Genomic Variation in Micropropagated *Robinia ambigua* ‘idahoensis’ Revealed by RAPD Markers

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Additional index words. *Robinia ambigua*, micropropagation, RAPD markers, somaclonal variation, sequence analysis

Abstract. The authors have previously reported an efficient in vitro system for mass micropropagation of *Robinia ambigua* ‘idahoensis’ (Idaho locust). Their method used enhanced branching of axillary buds from a single donor plant along with detection of somaclonal variation by the intersimple sequence repeat (ISSR) markers. Because ISSRs tend to be clustered to specific chromosomal regions in plant genomes, the extent and scope of the genomic variations and the sequences underlying the variation warranted further investigations. In this study, the authors analyzed the same set of 40 randomly selected micropropagated *R. ambigua* plants by a more general molecular marker—random amplified polymorphic DNA (RAPD) using 34 selected primers. In addition, they sequenced some of the variable bands. Of the 260 reproducible RAPD bands scored, 70 were polymorphic among the 41 plants (40 micropropagated and one donor), corresponding to a polymorphism level of 26%. Cluster analysis revealed a genetic similarity ranging from 0.61 to 0.97. Of the 20 sequenced bands underlying the variations, eight showed significant homology to known or predicted functional cellular genes or retroelements. These results clearly indicated that micropropagation of *R. ambigua*, even by enhanced branching of axillary buds, can be accompanied by extensive genomic variations, which should be taken into account for commercial propagation of this plant by in vitro means.

Robinia ambigua, commonly called Idaho locust, belongs to the legume family (*Fabaceae*), and is presumably a hybrid between the black locust (*R. pseudoacacia* L.) and either the shrub bristly locust (*R. hispida* L.) or the clammy locust (*R. viscosa* Vent.). This tree has combined features of both parental species, including the higher plant stature of the black locust (15 m compared with 9–30 m) and the vivid coloring of the bristly locust (Fowells, 1965).

Micropropagation through in vitro culture has been shown to be an efficient means for propagation of endangered or valuable plants (Osorio-Rosales and Mata-Rosas, 2005; Paredes and Lavin, 2005). Recently, we have established an efficient in vitro culture system for the micropropagation of *R. ambigua* by using enhanced branching of axillary buds (Guo et al., 2006). Surprisingly, molecular analysis by the intersimple sequence repeat (ISSR) markers showed genomic variations in these propagated plants (Guo et al., 2006).

Received for publication 31 Mar. 2006. Accepted for publication 7 June 2006. This study was supported in part by the Program for Changjiang Scholars and Innovative Research Team (PCSIRT) in University.

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Nonetheless, because the ISSR markers often confine to specific chromosomal regions, such as the disease-resistance gene analogous clusters (Ratnaparkhe et al., 1998), in plant genomes, the extent and scope of the genomic variations and the sequences underlying these variations remain unknown. This study was aimed to address these issues by using a more general molecular marker, the randomly amplified polymorphic DNA (RAPD), which is known to distribute and amplify unbiased loci throughout the genome (Sauve et al., 2005; Williams et al., 1990). We report that a high level of genomic variations indeed occurred in the *R. ambigua* var. *idahoensis* micropropagated plants. Moreover, cellular genes and retroelements appeared to underlie a substantial proportion of the variations.

Materials and Methods

The same set of 40 randomly chosen plants from a large micropropagated *R. ambigua* var. *idahoensis* population as well as the single mother donor plant (Guo et al., 2006) were used for this study. Genomic DNA was extracted by a modified cetyltrimethylammonium bromide (CTAB) protocol (Kidwell and Osborn, 1992) from fresh young leaves of 5-month-old micropropagated plants, and two independent samples of the single mother

donor plant. The RAPD amplifications were performed with selected 10-mer primers in a total volume of 25 µL containing 10× reaction polymerase chain reaction buffer [100 mM Tris-HCl (pH, 8.0), 400 mM KCl, and 15 mM MgCl₂], 2.5 mM MgCl₂, 0.25 mM deoxynucleotide triphosphates (dNTPs), 10 mM random decamer primer, 2% (v/v) deionized formamide, 10 ng genomic DNA, 1.0 U Taq DNA polymerase (The Sangon Biotech. Inc., Shanghai), and sterile, double-distilled water to volume. RAPD amplifications were performed in a PTC-100 thermocycler (The MJ Research, Waltham, Mass.) programmed as 94 °C for 4 min, followed by 45 cycles consisting of 94 °C for 1 min for a denaturation step, an annealing step of 1 min at 36 °C, and an extension step of 72 °C for 2 min, ending with 10 min at 72 °C.

