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Application of SSR Markers for Purity Testing of Commercial Hybrid Soybean (*Glycine max* L.)

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ABSTRACT

Exploitation of the full potential of any hybrid requires the possessing of genetically high-purity seeds. Commercial soybean hybrids have been developed using a cytoplasmic male sterility (CMS) system. In order to avoid reduction in yield caused by using low-purity seeds, development of a simple, rapid, and accurate method for hybrid purity assessment is of great essence and significance. Therefore, the parental lines of HybSoy 1 to 5 were screened using 160 Simple Sequence Repeat (SSR) makers, of which 8 markers exhibited polymorphism. A PCR-based assay with these markers detected both alleles of the parental lines in pure hybrids, proving their heterozygosity, whereas impurities were identified by the presence of only one parental allele. The confirmation of hybrid purity indicated that a single polymorphic marker was sufficient for detection of contaminations of these hybrids from their parents. It was also found that if a hybrid seed lot was contaminated by another hybrid or its parental lines, two or more appropriate markers could be used to easily detect such contamination. This method could accurately and effectively identify the hybrid purity in a predetermined sample of soybean hybrids constituted by deliberately mixing seeds of parental lines. This is the first report that demonstrates the utility of SSR markers for assessment of genetic purity of soybean hybrids.

Keywords: Cytoplasmic male sterility, Genetic purity, Molecular marker, Contamination, Polymorphism.

INTRODUCTION

Soybean is one of the most important oil crops in the world. However, the yield is less than one third of corn. Exploitation of heterosis can significantly improve the yield of soybean (Palmer *et al*., 2010). A soybean CMS system had been developed and a method for producing soybean hybrid was invented by our team (Sun *et al*., 1994 and 2001). Then, the world's first commercial soybean hybrid, HybSoy 1, was bred and released in China. The yield of HybSoy 1 was 20.9% higher than that of the CK (Jilin 30) (Zhao *et al*., 2004). Subsequently, seven soybean hybrids, designated as HybSoy 2, HybSoy 3, HybSoy 4, HybSoy 5, Zayoudou 1, Zayoudou 2 and Fuzajiaodou 1, respectively, have been developed and released for commercial production in China. Good promotion and application of any hybrid require high-quality seeds. Exploitation of the full potential of the high purity hybrid can improve farmers' enthusiasm for growing soybean hybrids, which, in return, increases the overall yield. In order to avoid reduction in yields caused by using low-purity soybean hybrid seeds, a simple, reliable and efficient method is developed for assessment of the genetic purity of hybrids.

Currently, a morphology-based identification method is used to assess the genetic purity of

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soybean hybrids (unpublished). The method relies on seeds or plants possessing different phenotypic traits to identify the individual, which is intuitive and simple. However, direct observation of agronomic traits is often difficult. For instance, some reproductive traits must be observed within a specific period in order to detect phenotypic differences, and some traits are environmentally labile, which make it particularly difficult to differentiate individuals that have a close genetic relationship. Color traits are only useful for identification of soybean hybrid purity when the parents possess a different color phenotype. If the parents possess the same stem, flower, and pubescence color phenotype, the purity of the hybrids cannot be identified on the basis of those traits. In addition, the morphological identification of rogue genotypes from differences in seed traits and growth form of individual plants is applied to avoid wastage of land, labor resources, and environmental resources (e.g., soil nutrients and water) by cultivating less-productive genotypes. In recent years, Marker Assisted selection (MAS) not only have been used for the analysis of genetic diversity (Naghavi *et al*., 2009), but also used extensively to test hybrid purity to overcome the shortcomings of morphology-based methods (Varshney *et al*., 2009). A variety of marker types, such as Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Sequence-related Amplified Polymorphism (SRAP), Inter-simple Sequence Repeat (ISSR), EST-SSR and SSR markers, have been used in hybrid purity test (Grzebelus *et al*., 2001; Ilbi 2003; Jang *et al*., 2004; Dongre and Parkhi 2005; Liu *et al*., 2007a; Asif *et al*., 2009; Naresh *et al*., 2009; Dongre *et al*., 2011). Given that SSRs are locus-specific and co-dominant markers, they have been used widely to assess hybrid purity in maize (Daniel *et al*., 2012), rice (Sundaram *et al*., 2008), sunflower (Iqbal *et al*., 2010), cabbage (Liu *et al*., 2007b), bunching onion (Tsukazaki *et al*., 2006), cauliflower (Zhao, *et al*., 2012) and other crops. However, the utilization of SSR markers for testing of seed genetic purity of soybean hybrids has not been reported previously.

