**	0.47ns	74.2**	30.02**	-37.50**	1141.30**	-39.17**	-37.45**	248.69**
Hb	-32.99**	-17.88**	4.73ns	-23.81**	-59.52**	1007.08**	-50.95**	-65.27**	84.58**

Table 5 (Continued)

Entry	Days first flowering	No. branches per plant	No. clusters per plant	No. pods per cluster	Pod length (cm)	No. pods per plant	No. seeds per pod	100-seed weight (g)	Yield per plant (g)
SKT	38.4b	4.7b	26.3b	3.78b	14.9a	103.7b	15.3a	8.3a	91.7b
W166	54.2a	6.2a	117.0a	0.19c	3.7c	22.2c	6.5c	1.5c	1.5c
SKT x W166 (F1)	38.3b	5.8ab	117.7a	5.54a	7.0b	547.2a	10.6b	3.1b	144.9a
H	-17.21**	6.41ns	64.24**	179.08**	-24.16**	768.80**	-3.19ns	-37.69**	211.01**
Hb	-29.34**	6.67ns	0.57ns	46.56**	-52.72**	427.57**	-31.04**	-63.17**	58.06**

* Entry means of each trait in each cross followed by the same letters are not significantly different as determined by DMRT at $P \leq 0.05$. T-tests for heterosis (H) and heterobeltiosis (Hb) were significant at P≤ 0.05 (*), significant at P≤0.01 (**) and ns = non-significant.

Table 6 Correlation coefficients among heterosis (H), heterobeltiosis (Hb) and phenotypes of yield, yield components and agronomic characters among four F_1 mungbeans.

Characters	Estimators	No. branches per plant	No. clusters per plant	No. pods per cluster	Pod length (cm)	No. pods per plant	No. seeds per pod	100-seed weight (g)	Yield per plant (g)
Days first flowering	H	0.931	-0.992**	-0.893	0.944	-0.994**	0.764	0.958*	-0.999**
	Hb	0.941	-0.301	-0.458	0.987*	-0.903	0.919	0.950*	-0.804
	Phenotypes	0.575	0.361	-0.497	-0.219	0.011	0.023	-0.502	0.358
No. branches per plant	H		-0.956*	-0.806	0.914	-0.944	0.762	0.959*	-0.938
	Hb		-0.601	-0.730	0.957*	-0.962*	0.975*	0.820	-0.733
	Phenotypes		0.712	0.124	-0.650	0.719	-0.591	-0.802	0.929
No. clusters per plant	H			0.841	-0.918	0.982*	-0.716	-0.986*	0.996**
	Hb			0.944	-0.399	0.631	-0.622	-0.125	0.281
	Phenotypes			-0.367	-0.989*	0.885	-0.919	-0.985*	0.886
No. pods per cluster	H				-0.977*	0.927	-0.953*	-0.744	0.875
	Hb				-0.511	0.675	-0.683	-0.220	0.241
	Phenotypes				0.310	0.082	0.098	0.357	0.049
Pod length (cm)	H					-0.974*	0.934	0.852	-0.934
	Hb					-0.956*	0.964*	0.950*	-0.868
	Phenotypes					-0.922	0.964	0.950*	-0.867
No. pods per plant	H						-0.828	-0.940	0.991**
	Hb						-0.999**	-0.848	0.865
	Phenotypes						-0.974*	-0.845	0.922

Table 6 (Continued)

Characters	Estimators	No. branches per plant	No. clusters per plant	No. pods per cluster	Pod length (cm)	No. pods per plant	No. seeds per pod	100-seed weight (g)	Yield per plant (g)
No. seeds per pod	H							0.618	-0.745
	Hb							0.853	-0.848
	Phenotypes							0.853	-0.848
100-seed weight (g)	H								-0.968*
	Hb								-0.913
	Phenotypes								-0.914

*, ** represent a significant at the 0.05 and 0.01 probability levels, respectively (*df* = 2).

Discussion

1. Study on induction of novel characters enabling commercial hybrid seed production in mungbean

Our finding agreed well with those reported earlier that mutants obtained from gamma radiation depended on both gamma rates and mungbean genotypes (Gupta, 1996; Singh and Sharma, 1993; Santos, 1969). The cultivar V1197 is more sensitive to low rate of gamma ray than the other accessions. Changing form of flower in mungbean may affect pollination rate and increase outcrossing. An effective and economic pollination control system is necessary in hybrid seed production. Our discovery on chasmogamous mutant can help promoting outcrossing rate similar to hybrid rice seed production (Virmani, 1994). However, the mechanism of outcrossing requires pollinators such as insects or wind, and synchronization between male and female flowers (Mao *et al*., 1998). We observed that major pollinators of close mungbean flowers were bees. Herein, the chasmogamous mutant has the stigma and anther emerging out of the flower and encouraged outcrossing so that hybrid seed production in mungbean can be, for the first time, commercially feasible.

