A novel cDNA from Parthenium argentatum Gray enhances the rubber biosynthetic activity \textit{in vitro}*

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Abstract

Natural rubber (cis-1,4-polyisoprene) is an isoprenoid compound produced exclusively in plants by the action of rubber transferase. Despite a keen interest in revealing the mechanisms of rubber chain elongation and chain length determination, the molecular nature of rubber transferase has not yet been identified. A recent report has revealed that a 24 kDa protein tightly associated with the small rubber particles of \textit{Hevea brasiliensis}, therefore designated small rubber particle protein (SRPP), plays a positive role in rubber biosynthesis. Since guayule (\textit{Parthenium argentatum} Gray) produces natural rubber similar in size to \textit{H. brasiliensis}, it is of critical interest to investigate whether guayule contains a similar protein to the SRPP. A cDNA clone has been isolated in guayule that shares a sequence homology with the SRPP, thus designated guayule homologue of SRPP (GHS), and the catalytic function of the protein was characterized. Sequence analysis revealed that the GHS is highly homologous in several conserved regions to the SRPP (50% identity). \textit{In vitro} functional analysis of the recombinant protein overexpressed in \textit{E. coli} revealed that the GHS plays a positive role in isopentenyl diphosphate incorporation into high molecular weight rubbers as SRPP does. These results indicate that guayule and \textit{Hevea} rubber trees contain a protein that is similar in its amino acid sequence and plays a role in isopentenyl diphosphate incorporation \textit{in vitro}, implying that it contributes to the enhancement of rubber biosynthetic activity in rubber trees.

Key words: Guayule, \textit{Hevea brasiliensis}, IDP incorporation, \textit{Parthenium argentatum}, rubber biosynthesis, rubber particle protein.

Introduction

Among more than 2000 plant species known to produce natural rubber (cis-1,4-polyisoprene), the Brazilian rubber tree (\textit{Hevea brasiliensis}) is the only commercial source at present (Backhaus, 1985). The diminishing acreage of rubber plantations, an increasing demand, and the life-threatening latex allergy to \textit{Hevea} rubber have prompted research interests in the development of alternative rubber sources. In recent years, guayule (\textit{Parthenium argentatum} Gray), which accumulates rubber in the parenchyma cells and contains high molecular mass rubbers comparable to \textit{H. brasiliensis}, has attracted research interest as an additional source for natural rubber (Bowers, 1990; Mooibroek and Cornish, 2000). In spite of some limitation as an alternative rubber crop due to its slow volume growth and low abundance of rubber particles, guayule has been proposed as a viable commercial alternative for hypoallergenic latex (Mooibroek and Cornish, 2000).

Despite its importance as a low material in manufacturing varieties of industrial and medical products, the molecular mechanism of rubber chain elongation and chain length determination in plants have not been studied in detail. A series of efforts has been given to isolate and characterize key enzyme(s) involved in rubber biosynthesis in \textit{H. brasiliensis} (Dennis and Light, 1989; Light and Dennis, 1989; Attanyaka et al., 1991; Goyvaerts et al., 1991; Cornish, 1993; Oh et al., 1999), guayule (Benedict

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Abbreviations: CPT, cis-prenyltransferase; FDP, farnesyl diphosphate; GHS, guayule homologue of SRPP; IDP, isopentenyl diphosphate; PAGE, polyacrylamide gel electrophoresis; SRPP, small rubber particle protein; WRP, washed rubber particle.
et al., 1990; Cornish and Backhaus, 1990; Backhaus et al., 1991; Cornish et al., 1994; Siler and Cornish, 1994; Pan et al., 1995), *Ficus elastica* (Siler and Cornish, 1993; Cornish and Siler, 1996), *Ficus carica* (Kang et al., 2000a), and *F. benghalensis* (Kang et al., 2000b). However, the function and role of the proteins suggested to be involved in rubber biosynthesis in these reports remain to be verified. Rubbers are synthesized by the sequential condensation of isopentenyl diphosphate (IDP) to the initiating allylic diphosphates such as geranyl diphosphate, farnesyl diphosphate (FDP), and geranygeranyl diphosphate. Rubber transferase (EC 2.5.1.20) or its complex with additional factors is believed to catalyse the elongation of rubber chains and ultimately to determine the rubber chain length in plants. In order to understand what determines the size of rubber in rubber-producing plants that synthesize a defined molecular mass of rubber depending on the plant species, it is of critical importance to investigate and characterize the enzymes involved in rubber biosynthesis in different rubber-producing plant species.