In the present study, we tested the utilization of SSR markers for detecting the genetic purity of five commercial soybean hybrid varieties released in China. The procedure of one or two SSR markers for assessment of genetic purity of the hybrids is demonstrated.

MATERIALS AND METHODS

Plant Material

Five commercial soybean hybrids together with their parental lines were used in the study (Table 1). For each hybrid, 10 seedlings of each parental line and 60 seedlings of hybrid were grown in pots under artificial light (12 h photoperiod at 26 °C) and irrigated with water. Two-week-old plants were collected, immediately immersed in liquid N_2, and stored at −70 °C until extraction of genomic DNA for assessments of parental polymorphism and hybrid purity.

SSR Marker Polymorphism

A total of 160 SSR markers (based on data in the Soybase; http://www.soybase.org) were used for a survey of polymorphism in bulks of DNA extracted from the 10 seedlings of each female and male parent of five hybrids, respectively. Polymorphism between each parental line and hybrid was detected for a total of eight SSR markers. The polymorphism of these markers was further confirmed using DNA from individuals of the parental lines. For hybrid purity assessments, the identified markers were amplified from DNA extractions of the five hybrids and their parental lines. The PCR procedure was performed with 20 ng of template genomic DNA in 0.2 ml PCR tubes, 10 pmol of each primer, 10 mM each dNTP, 1 U of Taq polymerase, 10× Taq polymerase buffer, and double-distilled H_2O in a total

Table 1. Descriptions of the plant materials used in the study.

Hybrids	Cytoplasmic male sterile lines	Restorer lines
HybSoy 1	JLCMS9A	JLR1
HybSoy 2	JLCMS47A	JLR2
HybSoy 3	JLCMS8A	JLR9
HybSoy 4	JLCMS47A	JLR83
HybSoy 5	JLCMS84A	JLR1

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volume of 25 µL with the following conditions: denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 8 min. All PCR products were separated by electrophoresis in a 6% denaturing acrylamide gel, and stained with silver nitrate solution.

RESULTS AND DISCUSSION

SSR Polymorphism in the Parental Lines

In a previous study, the restorer gene (*Rf*), which gametophytically restores soybean cytoplasm, was located on linkage group J (LG J) (Wang *et al*., 2010). It can be used for identifying SSR markers linked to *Rf* gene of soybean to distinguish hybrid soybean and parental lines. On this basis, a total of 160 SSR markers from LG J were selected and used for the survey of polymorphism among the parental lines of the five commercial soybean hybrids, using bulks of DNA extracted from the 10 seedlings of each female and male parent of five hybrids, respectively. Eight of these markers (Table 2) were polymorphic for the parental lines and they were screened. Among these markers, Satt547, Sctt_011 and BARC16_1102 showed polymorphism between the parental lines of three hybrids, BARC16_1124 showed polymorphism between the parents of two hybrids, and Satt529, BARC16_1095, BARC16_1097 and BARC16_1123 showed polymorphism between the parents of only one hybrid (Figure 1). Interestingly, BARC16_1102 and BARC16_1124 markers showed polymorphism between the parents of HybSoy 1 and HybSoy 3; Satt547 and Sctt_011 markers showed polymorphism between the parents of both HybSoy 2 and HybSoy 4, and Sctt_011 and BARC16_1124 markers showed polymorphism between the parents of both HybSoy 1 and HybSoy 4. The polymorphism patterns of the eight markers were confirmed using DNA extracted from 10 individual plants of the parental lines. These polymorphic SSRs were then assessed using DNA bulks of both hybrids and parents. For all these markers, a single allele was detected in the parents, whereas both parental alleles were detected in the hybrids, thus, indicating the heterozygosity of the hybrids (Figure 1). The markers were further tested with DNA extracted from individual hybrid plants to assess their utility for assessment of hybrid purity. For all individual hybrid plants, both alleles specific to the parents were detected, which confirmed the utility of these markers for genetic purity assessment of the hybrids (Figure 2).

