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## Molecular cloning and sequencing of a polymorphic band from rubber tree [*Hevea brasiliensis* (Muell.) Arg.]: the nucleotide sequence revealed partial homology with proline-specific permease gene sequence<sup>†</sup>

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Rubber tree (*Hevea brasiliensis* Muell. Arg.) is mainly cultivated in high rainfall areas (traditional). Identification of DNA marker or genes could be highly useful to develop drought-tolerant clones or screen *Hevea* seedling populations, which will eventually extend the rubber cultivation in non-traditional areas (under irrigation). RAPD analysis is a valuable tool in studying genetic variation and for identification of specific gene fragments. Genomic DNAs from 37 clones were used for RAPD analysis by polymerase chain reaction (PCR), where the total and unique number of polymorphic bands varied with the clones and primers. Certain amplified bands appeared to be common to some clones, whereas others were present only in a few clones. RAPD markers specific to one or few clones were identified, with the results being reproduced at least three times. There were two DNA markers (1.2 and 1.4 kb) scored by presence vs absence of specific amplification products with OPB-12 primer. Of the two bands, a 1.4 kb RAPD marker was the most promising DNA fragment which was isolated, cloned and the nucleotide sequence determined and revealed certain homology with *Saccharomyces cerevisiae* proline-specific permease gene. Specific DNA technology, such as the use of SCAR (sequence characterized amplified region) markers, can be used to differentiate among *Hevea* clones to validate this RAPD marker. SCAR marker was created by sequencing a single RAPD band and designing primers to amplify the band of specific size. The SCAR primers amplify a band of 1.4 kb from only 14 among 37 clones used under more stringent PCR conditions. This SCAR marker could be used as genetic marker to screen the *Hevea* seedling populations for specific trait or gene from single PCR-based method.

**Keywords:** Cloning, DNA amplification, *Hevea brasiliensis*, polymerase chain reaction, RAPD marker, rubber tree.

RUBBER (cis-1,4-polyisoprene), an isoprenoid polymer with no known physiological function to the plant, is an

<sup>†</sup>The sequence described in this work has been deposited in the NCBI GenBank database under accession number AY130828.

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important raw material for many industrial uses requiring elasticity, flexibility and resilience. The only commercial source of natural rubber, at present is the Brazilian rubber tree [*Hevea brasiliensis* (Mull.) Arg.]. The rubber is harvested by tapping into the pipe-like network of latex-containing laticifers that run beneath the bark, a labour-intensive procedure. The expense of tapping and the tropical growth requirements of the tree make *H. brasiliensis* unsuitable in the United States and other cold countries. The diminishing acreage of rubber plantations and various stresses to *Hevea* coupled with an increasing demand have triggered research interests in the study of development of molecular markers for biotic and abiotic stresses. Rubber tree is known to have a narrow genetic base and cultivated varieties are clones propagated vegetatively by grafting. Moreover, as plantation-grown *H. brasiliensis* is derived from clonal material grafted onto seedling rootstocks, all plants of a commercial line are genetically identical to each other. The eventual incorporation of the material obtained into on going breeding programmes implies, concurrent with an agronomical evaluation, an estimation of the genetic variability present in these new clones compared with that of cultivated material. The genetics of rubber tree has been poorly understood. This lack of knowledge is due to the heterozygous nature of the crop, its long growing cycle that includes five years before latex collection and its low seed set. As a rule, rubber tree displays inbreeding depression, making it difficult to develop appropriate progeny for classical genetic studies<sup>1,2</sup>.

