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Fundamental Similarities in Rubber Particle Architecture and Function in Three Evolutionarily Divergent Plant Species

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Rubber particles of the evolutionarily divergent and anatomically distinct species Ficus elastica, Hevea brasiliensis *and* Parthenium argentatum *were analysed and compared. We have shown that rubber particles of the three species have intriguing differences in size, relative proportions of long- and short-chain rubber, and their protein profiles although they share fundamental similarities in particle architecture and function. We propose a model for rubber particle architecture, in which proteins are located in large complexes at the particle surface. Our results suggest that successful rubber production in other species may entail many of the same commonalities observed here.*

All natural rubber currently used commercially is obtained from a single species, *Hevea brasiliensis*, the Brazilian rubber tree[1], which is unsuitable for cultivation in the United States. There is a strong incentive to develop a domestic rubber crop because natural rubber, a vital raw material, is one of the most costly raw materials imported. *Parthenium argentatum* (guayule) is currently being developed but has limitations because it is frost-sensitive, and must be at least three years old before harvest and processing for rubber extraction[2].

Over 2000 plant species produce the polymeric secondary product *cis*-1,4-polyisoprene (natural rubber) and they represent four out of the six super orders of the Dicotyledonae[3,4]. The Magnoliidae and Caryophyllidae seem to be without rubber-producing members[4]. At least two fungal species are also known to make natura rubber[5].

This paper, examines rubber production in three dissimilar species in order to determine commonalities in rubber particle architecture and function that may be useful in isolating those enzymes and structural proteins essential for rubber biosyntheis. Meaningful inter-specific comparisons are not possible using existing literature due to differences in material preparation and analytical methods.

The botanical classification of *Ficus elastica, H. brasiliensis and P. argentatum*[4] in the distinct super orders Dilleniidae, Rosidae and Asteridae, respectively, suggests that they are evolutionarily divergent. The species differ in geographical origin, rubber quality and yield, and in the anatomical location of their rubber biosynthesis. *H. brasiliensis* is a tropical hardwood tree, native to Brazil, that produces mainly long-chain rubber within a complex network of laticifers that run beneath its bark[1]. *F. elastica*, the Indian rubber tree, is a rapidly growing plant popularised in temperate regions as an ornamental. *F. elastica*, also produces its rubber in laticifers, and was one of the first plants of tropical Asia to be grown for rubber production[6]. However, *F. elastica* makes predominantly short-chain rubber which is of little commerical value. *P. argentatum* is a woody shrub native to semi-arid regions of the South-western United States and Mexico and, like *H. brasiliensis*, makes substantial amounts of long-chain commercial-grade rubber[2]. Unlike

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H. brasiliensis and *F. elastica, P. argentatum* does not utilise laticifers, producing its rubber in the cytoplasm of unspecialised parenchyma cells in the stem and root bark. For rubber to be extracted from *P. argentatum*, the entire plant must be sacrificed.

MATERIALS AND METHODS

Plant Material

H. brasiliensis materials were generously provided by J.R. Bugansky (Senior Botanist, Goodyear Tire & Rubber Co.) from plantation trees grown in Sumatra. *F. elastica* plants were obtained from a local nursery and grown in a greenhouse in Albany, CA. Mature *P. argentatum* plants were grown in the field at the US Water Conservation Laboratory, Phoenix, AZ.

Purification of Washed Rubber Particles

Washed rubber particles were prepared using a minor modification of a published technique[7] involving the use of slower centrifugation speeds and filtering to avoid the inclusion of coagulated particles in the washed rubber particle preparations.

H. brasiliensis and *P. argentatum* rubber particles were suspended in wash buffer (100 mM Tris-HCl at pH 7.5, 2.5 mM $MgSO_4$, 5 mM dithiothreitol) and centrifuged for 30 min at 200 × g at 4°C. The floated rubber particles were collected (the particles were scooped off with a spoon-shaped spatula) and the tubes were re-spun for 40 min at 300 × g. The floated particles were collected and pooled. The pooled rubber particles were re-suspended in wash buffer and spun for 40 min at 300 × g. The washed rubber particles were then filtered through four layers of cheese cloth dampened with deionised water, and the filtrate was re-spun for 40 min at 300 × g.