Genetic Purity Testing of Soybean Hybrids

Soybean hybrids can be produced by the use of hand crosses, gametocytes, and male-sterility. Because of being labor-intensive and time consuming, the hand crosses are used only for research purpose. Gametocytes have been used for producing hybrid seeds successfully in other crops such as wheat; however, there have been no adequate and effective gametocytes available to kill pollen grains completely in soybean. Currently, several nuclear male-sterile mutants have been found in soybean. Owing to the difficulty in removing 50% of the fertile plants in female parent, they cannot be used in hybrid seeds production in large scale. CMS male-sterile system is the only choice to avoid self-pollination in female parent in hybrid soybean production. The

Table 2. Descriptions of the polymorphic SSR markers used in the study.

Marker	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)
Satt529	GCGCATTAAGGCATAAAAAAGGATA	GCACAATGACAATCACATACA
Satt547	GCGCTATCCGATCCATATGTG	TGATTTCGCTAGGTAAAATCA
Sctt011	CTCCGTTGCTGAT	TAAGCTGAATTAGTAAAA
BARC16_1095	CACCAATTTAAAGTTTGATGGTC	TCCCACCACTTATTGGTTTCA
BARC16_1097	GTTGCAATTGTCAGCATCGT	GAGTCGGTTAACAACGGAAA
BARC16_1102	TTGCTTGTGACTCGAGAAAA	CGTGATTGCATATGACTGTTCA
BARC16_1123	AGAAAAGAAATATATTCCCCAGATAA	ACAATGTTAAAGTCATCCAATTATACA
BARC16_1124	TGGAGTTATGGTAACAGTGATGCT	CATGTTTGTACTTGGTGCATGA

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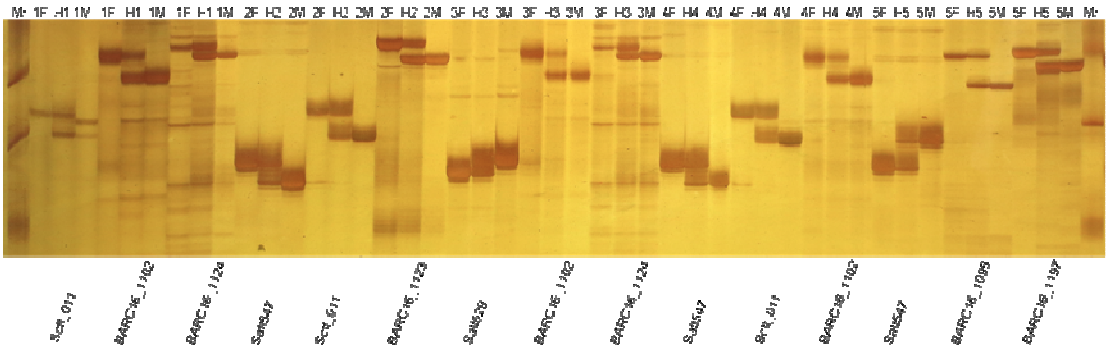