The amplification products were resolved by running through 1.5% agarose gels stained with ethidium bromide (0.5 mg·L⁻¹). The GeneRuler™ DNA Ladder Mix (Fermantas, Hanover, Md.) was used as a molecular size standard. Gels were visualized and photographed under an ultraviolet transilluminator. The presence or absence of bands was scored in a binary matrix way (1 for presence and 0 for absence of a band), and the data were analyzed for clustering using the NTSYS-pc version 2.02 software package (Numerical Taxonomy System, Exeter Software, Setauket, N.Y.) (Rohlf, 1997). The unweighted pair grouping method of averages (UPGMA) strategy (Rohlf, 1993) was used to analyze similarity estimates, and the resulting cluster was represented as a dendrogram.

Thirty variable bands in the RAPD gels were excised, eluted, cloned into the TA-cloning vector (The Promega, Madison, Wis.), and sequenced. The sequences were then queried against the Genbank databases by BlastX at the NCBI website (<http://www.ncbi.nih.gov/BLAST/>) to identify possible homologies.

Results

We tested a total of 68 10-decamer RAPD primers currently used in our laboratory on two independent samples of a single donor plant of *R. ambigua* var. *idahoensis* as replicated biological templates. Based on a criterion of the generation of multiple clear bands that were completely reproducible between the two replicates, 34 of the 68 RAPD primers were selected as suitable for *R. ambigua* ‘idahoensis’, and thus were used for the current study (primer information is available upon request). Using these 34 selected primers, we carried out RAPD analysis on the same set of 40 micropropagated plants and two independent samplings of the single donor plant of *R. ambigua* ‘idahoensis’, as detailed in an earlier study (Guo et al., 2006). A total of 269 clear and reproducible bands were scored, of which 70 were polymorphic among the samples. The number of amplified bands, sizes of the bands, as well as the level of polymorphism all varied substantially among the primers, suggesting the existence of a “hot spot” for genomic

changes (Fig. 1 and data not shown). For example, although primer R11 produced three bands that could be scored, R9 produced 15 (data not shown). Similarly, the percentages of polymorphic bands varied from 10% to 100%, with an average of 26% among the plants, suggesting a possible nonstochastic nature of genomic changes. A dendrogram generated by cluster analysis using the UP-GMA method based on Jaccard's coefficient of the RAPD markers indicated that the genetic similarity among the micropropagated plants ranged from 0.61 to 0.97. Thus, if at a similarity of 80% or higher, the plants could be divided into three distinct groups, with the donor plant being classified into the second group in both cases (Fig. 2).

Quality DNA sequences of 20 polymorphic bands were obtained and queried for homology against the Genbank database by BlastX analysis at the NCBI website. Results indicated that eight clones showed significant homology to known or predicted functional cellular genes or retrotransposons (Table 1). The cellular genes identified included a rice putative mitogen-activated protein (MAP) kinase, an *Arabidopsis* RNA-directed DNA polymerase homolog, and one putative protein, whereas the retrotransposons identified all belonging to the nonlong terminal repeat group (Table 1). Twelve of the 20 clones did not identify a match in the Genbank database (Table 1).

Discussion

Robinia ambigua var. *idahoensis* (Idaho locust), which probably resulted from a natural hybridization event between *R. pseudoacacia* L. and *R. hispida* L. or *R. viscosa* Vent., is an important horticultural plant widely used as street tree to overcome brittle wood and aggressive roots characteristic of its mother species, *R. pseudoacacia* L. Probably also

Fig. 2. Dendrogram derived from unweighted pair grouping method of averages cluster analysis using Jaccard's coefficient of similarity of the RAPD markers on 40 micropropagated plants (R1–R40) and two replicates of the single donor plant (donors 1 and 2) of *Robinia ambigua* var. 'idahoensis.'

because of its hybrid nature, Idaho locust is very tolerant to poor, dry, compacted soils, and is also famous in xeriscaping because of its drought and pest resistance (<http://canadagardens.com/2robiniaidahoensis.html>). Therefore, *R. ambigua* var. *idahoensis* is useful in reshaping xeric landscapes incited by pollution and overpopulation. In addition, it may have other uses, including ornamental and medicinal values. Thus, establishing an efficient micropropagation method may facilitate its utilization. Indeed, we have recently established such a method that can be used for massive propagation of *R. ambigua* var. *idahoensis* plants (Guo et al., 2006). Nonetheless,

an apparent concern for plants propagated by in vitro means is their genetic fidelity.

It is generally believed that somaclonal variations predominantly occur at the callus stage, which represents a highly stressful condition to a plant genome (Kaeppeler and Phillips, 1993; Larkin and Scowcroft, 1981), whereas micropropagation achieved by enhanced branching from preexisting primordial organs like shoot or axillary buds should largely preserve fidelity of the donor plants (Hao and Deng, 2003; Rahman and Rajora, 2001). Apparently, this general rule does not apply to *R. ambigua* var. *idahoensis*, because both ISSR (Guo et al., 2006) and RAPD

Fig. 1. (A, B) RAPD profiles of 40 micropropagated plants and two replicates of the single donor plant of *Robinia ambigua* generated by two primers R4 (A) and R39 (B). Black arrows denote de novo appearance of new bands whereas arrows refer to loss of original bands in the micropropagated plants relative to the donor plant. M is the GeneRuler™ DNA Ladder Mix (Fermantas, Hanover, Md.).

