Theor Appl Genet (2004) 109: 769–774
DOI 10.1007/s00122-004-1680-2

ORIGINAL PAPER

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SSR marker and ITS cleaved amplified polymorphic sequence analysis of soybean × *Glycine tomentella* intersubgeneric derived lines

Received: 4 February 2004 / Accepted: 28 March 2004 / Published online: 26 May 2004
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Abstract Wild perennial *Glycine* species are an invaluable gene resource for the cultivated soybean [*Glycine max* (L.) Merr., 2*n*=40]. However, these wild species have been largely unexplored in soybean breeding programs because of their extremely low crossability with soybean and the need to employ in vitro embryo rescue methods to produce F_1 hybrids. The objective of this study was to develop molecular markers to identify gene introgression from *G. tomentella*, a wild perennial *Glycine* species, to soybean. A selection of 96 soybean simple sequence repeat (SSR) markers was evaluated for cross-specific amplification and polymorphism in *G. tomentella*. Thirty-two SSR markers (33%) revealed specific alleles for *G. tomentella* PI 483218 (2*n*=78). These SSR markers were further examined with an amphidiploid line (2*n*=118) and monosomic alien addition lines (MAALs), each with 2*n*=40 chromosomes from soybean and one from *G. tomentella*. The results show that the use of SSR markers is a rapid and reliable method to detect *G. tomentella* chromosomes in MAALs. We also developed a cleaved amplification polymorphism sequence (CAPS) marker according to the sequences of internal transcribed spacer (ITS) regions in soybean and *G. tomentella*. Four MAALs that carry the ITS (rDNA) locus from *G. tomentella* were identified. The SSR and ITS-CAPS markers will greatly facilitate the introgression and characterization of gene transfer from *G. tomentella* to soybean.

Communicated by F.J. Muehlbauer

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Introduction

The genus *Glycine* Willd. consists of subgenera *Glycine* and *Soja*, with 23 wild perennial species and two annual species, respectively. The cultivated soybean and its wild annual progenitor (*G. soja*) belong to the subgenus *Soja*, contain 2*n*=40 chromosomes and are cross-compatible. On the other hand, the subgenus *Glycine* includes 23 wild perennial species and has a wide geographical distribution. Wild perennial *Glycine* species are an invaluable gene resource for the cultivated soybean (Singh and Hymowitz 1999). However in the past, these wild species have been largely unexplored in soybean breeding programs because of their extremely low crossability with soybean and the need to employ in vitro embryo rescue methods to produce F_1 hybrids. Most soybean researchers have been unable to proceed beyond the amphidiploid stage (Hymowitz et al. 1998). Thus far, only Singh et al. (1998) have successfully produced backcross-derived fertile progenies from the soybean and a wild perennial, *G. tomentella* PI 483218 (2*n*=78). A set of monosomic alien addition lines (MAALs) has been identified in this introgression program. These MAALs contain 40 chromosomes from *G. max* plus one chromosome from *G. tomentella*. They are excellent "bridge" materials to transfer useful genes from wild species.

The rapid and accurate identification of an alien genome would add to our knowledge of the evolutionary relationships and the interactions between soybean and its wild relatives. The transfer of novel traits would be greatly facilitated if the introgression could be characterized by molecular markers. Different marker systems, such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs) and simple sequence repeats (SSR or microsatellites), have been utilized in the wide hybridization of various plant species (Bommineni et al. 1997; Peil et al. 1997; Escalante et al. 1998; Yan et al. 2001; Hernandez et al. 2002). Well-saturated SSR maps, already available for numerous crops, are extremely useful in monitoring alien introgression because of their high reliability, prior knowledge of map

positions and simple procedures. In soybean, SSR markers have been used extensively in genetic and breeding programs, such as tagging disease resistance genes (Mian et al. 1999; Demirbas et al. 2001), mapping quantitative trait loci (QTLs) (Orf et al. 1999), identifying bacterial artificial chromosomes (BACs) (Marek et al. 2001), and assigning molecular linkage groups (MLGs) to soybean chromosomes (Cregan et al. 2001; Zou et al. 2003). Currently, over 1,000 SSR markers are available and positioned in the soybean genetic map (Song et al. 2004). If SSR markers developed in soybean could be utilized in the genetic analysis of its wild perennial relatives, such as *G. tomentella*, it would help to identify intersubgeneric gene introgression.

