Biotechnological development of domestic rubber producing crops

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Natural rubber is pervasive in modern life with more than 40,000 products and 400 medical devices containing the material (ref. 1). In many strategic and medical applications, no synthetic materials can achieve its unique combination of high performance and cost-effectiveness. Natural rubber also has the increasingly compelling advantage of being a renewable resource that will remain with us long after petroleum-derived polymers have disappeared.

Natural rubber (*cis*-1,4-polyisoprene) is made by more than 2,500 species of higher plants as well as by the occasional fungus (ref. 2). Despite this diversity, virtually all natural rubber used commercially for more than a hundred years has been derived from a single species *Hevea brasiliensis*, Muell. Arg., the Brazilian or *para* rubber tree (ref. 3). Initially, production was centered in South America, based on harvests of rubber from wild trees naturally dispersed in the native rain forest. Attempts to cultivate the tree in plantations eventually failed because of the devastating disease of leaf blight. The commercial enterprise was relocated to Southeast Asia, and this region has been the principle source of production ever since. The advanced lines in production are all very closely related to each other, contain little disease resistance, and are grown as clonal (genetically-identical) scions on seedling root stocks planted in close proximity to each other. This lack of genetic diversity, and the intermingling of roots and branches, puts the industry at serious risk of crop failure through pathogenic attack (ref. 4).

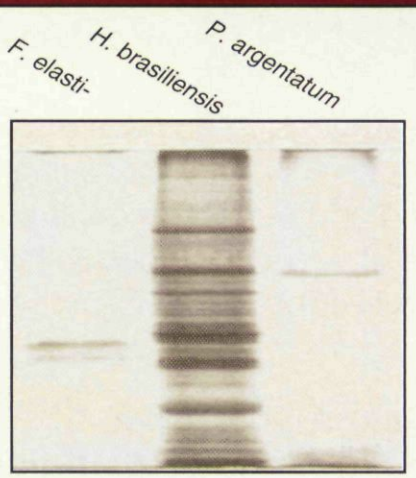
Thus, it has long been a goal (academic, industrial and federal) to have alternate sources of natural rubber production. However, except in times of war or high oil prices (and embargoes, and other causes of high price, come and go), such sources can only be developed successfully if they can attain and maintain a commercially-viable position in the global marketplace. The second natural rubber-producing crop now entering its commercial phase is guayule (*Parthenium argentatum* Gray) (ref. 5), which is able to provide a source of high-value non-allergenic latex safe for use by people suffering from Type I latex allergies to natural rubber products made from *H. brasiliensis* latex (refs. 6-10). Guayule's commercial competitiveness is supported by a quadrupling in yield achieved since the early 1980s through a combination of plant breeding and improved agronomic practice, coupled with a cost-effective aqueous latex extraction process (ref. 6) and a high-value market entry position.

Guayule latex yields should be further improved through additional plant breeding. It is well known that individual plants can contain 20% rubber (refs. 11 and 12), but the genetics of this species present substantial obstacles. Guayule is not only normally a tetraploid (although diploids, polyhaploids, triploids and octaploids all spontaneously occur), but it is also a facultative apomictic producing a mixture of seed types depending upon environmental conditions (ref. 13). Thus, direct optimization of the rubber biosynthetic pathway seems to present the most likely route to achieve significant gains in latex and rubber yield.

Interspecific comparison of rubber biosynthesis

An obvious target for genetic engineering is the rubber transferase enzyme, the biological catalyst that polymerizes natural rubber from isopentenyl pyrophosphate (IPP), an allylic pyrophosphate (usually farnesyl pyrophosphate, FPP, *in vivo*) being required to initiate the reaction. Considerable efforts have been, and still are, being concentrated on finding this enzyme in *H. brasiliensis,* but so far, these have met with little success. It is established that the rubber transferase is firmly associated with the monolayer biomembrane that surrounds each rubber particle (refs. 14-16). Earlier attempts to biochemically identify rubber transferase were hindered by the lack of solubilized enzyme activity. This prevented activity being tracked through successive rounds of purification and, coupled with the enormous number of proteins associated with *H. brasiliensis* rubber particles (figure 1), this made the task of identification virtually impossible (ref. 17). More recent genetic approaches were encouraged by the cloning of other microbial and then plant *cis*-prenyl transferases over the last decade (refs. 18-20). This led to considerable hope that the rubber transferase enzyme would be found by screening cDNA libraries for homologous sequences, but these searches

Figure 1 - protein profiles of purified rubber particles from *Ficus elastica*, *Hevea brasiliensis* and *Parthenium argentatum*



Lanes were standardized to the same dry weight of rubber. Figure reproduced from ref. 17.