Figure 1. SSR markers polymorphic between an individual hybrid and its parental lines for five hybrids. PCR amplifications were performed with SSR markers Sctt_011, BARC16_1102 and BARC16_1124 for HybSoy 1 (H1), female parent (1F) and male parent (1M); SSR markers Satt547, Sctt_011 and BARC16_1123 for HybSoy 2 (H2), female parent (2F) and male parent (2M); SSR markers Satt529, BARC16_1102 and BARC16_1124 for HybSoy 3 (H3), female parent (3F) and male parent (3M); SSR markers Satt547, Sctt_011 and BARC16_1102 for HybSoy 4 (H4), female parent (4F) and male parent (4M); SSR markers Satt547, BARC16_1095 and BARC16_1097 for HybSoy 5 (H5), female parent (5F) and male parent (5M). Marker: 100 bp DNA ladder.

major steps for hybrid seeds production include: (a) F_1 seeds production by planting female parent CMS A-line and male parent R-line alternatively in isolation plots, harvesting F1 seeds from female parent for farmer planting; (b) Seed increase of CMS A-line by planting female parent CMS-A line and B-line alternatively in isolation plots, harvesting male-sterile seeds from female parent for F_1 seeds production the next year (insect pollinators must be used in step a and b); and (c) Seed increase of R-line in isolation plots if needed, harvesting seeds for F_1 seeds production the next year. The seeds contamination mainly come from step a and b. One possible way is the mechanical mixture of male and female parents during planting, harvesting, and seed processing. Another possible way is the biological contamination. In case the A-line mixed with B-line in the A-line seed increase field, some female parent may be pollinated by B-line instead of R-line in F_1 seed production field and certain amount of male-sterile plants will appear in famer's production field. This is the number one problem and the serious situation we are facing now as far as seed purity is concerned.

SSR markers have been used for purity testing of many crop hybrids, and national and local standards for hybrid purity testing methods using SSR markers are being established. Some seed companies also appreciate the advantages of the method, and actively develop SSR polymorphic primers to control the seed genetic purity at the parental line and hybrid seed production stage. Over the past 10 years, major advances in soybean hybrid research have been achieved. Currently, we have released five commercial soybean hybrids, which have promoted hybrid soybean cultivation in the major soybean-producing areas of northeastern China. In order to ensure the hybrid purity of seeds available to farmers, and to demonstrate the utility of the eight polymorphic SSR markers for hybrid purity testing, a predetermined mixture of the hybrids HybSoy 1 to 5 were created by mixing 3 or 5 seeds of each parental line to make a sample of 50 seeds, approximately. The markers BARC16_1102, BARC16_1123, BARC16_1124, Sctt_011 and BARC16_1097, which are polymorphic between the hybrids' respective parents, were used to amplify the genomic DNA of HybSoy 1 to 5 seedlings by PCR. After separation of the amplified fragments by SDS-PAGE electrophoresis, impurities among these seedlings were clearly identified on the basis of fragment sizes (Figure 2). The impurities were identified on the basis of DNA marker-based genotyping, which corresponded with the proportion of parental-line seeds in the mixture. This result