Although considerable progress has been made to increase rubber yield in *Hevea* in the recent past, satisfactory resistance to biotic and abiotic stresses has not been achieved because of limited genetic resources within the *Hevea* gene pool<sup>3</sup>. Molecular markers serve as useful aids in understanding the genetics of *H. brasiliensis*. They can play a useful role in genotype selection and hence facilitate and hasten the progress of *Hevea* breeding. Molecular markers such as isozymes, minisatellites, RFLP, AELP and RAPD have already been applied to the rubber tree to investigate the polymorphism between clones and varietal identification<sup>2,4-14</sup>. Among techniques for the detection of DNA polymorphism, PCR-based RAPD analysis is a relatively simple and efficient technique, where a small quantity of DNA is required to develop DNA fingerprints. DNA molecular markers are considered to be superior to examine the genetic relationships between clones because of the availability of a large number of potential polymorphic sequences. PCR amplification products are then resolved in gels and recorded by a variety of techniques. Presence or absence of DNA bands in the gel may be used as RAPD markers to study inter- and intra-specific genetic variation<sup>15</sup> for the identification of specific genes<sup>16,17</sup> and to study the pattern of gene expression<sup>18</sup>. These markers do not depend on environment and development. More recently, SCAR (sequence characterized amplified region) DNA analysis was developed to produce more specific and re-

producible results<sup>19,20</sup>. SCAR markers are created using a longer primer (extended sequence of a RAPD primer) that has a specific sequence of approximately 20 bases. By increasing the specificity, the results are less sensitive to changes in reaction conditions and are more reproducible<sup>21</sup>. Reliable SCAR markers have already been successfully derived from RAPD fragments in *Lettuca*, *Vicia*, *Triticum* and *Agrostis*<sup>19,21-23</sup>. Therefore, we have attempted to study genetic diversity within the cultivated *Hevea* clones using RAPD analysis. This communication presents our results on identification, cloning and sequence analysis of a polymorphic band partially encoded by a proline-specific permease protein gene sequence from *Hevea* and development of SCAR marker for PCR screening of *Hevea* clones.

Young leaves from 37 cones of *H. brasiliensis* were collected from the nursery as well as experimental field of the Rubber Research Institute of India (RRII), Kottayam and preserved at -80°C until DNA extraction.

Total DNA was isolated from individual clones as previously described<sup>14</sup>. DNA prepared from all clones was used for PCR analysis. RAPD variations among clones were assessed using DNA from individual clones. DNA was individually primed with different oligonucleotide primers synthesized by Operon Inc. (USA). In a 20 µl volume, 25 ng plant DNA, 25 pmol of the operon primer and 250 µM of dNTPs were mixed with 10x reaction buffer and 0.5 U of Taq DNA polymerase. Samples were amplified on a DNA thermal cycler (Perkin Elmer). After an initial denaturation at 94°C for 4 min, 35 cycles of 60 s denaturation at 94°C, 90 s annealing at 37°C and 2 min extension at 72°C were performed before a final extension of 7 min at 72°C. An aliquot of the PCR product was characterized by electrophoresis on 1.5% agarose gel to detect polymorphic bands. RAPD assays with each primer were performed at least three times each, with only reproducible, amplified fragments being scored. For each clone, the presence and absence of fragments was recorded as 1 or 0 respectively and treated as discrete characters. Faint RAPD bands were not included in this analysis.

To convert the selected RAPD band to a SCAR marker, the bands were excised, cloned and sequenced following the procedure outlined by Sambrook *et al.*<sup>24</sup>. DNA bands produced by all clones were considered as common bands, whereas bands produced by some but not all clones were considered uncommon bands (polymorphic bands). Selected uncommon DNA band was excised from agarose gel and the DNA was purified. The purified DNA was then cloned into *EcoRV* site of the pBS vector. The recombinant plasmid DNA was used to transform *Escherichia coli* (DH5α) competent cells. Selection of transformed clones was performed by blue/white selection. Positive colonies (white) were grown overnight in 10 ml of Luria Bertani (LB) liquid medium containing 50 µg/ml ampicillin. Plasmid DNA was purified from five independent transformed clones. The size of cloned DNA inserts was

checked by double digestion with *EcoRI* and *BamHI* enzymes and separated in a 1.5% agarose gel. Plasmid was also used for PCR amplification with T7 and T3 sequencing primers. The complete sequence of the cloned fragment was obtained using an automated DNA sequencer (Indian Institute of Science, Bangalore). The DNA sequence was analysed for homology in the GenBank database using the BLASTN program<sup>25</sup>.