The *F. elastica* washed rubber particles were prepared by washing them three times in wash buffer for 8 min at 2500 × g for each step, decanting and re-suspending in fresh wash buffer after each spin. The washed rubber particles were filtered through four layers of dampened cheese cloth between the second and final spins. Concentrated washed rubber particle suspensions were mixed with equal volumes of 70% glycerol and stored frozen until analysed. Samples of washed rubber particles of all three species were dried on filters and weighed to determine their rubber particle content.

Particle Size Analysis

The size of particles purified from *F. elastica, H. brasiliensis* and *P. argentatum* was determined by NICOMP Particle Sizing Systems (Santa Barbara, CA) using both the NICOMP Model 370 Submicron Particle Sizer (which uses dynamic light scattering, also known as photon correlation spectroscopy[8]), and the Model 770 Accusizer (which employs single particle light obscuration[9]). Analysis of the particle distributions was done using intensity-weighted Gaussian analysis and the NICOMP multi-modal distribution analysis[8,9]. The concentrations of washed rubber particles analysed, expressed on the basis of washed rubber particles dry weight, were as follows: *F. elastica*, 0.16 mg/ml; *H. brasiliensis*, 0.10 mg/ml; and *P. argentatum* 0.07 mg/ml.

Determination of Rubber Molecular Weight

Thrice-washed rubber particles were prepared as described, then dried on filters overnight at 37°C and weighed. The rubber was peeled from the filters, and samples from *H. brasiliensis* (160 mg), *F. elastica* (180 mg) and *P. argentatum* (60 mg) were analysed at Polymer Laboratories, Inc., Foster City, CA. At Polymer Laboratories, the rubber was dissolved, at a concentration of 0.1%, in stabilised tetrahydrofuran and filtered through 0.2 μm filters. Aliquots of 50 μl were injected onto two PLgel 5 μm gel permeation columns (300 × 7.5 mm) at a flow rate of 1 ml/min. The molecular weight profiles were determined using an ACS evaporative mass detector (Model 750/14). The columns were calibrated against polyisoprene standards (Polymer Laboratories, Inc.).

Protein Quantification

Protein concentrations were determined using the micro-BCA protein assay according to the manufacturer's instructions (Pierce Rockford, IL) with the following modifications

to overcome the difficulty of determining protein concentrations in the presence of rubber particles, which scatter light. Washed rubber particles were prepared in the absence of dithiothreitol (which interferes with the assay), from *F. elastica, H. brasiliensis*, and *P. argentatum*. The preparations were diluted in 1% CHAPS and incubated at 25°C for 60 min, with gentle agitation to keep the particle suspended. This solubilised the surface proteins without destroying the rubber particles. Aliquots were mixed with an equal volume of bicinchoninic acid (BCA) reagent, consisting of a 2:48:50 mixture of Pierce Micro-Reagent C (4% cupric sulphate, pentahydrate in water), Pierce Micro-Reagent B (4% sodium bicinchoninate in water) and Pierce Micro-Reagent A (sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.2% NaOH), and incubated at 60°C for 1h. After colour development, samples were cooled to room temperature, and the rubber particles were disrupted by extraction with an equal volume of water-saturated hexane. The aqueous layer was transferred to a cuvette and the absorbance determined at 562 nm. Controls were prepared as described above, except that BCA reagent was replaced by 50% BCA Micro-Reagent A in water. The protein concentration of the samples were estimated by substracting the absorbance of controls, and comparing with a standard curve prepared with bovine serum albumin in 1% CHAPS. Standard curves prepared with and without hexane extraction were indistinguishable.

SDS-PAGE Analysis

Washed rubber particles of *H. brasiliensis, F. elastica* and *P. argentatum* were analysed on 1.5 mm 12% (w/v) and 0.75 mm 4%–20% (BioRad) polyacrylamide gels and electrophoresed as described[10]. Proteins were detected by silver-staining, using a Silver Stain Kit (BioRad) according to the manufacturer's instructions.