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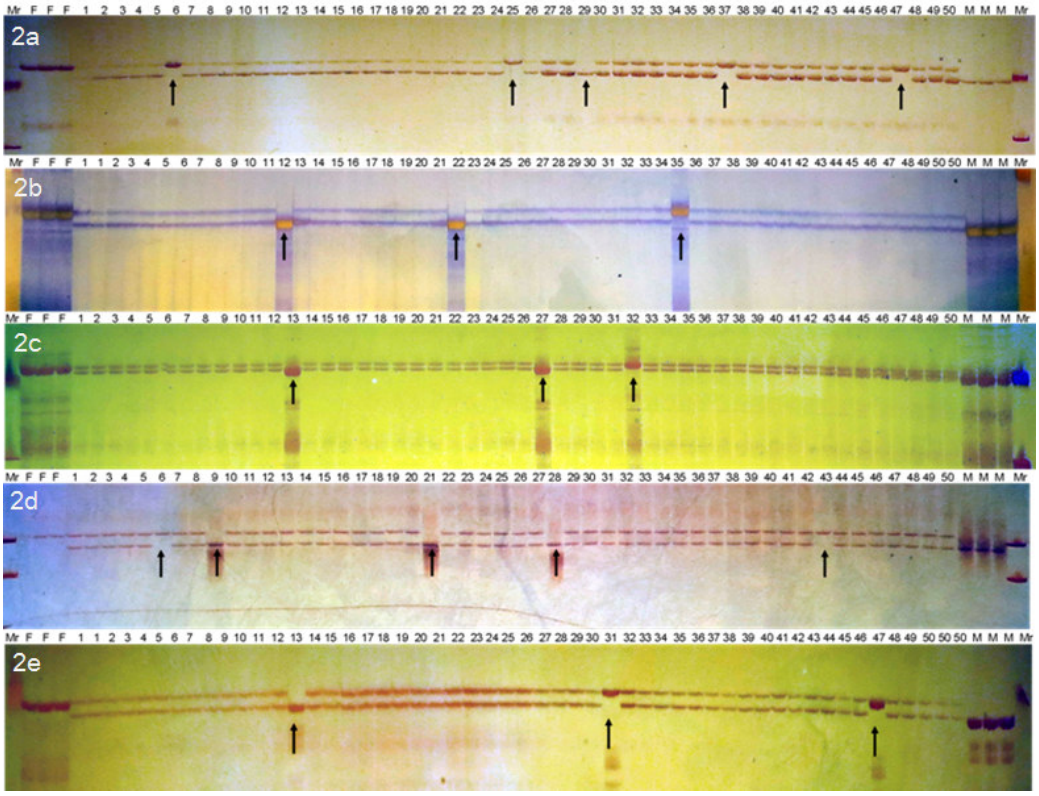

Figure 2. Single seedling assay for detection of purity of the five soybean hybrid. (2a) SSR marker BARC16_1102 for detected the contamination of 50 seeds, include 4 female parents and 1 male parent; (2b) SSR marker BARC16_1123 for detected the contamination of 50 seeds, include 1 female parent and 2 male parents; (2c) SSR marker BARC16_1124 for detected the contamination of 50 seeds, include 1 female parent and 2 male parents; (2d) SSR marker Sctt_011 for detected the contamination of 50 seeds, include 2 female parents and 3 male parents; (2e) SSR marker BARC16_1097 for detected the contamination of 50 seeds, include 2 female parents and 1 male parent. Mr indicates a 100 bp DNA ladder. F: Female parent; M: Male parent; Lanes 1–50, contamination of 50 seeds. The asterisk indicates impurities.

demonstrated the reliability of a marker-based method for soybean hybrid purity analysis. Identification of genetic impurity owing to hybridization with two or more genotypes can also be detected by SSR markers. For example, the SSR marker Satt529 only detected polymorphism in HybSoy 2 and its parents, thus, it can be used to detect whether a mixture contains other hybrids or parents other than those of HybSoy 2. Therefore, with the appropriate markers, such contamination can be easily detected. In the present study, the markers Satt547, Sctt_011, BARC16_1102, and BARC16_1124 amplified a similar band in multiple hybrids, which may be the reason that some hybrids derived from the same parent had similar genetic backgrounds. Under these conditions, such markers cannot be used to distinguish hybrids. However, by screening a large number of SSR markers, it is possible to identify one marker that can distinguish more than two hybrids at the same time. Because the current hybrid seeds production of soybean are produced in arid areas where commercial soybean production is seldom practiced. The contamination mainly comes from only parents of hybrid within one isolation area or other isolation area nearby. We know exactly the parents of every hybrid. Therefore, the method of this study can be

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used to identify the commercial hybrids, rather than developing more SSR makers.