The authors were interested in identifying and characterizing the enzymes, especially rubber transferase, involved in the chain elongation of natural rubber. In recent years, a gene from *H. brasiliensis* encoding a protein tightly associated with small rubber particle protein has been cloned and, therefore, designated small rubber particle protein (SRPP) (Oh et al., 1999). An *in vitro* rubber biosynthesis assay with the recombinant protein overexpressed in *E. coli* revealed that the SRPP plays a positive role in rubber biosynthesis. If the SRPP is a protein that controls the molecular size of rubber in *H. brasiliensis*, it is considered that other plant species producing a similar size of rubber to *H. brasiliensis* should contain the protein homologous to the SRPP. Guayule is a desert shrub that accumulates rubber in the parenchyma. Guayule is a species. It is homologous to those of the SRPP and the proposed homologue of SRPP (GHS). The deduced amino acid sequence was similar to the SRPP and the proposed rubber elongation factor (REF) that is abundantly associated with *Hevea* rubber particles (Dennis and Light, 1989; Attanyaka et al., 1991; Goyvaerts et al., 1991). An *in vitro* analysis of the recombinant protein overexpressed in *E. coli* revealed that the GHS plays a positive role in rubber biosynthesis, similar to the SRPP. The sequence and catalytic nature of the GHS were compared with those of the SRPP, and the possible role of the GHS in rubber biosynthesis was proposed.

**Materials and methods**

**Screening of the cDNA library**

A guayule cDNA library was provided by R Backhaus at Arizona State University, USA. From the amino acid sequence information of the SRPP and REF, degenerate PCR primers were designed corresponding to the sequences from 187 to 206 bp (upstream primer: 5’-GGICCI(T/C)TNA(A/G)CCGNGT-3’, where N=any nucleotide, I=inosine), and from 638 to 657 bp (downstream primer: 5’-GGIGGIA(A/G)NA(A/G)IGGIA(A/G)A/GTA-3’, where N=any nucleotide, I=inosine) in the SRPP. These primers were used to amplify target DNA from the guayule cDNA library. PCR was performed for 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 2 min at 72 °C, with a 5 min pre-heat and a 10 min final extension at 72 °C. The PCR product was used to screen 5×10³ plaques of the cDNA library. The cDNA clones hybridized to the probe were subjected to *in vivo* excision according to the protocol provided by the cDNA library kit (Stratagene). One clone carrying a full-length cDNA insert was designated as pGHS and chosen for sequencing and functional analysis.

**Sequencing of the cDNA clone**

Plasmid DNA for sequencing reactions was prepared by the alkaline lysis method (Sambrook et al., 1989) using Qiagen Spin Miniprep Kit (Qiagen). The sequencing reaction was performed with the ALFexpress AutoRead Sequencing kit (Amersham Pharmacia Biotech) using the fluorescent dye-labelled M13 universal or reverse primer (provided by the kit). The nucleotide sequences were obtained by electrophoresis on an ALF automatic sequencer (Perkin Elmer Co.).

**Heterologous expression and purification of the GHS protein in E. coli**

The GHS gene was cloned in the *HindIII-XhoI* site of pET-22b(+) (Novagen) to construct pET-GHS. The *E. coli* BL21 (DE3) transformed with pET-GHS was grown to mid-stationary phase in Luria Broth (LB) containing 50 mg l−1 ampicillin at 37 °C with vigorous aeration. The cultures were induced by adding the isopropyl β-D-thiogalactoside to a concentration of 1 mM and then incubated for another 5 h at 30 °C. All subsequent steps were carried out at 4 °C. The cells were harvested, washed with cell lysis buffer (50 mM TRIS-HCl, pH 8.0, 300 mM NaCl, and 10 mM imidazole) by centrifugation (5000 g, 10 min), and then disrupted by sonication. The Triton X-100 was added to a final concentration of 1%, and the lysate was mixed gently for 40 min to aid solubilization of the protein. The homogenate was centrifuged at 15 000 g for 20 min and the supernatant was subjected to further purification. Purification of the GHS-His-tag fusion protein was performed using the Qiagen purification system according to the manufacturer’s protocol. After extensive washing of the unbound protein with 40 mM imidazole, the GHS protein was eluted with 250 mM imidazole. All expression and purification were monitored by SDS-PAGE on 12% gel analysis of the bacterial extracts and recombinant proteins.

