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DETECTION AND ISOLATION OF GOOD QUALITY DNA FROM THE LATEX OF *HEVEA BRASILIENSIS*

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The laticiferous system of natural rubber (NR) *Hevea brasiliensis* has abundant transcripts of several genes, mainly those involved in rubber biosynthesis and plant defense. So far no detailed study to understand the mechanism behind this transcript abundance has been made. There is little evidence for the presence of DNA or active nuclei in large numbers in the latex expelled during tapping. There is also no protocol available for the isolation of genomic DNA from NR latex which contains large amounts of DNase. The present study explore the presence of genomic DNA in *Hevea* latex expelled on tapping and isolation of the same following a modified CTAB based DNA isolation protocol. Initially, the whole latex was treated with CTAB buffer and DNA isolation was carried out. Further different fractions of latex after seperated by centrifugation. High molecular weight DNA was obtained from the bottom fraction. No DNA was obtained from the whole latex or from the serum fraction of any of the treatments. The quality and concentration of the isolated DNA was better when latex was pre-treated with a latex specific DNA isolation buffer and the DNA was isolated from the bottom fraction. The DNA isolated was fully digested with DNase1 treatment. When this DNA was used as template for PCR using hevein gene specific primers, the amplified products were similar to those amplified from leaf DNA. This is the first report on detection of genomic DNA from NR latex and developing a successful protocol for the isolation of good quality genomic DNA from the latex of *Hevea brasiliensis.*

Keywords: DNA isolation, *Hevea brasiliensis*, Natural Rubber latex, PCR amplification

INTRODUCTION

Natural rubber (NR) is produced in the latex vessels present in the bark of the rubber tree, *Hevea brasiliensis* (Kekwick, 1989). NR latex is a milky cytoplasm which is exuded from specialized cells called latex vessels or laticifers. The detailed ultra structure of *Hevea* latex vessels indicates that the expelled latex is part of a specialized hydrated and progressively degenerating cytoplasm (Auzac and Jacob, 1989). The latex vessels of *H. brasiliensis* are specialized cells derived from the fusiform initials of the cambium (De-Fay *et al*., 1989) and they contain the machinery to convert simple sugars into rubber particles (cis-1,4-polyisoprene) (Chow *et al*., 2007), which is about 35% of the latex.

Being cytoplasm, NR latex contains several gene transcripts with abundance of those involved in rubber biosynthesis and plant defense genes (Kush *et al*., 1990; Chow *et al*., 2007; Ko *et al*., 2003). While several cellular organelles are seen in the latex, there

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is general agreement that active nuclei are scanty in it. It is said that the nuclei stick to the walls of the laticifers and they were found only occasionally in the tapped latex (De-Fay *et al*., 1989). Although, abundance of different types of RNA and protein synthesizing machinery is reported from *Hevea* latex, so far no genomic DNA was detected and isolated from NR latex. Thus the question of the source of the abundant mRNAs in the latex arises. The present study attempted to ascertain the presence of genomic DNA in the latex and to explore the possibility of isolation of good quality DNA from fresh latex. Extensive optimization experiments were also carried out to obtain good quality DNA in good concentration. Attempts were also made to PCR amplify specific gene sequences from the latex DNA and compare with the same sequence amplified from leaf DNA.

MATERIALS AND METHODS

Latex DNA isolation

DNA isolation from latex was attempted following the CTAB protocol reported by Doyle *et al.* (1990) after suitable modifications. Fresh latex was collected from *H. brasiliensis* (Clone RRII 105) plants growing in the experimental garden of Rubber Research Institute of India. The latex expelled on tapping was either collected directly into centrifuge tubes kept in ice or in tubes containing different buffers after leaving a small volume of initial flow. The collected latex was immediately brought to the laboratory and DNA isolation was carried out. Extensive optimization experiments were carried out for the release of DNA without degradation. Further, DNA precipitation steps followed were similar in all the experiments. Following are the different experiments carried out for the release of DNA.