The internal transcribed spacer (ITS) sequence of nuclear ribosomal RNA gene (rDNA) is a powerful method to assess phylogenetic relationships in *Glycine* species. For example, ITS sequences and cytogenetic analysis have been used to assign genomic symbols to different *Glycine* species (Kollipara et al. 1997) and to establish homologous evolution in the *G. tomentella* allopolyploid complex (Rauscher et al. 2004). So far, over 140 ITS sequences of *Glycine* species have been deposited in GenBank (as of 26 January 2004). However, this sequence information has not been utilized in any *G. tomentella* -soybean introgression program.

In this study, we focused on developing molecular tools to identify introgression from *G. tomentella* to soybean. SSR markers from soybean were used to detect *G. tomentella* genome in the soybean genetic background. We also developed *G. tomentella*-specific ITS cleaved amplified polymorphic sequence (CAPS) markers to identify the alien chromosome carried by soybean.

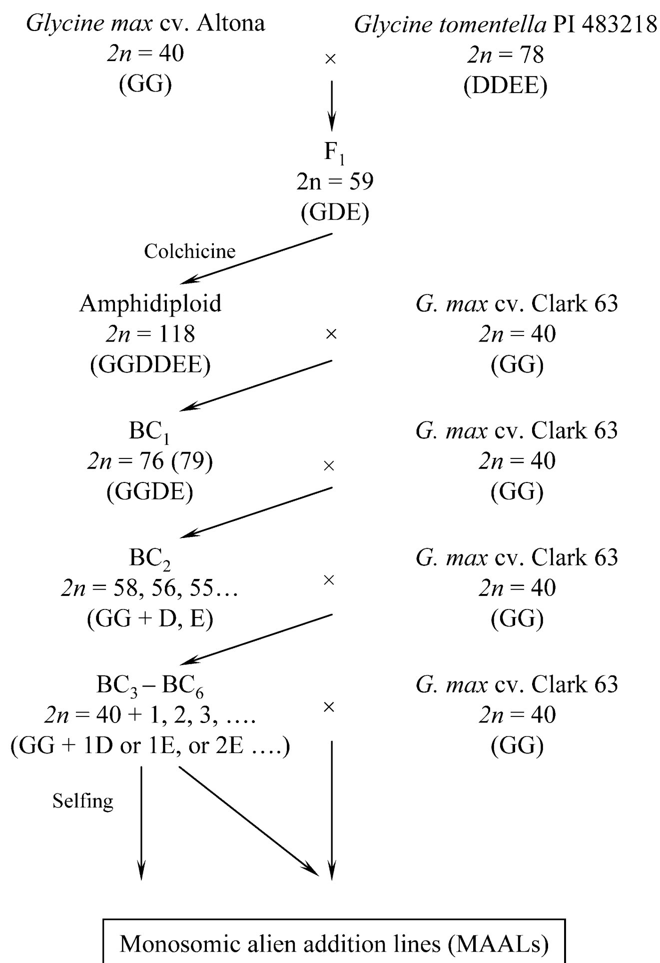

Fig. 1 Procedure for genome introgression from *Glycine tomentella* to soybean (adapted from Singh et al. 1998)

Materials and methods

Plant materials

The crossing scheme for genome introgression from *G. tomentella* to *G. max* (cultivated soybean) is shown in Fig. 1. *G. tomentella* ($2n$=78) PI 483218 is resistant to soybean rust (Schoen et al. 1992), soybean cyst nematode (Riggs et al. 1998), and bean pod mottle virus (R. Gergerich, University of Arkansas, 1997, personal communication). The origins of BC_1 plants [(*G. max* cv. Altona, $2n$=40, genome GG) × (*G. tomentella*, genome DDEE)→F_1, $2n$=59, genome GDE→colchicine treatment (CT)→$2n$=118, genome GGDDEE × soybean cv. Clark 63→BC_1 $2n$=76 (expected $2n$=79), genome GGDE], BC_2 to BC_4 derived lines were described by Singh et al. (1998). Thirty-three MAALs, which were obtained from BC_4, BC_5, or BC_4 F_2 plants with Clark 63 as the recurrent parent, were examined in this study. All the cytological analyses were conducted according to Singh et al. (1998).

Glycine tomentella is an extremely variable species distributed in Australia, China, Papua New Guinea, Philippines and Taiwan. Four cytotypes have been reported for *G. tomentella* ($2n$=38, 40, 78, 80). In this study, we examined 29 *G. tomentella* accessions which represent different chromosome numbers, genomes, and diverse geographical origins (Table 1).