RESULTS

Particle Size Analysis

Previous reports of particle size analysis of rubber particle suspensions have only described natural latex from H. brasiliensis[11]. Therefore, we examined the particle size distributions of washed rubber particles isolated from *F. elastica, H. brasiliensis* and *P. argentatum*. The two analytical methods used, the intensity-weighted Gaussian analysis (*Figure 1a*) and the NICOMP distribution analysis (*Figure 1b*), gave comparable particle size distributions. The mean particle sizes of the major peaks in these analyses are shown in the figure legend. The Gaussian analysis presents the normal distribution that provides the best fit to the data. However, although the Gaussian analysis supplies a good estimation of the range of particle size, the apparent peak mean would be altered by a skewed or multi-modal distribution. The NICOMP analysis provides more accurate estimations of peak position and peak height[8,9]. The very broad distribution seen for *H. brasiliensis* in the Gaussianm analysis (*Figure 1a*) is due largely to a few very large clumps of coagulated rubber in the suspensions and a subset of small particles. The poor fit of the normal distribution to the data is indicated by the large χ^2 of 3.44 forn *H. brasiliensis*. The Gaussian analysis provided a much better approximation of the particle distributions for *P. argentatum* ($\chi^2 = 0.15$) and *F. elastica* ($\chi^2 = 0.10$).

The large particle aggregates in the *H. brasiliensis* suspension were discerned by the Model 770 Accusizer (which employs single particle light obscuration) and then omitted from the NICOMP distribution analysis (*Figure 1b*). In this analysis it is clear that *H. brasiliensis* washed rubber particles are, on the average, a little smaller than those of *P. argentatum*. *H. brasiliensis* also has a distinct subset of smaller particles (*Figure 1b*) with a mean diameter of 0.22 μm. A bi-modal distribution of particle size has been reported for *H. brasiliensis*, where three natural latices had peaks[11] in the regions of 0.30 μm and 0.70 μm. *F. elastica* rubber particles are substantially larger than those of the other two species (*Figure 1*) with a mean diameter three times bigger. Only 0.175% of the *F. elastica* particles were sized between 0.96 μm and 1.01 μm on the NICOMP analysis, and no