Experiment 1. Fifteen ml latex was collected directly in 50 ml oak ridge tubes kept in ice and added different volumes (5, 10 or 15 ml) of CTAB buffer (2% CTAB, 1.4 M Na Cl, 20 mM EDTA, 0.1M Tris-HCl (pH 8.0), 1% polyvinyl polypyrollidone and 0.1% β- mercaptoethanol). The tubes were mixed thoroughly and incubated at 60^0C for 30 minutes, centrifuged at 10,000 rpm at room temperature and the middle serum fraction was taken for DNA isolation treatment (T 1.1). The middle serum fraction obtained after centrifugation of fresh latex at 10,000 rpm at 4^0C was also used either directly (T 1.2) or after CTAB treatment for DNA isolation (T1.3).

Experiment 2. Fifteen ml latex was collected in 50 ml oak ridge tube containing 15 ml of 0.2 M Tris buffer (pH 8.0), mixed well and centrifuged at 10,000 rpm for 30 min. The bottom fraction from each tube was re-suspended in 5 ml Tris buffer and added different volumes (2.5, 5.0 or 10 ml) of CTAB buffer (T 2.1, T 2.2 and T 2.3). The tubes were mixed and incubated at 60^0C for 30 minutes. The samples were centrifuged at 10,000 rpm and the supernatants were used for DNA isolation. The middle serum portion was also either directly used for DNA isolation (T 2.4) or mixed with equal volume of CTAB buffer (T 2.5), centrifuged at 10,000 rpm and the supernatants were used for DNA isolation.

Experiment 3. Fifteen ml latex was directly collected in 50 ml oak ridge tube containing 15 ml of a) 0.2 M Tris buffer (pH 8.0) containing 1.0 M mannitol (T 3.1), b) 0.2 M Tris buffer containing 1.0 M mannitol and 0.08 M EDTA (T 3.2) and c) 0.2 M Tris buffer

containing 0.5 M mannitol and 0.08 M EDTA (T 3.3). The contents of the tubes were mixed and centrifuged at 10,000 rpm for 30 minutes. The bottom fraction was re-suspended in 10 ml of the respective 0.5X buffers and centrifuged. The pellet was again suspended in 5 ml 0.5X of the respective buffers and further treated with CTAB buffer and incubated at 60ºC for 30 minutes, centrifuged at 10,000 rpm and used for DNA isolation.

The supernatant from the previous experiments were transferred to a new tube and treated with equal volume of Tris-saturated phenol-chloroform-isoamyl alcohol (25:24:1) and mixed by gentle inversions. The samples were spun at 10,000 rpm for 10 min. and the aqueous phase was transferred to a new tube. This process was repeated once again. RNA in the samples was removed by incubating at 37°C for 1 h after the addition of 25 µl of DNase free RNase (10 mg/ml, Sigma, USA). Proteinase K (20 mg/ml, Bangalore Genei) was added and incubated for another 1 h at 37°C to inactivate the RNase and other residual proteins. Equal volume of chloroform: isoamyl alcohol (24:1) was added to the sample, mixed gently and centrifuged at 8000 rpm for 10 min at room temperature. To the aqueous phase saved, 0.6 volume ice-cold isopropyl alcohol was added to precipitate the DNA. The tubes were kept in ice for 10 min and the precipitated DNA was pelleted by centrifuging at 3000 rpm for 5 min at 4°C. The pellet was washed twice in 70% ethanol. The pellet was air-dried and suspended in TE (10:1) buffer.

The quality and quantity of appropriately diluted DNA samples were checked in a UV spectrophotometer (Beckman, USA). The quality was checked by measuring the ratio of absorbance at 260 nm and 280 nm (260/280). Agarose gel electrophoresis was carried out following standard protocol (Sambrook *et al*., (2001) and DNA band was viewed under UV trans - illuminator and documented using EDAS 290 (Electrophoresis Documentation and Analysis System- Kodak, USA)

PCR amplification and characterization of genomic sequences coding for hevein protein from latex DNA