DNA extraction and SSR analysis

DNAs were extracted according to the procedure of Walbot (1988). Ninety-six SSR markers from 20 soybean MLGs (Cregan et al. 1999) were initially screened against Altona, Clark 63 and PI 483218. PCR reactions, amplification conditions and fragment detection were according to Zou et al. (2003). Successful transfer of soybean SSR to *G. tomentella* was based on a clear amplification product of the expected size and polymorphism. In order to ensure that non-amplification was not due to failed PCRs, most of the PCR amplifications were done twice. SSR analysis was also performed on different *G. tomentella* accessions.

Amplification of the ITS region and sequencing

Internal transcribed spacer regions of PI 483218, Clark 63, and Altona were amplified using the forward primer SSF

Table 1 *Glycine tomentella* accessions used in this study and their SSR amplification results. Accession numbers are given as both *IL*, a temporary number assigned at Urbana, IL, USA, and PI, the plant introduction number assigned by the USDA. The isozyme group assignments are based on Doyle and Brown (1985), with group T7 based on Kollipara et al. (1994); ? isozyme group not known. The collection sites are *NSW* New South Wales, *QLD* Queensland, *WA* Western Australia. The SSR fragment numbers (1 or 2) are based on PCR amplification results of Satt411

Code	Accession number		2*n*	Isozyme group	Collection site	SSR fragment
	PI	IL				
1	483218	428	78	T1	QLD, Australia	2
2	441001	489	78	T1	QLD, Australia	2
3	505232	719	78	T1	QLD, Australia	2
4	483220	451	78	T1	QLD, Australia	2
5	339657	363	78	T1	NSW, Australia	2
6	441008	520	78	T1	NSW, Australia	2
7	563876	869	78	T1	QLD, Australia	2
8	446985	606	78	T1	Port Moresby, Papua New Guinea	2
9	505286	798	78	T6	WA, Australia	2
10	509501	866	78	T5	NSW, Australia	1
11	509502	867	78	T5	NSW, Australia	1
12	373988	368	78	?	NSW, Australia	1
13	483220	451	78	T1	QLD, Australia	2
14	563879	874	80	T4	Haikou, Taiwan	2
15	505304	816	80	T3	WA, Australia	2
16	446988	609	80	T3	Port Moresby, Papua New Guinea	2
17	441005	485	80	T2	QLD, Australia	2
18	563878	872	80	T4	Ta Kuang, Taiwan	2
19	330961	360	80	T4	Bataan Island, Philippines	2
20	563881	888	80	T7	Timor, Indonesia	2
21	583968	887	80	T7	Timor, Indonesia	2
22	546958	886	80	T7	Timor, Indonesia	2
23	483219	442	80	T3	QLD, Australia	2
24	441000	448	40	D4	QLD, Australia	1
25	505222	709	40	D3	QLD, Australia	1
26	440998	398	38	D1/D2	QLD, Australia	1
27	505203	683	40	D5	WA, Australia	1
28	505301	813	40	?	WA, Australia	1
29	505210	697	40	?	QLD, Australia	1

(5′-AAGTCGTAACAAGGT TTCCGTAG-3′) and the reverse primer LSR (5′-GTTAGTTTCTTTTCCTCC-3′). PCR reactions were undertaken in 100 µl volumes containing 50 ng of template DNA, 4.0 pmol of each primer, 0.2 mM dNTPs (Pharmacia Biotech Inc., Piscataway, NJ, USA), 1.5 mM $MgCl_2$, 1×PCR buffer and 2 U *Taq* polymerase (Gibco BRL Life Technologies, Gaithersberg, Md., USA). Temperature cycling was performed in an MJ Research PTC 100 Thermal Controller using 'touchdown' PCR. The amplification profile was set to run at 94°C for 3 min followed by six cycles of denaturing at 94°C for 30 s, annealing for 30 s at temperatures from 55°C to 50°C with a decrease of 1°C per cycle, and extending at 72°C for 1 min. The final cycle (94°C for 30 s, 50°C for 30 s, and 72°C for 1 min) was repeated 35 times. After confirming the amplification of a single size fragment (~700 nucleotides) as revealed by the presence of a single band on a 1.2% agarose gel, the fragment was purified by spin column chromatography (QIAquick Spin, Qiagen, Chatsworth, Calif., USA). PCR products were sequenced using an automated DNA sequencer (model 3700, Perkin Elmer, Foster City, Calif., USA).