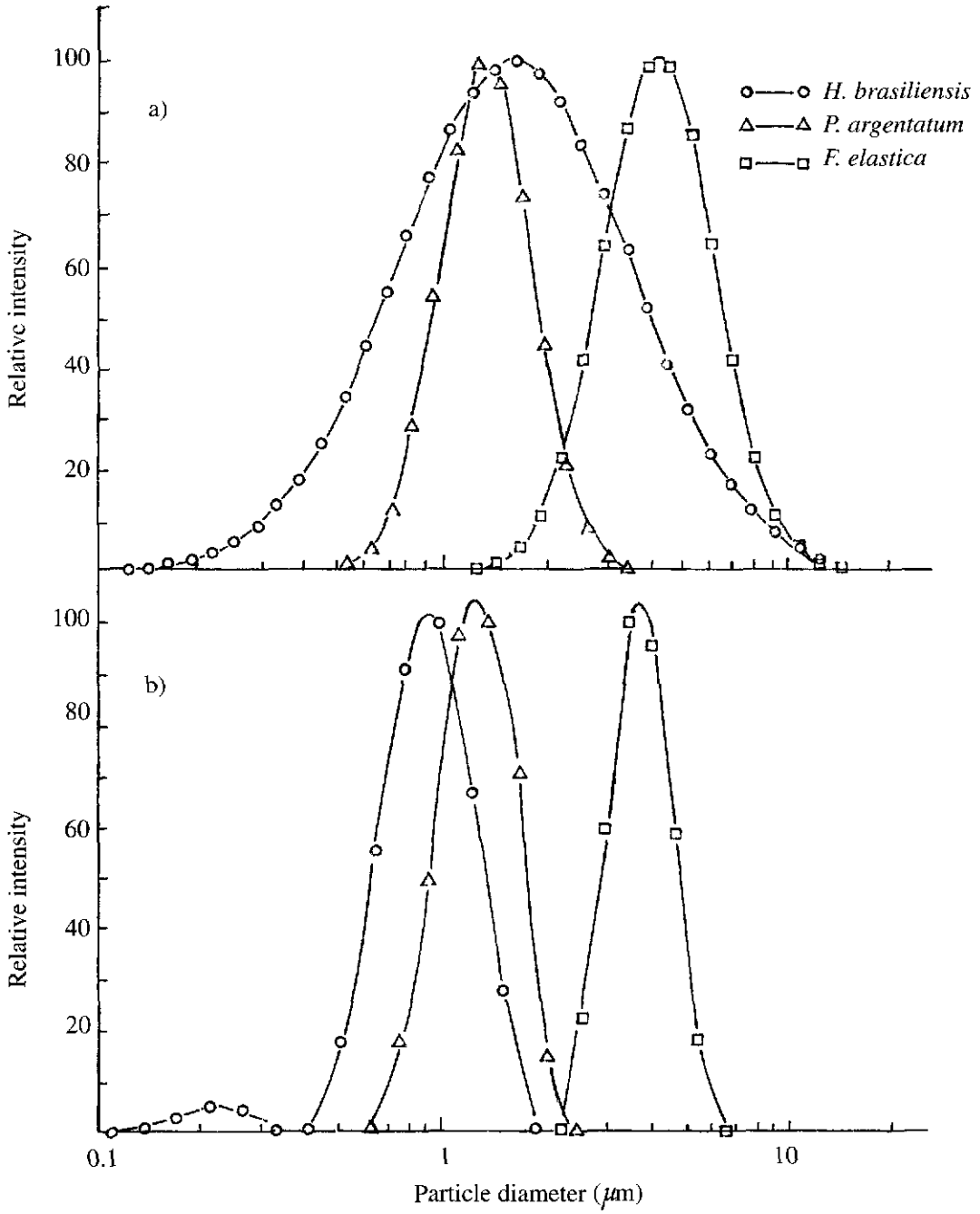

Figure 1. Particle size analysis of washed rubber particles isolated from F. elastica, H. brasiliensis *and* P. argentatum, *using a) intensity-weighted Gaussian analysis and b) the NICOMP distribution analysis. The mean particle diameters determined from each analysis are: F. elastica (a) 3.91 μm (b) 3.80 μm;* H. brasiliensis *(a) 1.23 μm (b) 0.96 μm;* P. argentatum *(a) 1.27 μm (b) 1.41 μm.*

smaller ones were detected. This lower limit for *F. elastica* is similar to the mean particle size for the other two species (0.96 μm for *H. brasiliensis* and 1.41 μm for *P. argentatum* as determined by the NICOMP analysis). The lack of small particles in *F. elastica* reflects a virtual absence of particles below 0.96 μm rather than technique sensitivity limits; particles of 0.20 μm diameter were detected in *P. argentatum* and those as small as 0.12 μm in *H. brasiliensis*.

Rubber Molecular Weight Analysis

Natural rubber average molecular weight varies considerably among species. Very few species produce, predominantly, the long-chain molecules required for high performance commercial grades[2,12]. The regulation of chain length is not understood. We directly determined, using the same analytical method, the different rubber sizes from the three species.

The rubber from particles of all three species was found to contain both high and low molecular weight components (*Figure 2*). However, the relative proportion varied greatly. *H. brasiliensis* rubber is predominantly long chain with a mean molecular weight of 1.5 million Da, which is consistent with earlier reports[13,14]. The maximum size is over 9 million Da. Small amounts of short-chain components are also present. *P. argentatum* produces rubber with a very similar size distribution to that of *H. brasiliensis* (*Figure 2*) including a maximum size of over 9 million Da. In contrast, *F. elastica* contains substances generating two main peaks with mean molecular weights of 1000 Da and 200 Da. A molecular weight of 1000 Da would reflect an average chain length of 15 isoprene units and the 200 Da about 3 units. Of considerable interest, in *F. elastica*, is the small amount (2%) of high molecular weight product revealed by the mass detector (*Figure 2*). High