**Western blot analysis**

The recombinant GHS protein was separated by SDS–12% PAGE. The gels were subsequently stained with Coomassie Blue or used for western blotting where the proteins in the gel were transferred to a polyvinylidene difluoride membrane. For the detection of GHS-His-tag fusion protein, the membrane was incubated with the buffer containing Penta Anti-His antibody (Qiagen). The membrane was also incubated with the buffer containing the polyclonal rabbit antibody raised against the SRPP from *H. brasiliensis*. After three cycles of washing with TBS-T buffer or PBS-milk, respectively, the membrane was incubated for 1 h with anti-IgG antiserum conjugated...
to horseradish peroxidase (Amersham Pharmacia Biotech). After a further three cycles of washing with the same buffer, the proteins on the membrane were detected by an enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech).

**In vitro rubber biosynthesis assay**

The recombinant protein was incubated in 50 μl of reaction mixture containing 100 mM TRIS-HCl, pH 7.5, 80 μM [14C]IDP (55 mCi mmol⁻¹; Amersham), 20 μM FDP, 1 mM MgSO₄, 1 mM DTT, and indicated amounts of washed rubber particle (WRP) and recombinant enzyme solution at 25 °C for 5 h. Washed rubber particles were prepared by the repeated centrifugation/floatation procedure as described (Cornish and Backhaus, 1990; Siler and Cornish, 1993). For control experiments, 25 mM of EDTA was added to the reaction mixture to chelate the Mg²⁺ ion necessary for rubber transference activity. The resulting [14C]IDP-incorporated rubber was quantified by using a benzene extraction method. The reaction mixture was extracted three times with four volumes of benzene, the benzene extract was mixed with a Ready Solv HP scintillation cocktail (Beckman), and the radioactivity was determined by a liquid scintillation counter (Oh et al., 1999).

**Gel permeation chromatography**

For the analysis of molecular size of *in vitro* synthesized rubber, [14C]-labelled rubber in the reaction mixture was extracted with benzene and was concentrated to a small volume under N₂. Gel permeation chromatography was carried out in a Waters HPLC model using three columns in series, a mixed-bed polydicylbenezene column with molecular weights cut-off from 100 to 10 000 k (Jordi), and two polystyrene-divinyl-benzene copolymer gels having an exclusion limit of 4 × 10⁷ and 6 × 10⁴ (Supelco). Measurements were made at 35 °C using tetrahydrofuran as the eluent at a flow rate of 0.5 ml min⁻¹, and the rubber was collected at 0.5 min intervals and assayed for radioactivity. The weight average molecular mass (Mₐ) of the rubber was estimated by comparing the elution profile of the sample to the molecular weight distribution profile of standard polystyrene and polyisoprene.

**Results**

**Isolation and characterization of the GHS gene**

The primary cDNA library containing 5 × 10⁶ recombinant phages was screened to clone the gene. A cDNA clone containing an insert of approximately 1.0 kb in size was selected and subjected to *in vivo* excision for further study. The resulting phagemid was designated pGHS. The nucleotide sequence of the GHS has been deposited in the GenBank database under GenBank accession no. AF541942. Sequence analysis showed that the cDNA insert was 937 bp long (Fig. 1) and contained a 723 bp open reading frame, flanked by a 69 bp 5'-UTR and a 145 bp 3'-UTR including a poly(A) tail of 59 bp. The open reading frame codes for a 241 amino acid polypeptide with a predicted molecular mass of 26.2 kDa. The predicted amino acid sequence of the GHS has high similarity to those of the SRPP (accession no. A223388; 50% identity), the REF (accession no. P15252; 41% identity), *P. vulgaris* stress-related protein (PvSRP) (accession no. 1326163; 47% identity), and *A. thaliana* stress-related protein (AtSRP) (accession no. 152(0426; 33% identity) (Fig. 2).