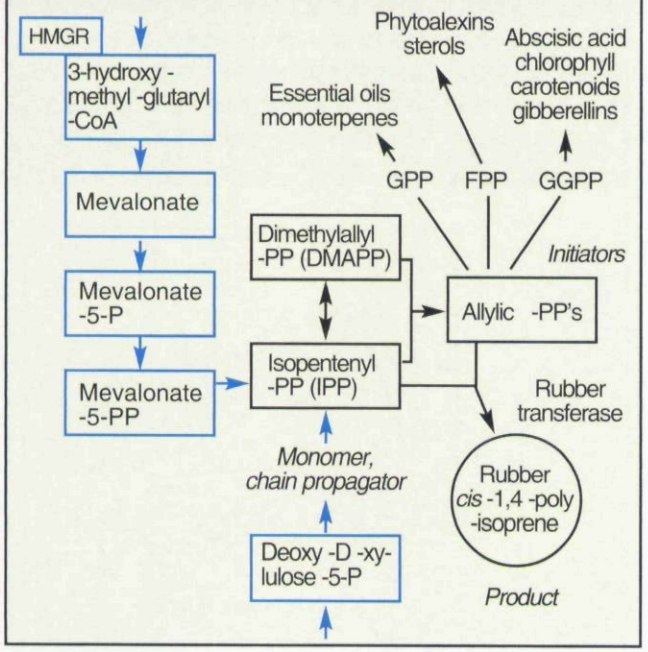
have only discovered additional, usually soluble, *cis*-prenyl transferase enzymes and not the membrane-bound rubber transferase itself.

In an attempt to improve the selectivity of biochemical approaches, the USDA-ARS project, established in 1989, has employed an interspecific approach (ref. 17). Three rubber-producing species of higher plant were chosen from different Super Orders of the Dicotyledoneae to reflect as distant a phylogenetic relationship as possible. The rationale was that this would minimize the genetic commonality between them and might allow similarities in rubber production machinery to be highlighted, assuming that all three species make natural rubber in essentially the same way. *H. brasiliensis,* from the Rosidae, was selected as the industry standard; *P. argentatum*, from the Asteridae, because it was known to provide one of the simplest rubber-producing systems known; and *Ficus elastica* Roxb., from the Dilleniidae, because it was readily available, easy to grow and produces a lower molecular weight, poorer quality polymer. *H. brasiliensis* and *F. elastica* both produce their rubber in the form of rubber particles in laticifers, a complex anastomasized cell system that forms pipes, which can then be tapped allowing latex to bleed out. In contrast, *P. argentatum* forms rubber in bark parenchyma cells, although still in the form of membrane-bound rubber particles.

It became clear that *H. brasiliensis* has one of the most complex rubber particle protein profiles yet observed, and the protein profiles of both *F. elastica* and *P. argentatum* were considerably simpler (figure 1), presenting fewer rubber transferase candidates.

Interspecific comparison of rubber biochemistry also can provide information as to the likelihood of being able to overexpress or suppress specific rubber biosynthetic genes within (1) the host plant and (2) within a different species, to achieve the desired effects on rubber yield and quality.

Figure 2 - a section of the isoprenoid pathway, including the biosynthesis of NR from isopentenyl pyrophosphate and allylic pyrophosphate - downstream of rubber biosynthesis are at least 75,000 compounds



Regulation of yield

Rubber yield is a function of the availability of substrate and cofactor, the rate of reaction, and the number of reactions occurring at any one time. The supply of initiator and monomer is regulated by the isoprenoid pathway (figure 2), with hydroxyl-methyl-glutaryl co-enzyme A reductase (HMGR) often being a rate limiting step. In all three species, polymerization rate is affected by both the concentration and size of the initiator and by the concentration of the monomer (ref. 21). Many different allylic pyrophosphates are effective initiators, but FPP seems to be the one primarily used *in vivo*.

The rate differences seem due to differences in the binding affinity of the rubber transferase for the different initiators, and by the different initiators affecting the affinity of the rubber transferase for the monomer. High concentrations of initiator inhibit the rate (data not shown). In *P. argentatum*, negative cooperativity inhibits the chain transfer reaction across a wide range of FPP concentrations, a property possessed in far less degree by *H. brasiliensis* and *F. elastica* (ref. 22). (Editor's note - definitions: *in vivo* - takes place within a living biological organism; *in vitro* - in an artificial environment outside the living organism.)

