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Application of ISSR markers for verification of F_1 hybrids in mungbean (*Vigna radiata*)

P. Khajudparn[1,2], T. Prajongjai[1], O. Poolsawat[1] and P.A. Tantasawat[1,2]

[1]School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand
[2]Center of Excellence on Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok, Thailand

Corresponding author: P.A. Tantasawat
E-mail: piyada@sut.ac.th

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ABSTRACT. Mungbean improvement via hybridization requires the identification of true F_1 hybrids from controlled crosses before further generations of selfing/crossing and selection. We utilized inter-simple sequence repeat (ISSR) markers for identifying putative F_1 hybrids from six cross combinations whose morphological characteristics were very similar to those of their respective female parents and could not be visually discriminated from the self-pollinated progeny. Based on 10 ISSR primers, polymorphisms were found between female and male parents of all six cross combinations. The highest value of genetic differentiation (21.4%) was found between male and female parents of the SUT3 x M5-1 cross. These 10 ISSR primers gave 2.8-25.0% polymorphism between male and female parents, with a mean of 12.1%, and 0-13.0% polymorphism between F_1 hybrid and female parents, with a mean of 4.8%. F_1 hybrids of all six cross combinations could be differentiated from the self-pollinated progeny of their female parents by using only either ISSR 841 or 857 primers, together with the ISSR 835 primer. We conclude that ISSR markers are useful and efficient for identifying mungbean F_1 hybrids in controlled crosses

from different genetic background.

Key words: Inter-simple sequence repeat; Molecular marker; Hybrid identification; Polymorphism

INTRODUCTION

Mungbean (*Vigna radiata* [L.] Wilczek) is a member of the subgenus *Ceratotropis* and is considered one of the ancestral species of the genus *Vigna* (Ajibade et al., 2000). It is an important legume crop in Asia because of its short growth duration and adaptation to low soil fertility and drought. It can also be used in crop rotation practices to restore soil fertility by fixing atmospheric nitrogen (Somta and Srinives, 2007). Moreover, it is rich in vitamins and protein, and its starch contains 32-35% amylose (Yu et al., 2011). It is widely cultivated in many Asian countries, including Bangladesh, India, Japan, Kampuchea, Korea, Laos, Pakistan, Thailand, and Vietnam (Lakhanpaul et al., 2000). In Thailand, the Office of Agricultural Economics estimates that in 2011-2012, the cultivated mungbean area will be 137,280 ha yielding 99,000 tons of grain (Potiwan, 2011). This amount is insufficient for internal consumption, which highlights the need for new varieties with higher yields.

In self-pollinated crops such as mungbean, yield improvement has been achieved through selection, hybridization, mutation breeding, and biotechnological approaches (Bisht et al., 1998; Tomooka et al., 2005; Somta and Srinives, 2007; Ngampongsai et al., 2009). To increase genetic variation and produce novel genotypes, a controlled cross between 2 genetically distinct genotypes may be made to produce F_1 hybrids, which are then selfed/crossed to generate populations of progeny segregated for various traits of interest. In self-pollinated crops, the production of F_1 hybrid seeds in controlled crosses requires floral emasculation, which if improperly performed, can result in self-pollinated seeds. The F_1 hybrids are usually identified based on their morphological characteristics, a process that is successful if at least one of these characteristics is intermediate between the male and female parents or highly similar to the respective male parents. However, identification based on morphological characteristics can be difficult (especially at early stages), ambiguous, time-consuming, and dependent on environment (Asif et al., 2006; Lin et al., 2010). In addition, the parents in some crosses might have highly similar morphological characteristics or produce F_1 hybrids with morphological characteristics that cannot be visually distinguished from their respective female parents and hence progeny arising from self-pollination. In such cases, morphological characteristics alone are insufficient for the identification of F_1 hybrids, and analysis at the DNA level using molecular techniques is required. The application of molecular markers allows rapid identification of plant genotypes (hybrids, clones, somaclonal variants, and cultivars) with high efficiency and low labor cost (Reddy et al., 2002).