The SCAR marker sequences were designed by identifying the original 10 bp sequence of the RAPD primer and adding the next approximately 10 bp in the DNA sequence. These SCAR primers were synthesized by Sigma, Bangalore. PCR amplification was achieved in 20 µl reaction as described above for RAPDs, except that (1) the RAPD primer was replaced by the specific primer pair (forward 5'-CCTTGACGCATGGAGGGGAA-3' and reverse 5'-CCTTGACGCACTTAATCCTC-3') and (2) thermal cycler conditions used an initial denaturation at 94°C (4 min), annealing of primers at 65°C (1 min) and primer extension at 72°C (2 min) followed by 30 cycles of 94°C (1 min), 65°C (1 min) and 72°C (1.30 min). A final elongation was performed for 7 min at 72°C to ensure a double-strand amplicon. SCAR marker was optimally amplified under various PCR programmes. PCR products were analysed by agarose gel (1.5%) electrophoresis. Presence and absence of the SCAR band was visually scored and compared with samples of each clone.

DNA isolated from 37 clones was used for the initial RAPD-PCR analysis. RAPD-PCR conditions have been optimized as previously described<sup>14</sup> to allow reproducible amplification of DNA. We screened 140 decamer primers to amplify the genomic DNA of *Hevea*. Many of them failed to amplify *Hevea* DNA. Of these, only 17 primers generated robust and reproducible bands. However, primer OPE-08 revealed monomorphic banding pattern for 37 clones. Finally, 16 primers were selected and used to analyse all 37 accessions for DNA polymorphism. All reactions were duplicated and only highly reproducible bands were considered for analysis. The base sequences of the primers and their G + C contents are given in Table 1. In this study, all the primers have a GC content >60% and the optional annealing temperature was 37°C. As expected, a considerable degree of DNA polymorphism was detected among the clones with 16 primers. Primer OPG-04 amplified the highest number of bands (22), while OPD-9 amplified only seven bands. Different number of bands was amplified in different clones. These primers differed greatly in their efficiency for revealing polymorphism. Since the reaction conditions were kept uniform for all primers, differences in the amplification resolution and clarity in the banding patterns were probably due to specific requirements of the primers. The significance of the G + C content of the primer on detectable amplification products has also been reported<sup>26</sup>. However, our results did not show any relation between the G + C content of the primer and the number of bands amplified. We found

that if the amplification conditions (reagents and thermal cycler parameters) were identical for all reactions, the results were highly reproducible. A similar result was also obtained by Sharma and Jana<sup>27</sup> in a study of the species relationships in *Fagopyrum*.