The identity and concentration of the divalent cation cofactor also affect rate. As covered in more detail in a companion work (ref. 22a), both manganese and magnesium cations are effective cofactors *in vitro*, but magnesium is the one used *in vivo* (ref. 23). The maximum stimulation of rubber biosynthetic rate appears to be the result of a conformational change that greatly increases the binding affinity of the enzyme for the monomer, an effect most pronounced in *H. brasiliensis* (ref. 24).

Regulation of molecular weight

Molecular weight is an important component of quality, with high molecular weights being required for high performance rubber products. The three species include two high molecular weight species (*H. brasiliensis* and *P. argentatum*) and one low molecular weight species (*F. elastica*). As was the case for rate, the molecular weight of the rubber produced in *in vitro* assays varied with the concentration and size of the initiator and with the concentration of the monomer (ref. 21). All three species made a range of molecular weights from low to high, depending upon substrate concentrations and ratios (refs. 21, 22 and 24). In general, the higher the initiator concentration the lower the molecular weight, whereas high monomer concentrations increase molecular weight both when the initiator concentration is limiting and under conditions where a negative cooperative is operating, i.e., whenever the chain transfer reaction is limited. Also, under most concentrations of initiator and monomer, *F. elastica*, the species producing the lowest molecular weight *in vivo,* made rubber, *in vitro*, of twice the molecular weight of that produced by *H. brasiliensis* or *P.*

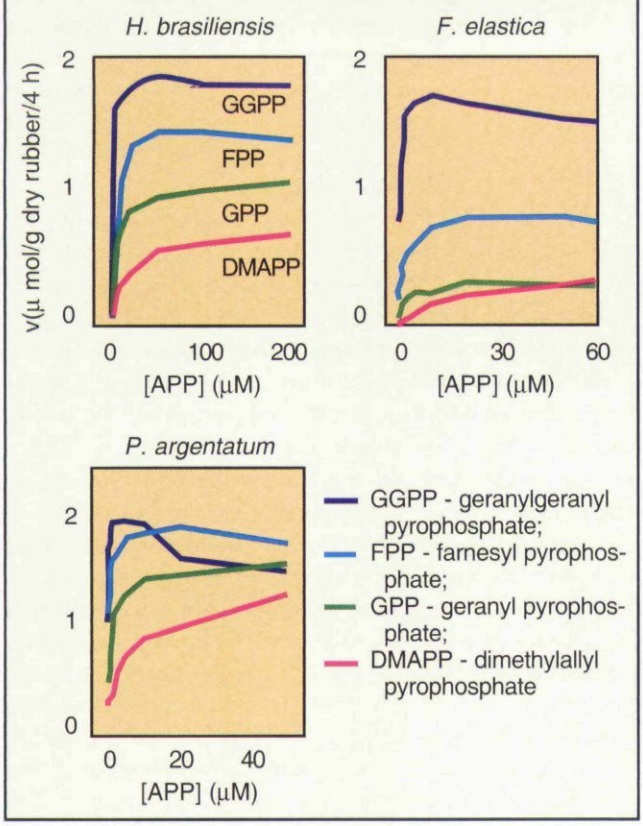
argentatum. This is apparently caused by the *F. elastica* rubber transferase having three times the affinity for the monomer, IPP, in the presence of the FPP initiator. One can infer that a three-fold greater affinity for the monomer could readily translate into a doubling of polymerization rate, and thus a doubling of molecular weight (ref. 24). The lack of correlation between the *in vivo* molecular weights and the *in vitro* ones implies that the rubber transferase, per se, is not the principle regulator of molecular weight *in vivo*. Other possible regulatory factors include the presence of termination inhibitors or enhancers or a regulatory role of the magnesium cofactor.

Environmental and developmental regulation of rubber yield and quality

In addition to the clear effects of substrate and cofactor identity and concentration on rubber biosynthesis, there are developmental and environmental effects to factor in as well. For example, *H. brasiliensis* rubber production declines, as might be expected, when assimilate is reduced during the leaf drop cycle. However, ethephon (a synthetic ethylene mimic plant growth regulator) is used to enhance production (ref. 25). Also, experimental work suggests that soil magnesium levels may impact yield and quality of the latex rubber. Young plants are too small to be worth tapping for latex, and panel tapping dryness can seriously impact latex yields (ref. 26).