Several molecular markers have been used in mungbean, including amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and inter-simple sequence repeats (ISSR) (Chaitieng et al., 2002; Afzal et al., 2004; Tangphatsornruang et al., 2009; Tantasawat et al., 2010b; Raturi et al., 2012). Afzal et al. (2004) have used RAPD to evaluate the genetic diversity of 21 cultivated mungbeans from Thailand, Bangladesh,

and India. Recently, we found that ISSR markers were more efficient than morphological markers for variety identification and estimation of genetic relationships among 22 mungbean and blackgram (*Vigna mungo*) genotypes in Thailand (Tantasawat et al., 2010b). El-Hady et al. (2010) have shown that individual or combined RAPD and ISSR markers can be used effectively to determine genetic relationships among *Vigna* species. Similarly, both RAPD and ISSR markers have been used in genetic diversity analysis and genotype identification in plants such as banana, betel vine, castor, elephant grass, rice bean, shisham, and tea (Devarumath et al., 2002; Lakshmanan et al., 2007; Muthusamy et al., 2008; Arif et al., 2009; Gajera et al., 2010; de Lima et al., 2011; Patra et al., 2011). Many authors have found that ISSR markers are highly polymorphic and exhibit higher levels of efficiency, reproducibility, and accuracy than those of RAPD markers (Souframanien and Gopalakrishna, 2004; Ghalmi et al., 2010; Ali et al., 2011).

ISSR uses a single SSR-containing primer to amplify regions between adjacent, inversely oriented SSRs in a polymerase chain reaction (PCR) to generate multi-locus markers (Reddy et al., 2002). Because of its simplicity, efficiency, high reproducibility, and non-reliance on prior genomic sequence information, it has been widely used in applications including genetic diversity, genetic relationship, and germplasm analyses, seed purity evaluations, gene/quantitative trait loci mapping, marker-assisted selection, and evolutionary study in various plants including mungbean (Ajibade et al., 2000; Reddy et al., 2002; Wong et al., 2005; Somta and Srinives, 2007; Abbas et al., 2010; Tantasawat et al., 2010a,b; Vir et al., 2010; Li et al., 2011; Mudibu et al., 2011). Its application in the identification and confirmation of hybrids and parentage has also been reported in artichoke, bamboo, clover, mandarin, *Penstemon*, and the Triticeae tribe (Wolfe et al., 1998; Scarano et al., 2002; Carvalho et al., 2005; Dabkevičienė et al., 2008; Lin et al., 2010; Bianco et al., 2011). In this study, we used ISSR markers to verify 6 putative F_1 hybrids with morphological characteristics that could not be visually discriminated from the self-pollinated progeny of their female parents.

MATERIAL AND METHODS

Plant material

F_1 hybrids from 6 crosses (SUT3 x M5-1, SUT3 x V4758, CN72 x V1946, KPS1 x V1415AG, M4-2 x KPS2, and CN36 x V6009) that were morphologically undistinguishable from the self-pollinated progeny of female parents were grown on a farm at Suranaree University of Technology Farm, Nakhon Ratchasima, Thailand. Each hybrid and its male and female parents were planted in a single row of 30 plants per row with spacing of 50 cm between rows and 20 cm between hills.

Verification of F_1 hybrids using ISSR markers

Young flowers or leaves of parents and 3 randomly selected F_1 hybrids were collected from each cross for ISSR analysis. DNA extraction was performed according to the method of Tantasawat et al. (2010b). The concentration and purity of DNA were determined using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) at A_{260} and A_{280}. Ten ISSR primers homologous to microsatellite repeats and

containing other selective anchor nucleotides that were developed at the University of British Columbia were chosen for the analysis (Table 1). These primers were homologous to microsatellite repeats (AC, AG, CA, GA, or TG) anchored at the 3'-end by 1 or 2 nucleotides. Eight of these primers have been used successfully to identify varieties and assess genetic relationships of mungbean and blackgram in Thailand (Tantasawat et al., 2010b). Each 20-µL PCR contained 50 ng genomic DNA template, 1X buffer [75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$], 250 µM of each deoxyribonucleotide triphosphate, 3.5 mM $MgCl_2$, 0.4 µM of each ISSR primer (807, 808, 825, 835, 836, 841, 847, 856, 857, and 858), and 1 U Biotools DNA Polymerase (Biotools B and M Labs, S.A., Madrid, Spain). The PCRs were subjected to amplification with an initial denaturation at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 4 min, and a final extension at 72°C for 10 min in a ThermoHybaid Px2 thermocycler (Thermo Fisher Scientific, Inc., Waltham, MA, USA). PCR products were electrophoresed on 6% denaturing polyacrylamide gel at 250 V for 65 min. The gel was stained with silver nitrate following the method of Vari and Bell (1996). Molecular weights of the DNA bands were estimated by using 1 kb Plus DNA Ladder (Gibco-BRL, Gaithersburg, MD, USA) as a standard.