Hevein is a chitin binding lectin like protein involved in the coagulation of latex leading to plugging of latex vessels after tapping. Hevein protein also inhibits the proliferation of various chitin binding fungi. High level expression of this gene is observed in the latex vessels. For the amplification of hevein gene, primers were designed based on a previously reported cDNA sequence (Genbank accession No: M36986). The sequences of the designed primers were as follows: Forward primer: 5'GGAAGAGTTAT-GTTTATATT3' and Reverse primer: 5'CATACATCCAATCCAATGT3'. PCR was performed in 20 µl reactions which contained 1X buffer having 50 mM KCl and 1.5 mM $MgCl_2$ (pH 8.3), 100 µM each of dNTPs, 0.5 units of Taq DNA polymerase (M/s Bangalore Genei, India), 20 ng of template DNA and 250 nM each of forward and reverse primers. The reaction mixture was overlaid with mineral oil. The amplification was carried out in a thermal cycler (Perkin Elmer 480, USA). The PCR profile consisted of an initial denaturation at 94 ^{0}C for 3 minutes followed by 36 cycles with denaturation at 94 ^{0}C for 1 minute, annealing at 55^{0}C for 1 minute and an extension at 72^{0}C for 2 minutes. The final extension was carried out for 10 minutes.

Amplified products were separated on 1.5% agarose gel and the DNA band was eluted using the DNA gel band purification kit (M/S Amersham Pharmacia Biotech, USA), following the manufacturer's instructions. The purified fragments were ligated into TOPO –TA® cloning plasmid vector (M/S Invitrogen Life Technologies, USA). The ligated plasmids were transferred into *E. coli* cells (DH5∝) supplied along with the kit and plated onto LB agar plates containing 50 µg/ml ampicilin and 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 40 µg/ml 5-bromo-4-indolyl-β-d-lacto-pyranoside (X-Gal) for blue-white screening. The presence of the insert was confirmed by selecting a few recombinant colonies after 16 hours of incubation and colony PCR was carried out to identify the positive clones. Plasmids were isolated from positive colonies using the Perfect prep Plasmid mini kit (M/S Eppendorf, USA). The confirmation of ligated plasmids was done through PCR using hevein gene specific primers. The nucleotide sequence of the cloned DNA fragment was determined at Macrogen, Korea. The sequence was compared with published sequences in the NCBI database using blastn programme (Altschul *et al*., 1990). Sequence alignment was also made using the Clustal W (Thomson *et al*., 1994).

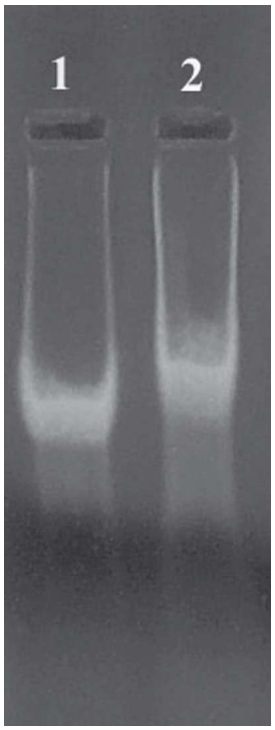

Fig. 1.

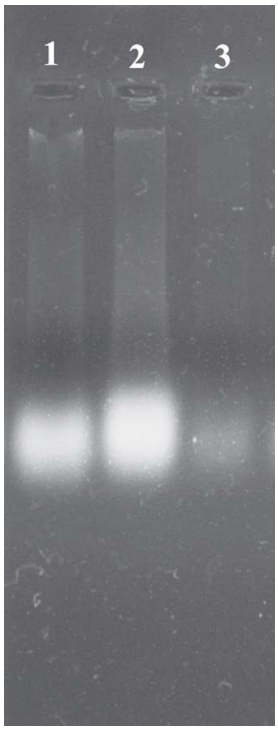

Fig. 2.

Fig. 1. DNA isolated by direct treatment of latex with CTAB buffer; Lane 1 & 2 shows no DNA bands

Fig. 2. DNA isolated from the bottom fraction collected after diluting latex with equal volume of 0.2 M tris buffer. Lane 1- bottom fraction treated with one vol. of CTAB buffer; Lane 2- treated with 0.5 vol. of CTAB buffer; Lane 3- treatment with 2.0 vol. of CTAB buffer