Rubber biosynthesis in *P. argentatum* is environmentally-regulated, and most rubber is produced during the winter months when the plant is essentially dormant. The amount of rubber transferase increases (ref. 27), as does the level of the HMGR that supplies the IPP monomer (ref. 28). Very young plants do not appear to be responsive to cold induction, although young branches on mature plants are fully capable (ref. 27). Thus, a relatively small broadening of the rubber production season could have a tremendous impact on overall yield. This could be accomplished through genetic engineering, for example, by constitutive expression of the rubber transferase. Also, exogenously applied synthetic plant growth regulators, such as DCPTA, may enhance rubber biosynthesis under some environments (ref. 29).

Figure 3 - the effect of concentration and identity of four allylic pyrophosphate initiators (APP) on the rate of rubber biosynthesis in three different rubber-producing species

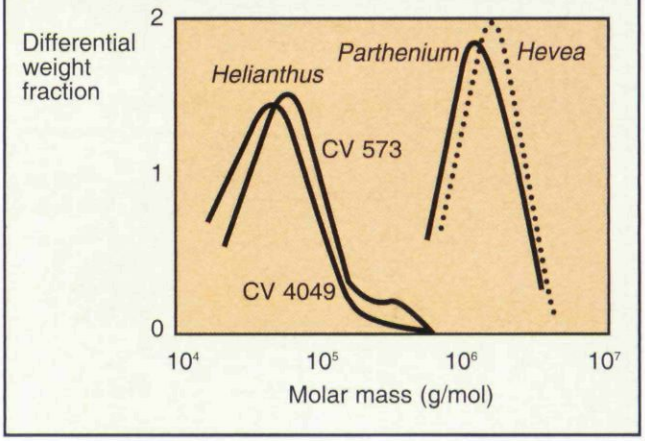


Genetic engineering of rubber yield and quality

It is clear that rubber biosynthesis proceeds biochemically in fundamentally the same way in all rubber-producing species, and recent, detailed biochemical studies of rubber biosynthesis in *Taraxacum kok-saghz* (Russian dandelion) have added another species to our list.

However, the species-specific differences in rubber biochemistry described have considerable relevance to genetic engineering programs. For example, *Helianthus annus* (sunflower) makes a small amount of low molecular weight rubber (figure 4). If we overexpressed a rubber transferase from *H. brasiliensis* in *H. annuus*, and did nothing else, we likely would get more rubber, but of the low molecular weight (and poor quality) normally produced by *H. annuus*. The *P. argentatum* rubber transferase, with its intrinsic property of negative cooperativity, is more likely to produce high molecular weight rubber in a wide range of genetic backgrounds (i.e., in other species) than the rubber transferases from either *H. brasiliensis* or *F. elastica*. Also, metabolic engineering could

Figure 4 - molecular weight of latex rubber extracted from leaves of two cultivars of *Helianthus annuus*, compared with latex rubber from *Parthenium argentatum* and *Hevea brasiliensis*



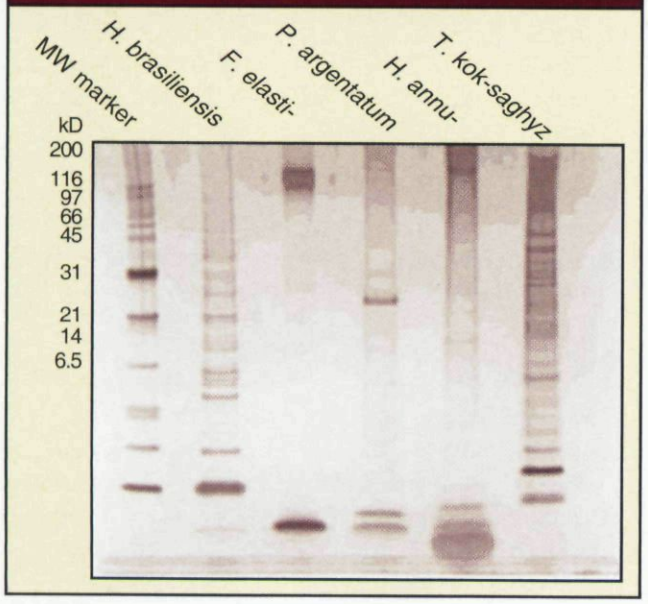
be performed to adjust the levels of substrate to affect the molecular weight, but care must be taken to not harm the plant through diversion of essential substrates to rubber biosynthesis, because of the central importance of the isoprenoid pathway in plant growth and development. In this context, the first round of *P. argentatum* transgenics, in three different lines, constitutively overexpressing non-native genes encoding three different *trans*-prenyl transferases that synthesize different allylic pyrophosphate initiators produced healthy plants. Overall, no more rubber was accumulated, but there was some indication that more rubber molecules were synthesized in those transgenic lines with the highest prenyl transferase activities. These data suggest that the monomer was limiting in the plants, and if IPP levels were also increased, the desired yield increases might be obtainable. In addition, secondary product analysis of parts of the downstream portion of the sesquiterpene and triterpene pathways revealed substantial suppression of the major cinnamic and aniscic acid ester secondary product levels compared to non-transgenic and empty vector controls (ref. 30). Total resin levels, which include these compounds, were not suppressed and were enhanced in some lines. Thus, a redirection of substrate occurred, but this did not adversely affect plant health, or growth and development (ref. 30). A more detailed analysis of the isoprenoid pathway is needed to determine what metabolites replaced the esters in the transgenic plants.