The patterns of DNA bands resulting from the amplification using all 10 primers were compared between male and female parents and between F_1 hybrids and their respective female parents in each cross, and the similarities and differences were identified. The experiment was repeated at least twice, and only the DNA bands that gave consistent results were recorded. The total number of scorable DNA bands for both male and female parents in each cross was recorded. Two indices were used to estimate the efficiency of each marker in detecting DNA polymorphisms. The percentage of polymorphism between male and female parents of each hybrid, which was used to estimate the ability of each marker to differentiate between male and female parents, was calculated as follows: (number of polymorphic DNA bands between male and female parents / total number of scorable DNA bands) x 100. Similarly, the percentage of polymorphism between the F_1 hybrid and the female parent, which reflected the efficiency of each marker to distinguish the F_1 hybrid from self-pollinated progeny, was calculated as follows: (number of polymorphic DNA bands between the F_1 hybrid and the female parent [number of male parent-specific DNA bands] / total number of scorable DNA bands) x 100.

RESULTS

Mungbean F_1 hybrids from 6 crosses that displayed morphological characteristics similar to those of their respective female parents were verified using ISSR markers. In total, 1038 ISSR fragments were clearly amplified from all hybrids using 10 ISSR primers. Among these amplified fragments, 131 DNA bands (12.6%) were found to be polymorphic between male and female parents. The total number of polymorphic DNA bands between F_1 hybrids and their respective female parents, which can be used to distinguish the true F_1 hybrids from the self-pollinated progeny of female parents, was 49 (4.7%).

Total number of scorable DNA bands amplified by each primer ranged from 9 (ISSR 856) to 24 (ISSR 825, 841, and 858) with an average of 17.3 bands/primer. The size of amplified products was in the range of 220-2400 bp (Tables 1 and 2). Nine of the primers could

effectively differentiate between male and female parents in all 6 crosses, and could distinguish true F_1 hybrids from the self-pollinated progeny of female parents; however, ISSR 847 yielded similar DNA patterns in F_1 hybrids and female parents. Either ISSR 841 or 857 could distinguish most of the F_1 hybrids from the self-pollinated progeny of female parents, except in the KPS1 x V1415AG cross. However, the F_1 hybrids from this cross could be identified using ISSR 835 (see Table 2). Therefore, only 2 ISSR primers (ISSR 841 or 857 combined with ISSR 835) were sufficient for the identification of true F_1 hybrids from these 6 cross combinations. In each cross combination, at least one in 3 putative F_1 hybrid plants was verified as a true F_1 hybrid for future use in mungbean breeding programs. These true F_1 hybrids displayed polymorphic DNA bands specific for both male and female parents and could be distinguished from the self-pollinated progeny of female parents by the presence of male-parent-specific ISSR bands. The number of ISSR bands useful for confirming the hybridity of these 6 mungbean crosses ranged from zero (KPS1 x V1415AG) to 3 (M4-2 x KPS2) using ISSR 841 primer, from zero (KPS1 x V1415AG) to 4 (SUT3 x M5-1) using ISSR 857 primer, and from zero (SUT3 x M5-1, SUT3 x V4758, CN72 x V1946, M4-2 x KPS2, CN36 x V6009) to one (KPS1 x V1415AG) using ISSR 835 primer (see Table 2).

The highest percentage of polymorphism between male and female parents (25.0%) was achieved using ISSR 857 primer; next highest were ISSR 841 (21.4%) and ISSR 807 (17.7%). Similarly, these 3 primers displayed the highest percentages of polymorphism between the F_1 hybrid and female parents: ISSR 807 (13.0%), ISSR 857 (12.5%), and ISSR 841 (6.0%; see Table 1).