The deduced protein is neutral with an isoelectric point of 6.42, which is different from that of the SRPP (pI=4.8) and the REF (pI=5.04). Hydrophathy analysis (Kyte and Doolittle, 1982) of the deduced amino acid sequence showed that the GHS is amphiphilic, in contrast to the hydrophobic nature of the SRPP (data not shown).

**Heterologous expression of the recombinant GHS protein**

In order to obtain the recombinant GHS proteins needed to test its involvement in rubber biosynthesis *in vitro*, the GHS was expressed in *E. coli* as a GHS-His-tag fusion protein by using the pET-22b(+) expression vector (Fig. 3). The GHS-His-tag fusion proteins were purified on a His-tag column. The proteins from each purification step were analysed on SDS-12% PAGE. After extensive analysis of induction conditions including incubation time, temperature, and IPTG concentration, the GHS-His-tag fusion protein could be expressed and purified in *E. coli*.

**Western analysis of the recombinant GHS protein**

Since the GHS shares a high sequence homology with the SRPP, it is of interest to test whether the antibody raised against the SRPP could recognize the GHS. The purified recombinant GHS, recombinant SRPP, and *Hevea* latex containing native SRPP and REF were separated on SDS-PAGE, transferred to a membrane, and were reacted with the SRPP-antibody. As shown in Fig. 4, SRPP-antibody bound to both the SRPP and GHS, indicating that the GHS and SRPP contain a similar structural motif recognized by a single antibody. These results suggest that the GHS is similar to the SRPP in overall conformation, and may play a role in rubber biosynthesis as SRPP does (Oh et al., 1999).

**Positive role of the GHS in rubber biosynthesis in vitro**

To test the effect of the GHS on rubber biosynthetic activity *in vitro*, the reactions were carried out using the *Hevea* WRP and recombinant GHS as enzyme sources. Washed rubber particle from *H. brasiliensis* per se contains native SRPP as well as REF, and exhibited a fairly high level of rubber biosynthetic activity (Fig. 5). WRP compared with the control reaction in which rubber transference was inactivated by the addition of 25 mM EDTA that chelates the Mg²⁺ ion necessary for the activity of the enzyme (Fig. 5, +EDTA). Addition of the bacterial extracts containing 60 μg total protein expressing the GHS with IPTG induction resulted in a marked increase in [14C]IDP incorporation (Fig. 5, +GHS). Next, it was necessary to test whether this increase in [14C]IDP incorporation resulted specifically from the GHS or non-specifically from other proteins present in the bacterial extracts. Different amounts of bacterial extracts without IPTG induction were added to the reaction mixture, and no
increase in $[^{14}\text{C}]$IDP incorporation was detected in the reaction mixture supplied with the bacterial extracts containing 20, 40, and 60 $\mu$g total protein (Fig. 5, +vector1, +vector2, and +vector3). In a control reaction removing FDP, which is a necessary initiating molecule for rubber biosynthesis, no $[^{14}\text{C}]$IDP incorporation was observed (Fig. 5, ±FDP), indicating that the enzymes catalysed the condensation of IDP into newly synthesized rubber chains. The enhancing activities of GHS in rubber biosynthesis were further investigated with the purified recombinant GHS protein. As shown in Fig. 6, the amounts of $[^{14}\text{C}]$IDP incorporated into rubber were substantially increased with the addition of the recombinant GHS. The incorporation of $[^{14}\text{C}]$IDP was increased about 3-fold by the addition of 40 $\mu$g of the GHS-His-tag fusion protein to the reaction mixture containing 10 $\mu$g of the Hevea WRP. In time-course experiments using the Hevea WRP and the recombinant GHS as enzyme sources, $[^{14}\text{C}]$IDP incorporation into rubber increased with incubation time. By contrast, no increase in $[^{14}\text{C}]$IDP incorporation was observed in the control reaction containing 25 mM EDTA. These observations indicate that the GHS plays a positive role in IDP incorporation, and the increase in IDP incorporation is specific to the presence of the recombinant GHS.

