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Sequencing of the chloroplast genomes of cytoplasmic male-sterile and male-fertile lines of soybean and identification of polymorphic markers

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ABSTRACT

The RN-type cytoplasmic male sterility (CMS) system used to develop Hybsoy-1, the first commercial hybrid soybean, has been subsequently applied to generate nearly all released soybean hybrids. Although more than 3 years are needed to classify sterile (S) and normal male-fertile (F) cytoplasms by conventional crossing, such classifications can be performed rapidly using organellar DNA-based molecular markers. Except for fertility, the agronomic traits of CMS hybrid soybean sterile and maintainer lines are identical. Consequently, it is difficult to distinguish them by routine visual inspection in the mixture arising in the course of field planting and harvesting during breeding. In this study, we performed next-generation sequencing of chloroplast DNAs of F- and S-cytoplasmic soybeans, assembled and annotated the genomes, and identified polymorphisms distinguishing them. Chloroplast DNAs of F and S cytoplasms were very similar in size (152,215 and 152,222 base pairs) and GC contents (35.37%). Among 23 shared SNPs in gene coding regions, we identified four that could be used in conjunction with restriction endonucleases to distinguish S and F cytoplasms. Although CMS is likely associated with mitochondrial DNA, maternal transmission of mitochondrial and chloroplast DNAs allows polymorphisms in either genome to be used to classify soybean cytoplasms, aiding hybrid soybean cultivar development.

1. Introduction

Although Davis published a patent for the cytoplasmic male sterility system in soybean (*Glycine max* [L.] Merrill) in 1985 [1], the patent has rarely been applied to hybrid breeding. Since the 1980s, cytoplasmic male-sterile lines RN, ZD, XXT, N8855, and N21566 have been identified in China [2–7]. Because RN-type male-sterile cytoplasms were used for initial hybrid seed production in *G. max*, almost all hybrids of soybean possess the RN genotypes. As of 2013, over 200 pairs of stable sterile and maintainer lines have been bred from *G. max* line Ru Nan Tian E Dan, which possesses RN-type male-sterile cytoplasm. The first soybean CMS three-line system, comprising of a male-sterile line, a maintainer line, and a restorer line, was developed by Sun [8]. The biggest advantage of CMS is that the maternal lines with their 100% male-sterile plants eliminate the need for artificial emasculation, thereby reducing labor requirements and costs, and also ensuring seed purity. Consequently such systems have been widely applied for the exploitation of crop heterosis [9].

Although the use of hybrids can greatly increase soybean yields, the application of hybrid breeding production technology has not been widely promoted in soybean. This situation is partially caused by limited amounts of existing hybrid seed, but other factors are also responsible. For example, the available pool of sterile and fertile germplasm resources needs to be expanded; however, the identification of fertile and sterile cytoplasms is a slow process [10]. As RN-type male-sterile cytoplasm is associated with gametophyte sterility, over 3 years are needed to classify sterile and normal male-fertile cytoplasms using conventional crossing techniques. Another problem involves seed propagation of the three CMS lines. In the

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CMS three-line system, a male-sterile line as the maternal parent is crossed with a paternal restorer line to obtain hybrid seeds. The maintainer and restorer lines are self-reproducing, but male-sterile line seeds can only be propagated from the female parent by crossing it with its isotype maintainer line – the latter as the male parent. During this process, no good method exists to distinguish between the mixture of male-sterile and maintainer lines inevitably arising during field planting, harvesting, and threshing: other than fertility, these lines all possess the same agronomic characteristics [9].

Molecular markers used to differentiate between cytoplasmic male sterility and fertility [11] can be applied to distinguish sterile from maintainer lines. Such an approach should provide a convenient method to broaden CMS germplasm resources and separate out maintainer lines mixed with sterile ones. In this study, we sequenced and compared a soybean RN-type male-sterile CMS line and its maintainer (containing male-fertile cytoplasm) to identify single-nucleotide polymorphism (SNP) and insertion–deletion (InDel) differences. As a result, we identified four PCR product fragments that could be used in conjunction with restriction endonucleases to distinguish RN-type male-fertile and male-sterile cytoplasms. This paper also provides a new and direct way to distinguish the cytoplasm of sterile and maintainer lines.

2. Materials and methods

2.1. Plant materials

In this study, we sequenced soybean plants of the CMS line JLCMS9A (S; possessing the cytoplasmic male-sterile RN genotype) and its contrasting maintainer line JLCMS9B (F) [12]. JLCMS9A, JLCMS9B; other populations of cytoplasmic male-sterile RN genotypes used in this study (see Table 1) were obtained from the Jilin Academy of Agricultural Sciences, China. The RN genotypes were derived from a cross between a cultivated soybean and an annual wild soybean; they appeared to be stable, and their sterility was unaffected by different temperatures and photoperiods [13,14]. Importantly, JLCMS9A and JLCMS9B have almost identical nuclear genomic backgrounds [15]. The enzymatic lysis buffer method [16] was used to isolate chloroplast genomic DNA from leaves of field-grown JLCMS9A and JLCMS9B plants.