SSR markers exhibit a high degree of polymorphism, co-dominant inheritance, are reproducible and provide accurate results, and thus are far preferable to RFLP, RAPD, and AFLP molecular markers. SSR marker technology requires only small volumes of DNA samples and does not have stringent DNA purity requirements, which allows shortening of the detection time. Although it is expensive and requires experience to screen SSR primers, many primer sequences for SSR loci are available in public databases for a variety of crops, including soybean. In addition, SSR markers for seed purity identification have potential for broad application. However, this method can only test the mixture of the studied hybrids with each other or with their parental lines. If the sample is contaminated with another unknown genotype carrying the same alleles this method would not be able to detect. Thus, more than one PCR is needed for detection of contaminations from other unknown genotypes, and the more the number of markers, the better will be the judgment about purity.

CONCLUSIONS

The present study is the first report of the application of SSR markers for detection of genetic purity in commercial soybean hybrids. The SSR markers were able to detect identifiable non-soybean hybrids, such as paternal lines and mechanical mixtures of genotypes, and by using one or more of the SSR markers, distinguishing among different parental or hybrid genotypes in genetically impure seed mixtures was possible.

The developments of SSR makers specific for each parent will simplify the procedure of purity test and the method is rapid and accurate. It will be beneficial for seed companies for implementation during processing, packaging, or dispatch of shipments since assessment using SSR markers is achieved much earlier than at a post-germination stage. There is no lag between seed production and sale of the seeds, which is a problem with field-based purity assessment methods. The method will not only help to maintain quality standards by seed production companies, but also increase farmers' enthusiasm for planting hybrids. Ultimately, it will play a positive role in promoting the popularization and utilization of soybean hybrids.

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کاربرد نشانگرهای اس اس آر برای آزمون خلوص هیبرید های تجاری سوژا (*Glycine max L.*)

س. ب. ژانگ، ب. پنگ، و. ل. ژانگ، س. م. وانگ، ه. سان، ي. س. دانگ، و ل. م. ژاوو

چکیده

لازمه بهره مندی کامل از پتانسیل هر هیبریدی داشتن بذرهایی است که خلوص ژنتیکی بالایی داشته باشند. هیبرید های تجاری سوژا با استفاده از نر عقیمی سیتو پلاسمی (CMS) ایجاد شده اند. به منظور جلوگیری از کاهش عملکرد به علت خلوص کم بذرها، ابداع روشی ساده، سریع و صحیح برای ارزیابی خلوص بذر از اهمیت بسیاربرخوردار است.بنا بر این،رگه های والدی HybSoy 1 **تا** HybSoy 5با استفاده از ۱۶۰ نشانگر اس اس آر که ۸ تای آنها چندشکلی بودند غربال شدند. یک آزمون مبتنی بر پی سی آر با این نشانگر ها هر دو آلل رگه های والدی را در هیبرید خالص تجسس کرد و ناخالصی آنها را ثابت کرد در حالیکه ناخالصی ها با حضور فقط یک آلل والد مشخص شد. تایید خلوص هیبرید چنین اشارت داشت که یک نشانگر چندشکلی برای تجسس آلودگی(نا خالصی) این هیبریدها از والدهایشان کافی است. یافته دیگر این بود که چنانچه توده ای از بذر یک هیبرید با هیبرید دیگری یا با والدش آلوده شد، دو نشانگر مناسب یا تعداد بیشتری از آنها را می توان به کار برد تا به سادگی چنین ناخالصی ها را تشخیص داد. این روش می تواند به طور صحیح و موثر خلوص هیبرید را در یک نمونه از هیبریدهای سوژا که عامدانه از مخلوط بذر رگه های والد از پیش تهیه شده است تشخیص دهد. این اولین گزارشی است که کاربرد نشانگر های اس اس آر را در ارزیابی خلوص ژنتیکی هیبرید های سوژا نشان می دهد.

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