Table 1. Characterization of the sequenced clones underlying genomic changes in the 40 micropropagated plants of *Robinia ambigua* var. 'idahoensis'.

Clone ID	Primer	Size (bp)	Homology or protein function predicted by BlastX	Accession number and E value
XR3-1	R3: 5'-GGGCGCCTAG	510	No	
XR6-2	R6: 5'-GGGTGCGGTA	520	Putative nonLTR retroelement reverse transcriptase (<i>Arabidopsis thaliana</i>)	AC007730.3; 2E-11
XR9-2	R9: 5'-CCGAATCACT	1000	Hypothetical protein (<i>Olea europaea</i>)	Z70241.2; 2E-76
XR9-4	R9: 5'-CCGAATCACT	380	Putative retrotransposon [<i>Oryza sativa</i> (japonica cultivar, group)]	XM_470061.1; 1E-46
XR10-1	R10: 5'-CCCGCAGAGT	631	Putative nonLTR retroelement reverse transcriptase (<i>A. thaliana</i>)	AC005825.4; 3E-9
XR10-2	R10: 5'-CCCGCAGAGT	520	RNA-directed DNA polymerase homolog (<i>A. thaliana</i>)	T00833; 1E-09
XR11-1	R11: 5'-CCATCGTACC	670	No	
XR13-1	R13: 5'-CCAGATGCAC	499	No	
XR14-1	R14: 5'-TGCGGGTCTCT	1092	No	
XR20-3	R20: 5'-GGTGAACGCT'	621	No	
XR21-2	R21: 5'-GATGCCAGAC	540	SocE (<i>Myxococcus xanthus</i>)	AF263243.1; 7E-20
XR23-1	R23: 5'-CTACGCTCAC	701	No	
XR23-4	R23: 5'-CTACGCTCAC	413	No	
XR26-1	R26: 5'-CACTGGGCCCA	636	No	
XR35-2	R35: 5'-CTGGCGAACT	520	No	
XR39-1	R39: 5'-ACCTTTGCGG	714	OSJNBa0017B10.12 [<i>O. sativa</i> (japonica cultivar, group)]	AL606628.3; 1E-70
XR40-3	R40: 5'-CCTGCTCATC	610	No	
XR43-1	R43: 5'-AGACCCAGAG	562	No	
XR45-1	R45: 5'-GGCTGGTTCC	1248	Putative MAP kinase [<i>O. sativa</i> (japonica cultivar, group)]	AP004160.2; 1E-35
XR45-2	R45: 5'-GGCTGGTTCC	565	No	

LTR, long terminal repeat; MAP, mitogen-activated protein.

(current study) markers showed occurrence of genomic variations at a high frequency. Similar findings have also been made in some other plant species, including one of the putative progenitor species of *R. ambigua* (Bindiya and Kanwar, 2003; Modgil et al., 2005; Renau-Morata et al., 2005). In a sharp contrast, genetic stability was observed on micropropagated plants of a tetraploid cultivar of *R. pseudoacacia* L., also based on RAPD analysis (Shu et al., 2003). This may suggest that the occurrence and extent of genomic variation might be influenced by multiple factors, such as genotype of the donor mother plant or medium composition, as demonstrated in some cases (Devarumath et al., 2002; Modgil et al., 2005).

Although the RAPD marker has been widely used in detecting genomic variations in regenerated or micropropagated plants, reproducibility of this marker has been cautioned in some cases (Devarumath et al., 2002). However, the results of this study clearly showed that by using high-quality DNA and including appropriate controls for reproducibility, RAPD analysis is reliable and robust (Fig. 1). An added support to this conclusion is that very similar dendrograms have been generated based on genomic variations detected by two independent markers: RAPD (Fig. 2) and ISSR (Guo et al., 2006).

Because a large fraction of plant genomes is noncoding, it is important to know the kinds of sequences that underlie genomic variations. We showed in this study that of the 20 sequenced bands manifesting genomic changes, eight had significant homology to known or predicted cellular genes or retroelements. This implicates possible functional consequences of the genomic changes that occurred in the micropropagated *R. ambigua* var. *idahoensis* plants, although at the first generation no phenotypic variation was ob-

served among these plants (Guo et al., 2006). In any case, large-scale commercial propagation of this plant species will have to take into consideration of the occurrence of genomic variations and their associated morphological and physiological changes. On the other hand, the somaclonal genomic variations might be exploitable for breeding purposes in this plant.

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