2.2. Sequencing and data analysis

Using the isolated chloroplast DNA, genomic libraries were prepared for sequencing on a Hiseq 2000 platform, following manufacturer's recommended protocols (Illumina, California, USA). Paired-end sequencing of the resulting 500-bp fragment libraries by the whole shotgun method generated 100-bp-long reads. After removal of nuclear and mitochondrial genome sequences [17,18], ABySS, SSPACE, and GapCloser assembly software were used separately to assemble the reads from each F- and S-chloroplast DNA library (see Table 2). The primary assembly yielded a complete circular chloroplast DNA sequence [19,20]. After annotation of regions by alignment against the PI 437654 soybean chloroplast reference genome [21], oligonucleotide primers were designed to amplify across problematic and remaining gap regions. To verify the resulting sequences, amplicons were subjected to Sanger sequencing [22] and aligned using Sequencer version 5.1 (GeneCodes, Ann Arbor, MI, USA). Aligned reads listed in Table 2 were visualized using genomeVx software (http://www.wolfe.gen.tcd.ie/genomeVx/), and performed final annotations using DOGMA [23].

The software mVISTA [24] was used to visualize the differences between JLCMS9A (S) and JLCMS9B (F) chloroplast sequences. SNPs

Table 1
Soybean accessions, origins, and cytoplasmic status (male-fertile [F] or male-sterile [S]).

Accession	Origin	Cytoplasm
JLCMS5B	Jiamusi, Heilongjiang, China	F
JLCMS6A	Zhenzhou, Henan, China	S
JLCMS6B	Zhenzhou, Henan, China	F
JLCMS7A	Changchun, Jilin, China	S
JLCMS7B	Changchun, Jilin, China	F
JLCMS8B	Suihua, Heilongjiang, China	F
JLCMS9A	Jiuzhan, Jilin, China	S
JLCMS9B	Jiuzhan, Jilin, China	F
JLCMS11B	Jiamusi, Heilongjiang, China	F
JLCMS14B	Suihua, Heilongjiang, China	F
JLCMS16B	Jiuzhan, Jilin, China	F
JLCMS18A	USA	S
JLCMS18B	USA	F
JLCMS22A	Buyang, Anhui, China	S
JLCMS22B	Buyang, Anhui, China	F
JLCMS24A	USA	S
JLCMS24B	USA	F
JLCMS27B	Baicheng, Jilin, China	F
JLCMS29B	Jiamusi, Heilongjiang, China	F
JLCMS34B	Jiamusi, Heilongjiang, China	F
JLCMS36B	Jiuzhan, Jilin, China	F
JLCMS42B	USA	F
JLCMS44B	Suihua, Heilongjiang, China	F
JLCMS47A	Gongzhuling, Jilin, China	S
JLCMS47B	Gongzhuling, Jilin, China	F
JLCMS48B	Haerbin, Heilongjiang, China	F
JLCMS51B	Hefei, Anhui, China	F
JLCMS54B	Jiamusi, Heilongjiang, China	F
JLCMS55B	Suihua, Heilongjiang, China	F
JLCMS61B	Haerbin, Heilongjiang, China	F
JLCMS68A	Japan	S
JLCMS68B	Japan	F
JLCMS71B	Gongzhuling, Jilin, China	F
JLCMS73B	Gongzhuling, Jilin, China	F
JLCMS82B	Shenyang, Laoning, China	F
JLCMS84A	Jiamusi, Heilongjiang, China	S
JLCMS84B	Jiamusi, Heilongjiang, China	F
JLCMS85B	Tieling, Liaoning, China	F
JLCMS87A	USA	S
JLCMS87B	USA	F
JLCMS96B	Jiamusi, Heilongjiang, China	F
JLCMS98B	Haerbin, Heilongjiang, China	F
JLCMS101B	USA	F
JLCMS103B	Haerbin, Heilongjiang, China	F
JLCMS110B	Zhoukou, Henan, China	F
JLCMS112B	Changchun, Jilin, China	F
JLCMS115B	Yanjin, Henan, China	F
JLCMS116B	Jiuzhan, Jilin, China	F
JLCMS120B	Zhenzhou, Henan, China	F
JLCMS123B	Yingkou, Liaoning, China	F
JLCMS124B	Zhenzhou, Henan, China	F
JLCMS126B	Gongzhuling, Jilin, China	F
JLCMS128B	Gongzhuling, Jilin, China	F
JLCMS134B	Zhenzhou, Henan, China	F
JLCMS136B	DIFANGPINZHONG, China	F
JLCMS139A	Japan	S
JLCMS139B	Japan	F
JLCMS147B	Jiamusi, Heilongjiang, China	F
JLCMS160B	Sihong, Jiangsu, China	F
JLCMS171A	USA	S
JLCMS171B	USA	F
JLCMS172B	USA	F
JLCMS174B	Buyang, Anhui, China	F
JLCMS181B	USA	F
JLCMS182B	Changchun, Jilin, China	F
JLCMS188B	Gongzhuling, Jilin, China	F
JLCMS189B	Hefei, Anhui, China	F
JLCMS199B	Gongzhuling, Jilin, China	F
JLCMS213B	Gongzhuling, Jilin, China	F
JLCMS225B	Zhenzhou, Henan, China	F
JLCMS235B	Yan'an, Shanxi, China	F
JLCMS236B	Taiyuan, Shanxi, China	F