ITS sequence alignment and selection of restriction enzymes

Internal transcribed spacer sequences of PI 483218, Clark 63, and Altona were aligned with those published sequences of the 18 *Glycine* species (Kollipara et al. 1997; GenBank accession numbers U60533–U60551). Restriction sites of the ITS sequences from soybean and *G. tomentella* PI 483218 were surveyed using Vector NTI 7.0 (Informax, Frederick, Md., USA). The enzymes that had restriction sites that varied within the ITS sequences were chosen for RFLP analyses. Five microlitres of each PCR product was digested in a 10 µl reaction containing 2 U of the relevant restriction enzyme. Digested PCR products were electrophoresed on 1.2% TBE agarose gels. The gels were stained with ethidium bromide, visualized, and photographed under UV light.

Table 2 Soybean SSR markers that revealed polymorphism between soybean cultivars Altona and Clark 63 and *G. tomentella* PI 483218. The linkage group assignments are based on Cregan et al. (1999). The amplification types are based on the amplification results of *G. tomentella* PI 483218. *1* one fragment, specific for *G. tomentella*, *2* two fragments, with one fragment specific for *G. tomentella*, *3* two fragments, both specific for *G. tomentella*

SSR	Linkage group	Amplification type	SSR	Linkage group	Amplification type
Satt236	A1	2	Satt566	G	3
Satt177	A2	1	Satt353	H	1
Satt589	A2	1	Satt440	I	1
Sct_026	B1	1	Satt367	I	2
Satt509	B1	2	Satt244	J	2
Sct_094	B2	1	Sat_020	K	1
Satt126	B2	1	Satt381	K	3
Satt565	C1	2	Satt196	K	2
Satt286	C2	1	Satt462	L	1
Satt371	C2	1	Satt551	M	1
Satt360	D1a+q	1	Sat_003	M	3
Satt311	D2	3	Satt584	N	3
Sct_137	D2	3	Sat_091	N	1
Satt411	E	3	Satt521	N	3
Satt369	E	2	Satt358	O	1
Satt472	G	1	Satt478	O	3

Results

Polymorphism of soybean SSR markers in *G. tomentella*

Simple sequence repeat analysis was based on marker location on the soybean molecular linkage map (Cregan et al. 1999). Initially, 3–9 markers were selected from each of the 20 linkage groups. We screened against the soybean cultivars Clark 63 and Altona and *G. tomentella* PI 483218 that were involved in the intersubgeneric hybridization scheme (Fig. 1). Of the 96 markers, a total of 40 (42%) gave clear amplification products from *G. tomentella*. Eight markers gave clear amplification from *G. tomentella*, but no polymorphism with the soybean parents. Thirty-two SSR markers (Table 2) showed *G. tomentella* -specific alleles in this introgression program.

Primer pairs for five SSR loci, Satt584, Satt411, Satt244, Satt462, and Satt509, were used to analyze 29 *G. tomentella* accessions (Table 1). They all revealed polymorphisms between soybean cv. Altona and different *G. tomentella* accessions. We observed a possible tendency for the number of DNA fragments to increase with the ploidy level at loci Satt411 (Table 1). All six diploid ($2n$=40) and aneudiploid ($2n$=38) *G. tomentella* accessions showed a single fragment. Most of the 78 and 80 chromosomes accessions showed two fragments, except three accessions (PI 509501, PI 509502 and PI 373988) which showed a single fragment. These three accessions were originally collected from same location (Northern New South Wales, Australia). Up to 26 of the 29 (90%) *G. tomentella* accessions could be uniquely fingerprinted using one pair of SSR primers and all of the individuals could be fingerprinted by their genotypes revealed by two pairs of primers. The presence of a high level of unique single- and multi-locus genotypes makes SSR markers an excellent tool for the fingerprinting and identification of *G. tomentella* individuals.