RESULTS AND DISCUSSION

It is reported that latex of many plants has high DNase activity. Since DNA was not isolated or detected from *Hevea* latex so far, extensive optimization experiments were carried out to isolate good quality and quantity of DNA from fresh latex. Initially DNA isolation was attempted by directly treating fresh latex with equal volume of CTAB buffer following the protocol reported by Doyle *et al.*, (1990) (T 1.1). When the precipitate obtained in this experiment was dissolved in 200 µl TE buffer and even after loading 20 µl in a gel, no detectable DNA band was observed (Fig.1). However, some sort of fluorescence was observed in the ethidium bromide gel away from the well. Such fluorescence remained even after treating the sample with DNase indicating that the glow observed is not due to the presence of DNA in the sample. DNA was also not observed in the serum fraction obtained after centrifugation of whole latex (T1.2 & T1.3). Further, DNA isolation was attempted using the bottom fraction and the

middle fraction obtained after centrifugation of fresh latex (T 2). In these experiments the latex was diluted with equal volume of 0.2 M Tris buffer (pH-8.0). After centrifugation the bottom fraction was re-suspended in 5.0 ml of the same buffer (0.1 M) and different volumes of CTAB buffer were added followed by DNA isolation. Fade bands could be observed near to the well when the re-suspended bottom fraction was treated with either 0.5 vol. or 1 vol. of CTAB buffer (T 2.1 & T 2.2) (Fig. 2). When the bottom fraction was treated with 2 vol. of CTAB (T 2.3) no DNA band was observed (Fig. 2 Lane 3). No detectable band could be observed in the serum fraction under treatments T 2.4 and T 2.5 also.

Since DNA was obtained from the bottom fraction, attempts were continued to refine this procedure. In the dilution buffer, mannitol and EDTA were provided (T 3). Mannitol was given as an osmoticum to prevent bursting of nucleus or any other organelles containing DNA. EDTA was supplied to prevent DNase activity if any, present in the latex. In these experiments DNA of good quality and quantity was obtained from the bottom fraction. Among the different treatments in experiment 3, the quality of DNA was better when the latex was pre-treated with 0.2 M Tris-HCl buffer containing 1.0 M mannitol and 0.08M EDTA and the bottom fraction was suspended in 0.5X of the same buffer (T 3.2; final concentrations of 0.5 M mannitol and 0.04 M EDTA). Among the different protocols tried, this protocol was found to be the most suitable (Fig. 3). The quantity of DNA obtained through this protocol was about 2.72 µg/ ml of latex. To confirm whether the band observed in the gel is DNA itself, the isolated product was treated with DNase1 and also PCR amplified with gene specific

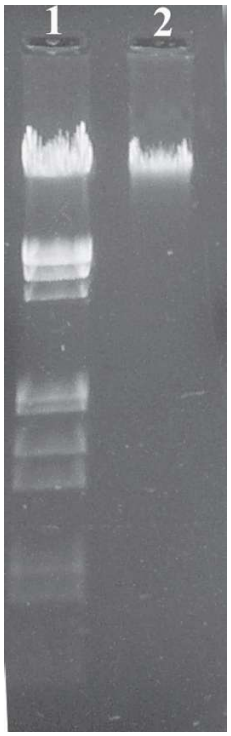

Fig. 3.

Fig. 4.

Fig. 3. Latex DNA isolated from bottom fraction collected after diluting the latex with dilution buffer containing in 0.2 M Tris buffer containing 1.0 M mannitol and 0.08 M EDTA. Lane 1- Mol. Wt. marker (λ DNA Eco R1, Hind3 double digest); Lane 2- isolated DNA

Fig. 4. DNase1 treatment of latex DNA. Lane 1- genomic DNA isolated from latex; Lane 2- genomic DNA from latex after overnight digestion with DNase1

primers. When 5.0 µg of DNA was treated with 1.0 µl of DNase1, the DNA was completely digested by incubating overnight (Fig.4).

When PCR was carried out with hevein gene specific forward and reverse primers using 20 ng of latex DNA isolated from the clone RRII 105 as template, two bands were observed in close proximity. Similar bands were amplified earlier when leaf DNA was used as the template. The two bands amplified with latex DNA were eluted separately from the gel (2% agarose gel),