The total number of scorable DNA bands amplified by the 10 ISSR primers in each cross combination varied from 167 (SUT3 x V4758) to 181 (CN72 x V1946), with an average of 173 bands (Table 3). The percentage of polymorphism between male and female parents could reveal the genetic difference between male and female parents in each cross combination. When considering all 10 primers, the highest percentage of polymorphism between male and female parents (21.4%) was found in the SUT3 x M5-1 cross; the next highest were the SUT3 x V4758 (14.7%) and CN72 x V1946 (12.9%) crosses (see Table 3). These results will be useful for parental selection in future mungbean breeding programs.

Table 1. Primer sequences, DNA size, number of scorable DNA bands, percentages of polymorphism between male and female parents, and percentages of polymorphism between F_1 hybrid and female parent for each ISSR primer in all 6 crosses of mungbean.

Primers	Primer sequences	DNA size (bp)	No. of scorable DNA bands	% Polymorphism (male-female parents)	% Polymorphism (F_1 hybrid-female parent)
807	$(AG)_8T$	250-2200	12-15	17.7	13.0
808	$(AG)_8C$	270-2100	14-17	14.9	4.3
825	$(AC)_8T$	300-2300	13-24	9.8	2.2
835	$(AG)_8Y$[1/]C	280-2300	20-21	4.8	0.8
836	$(AG)_8YA$	220-2100	17-23	13.7	3.8
841	$(GA)_8YC$	330-2000	18-24	21.4	6.0
847	$(CA)_8R$[2/]C	380-2400	12-20	2.9	0.0
856	$(AC)_8YA$	360-2200	9-16	2.8	1.4
857	$(AC)_8YG$	300-2000	12-17	25.0	12.5
858	$(TG)_8RT$	270-2100	17-24	8.2	3.7
Average			17.3	12.1	4.8

[1/]Y = pyrimidines (C, T). [2/]R = purines (A, G).

Table 2. Number of scorable DNA bands, number of polymorphic bands between male and female parents, number of polymorphic bands between F_1 hybrid and female parent, percentages of polymorphism between male and female parents, and percentages of polymorphism between F_1 hybrid and female parent for each ISSR primer in each cross of mungbean.

Crosses/primers	No. of scorable DNA bands	No. of polymorphic bands (male-female parents)	% Polymorphism (male-female parents)	No. of polymorphic bands (F_1 hybrid-female parent)	% Polymorphism (F_1 hybrid-female parent)
SUT3 x M5-1					
807	14	4	28.6	2	14.3
808	15	6	40.0	2	13.3
825	13	1	7.7	0	0.0
835	20	0	0.0	0	0.0
836	18	2	11.1	1	5.6
841	21	6	28.6	2	9.5
847	17	3	17.6	0	0.0
856	9	0	0.0	0	0.0
857	17	8	47.1	4	23.5
858	24	8	33.3	4	16.7
SUT3 x V4758					
807	13	3	23.1	3	23.1
808	15	3	20.0	1	6.7
825	13	2	15.4	0	0.0
835	20	1	5.0	0	0.0
836	23	9	39.1	4	17.4
841	23	5	21.7	1	4.3
847	12	0	0.0	0	0.0
856	12	2	16.7	1	8.3
857	16	1	6.3	1	6.3
858	20	0	0.0	0	0.0
CN72 x V1946					
807	14	3	21.4	1	7.1
808	17	3	17.6	0	0.0
825	22	1	4.5	1	4.5
835	21	0	0.0	0	0.0
836	21	1	4.8	0	0.0
841	24	9	37.5	1	4.2
847	15	0	0.0	0	0.0
856	16	0	0.0	0	0.0
857	14	6	42.9	2	14.3
858	17	0	0.0	0	0.0

Continued on next page

Table 2. Continued.