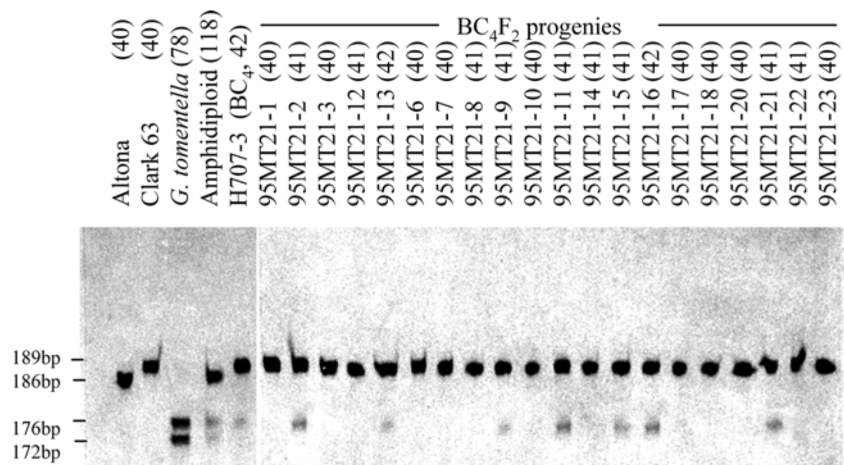

Fig. 2 Identification of *G. tomentella* chromosome in the BC_4F_2 progenies with SSR (Satt584) and cytological analysis. Chromosome numbers are shown in *parentheses*

Detection of *G. tomentella* genome in soybean × *G. tomentella* derived lines by SSR markers

To examine the usefulness of SSR markers in soybean × *G. tomentella* derived lines, SSR markers that revealed *G. tomentella*-specific alleles (Table 2) were used to analyze an amphidiploid line (2*n*=118) and 33 MAALs. The amphidiploid line displayed a combination of parental fragments in most cases, confirming their hybrid character. In several cases, parental genome patterns were not obtained in the amphidiploid line, suggesting a loss of the donor genome during the meiotic process. Six MAALs could be identified by their unique SSR genotypes: 91H721-2 (Satt462), 95MT27-4 (Satt472), 92H760-3 (Sct_094), 95MT25-2 (Sct_137), 95MT27-6 (Satt244), and 92H771-2 (Satt411).

The chromosome numbers of a BC_4 plant (H707-3, 2*n*=42) and its self-derived BC_4 F_2 progenies (2*n*=40, 41 or 42) were counted and listed in Fig. 2. Among the 32 SSR markers, only Satt584 showed a *G. tomentella*-specific fragment (176 bp) in H707-3. In BC_4 F_2 progenies, this fragment was revealed in all the 42 chromosome plants and five of the nine 41 chromosome plants. All the 40 and the rest of the four 41 chromosomes plants did not show this fragment (Fig. 2). This suggested that in the progenies of H707-3 five MAALs (95MT21-2, 95MT21-9, 95MT21-11, 95MT21-15, and 95MT21-21) carried the same chromosome (containing the 176 bp fragment) from *G. tomentella* and four MAALs (95MT21-12, 95MT21-8, 95MT21-14, and 95MT21-22) carried the other *G. tomentella* chromosome. We observed that this fragment was prominent in the *G. tomentella* SSR profile while very faint in both the amphidiploid line and the corresponding MAALs (Fig. 2). This is probably attributable to competition from soybean sequences with higher homology to the primers. It was also observed that Altona genome (186 bp fragment) was replaced by the Clark 63 genome (189 bp fragment) after backcrossing 4 times (Fig. 2).

Development of CAPS marker and Identification of *G. tomentella* genome in MAALs

Using ITS specific primers, we amplified a single fragment (~700 nucleotides) from soybean cv. Clark 63, Altona, and PI 483218. There are no sequence differences between Clark 63 and Altona at this region. The ITS sequence of PI 483218 was very similar (96% identity) to that of a diploid *G. tomentella* accession PI 505222 (2*n*=40), which was assigned the genome symbol DD in a previous study (Kollipara et al. 1997). With Vector NTI 7.0, we surveyed 229 enzymes for their restriction sites. Fifty-eight of those enzymes showed up to three restriction site difference between PI 483218 and Clark 63. For convenience, we used only *Hinc* II in the following studies.

As expected, PI 483218 showed two fragments of 200 bp and 500 bp after digestion with *Hinc* II (Fig. 3). However, PI 483218 consistently showed the original 700 bp fragment after digestion. A similar result was obtained when PI 483218 was digested with *Sal* I. This might be caused by the locus complex in G. *tomentella* genome. Nevertheless, the *G. tomentella* genome can be clearly identified by the two extra fragments. An example of the digestion result is shown in Fig. 3. An amphiploid plant showed the fragments from *G. tomentella*. This marker was further examined against 33 MAALs. Four MAALs (92H744-1, 92H744-3, 94H830-1, and 95MT25-2) showed fragments from *G. tomentella*. Upon the pedigree analysis, all of four of these MAALs (BC_5 or BC_4F_2) were derived from same plant H698-3 (BC_3), which carried 46 chromosomes.