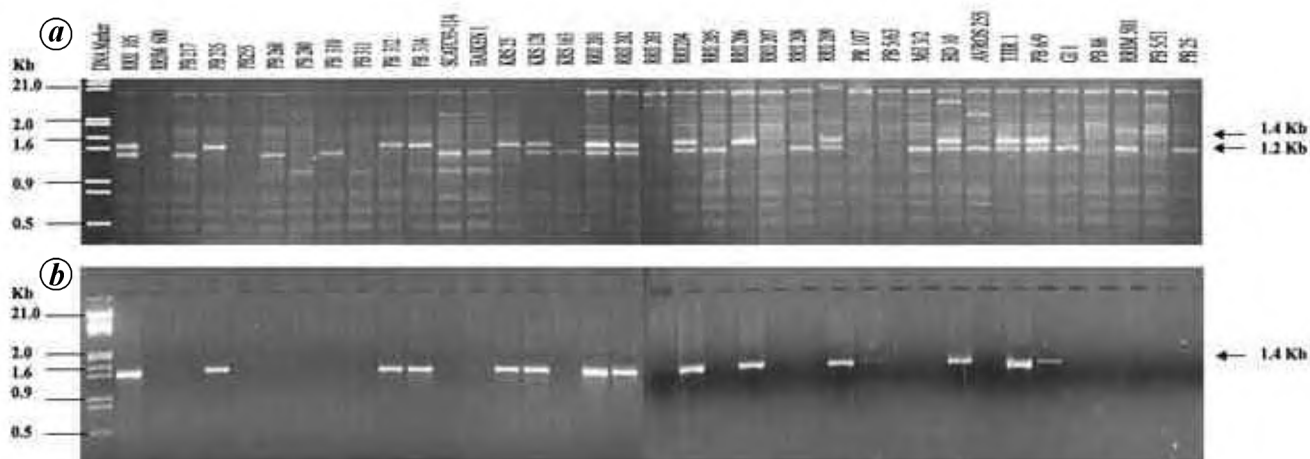
Results indicated that the primers amplified reproducible band patterns among 37 clones used. Different primers generated bands of variable intensity from different clones; however, most primers generated common bands. Primer OPB-12 yielded two recognizable uncommon bands with DNA from some of the clones, along with several common bands with each clone. The molecular size of the two uncommon bands was 1.4 and 1.2 kb (Figure 1a). Among the 37 clones used, a 1.4 kb polymorphic band alone was detected only in five clones (PB 235, PB 312, PB 314, KRS 25 and RRII 206), whereas 1.2 kb band alone was noticed in 13 clones (PB 217, PB 260, PB 310, SCATC 93-114, Haiken 1, KRS 163, RRII 205, RRII 208, Mil 3/2, AVROS 255, Gl 1, RRIM 501 and PB 25). However, it is interesting to note that both the 1.4 as well as 1.2 kb bands were amplified together in nine clones (RRII 105, KRS 128, RRII 201, RRII 202, RRII 204, RRII 209, BD 10, TJIR 1 and PB 6/9). On the other hand, these two uncommon bands were not amplified in the remaining 10 clones (RRIM 600, PB 255, PB 280, PB 311, RRII 203, RRII 207, PR 107, PB 5/63, PB 86 and PB 5/51; Table 2).

In the present study, both hybrid as well as primary clones (seedlings) were used for RAPD analysis. The RRIM 600 hybrid clone does not contain these polymorphic bands, but two bands were amplified with its parental clones (TJIR 1 and PB 86). Although the 1.2 kb polymorphic band was amplified in Mil 3/2 parent, this polymorphic band did not exist in RRII 203 hybrid clone. The 1.4 kb polymorphic band was present in the hybrid clone PB 235, which is one of the parents of the PB 311 clone and this clone does not produce both polymorphic bands. In the case of RRII 207 hybrid clone, the 1.2 kb polymorphic band was detected in both the parents. However, this band was not amplified in RRII 207 clone. The 1.2 kb band was obtained from RRII 205 clone, but the 1.4 kb amplicon was absent. However, one of the parents (BD 10) of the RRII 205 clone produced both polymorphic bands. These two polymorphic bands were not detected in some of the hybrids (PB 255, PB 5/63 and PB 5/51) as well as primary clones (PB 280, PR 107 and PB 86). Loss or absence of a polymorphic band in the clones could be due to deletion or translocations in the genome during chromosome rearrangements<sup>28</sup>. Our results indicate that both polymorphic bands were not detected in at least one of the parents of the respective clones.

In order to characterize these markers, we made an attempt to clone these two DNA markers. However, a 1.4 kb fragment was successfully cloned into the *EcoRV* site of pBluscript vector. Despite several attempts, cloning of the 1.2 kb fragment was unsuccessful. The recombinant plasmids were tested for authenticity of inserts by PCR

**Table 1.** DNA sequence of random decamer oligonucleotide primers used for *Hevea* DNA amplification, their G+C content and per cent polymorphism

Primer no.	DNA sequence 5' to 3'	Per cent G + C content	No. of bands produced	Per cent polymorphic bands
OPA-01	CAGGCCCTTC	70	13	30.7
OPA-04	AATCGGGCTG	60	21	33.3
OPA-07	GAAACGGGTG	60	10	50.0
OPA-16	AGCCAGCGAA	60	6	33.3
OPA-17	GACCGCTTGT	60	18	55.5
OPA-18	AGGTGACCGT	60	13	61.5
OPB-12	CCTTGACGCA	60	12	58.3
OPC-05	GATGACCGCC	70	12	50.0
OPD-09	CTCTGGAGAC	60	7	42.8
OPE-12	TTATCGCCCC	60	15	33.3
OPF-03	CCTGATCACC	60	16	37.5
OPF-10	GGAAGCTTGG	60	17	29.4
OPG-02	GGCACTGAGG	70	16	31.2
OPG-04	AGCGTGTCTG	60	22	45.4
OPG-08	TCACGTCCAC	60	13	46.1
OPG-10	AGGGCCGTCT	70	16	37.5