In order to estimate molecular weight of the enzymatically-synthesized rubber, the rubbers extracted with benzene from the reaction mixture were analysed by gel
permeation chromatography. Figure 7 compares the molecular weight distributions of 14C-labelled rubber synthesized in vitro by using the Hevea WRP alone, and both the Hevea WRP and recombinant GHS. Arabidopsis cis-prenyltransferase (Oh et al., 2000) was also used as a control for the biosynthesis of small molecular weight isoprenoid compound. As shown in Fig. 7, the molecular mass of the radiolabelled rubber synthesized by using Hevea WRP alone distributed from about 200 kDa to 4000 kDa with the mean average molecular mass of 1500 kDa. Similar molecular mass distribution was observed for the reaction containing both Hevea WRP and the recombinant GHS as enzyme source. Substantially smaller sized polymers were produced in the reaction mixture containing the Arabidopsis cis-prenyltransferase as an enzyme source.
Rubber transferase activity has been reported from the rubber particle-bound proteins of rubber-producing plants including *H. brasiliensis*, *P. argentatum*, *F. elastica*, *F. carica*, and *F. benghalensis*, and the similarities and differences in rubber biosynthesis among these plant species have been reviewed (Cornish, 2001). Despite substantial amounts of the reports investigating the proteins associated with the rubber particles and involved in rubber biosynthesis, the report investigating the genes encoding rubber biosynthesis-related proteins is relatively scanty. Since many plant species produce various sizes of rubber, the important question in rubber biosynthesis is what controls the size of rubber in plants. It is possible that rubber transferase itself or rubber transferase in complex with additional factors controls the molecular weight of rubber. Among many latex proteins in *H. brasiliensis*, two main proteins are tightly associated with rubber particles: the REF (14 kDa) with large rubber particles and the SRPP (23 kDa) with small rubber particles (Yeang et al., 1996).

A full-length cDNA encoding the REF has been cloned (Attanyaka et al., 1991; Goyvaerts et al., 1991), and it has been suggested that the REF plays a functional role in rubber polymerization (Dennis and Light, 1989; Light et al., 1989). However, the actual role of the REF in cis-1,4-polyisoprene elongation has not been fully assessed. In recent studies, Oh et al. (1999) have cloned a gene encoding the SRPP from *H. brasiliensis*. It has been suggested that the SRPP has high amino acid sequence homology to the REF and plays a positive role in IDP incorporation into high molecular weight polymers. From these findings, together with the fact that it is tightly associated with small rubber particles and is abundantly expressed in latex, they proposed that the SRPP plays a role in rubber synthesis and could potentially constitute a rubber transferase complex. If the SRPP is a part of the...
rubber transferase complex, it is assumed that other plant species that produce similar sizes of rubber to *H. brasiliensis* also contain a similar enzyme. It is shown here that guayule, which produces a similar size of rubbers comparable with *Hevea* rubbers, contains a gene that is similar to the SRPP.

The GHS has both similar and different properties compared with the SRPP. Although the GHS is longer by 37 amino acids than the SRPP, the primary sequence of the GHS is highly homologous to that of the SRPP (Fig. 2). The hydrophobic nature of the two proteins differs from each other in that the GHS is amphiphilic (data not shown), in contrast to the hydrophobic nature of the SRPP (Oh *et al.*, 1999). Neither protein contains any signal peptides targeting specific cellular compartments, suggesting that both proteins localize in the cytoplasm where the rubber biosynthesis takes place. The cross-reactivity of the SRPP-antibody with the GHS (Fig. 4) suggests that the GHS shares similar structural motif or antigenicity with the SRPP. The increase in IDP incorporation by the addition of the bacterial extracts expressing the GHS with IPTG induction (Fig. 5) implies that the GHS contributes to the rubber biosynthesis *in vitro* as the SRPP does. No increase in IDP incorporation was observed by the addition of the bacterial extracts without IPTG induction (Fig. 5), suggesting that the increased rubber biosynthetic activity is a specific effect of the GHS. In this study, the effect of the GHS on rubber biosynthesis was tested further by using the purified enzyme, which was not demonstrated in the case of the SRPP. Although it is not known at this stage whether the GHS is directly involved in rubber chain elongation, it is clear that the purified GHS contributes to the IDP incorporation in rubber biosynthesis *in vitro* (Fig. 6).