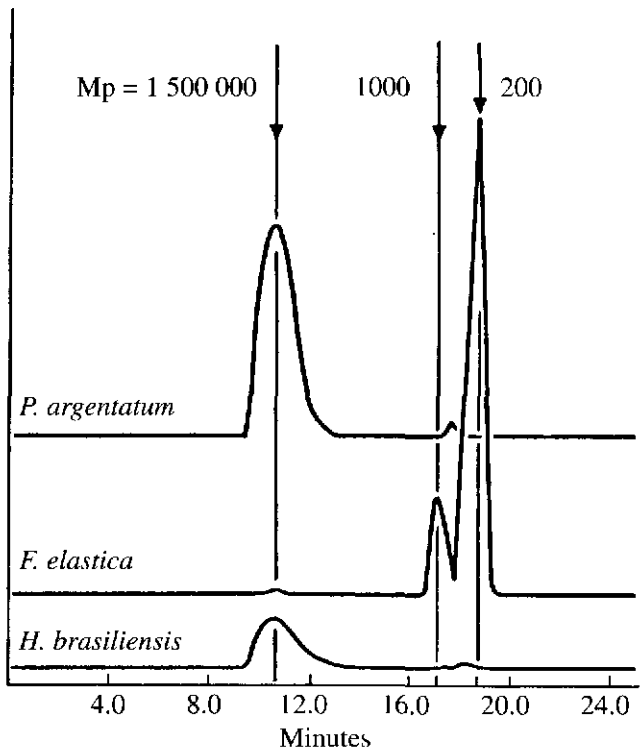

Figure 2. Molecular weight analysis of rubber from washed rubber particles isolated from F. elastica, H. brasiliensis and P. argentatum. M_p = molecular weight of the peak maxima.

and low molecular weight components of *F. elastica* rubber have been observed previously[15] although in different proportions to our results. The *F. elastica* long-chain rubber we observed (*Figure 2*) represents a product of comparable size to the *H. brasiliensis* and *P. argentatum* high molecular weight rubber. Thus, the difference in chain length among the species appears to be quantitative and not a qualitative one.

Quantification of Rubber Particle Protein

We compared the amount of surface protein for *F. elastica, H. brasiliensis* and *P. argentatum*, as this has direct relevance to particle architecture and functioning. Rubber particles of *F. elastica* and *P. argentatum* were found to contain similar amounts of protein, 0.39% ± 0.03% and 0.33% ± 0.04% respectively, expressed as percentage of protein per dry weight of rubber particles. *H. brasiliensis* rubber particles had much protein, at 1.49% ± 0.37%.

Electrophoretic Analysis of Rubber Particle Proteins

The rubber particle proteins were analysed, by SDS-PAGE, after extensive washing of the particles to remove any contaminating cytosolic proteins. In *P. argentatum*, several rubber particles proteins are visible (*Figure 3, lane c*), which is in agreement with the published reports of four to eight characteristic proteins of this species[7,16]. The 50 kD RPP is the most abundant protein in this species[16] and its position in *lane c* (*Figure 3A*) is indicated. The rubber particles from *F. elastica* (*Figure 3A, lane a*) have the simplest protein complement of the three species with only two proteins clearly visible (about 30 kD). A simple protein profile has been reported for *H. brasiliensis* gel-filtered particles, using Coomassie Blue-staining, showing only the abundant 14.6 kD particle protein, REF (rubber elongation factor)[17]. However, Coomassie Blue does not detect all rubber particle proteins[18] and is also 100 times less sensitive than silver-staining[19]. Silver-staining reveals a very complex profile in *H. brasiliensis* (*Figure 3A, lane b*) with at least thirty associated proteins. Thus, *H brasiliensis* rubber particles are far more complex, with respect to their protein complement, than the other two species. The position of REF is indicated on the gel (*Figure 3A, lane b*). In addition to the proteins described above, Siler and Cornish[18] have previously characterised an additional very large rubber particle protein (LPR, 376 kD) in *F. elastica*, which is too large to migrate into the 12% gel shown here (*Figure 3A, lane a*).

Separate 4%–20% SDS gels of washed rubber particles (*Figure 3B*), demonstrate small amounts of the same proteins indicated by the arrows in *gel A* as well as large proteins. In *F. elastica* (*Figure 3B, lane a*), the 376 kD protein has clearly moved into the gel (see arrow) and the 30 kD bands shown on *Figure 3A, lane a*, are also visible. Similarly, in *P. argentatum* (*Figure 3B, lane c*), a large protein is visible, in addition to the 50 kD RPP indicated by the arrow. A large protein is also clearly shown in *H. brasiliensis* (*Figure 3B, lane b*), as well as the 14.6 kD REF indicated by the arrow. However, most of the proteins revealed on the 12% gel (*Figure 3A, lane b*) are not apparent here. This paucity of bands supports a model in which the large proteins are actually protein complexes which contain most of the rubber particle proteins. The relative amount of large and small proteins from the rubber particles of the three species appears to depend on the degree of denaturation. Under non-denaturing conditions (native-PAGE) the large proteins are always seen (data not shown), and the native molecular weight[18] of the *F. elastica* LPR is 750 kD. The native molecular weights of the large proteins, or protein complexes, from rubber particles of *H. brasiliensis* and *P. argentatum* have yet to be determined.