Crosses/primers	No. of scorable DNA bands	No. of polymorphic bands (male-female parents)	% Polymorphism (male-female parents)	No. of polymorphic bands (F_1 hybrid-female parent)	% Polymorphism (F_1 hybrid-female parent)
KPS1 x V1415AG					
807	12	0	0.0	0	0.0
808	14	0	0.0	0	0.0
825	19	0	0.0	0	0.0
835	21	3	14.3	1	4.8
836	17	0	0.0	0	0.0
841	18	0	0.0	0	0.0
847	20	0	0.0	0	0.0
856	16	0	0.0	0	0.0
857	12	1	8.3	0	0.0
858	19	0	0.0	0	0.0
M4-2 x KPS2					
807	12	0	0.0	0	0.0
808	14	0	0.0	0	0.0
825	21	3	14.3	1	4.8
835	21	2	9.5	0	0.0
836	22	6	27.3	0	0.0
841	22	6	27.3	3	13.6
847	19	0	0.0	0	0.0
856	16	0	0.0	0	0.0
857	12	2	16.7	2	16.7
858	19	0	0.0	0	0.0
CN36 x V6009					
807	15	5	33.3	5	33.3
808	17	2	11.8	1	5.9
825	24	4	16.7	1	4.2
835	21	0	0.0	0	0.0
836	17	0	0.0	0	0.0
841	23	3	13.0	1	4.3
847	15	0	0.0	0	0.0
856	11	0	0.0	0	0.0
857	14	4	28.6	2	14.3
858	19	3	15.8	1	5.3

Table 3. Number of scorable DNA bands, percentages of polymorphism between male and female parents, and percentages of polymorphism between F_1 hybrid and female parent for all 10 ISSR primers in each cross of mungbean.

Crosses	No. of scorable DNA bands	% Polymorphism (male-female parents)	% Polymorphism (F_1 hybrid-female parent)
SUT3 x M5-1	168	21.4	8.3
SUT3 x V4758	167	14.7	6.6
CN72 x V1946	181	12.9	3.0
KPS1 x V1415AG	168	2.3	0.5
M4-2 x KPS2	178	9.5	3.5
CN36 x V6009	176	11.9	6.7
Average	173	12.1	4.8

DISCUSSION

Our results confirm that ISSR markers are efficient tools for the discrimination of F_1 hybrids from the self-pollinated progeny of female parents in controlled crosses. They can also be effectively used to fingerprint and differentiate plants with highly similar morphological characteristics. The hybridity status of F_1 hybrids can be easily verified by comparing amplified polymorphic bands between F_1 hybrids and female parents (bands specific to the male parents). ISSR 807, 841, and 857 primers produced the highest percentages of polymorphism between male and female parents and between the F_1 hybrid and female parents in all 6 crosses analyzed. These primers are dinucleotide AG, GA, and AC repeats, respectively. Two of them (ISSR 841 and 857) have been previously reported as having high polymorphism information content (0.34 and 0.36, respectively). These primers used together with 4 additional ISSR primers have fully distinguished 22 mungbean and blackgram genotypes (Tantasawat et al., 2010b). ISSR primers with GA repeats have also been shown to have high polymorphism among *Vigna* genotypes (Ajibade et al., 2000).

In addition, ISSR markers allow the easy, fast, inexpensive, accurate, reliable, and simultaneous detection of polymorphisms at multiple loci in the genome using low quantities of DNA. These properties have made the markers useful for the genetic analysis of various plants (Reddy et al., 2002). Our results agreed with those of Ruas et al. (2003), who reported the use of 14 ISSR primers to evaluate the genetic similarity of 8 species of coffee and to identify the parentage of 6 interspecific hybrids. Similarly, Carvalho et al. (2005) have used 30 ISSR primers to confirm the interspecific hybrids of 3 crosses of the Triticeae tribe and found that 13-20 ISSR primers gave polymorphic DNA bands between male and female parents in each cross and could verify their interspecific hybrids. Eight ISSR primers have also been used successfully in the identification of interspecific bamboo hybrids (Lin et al., 2010). Moreover, the use of ISSR to distinguish hybrids has been reported in artichoke, clover, and mandarin (Scarano et al., 2002; Dabkevičienė et al., 2008; Bianco et al., 2011). ISSR markers are also useful for the estimation of seed purity and prediction of F_1 hybrid characteristics in several plants, including artichoke (Bianco et al., 2011).

In conclusion, only 2 ISSR primers (ISSR 841 or 857 combined with ISSR 835) could generate sufficient data to ascertain the hybridity of F_1 hybrids from 6 mungbean cross combinations. To our knowledge, this research is the first to use ISSR markers for hybrid verification in mungbean. Rapid and early verification of hybridity and identification of parentage of hybrids will be useful for mungbean improvement.

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