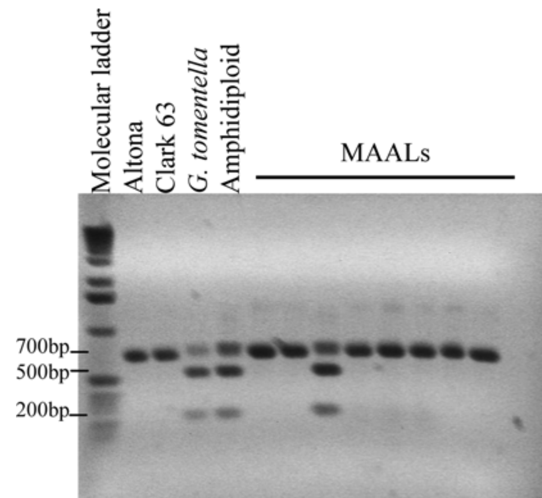

Fig. 3 *Hinc* II restriction endonuclease fragment patterns of the internal transcribed spacer (ITS) sequences in soybean, *G. tomentella*, and their derived lines. Two fragments of 200 and 500 bp were revealed in *G. tomentella*, amphidiploid, and one monosomic alien addition line (MAAL)

Discussion

Despite the prevailing tendency for developing species-specific SSRs, research based on the use of SSRs sourced from closely related species is becoming increasingly frequent (Peakall et al. 1998; Rossetto 2001). The large numbers of SSR markers in soybean provide a valuable marker resource for use with other species in the genus *Glycine*. The current results clearly demonstrate that soybean SSRs constitute efficient and cost-effective sources of molecular markers for *G. tomentella*. Forty (42%) soybean primer pairs amplified products from *G. tomentella* PI 483218. Similar results were observed in other *Glycine* species. For example, Peakall et al. (1998) investigated the transferability of 31 soybean SSR loci with wild perennial *Glycine* species *G. falcata, G. clandestina*, and *G. microphylla*. The successful amplification rate in their study was 48–61%.

The only soybean chromosome that can be identified at mitotic metaphase is the satellite chromosome, which has been designated as chromosome 13 by Singh and Hymowitz (1988). The rDNA locus in soybean and perennial *Glycine* species was located on the satellite chromosome by pachytene analysis and in situ hybridization (Singh and Hymowitz 1988; Singh et al. 2001). The soybean satellite chromosome has been associated with molecular linkage group F using primary trisomic and

SSR markers (Cregan et al. 2001; Zou et al. 2003). Several loci conferring resistance to diverse pathogens were mapped as a cluster on this chromosome (SoyBase, http://soybase.agron.iastate.edu/). In the current report, we developed a CAPS marker that was specific for the *G. tomentella* ITS region of the rDNA locus. Four MAALs ($2n$=41) that carry the ITS sequence from *G. tomentella* as the alien chromosome can be clearly identified. There is a possibility that this alien chromosome also carries a cluster of resistance genes. These MAALs are excellent materials for studying the interaction of the *G. tomentella* chromosome with *G. max* and comparing with soybean primary trisomic 13, which carries satellite chromosome as its extra chromosome.

Production of inter-subgeneric hybrids through embryo rescue is cumbersome and time-consuming. Confirmation of hybridity at a very early stage is advantageous as the contaminants or selfs can be discarded at an early stage. In this study, a set of soybean SSR markers have been identified that revealed a high degree of polymorphism between soybean and *G. tomentella* and among different *G. tomentella* accessions. These SSR markers are being used to confirm the hybrids in the early stage of embryo rescue (RJ Singh, unpublished data).

In conclusion, our results demonstrated that soybean SSRs are a valuable source of molecular markers for analyzing the relatively unknown *G. tomentella* gene pool and for studying *G. tomentella* introgression into the soybean. The SSR and ITS-CAPS markers developed in this study will greatly facilitate the introgression and characterization of gene transfer from *G. tomentella* to soybean.

Acknowledgements This research was supported in part by Illinois Agriculture Experimental Station.

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