DISCUSSION

Any universal model of rubber particle structure requires that fundamental features exist regardless of the species producing the rubber. We must account for the roles of the rubber, protein and lipid components in order to understand the structure, operation and ontogeny of rubber particles.

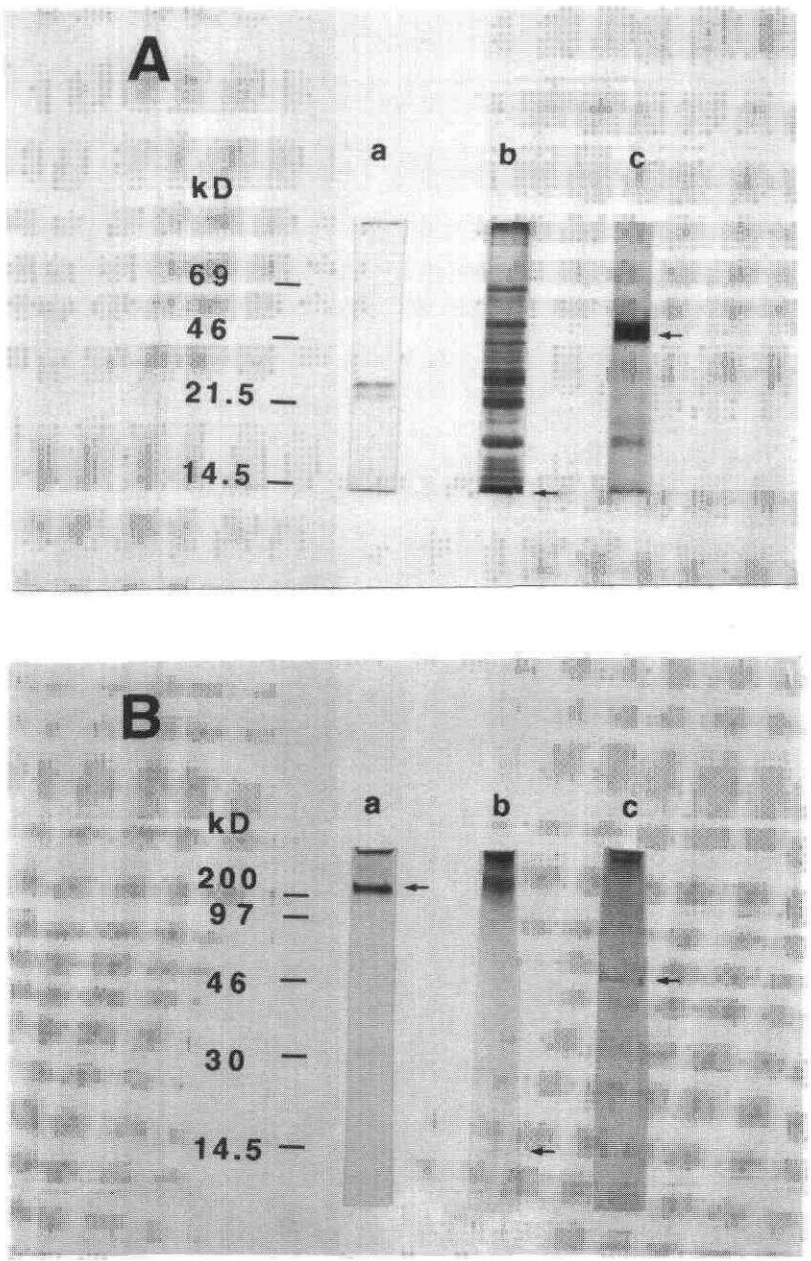

Figure 3. Silver-stained SDS-PAGE of proteins from washed rubber particles of three species on A) 12% and B) 4%-12% gels. In each case: lane a, F. elastica*; lane b,* H. brasiliensis*; lane c,* P. argentatum. *Similar amounts of washed rubber particles were analysed for all the species. The arrows indicate the positions of LPR (376 kD) in* F. elastica *in gel B, of REF (14.6 kD) in* H. brasiliensis *in gels A and B, and of RPP (50 kd) in* P. argentatum *in gels A and B. The high molecular weight LPR-like proteins visible in all lanes in gel B are too large to migrate into the 12% gel A. The M_r of protein standards are indicated.*

The rubber transferases are firmly bound to the rubber particle in *H. brasiliensis*[20,21], *P. argentatum*[7,22] and *F. elastica*[18]. A soluble rubber transferase has been reported in *H. brasiliensis*[23], but recent work has shown that although this enzyme may play a role in rubber molecule initiation it does not polymerise rubber[24].

Little is known about the ontogeny of rubber particles partly because the smallest rubber particles (<0.02 μm) are indistinguishable from other small electron-dense cytoplasmic bodies. The lack of particles under 1 μm in diameter in *F. elastica* washed rubber particles stands in contrast to the other two species (*Figure 1*). The lack of small particles has interesting implications on the ontogeny in *F. elastica* as, presumably, the particles over 1 μm in diameter began as much smaller particles. It is possible that the rubber particles are not released into the free latex until they reach a certain maturity. This would be analogous to *H. brasiliensis*, in which the nuclei and mitochondria are retained in a parietal position in the laticifers and are rarely visible in the tapped latex[1]. The preparation of *F. elastica* washed rubber particles entailed collecting the latex rubber particles from a pellet under a clear supernatant. This methodology makes it very unlikely that a significant subset of small particles was excluded from the washed rubber particles.