Virmani (1994) reported in rice, a self-pollinating crop with outcrossing rate between common cultivars generally less than 1%, that cultivars with open florets may have up to 6.8% outcrossing rate. In addition, male sterility can promote natural outcrossing up to 41.7%, depending on synchronization of flowering between male and female plants (Azzini and Rutger, 1982) as well as variation in flowering behavior, floral characteristics and pollen parents (Virmani and Athwal, 1973). Likewise, our mutant mungbean shows exerted stigma which has the potential to be utilized in a similar way to rice. Douglas and Freyre (2010) suggested that outcrossing in *Nolana* spp. was affected by the levels of stigma receptive to pollen over a wide range of floral developmental stages prior to anthesis. Thus, the open flower and exerting stigma in our chasmogamous mutant line are promising to use for promoting higher outcrossing in mungbean.

2. Comparison of hybrid vigor based on parental distance in SSR markers and agronomic traits in mungbean

Although hybrid varieties were developed in maize (Reif *et al*., 2003), wheat (Corbellini *et al*., 2002) and rice (Riday *et al*., 2003) base on genetic diversity, heterosis and molecular technique. A little research has been conducted in mungbean on heterosis, heterotic grouping and the criterion for selecting parents using molecular markers. Identification of good combiners from diverse germplasm is essential in heterosis breeding. This experiment showed that agronomic characters as well as SSR markers were efficient in classifying heterotic groups, and confirmed the previous study by Chen *et al*. (2003), Sawale *et al*. (2003) and Soehendi and Srinives (2005) that mungbean has high level of heterosis. In this study, although it would be difficult to choose a high yield hybrid with desirable traits from cultivar x wild crosses, heterosis in number of pods per plant was higher in the hybrids with the wild accessions than with the cultivated ones. In rice, Luo *et al*. (2011) reported that genetic diversity in wild rice showed a great potential for hybrid rice breeding. Thus in mungbean, this relationship gave the breeder a major question on how to compromise between the desirable traits during forming of hybrids from a given set of parental mungbeans.

In earlier studies, Soehendi and Srinives (2005) reported a mungbean hybrid with the maximum heterosis in seed yield up to 78.5% over the better parent, as compared to hybrid rice that possessed a yield advantage of 15 - 20% over the best pure line varieties (Virmani *et al*., 2003) and its commercial production has recently been successful in China (Yuan, 1997). In mungbean Chen *et al*. (2003) reported several mungbean lines that produced F_1 with significant heterosis (H) over mid-parent, and heterobeltiosis (Hb) over high parent in seed yield. Similarly, Sawale *et al*. (2003) reported a significant heterosis over mid- and better-parents in seed yield and yield components. The greatest heterosis in mungbean was obtained from the F_1 whose parental genotypes showing the highest level of divergence in phenotypic characters (Ramanujam *et al*., 1974). However, those reports were compared based on cultivar crosses and gave similar yield heterosis to the hybrids obtained from parents with close genetic distance in our study, i.e. ranging between 51.65-64.01%. Crosses of more distant parents show higher level of heterosis, ranging 211.01-248.69%. Moreover, our research found very high number of pods per plant in the F_1 of SKT and wild parents. They are vigorous with intermediate plant type. The number of pods per plant in SKTxTC1965 is as high as 1148.2 compared to their mid-parent value (92.5 pods), and the better parent (103.7 pods). This gives high level of heterosis and heterobeltiosis of 1141.3% and 1007.08%, respectively. The result reveals that the higher genetic distance between mungbean parents the higher degree of heterosis in the F_1 hybrids. However, the low genetic distance as the cultivars type as well as high adaptive inbred may reduce percentage of heterosis per se (Troyer, 2006).

In this study, clustering based on SSR markers (Figure 3) gave a similar relationship between hybrids and parental lines as clustering by phenotypes (Figure 4), although phenotypic clustering showed different relationship between parents and their F_1s. For example, morphological clustering grouped SKTxC357 and SKTxH192 together with H192, but rather far from C357 which was another non-recurrent parent. Similarly, morphological clustering of crosses having wild mungbeans as non-recurrent parents grouped the hybrids SKTxTC1965 and SKTxW166 together but far from their parents (Figure 4), since most traits of the hybrids fell between their parents. Some molecular foundation of phenotypic diversity may reside in the variability of gene and marker expression, which would be related to hybrid vigor. Teklewold and Becker (2006) reported that parents with low molecular distance also had low phenotypic distance, but parents with high molecular distance had either high, intermediate, or low phenotypic distance. The SSR clustering revealed a clear genetic relationship between the cultivars and their F_1s by assigning them into the same cluster, where SKTxTC1965 was near to TC1965 and SKTxW166 was near to W166 (Figure 3) which is reasonable in term of SSR grouping. Similarity, Mohammadi *et al*. (2008) revealed that SSR relationship between parental lines could be used to predict performance of maize hybrids. Later, Schrag *et al*. (2010) used AFLP and SSR markers for joint analyses of hybrids and parental inbreds to predict performance of untested maize hybrids. This marker-assisted parental selection consumed less time and money, and yet less influential by environmental factors as compared to conventional identification of combining ability based on morphological characters. However, both methods complimented each other in facilitating mungbean breeders because this crop has rather limited variation in both morphological and molecular markers (Karuppanapandian *et al*., 2006; Yimram *et al*., 2009).