**Figure 1.** *a*, RAPD amplification products showing polymorphism. Genomic DNA from *Hevea* clones was amplified using OPB-12 primer. Arrow indicates positions of the two polymorphic bands at 1.4 and 1.2 kb. *b*, SCAR marker amplification products obtained using 20-mer primers. A band approximately 1.4 kb was amplified from 14 clones.**Table 2.** Details of presence or absence of polymorphic bands specific to different *Hevea* clones by RAPD assay

Size and presence or absence of polymorphic bands	<i>Hevea</i> clones produced by polymorphic bands
1.4 kb band amplified	PB 235, PB 312, PB 314, KRS 25 and RRII 206
1.2 kb band amplified	PB 217, PB 260, PB 310, SCATC 93-114, Haiken 1, KRS 163, RRII 205 RRII 208, Mii 3/2, AVROS 255, GI 1, RRIM 501 and PB 25
1.4 + 1.2 kb	RRII 105, KRS 128, RRII 201, RRII 202, RRII 204, RRII 209, BD 10
Both bands amplified	TJIR 1 and PB 6/9
1.4 + 1.2 kb	RRIM 600, PB 255, PB 280, PB 311, RRII 203, RRII 207, PR 107
Both bands not amplified	PB 5/63, PB 86 and PB 5/51

analysis using the OPB-12 primer and T7 as well as T3 sequencing primers. The cloned DNA fragment nucleotide sequence was determined from both directions. The sequence of the 1.4 kb RAPD marker is shown in Figure 2 and is available in NCBI GenBank database. This RAPD

marker contains 1418 bp sequences. It revealed several short open reading frames for protein translation on both strands (data not shown). A BLAST search revealed that this sequence showed some homology with proline-specific permease gene. The cloning and sequencing of DNA

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1  ccttgacgca tgagggggaa atggtgcaaa catgacggaa tcctcatatg aagatttaat
61  tatagaaatg aatcagctgg ttctgtactt taacgagatc ttctgtgatg cagtacaaga
121 agggatatata agtccatggg aatccaactt tgaaagtggg tggcatagct caggatgtcc
181 ttaccatgga gaaataacca ataatgagct ggaaaactac agcaaattta aagaagaaat
241 tttgagaatg ttatcaactca gaatcttgag gtgttaagtt aggtcgaaaa atagagaagg
301 aagtatatac agccagagat ggtgaggacg cctcaaaacta tactccaaag atggtcttct
361 ctgtgcctac caccocaaca ccaccacca gaacaattat ccaaactcca cccagctcc
421 cagtcaccaaa taccatgct attccttgga attataaatt tcagggtttta ctcaaagca
481 tcagcaaacac ttctactcct actctcaatg atacctacac tcaacctatc acaacccta
541 aaccatgttt taccacacat gtcattttta aaatgagttt tatgtgacct tctaccctt
601 ccaatgatac tacttcttag ccagcaccaa aacctattgc aattagtaac ccaccgccac
661 ttgatcaaga ggtagaattt atcactagga gtgggagatg ctatgggaat gatgaagcag
721 aaaagaaaaa agggaaagta aagggtgggag agtcgcctga gaatgtggag aaagatattg
781 aaaaagggga gtctagttaa aagaaagaag aggaggaatt gctattacaa atcataaagc
841 agagtgaata tgatgtgatt gaacaattaa gaaagacatt tactcaaatc tccttgctgt
901 cattgatact gagctcagaa gtgcatacac atgccttaca gaggggatca agcctttgtg
961 acttaagaca tcacaccggg acaattttga gaagatagtg ggacaaatcc aggcattctaa
1021 tttcatcact ttctotgaag atgaaataga cctggcaagc ttgaagcaca ccaaagctct
1081 tcatgtgaca atcaagtgtg aaggatgtat ggttactaaa gttttgatcg acaatggatc
1141 ggccctcaac atttttctta atgcagcctt ggcaagactc ccaattgact catctactat
1201 acgcgaaagt acaatgatag taagggcatt tgacggtaac aagagagata tactaggaga
1261 cactgagttg ccacttcaga ttggggcatg tacatttaac gttacattcc aggtgataga
1321 tattaacccg gcatacacta tgetgttagg aagaccctgg atctattcgg ctaatgcagt
1381 gccctcaact ctacaccaga ggattaagtg cgtcaagg