Several different possibilities may explain the much smaller proportion of long-chain rubber in *F. elastica* rubber particles, compared with the other two species. Chain length may simply reflect the relative availability of the substrates. However, it is possible that two rubber transferase forms are present in *F. elastica*, one capable of long-chain and the other of short-chain rubber biosynthesis. The small quantities of high molecular weight rubber may be the result of the long-chain rubber transferase being present in only small quantities relative to the short-chain rubber transferase. Also, the long-chain enzyme may only be expressed during a brief period of time, under a specific set of environmental conditions, or at a particular developmental stage. Another possibility is that only one form of rubber transferase may exist in conjunction with a separate factor, or 'terminator', that ends the synthesis of a rubber polymer. It does seem likely that termination of the rubber molecule is an event separate from the elongation process. In this case, the small quantity of high molecular weight rubber in *F. elastica* may reflect a greater abundance of terminator overall than is present in either *H. brasiliensis* or *P. argentatum*. Alternatively, similar amounts of rubber transferase and terminator could exist in all three species. The observed differences in rubber molecular weight could still occur if most of the rubber transferase molecules in *H. brasiliensis* and *P. argentatum* but only a few of those in *F. elastica*, are positioned in locations on the rubber particle inaccessible to the terminator.

Rubber particles contain both rubber and protein and a logical arrangement would place the protein at the particle surface as a suitable interface between the hydrophobic rubber and the cytosol. The *P. argentatum* 50 kD protein[7,16] (RPP) and the *F. elastica* 376 kD protein[18] (LPR), which are both glycosylated, are known to be positioned at the particle surface. If all the proteins are located at the particle surface, the question arises of how much of the rubber particle is coated with protein in the different species. Our data on particle size and protein amount allow the calculation of how much of the particle surface is covered by proteins of specific size.