The rubber particle-bound proteins in guayule and their rubber transferase activities have been previously studied in detail (Madhavan and Benedict, 1984; Madhavan *et al.*, 1989; Benedict *et al.*, 1990; Cornish *et al.*, 1994; Siler and Cornish, 1994). From the SDS-PAGE analyses of rubber particle proteins in guayule, it has been noticed that one or two major proteins are associated with the rubber particles and a protein of 48.5 kDa in size is the most abundant protein (Cornish and Backhaus, 1990; Backhaus *et al.*, 1991). The gene encoding this protein has been cloned and was found to be a cytochrome P450, an oxide synthase with a molecular mass of 52 kDa (Pan *et al.*, 1995). In the same SDS-PAGE for guayule rubber particles, it was clear that the next abundant protein was a protein of about 26 kDa in size (Cornish and Backhaus, 1990; Backhaus *et al.*, 1991). However, the identity and catalytic activity of this protein have not been discovered. Although it was not possible to test the second most abundant protein on the SDS-PAGE gel due to the unavailability of the guayule plant materials, it is speculated that the cDNA clone that has been isolated may correspond to the second most abundant protein (26 kDa in size) in guayule rubber particles. Further investigation of the rubber particle-bound proteins in guayule by western analysis using the SRPP-antibody should help to probe the existence of the GHS on the guayule rubber particles.

The most important unsolved and challenging question in rubber biosynthesis is what the nature of rubber transferase is. Rubber transferase belongs to the family of *cis*-prenyltransferase (CPT) that catalyzes a sequential condensation of IDP with allylic diphosphate. It has been reported that the CPTs from micro-organisms produce the shorter chain length polypropenyl diphosphates ranging in carbon number from C50 to C120 (Ogura and Koyama, 1998). A recent report by Oh *et al.* (2000) demonstrated that the *Arabidopsis* CPT itself could not catalyse the formation of high molecular weight rubber. These results suggest that if the CPT is a key enzyme in a rubber transferase complex, additional factors are required to produce high molecular weight rubbers in plants. However, it is unlikely that the CPT is a core enzyme in a rubber transferase complex, because CPT is a ubiquitous protein found in micro-organisms and non-rubber-producing plant species as well. It is believed that the plants producing different sizes of rubber should contain specific factors that control the rubber biosynthesis and determine the molecular weight of the rubber. No homology was observed in the amino acid sequences between the CPT and GHS or SRPP, and it is speculated that the GHS or SRPP could be such a factor that plays an additional role in helping rubber transferase to produce high molecular weight rubber. However, in the assay using the GHS or SRPP, the bacterial extracts expressing the GHS or SRPP protein were used in the rubber biosynthesis assay. Since the bacterial extracts contain many other proteins and cellular factors that might contribute to rubber biosynthesis by stabilizing a rubber transferase complex, it is not clear whether the GHS or SRPP is a core component for a rubber transferase complex. The present study using the purified GHS, together with the *Hevea* washed rubber particles, could not give direct evidence that the GHS constitutes part of the rubber transferase complex. In a recent study, Singh *et al.* (2003) demonstrated by immunocytochemical analysis that the SRPP-antibody did not show any cross-reactivity with the rubber particle proteins in *Ficus carica* and *Ficus benghalensis* that produce substantially lower molecular weight rubber compared with *H. brasiliensis*. This observation suggests that the GHS, SRPP, or its homologue is present in the rubber trees that produce high molecular weight rubbers. These considerations further support the notion that, although they are not the core enzyme for rubber polymerase, the GHS or SRPP may constitute part of the rubber transferase complex and play a role in IDP incorporation into high molecular weight rubbers.

The present study identified the GHS as a potential factor for enhancing the rubber biosynthetic activity in
guayule, and strengthened the authors previous finding for suggesting the SRPP as a positive contributor for rubber biosynthesis in H. brasiliensis. However, it remains to be solved if these proteins play a direct role in elongating the rubber chain or contribute indirectly by stabilizing the rubber transferase complex during rubber biosynthesis. Further studies, such as the reconstitution of the rubber particle-like environment using the purified GHS or SRPP, a mixture of lipids, or additional protein factors, and transgenic approaches introducing the GHS or SRPP into the plants that produce a smaller size of rubber molecules could provide more direct clues to identify the role of the GHS or SRPP and the nature of rubber transferase. Identification of rubber transferase and/or its complex should provide crucial knowledge to develop alternative rubber crops with no latex allergy that is a problematic in H. brasiliensis latex by transgenic expression of the gene(s) responsible for the formation of high molecular weight rubbers.

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