Genetic engineering also requires the development and exploitation of effective tissue culture and transformation procedures. These have been developed for *H. brasiliensis* and *P. argentatum*, but *H. annuus* is notoriously recalcitrant, hampering the development of commercially-viable rubber-producing sunflower crops.

Genomics and proteomics

Modern molecular approaches are employing a functional identification methodology, using both overexpression and down-regulation of genes possibly involved in rubber biosynthesis. In brief, these genes include those encoding known substrates and genes encoding rubber particle-bound protein of both known and, as yet unknown, function. This type of functional approach is not very amenable to species such as *H. brasiliensis*, in which several years of growth are needed before a meaningful phenotype can be determined. Even *P. argentatum* can only be accurately assessed after two years. This is where the use of *T. kok-saghyz*, as a model rubber-producing system, comes into its own. This plant produces good yields of high molecular weight rubber, is easy to tissue culture and transform, and meaningful rubber phenotypes can be obtained within six months. Use of this species allows many genes to be tested, and a shortlist to be generated of the most promising functional genes to be tested in *P. argentatum*.

Figure 5 - protein profiles of rubber particle proteins from several different species



Prospects for other rubber-producing crops

Many rubber-producing plants produce natural rubber, although few do so in high yields or high molecular weights. In addition, the conversion of a wild rubber-producing plant into a cultivated crop is a far from trivial task. Even *P. argentatum* can only be considered partially-domesticated, and candidates such as the Russian dandelion species, the rubber vine or the Japanese rubber mushroom are very far from commercialization. Nonetheless, as with crops produced essentially for starch, such as rice, wheat, corn and potatoes, etc., there is much to be said for increasing the biodiversity of commercial rubber production beyond *H. brasiliensis* and *P. argentatum*. Sunflower, already a commercial crop for its oil and seed, faces fewer barriers than the introduction of wild species, because its agronomy and production practices are well understood. Unfortunately, its barriers to commercial rubber production are centered on the extreme difficulty of genetically transforming this species. It should also be borne in mind that all new rubber-producing species should be seriously evaluated both for their cross-reactivity to *H. brasiliensis* Type I latex allergy and for their potential to induce Type I allergies of their own. As may be seen (figure 5), *T. kok-sahgyz* has many rubber particle proteins, and possibly as much, if not more, than *H. brasiliensis*.

Conclusions

Molecular approaches appear to offer the most promise for making substantial improvements in rubber yield and quality in alternative rubber-producing plant species suitable for cultivation in temperate, rather than tropical, regions. *P. argentatum* lines developed from conventional plant breeding programs are already being produced on a commercial scale. However, a combination of biochemistry-based approaches, and genomics and proteomics methods capitalizing on model systems, promise to generate the fundamental breakthroughs in functional understanding needed to fully exploit molecular methods and generate significantly improved new lines. In addition, improvements in tissue culture and transformation methods, especially designed for recalcitrant species, are being aggressively pursued by academic, federal and industrial

laboratories, and their success should greatly facilitate the development and introduction of additional rubber-producing crops, such as sunflower.

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Additive influences

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ed., Interscience Publishers, 1965.

Correction

In the July, 2005, issue article, "Electron beam processing of elastomers," the second sentence under sub-heading Gamma-radiation, should have read: The γ-radiation has a very high penetration, but exhibits a low dose rate when compared to electron beam radiation."