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**Figure 2.** Nucleotide sequence of an RAPD-PCR fragment obtained from *Hevea*. Regions underlined were used for development of SCAR marker presented in this study.

markers to design gene or trait-specific markers would be justified to obtain a rapid and efficient method for screening and identification of clones. Therefore, the SCAR marker development could also improve the reliability of the current RAPD marker linked to a specific gene and allele. Proline accumulation is a widespread response of higher plants, algae, bacteria and animals to low water potential<sup>29</sup>. Proline oxidation to glutamate in mitochondria is catalysed by P5C dehydrogenase and proline dehydrogenase (PDH). Both PDH activity and its transcript levels are rapidly and strongly decreased in response to water stress<sup>30</sup>. In the last decade, several attempts were made to increase the level of proline accumulation in plants by transferring the genes associated with the biosynthetic pathway<sup>31</sup>. Therefore, proline-specific permease gene could be involved in drought tolerance in plants. In order to study the drought stress regulation involvement of this gene, it is essential to isolate full-length cDNA and characterize the role of this gene from *Hevea*.

To validate this RAPD marker, two SCAR primers were designed from the sequenced 1.4 kb DNA marker. Each primer was designed 20 bp long. With this primer set, a different PCR programme was optimized for ideal SCAR amplification products. The annealing temperature and number of PCR cycles had the greatest effect on the number and intensity of PCR products, i.e. lower annealing temperatures produced more PCR products, whereas more PCR cycles produced more intense bands. After 30 thermocycles, the primer pair yielded the predicted size of 1.4 kb amplicon with 14 clones, but not with the remaining 23 clones used in this study (Figure 1 b). This result con-

firms that the amplified SCAR marker is confined to specific gene locus of the *Hevea* genome and not artifacts. Similar results were also noticed earlier by Vidal *et al.*<sup>22</sup> in grapevine and Scheef *et al.*<sup>23</sup> in bentgrass. The SCAR marker designed in this study could be used for initial screening of *Hevea* seedlings taken from naturalized populations, identifying drought-susceptible vs tolerant seedlings, and potentially for identifying their natural hybrids with drought tolerance using reverse genetics approach (DNA sequence or gene to trait). More recently, breeders have been using interspecific hybridization to combine important traits<sup>23</sup>. Hybridizations of different species can be useful for incorporating traits such as disease or drought resistance into new clones/cultivars, making it critically important to identify the hybrids. This designed SCAR marker has the potential for identifying and determining which offspring are true hybrids.

In conclusion, this study provides information about the identification and characterization of RAPD marker with certain homology to proline-specific permease gene. However, it is necessary to isolate full-length gene for further characterization. This is the first report of the isolation, cloning and nucleotide sequence analysis of partial proline-specific permease gene fragment from *Hevea* plant genome. Morphological and agronomical evaluation of *Hevea* clones showed significant variations for several traits. The clones studied here are also well differentiated from one another with respect to such characteristics as tolerance to drought, wind damage, diseases and latex yield. Molecular markers are equally important to select better clones for future breeding or cultivation purposes. Sequencing

data confirm the reliability of this DNA marker among clones as determined by RAPD analysis. Further, development of SCAR marker may provide useful information to determine trait-specific markers for abiotic stress tolerance, including drought tolerance in *Hevea*.

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