Due to a limitation on number of markers and number of hybrid seeds obtained by hand-pollination in mungbean, Kumar *et al*. (2003) classified two major groups of mungbean by RAPD primers and forecasted hybrid vigor by crossing between those with high genetic distance. However, they could not prove this hypothesis. Our research is the first in mungbean to assess parental diversity based on phenotypes and SSR markers and found higher vigor from crosses between the parental lines with wider genetic distance. The SSR markers were able to group a large mungbean germplasm and identify the parental lines with promising heterotic patterns rather than evaluating the whole set of germplasm in more expensive field trials

CONCLUSION AND RECOMMENDATION

Conclusion

In the first experiment, new chasmogamous mutants were induced by gamma irradiation at the rate of 100 and 200 Gy. A study on inheritance of this character showed that all F_1 plants had normal flowers, while the F_2 plants segregated well with 3 normal: 1 chasmogamous ratio. When the F_1 was backcrossed to the chasmogamous parent, the progeny gave a ratio of 1 normal to 1 chasmogamous. Thus, chasmogamy was controlled by a single recessive gene, *cha*.

In the second experiment, four F_1 hybrids were formed from parental lines with different genetic distance. The higher genetic distance between the parents the higher degree of heterosis in the F_1 hybrids. Thus agronomic traits and molecular markers, expressed as phenotypic and genetic distances are useful to classify the parents into heterotic groups and patterns.

Recommendation

The chasmogamous mutant mungbean obtained from the first experiment shows exerted stigma, thus has the potential to be utilized in hybrid production in a similar way to rice. The open flower and exerting stigma characters are promising to use for promoting higher outcrossing in mungbean.

The second experiment revealed that higher hybrid vigor can be obtained from crosses between the parental lines with wider genetic distance. The SSR markers were able to group a large mungbean germplasm and identify the parental lines with promising heterotic patterns rather than evaluating the whole set of germplasm in more expensive field trials.

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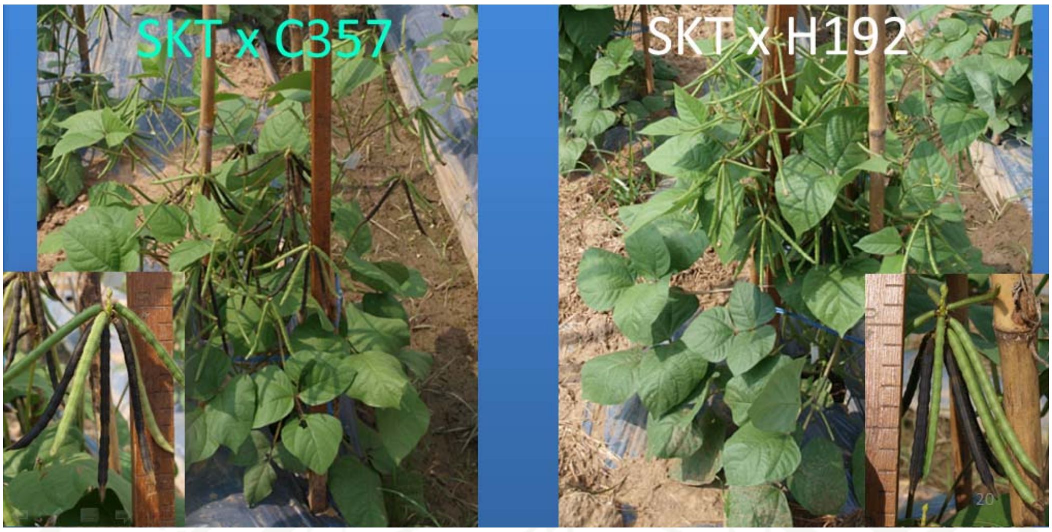
APPENDIX

Appendix Figure 1 The cultivated mungbean accessions (*V. radiata* var. *radiata*), Sukhothai (SKT), C357 and H192.

Appendix Figure 2 The wild accessions (*V. radiata* var. *sublobata*), TC1965 and W166.



Appendix Figure 3 The F_1 hybrids of cultivar group, SKTxC357 and SKTxH192.

Appendix Figure 4 The F_1 hybrids of wild accessions, SKTxTC1965 and SKTxW166.

Appendix Figure 5 Leaflet mutants found in V1197 treated by gamma ray at 200 Gy: (a) narrow rugose, (b) unshaped leaves.

Appendix Figure 6 (a) Triplet pod mutant from V1197, (b) evergreen seed and pod mutant from SKT. Both mutants were recovered from seeds treated with gamma ray at 200 Gy.

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