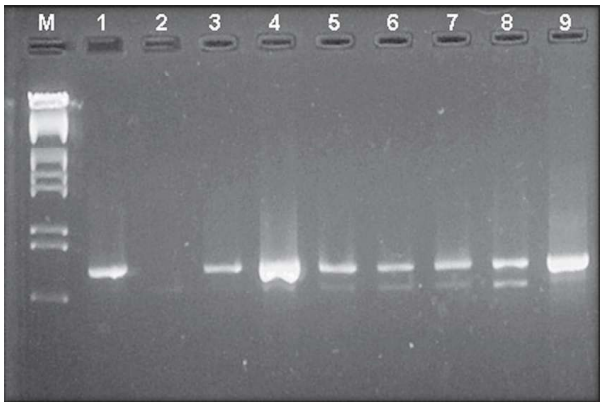

Fig. 5. Hevein gene amplified from latex DNA. Lane M- Mol. Wt. marker (λ DNA Eco R1, Hind3 double digest); Lanes 1-9, Latex DNA showing hevein gene amplification

cloned and sequenced. Sequence data revealed that the large fragment contained 748 nucleotides and the small fragment contained 645 nucleotides. When the two sequences were aligned with the sequences obtained earlier from leaf DNA through clustal W, 100% homology was observed. The 748 bp sequence contained an intron of 103 bp as observed in the genomic DNA amplified with hevein gene specific primers using genomic DNA isolated from the leaf. Except for the region of intron this sequence showed 100% homology with the 645 bp sequence, which emphasize that the 645 bp is an intron less genomic form of hevein gene (Fig.6). Two isoforms of hevein gene, one with an intron and another without an intron have been reported earlier from leaf DNA (Saleena, 2009)

No information is available regarding the source of templates for the transcript abundance of several genes in the laticiferous system of *Hevea*. Latex regeneration must be substantially active to compensate the loss of cellular materials through tapping. According to several investigators, presence of nuclei and mitochondria was very scanty in the *Hevea* latex collected upon tapping. It is suggested that they adhere to the plasma lemma while the latex is oozing out. Retaining nuclei and mitochondria within the latex vessels helps regeneration of latex between successive tappings. According to Dickenson (1969) nuclei persisted initially in their original positions inside the laticifers and as maturation proceeds, they become progressively displaced and confined towards the peripheral regions of the vessel, eventually to become vestigial. Since the nuclei degenerated progressively during the maturation of the vessels, they became increasingly infrequent and finally disappeared altogether (D'Auzac, 1989). Therefore, nuclei are extremely rare in the latex collected from mature trees. This hypothesis is not valid since genomic DNA and transcript abundance of several genes are present in the latex of mature trees also. Since DNA could be observed only in the bottom fraction of the whole latex while centrifuging the DNA might be present in active nuclei which are suspended in the latex.

A positive relation between the total nucleic acid content and rate of latex regeneration was observed by Tupy (1969). The rate of nucleic acid synthesis, particularly mRNA in the latex vessels may increase with increased rubber production. The DNA in the tapped latex may originate from the nuclei which are occasionally expelled from vessels along with the latex. There is a greater chance of nuclear bursting as they are exposed to a sudden depletion in turgor pressure during tapping. Intact latex vessels are under high turgor pressure (10-15 atm). Latex extraction by wounding the bark around half the circumference of the tree trunk reduces the turgor pressure of latex vessels in the drainage area during the initial period of latex flow (Pakianathan *et*