In our proposed model, most of the rubber particle proteins are contained within large complexes. The *F. elastica* large complex has been characterised (native molecular weight[18] 750 kD) and the large protein complexes of the other two species appear to be similar in size (unpublished data). Thus, a calculation using 750 kD probably reflects the distribution of the large protein complexes over the surface of the living particle. However, because we have no knowledge of the tertiary structure of the complexes, we also calculate particle coverage by the most abundant monomeric protein in each species. These are the *F. elastica* 376 kD LPR[18],

H brasiliensis 14 6 kD REF[17], and the *P argentatum* 50 kD RPP[16] All the calculations assume that the proteins and protein complexes are spherical in shape

The surface areas of the particles, based on the mean diameter (*Figure 1b*, are 45 5 μm^2 for *F elastica*, 2 90 μm^2 for H brasiliensis, and 6 25 μm^2 for *P argentatum* We estimated the amount of protein that would be required to form a mono-layer on the surface of the rubber particles The maximum cross-sectional area of a 14 6 kD spherical protein is 837 nm^2, a 50 kD protein 19 0 nm^2, a 376 kD protein 73 0 nm^2 and a 750 kD protein 116 nm^2 For our calculations, we used the particle diameters derived from the NICOMP analyses (*Figure 1B*) as these provide the more accurate particle diameter, and the amounts of protein determined using the micro-BCA protein assay (see Results)

Assuming an average protein density of 1 33 g cm^3, we first calculate the amount of protein that would be required to coat the particles in a contiguous mono-layer The amount of a 750 kD spherical protein complex required to form a contiguous mono-layer on the surface of the rubber particles of all three species is *H brasiliensis*, 31 1 fg, *P argentatum*, 68 1 fg *F elastica*, 488 fg In contrast, the amount of 14 6 kD protein required to form a mono-layer covering the surface of a *H brasiliensis* rubber particle is 8 37 fg, that of 50 kD protein on a *P argentatum* rubber particle 27 5 fg, and that of 376 kD protein required to coat a *F elastica* rubber particle 388 fg

The mean particle weights were calculated, assuming a particle density of 1, to be 28 7 pg for *F elastica*, 0 46 pg for *H brasiliensis*, and 1 47 pg for *P argentatum* The value for *P argentatum* is in agreement with a range of particle weight determinations obtained by Cornish and Backhaus (unpublished data) on particles isolated from three lines and three ages of stem bark In these experiments the number of particles in a suspension was estimated using haemocytometry and light microscopy and weight per particle was determined from the dry weight of the rubber particles from a known volume of the suspension Particle weights ranged from 0 56 pg to 1 53 pg in twenty three washed rubber particle isolations (Cornish and Backhaus, unpublished results)

Using the protein percentages and the mean particle weights, the calculated amount of protein per particle for *F elastica* is 118 fg, *H brasiliensis* 8 42 fg, and *P argentatum* 7 35 fg These amounts of protein, when expressed as a percentage of the estimated mono-layers (see above), reflect the percentage of the surface of the particles that the available protein would cover if it were all present at the particle surface When the calculation is based on the 750 kD large protein complex, then the surface covered by the available protein is *F elastica*, 24 2%, *H brasiliensis*, 27 1%, *P argentatum*, 10 8% When the calculation is based on the most abundant monomeric proteins for each species, the surface covered by the available protein is *F elastica* 376 kD, 59 6%, *H brasiliensis* 14 6 kD, 100 6%, and *P argentatum* 50 kD, 26 9% A mono-layer of the 14 6 kD REF protein, in *H brasiliensis*, seems an unlikely scenario as the entire particle surface would be coated with the protein, and the rubber particles are known to contain both neutral lipids and phospholipids[25 26] presumably, in part, at the particle surface Also, it is interesting to note that the rubber particles of *P argentatum*, the only non-laticiferous species of the three, appear to have markedly less surface protein than the other species irrespective of the method of determination

CONCLUSION

The three rubber-producing species in this study are both evolutionarily divergent and anatomically distinct However, despite the lack of relationship, their rubber production systems appear to be remarkably similar All three species possess rubber particle-bound rubber transferases and all synthesise high and low molecular weight rubber compartmentalised within the particle The associated proteins, including rubber transferase, in all three species, appear to be embedded in the particle surface as protein complexes

Given our demonstration of the remarkable inter-specific similarity of rubber production systems it is probable that genes encoding enzymes and structural proteins necessary for the synthesis of high quality rubber in one species will function in another. Studying species which differ in rubber quality and quantity will lead to an understanding of the detailed regulation of rubber biosynthesis Isolating and manipulating selected genes should enable us to improve rubber quality and yield in *H. brasiliensis, P argentatum* and alternative rubber-producing species

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