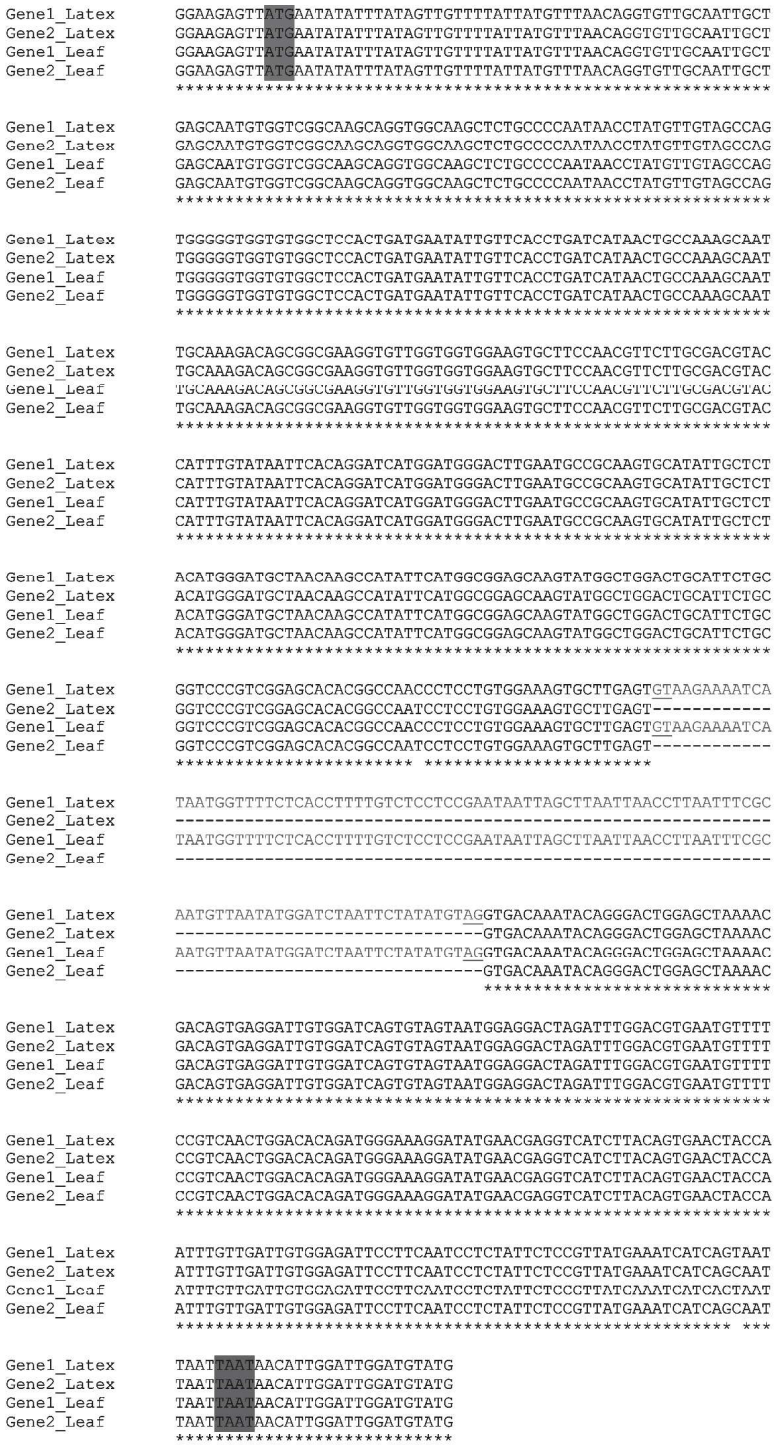

Fig. 6. Nucleotide sequence comparison of hevein gene sequences from latex DNA with sequences obtained from leaf DNA. Gene1_Latex- hevein genomic sequence with introns isolated from latex DNA; gene2_latex- hevein genomic sequence without introns isolated from latex DNA; Gene1_leaf - hevein genomic sequence with introns isolated from leaf DNA, Gene2_leaf - hevein genomic sequence with out introns isolated from leaf DNA, hevein genomic sequence with introns isolated from leaf DNA,Translation initiation codon (ATG) is given in green; Intron sequence in red with splice donor & acceptor are underlined and stop codon shaded in red

al., 1975). This combined with high levels of DNase activity in the latex and that may prevent the easy isolation of good quality DNA from whole latex. Later, latex collection as well as re-suspension of the bottom fraction in buffers containing osmoticum might have protected the nuclei from bursting. The presence of EDTA also helped protecting from DNase activity leading to isolation of good quality high molecular weight DNA.

In order to understand the transcript abundance in latex, it is essential to undertake an investigation on how enough DNA templates is made available in accordance with the high level of transcription after each tapping. Earlier investigators even suggested that the site of transcript synthesis may be remote from the latex of the mature vessel system (Dickenson, 1965). It is generally accepted that the latex yield is limited by the duration of latex flow after tapping and the capacity of laticiferous system to regenerate latex (D'Auzac, 1982). An interesting feature of latex production in *H. brasiliensis* is the increase in metabolism in the laticifers in response to tapping. The *in situ* latex regeneration mechanism allows the regeneration of the expelled latex before next tapping. There may exist an efficient mechanism for nuclei/DNA replication in the latex vessels to reconstitute the loss due to tapping. The current attempt of isolation and characterization of high molecular weight latex DNA opens a new avenue towards the study of molecular mechanisms of latex regeneration and associated gene expression. Latex also can be used for DNA isolation for various downstream applications when leaves are not available due to disease or wintering.

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