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Regulation of initiation and polymer molecular weight of *cis*-1,4-polyisoprene synthesized in vitro by particles isolated from *Parthenium argentatum* (Gray)

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Abstract

Parthenium argentatum is a prime candidate for development as a new crop for the production of natural rubber (*cis*-1,4-polyisoprene). The rubber obtained from *P. argentatum* is comparable in molecular weight (and quality) to that obtained from *Hevea brasiliensis* (M_r ca. 10^6 Da), which is currently the sole commercial source of natural rubber. Most of the 2500 rubber-producing plant species known make much lower molecular weight rubber, although the regulation of polymer molecular weight is not understood.

In the experiments reported here, we investigated the regulation of rubber biosynthesis and polymer molecular weight using purified, enzymatically-active rubber particles isolated from *P. argentatum*, under various concentrations of the allylic diphosphate (allylic-PP) rubber molecule initiators farnesyl diphosphate (FPP, a 15-carbon molecule), or geranyl diphosphate (GPP, a 10-carbon molecule) and the elongation substrate isopentenyl diphosphate (IPP). Our results show that the rates of both rubber molecule initiation and polymerization, and the final polymer molecular weight, were greatly affected by the concentration of initiator and IPP. Increasing allylic-PP initiator concentrations caused an increase in the amount of rubber synthesized and the number of molecules initiated but a decrease in the mean polymer molecular weight; increasing IPP concentrations increased the amount of rubber and the mean polymer molecular weight. At identical substrate concentrations of IPP and allylic-PP, GPP initiated about one third of the rubber polymers initiated by FPP but incorporated about two thirds the amount of IPP, compared to FPP. Consequently, while the amount of rubber synthesized in the presence of GPP was less than with FPP, the rubber polymers synthesized with GPP initiator were, on average, about twice the molecular weight of those with FPP.

Keywords: *Parthenium argentatum*; Asteraceae; *cis*-Prenyl transferase; Allylic diphosphate; *cis*-1,4-Polyisoprene; Isopentenyl diphosphate; Polymer molecular weight; Rubber; Rubber particles; Rubber transferase

1. Introduction

More than 2500 plants produce natural rubber (*cis*-1,4-polyisoprene) although polymer molecular weight varies greatly between species. In all rubber-producing species investigated so far, natural rubber biosynthesis is catalyzed by a membrane-bound rubber transferase (*cis*-prenyl transferase, EC 2.5.1.20) associated with cytoplasmic rubber particles (Archer, Audley, Cockbain, & McSweeney, 1963; Madhavan, Greenblatt, Foster, & Benedict, 1989; Cornish & Backhaus, 1990; Cornish, 1993; Cornish, Siler, Grosjean, & Goodman, 1993; Cornish & Siler, 1996b). This enzyme produces the rubber polymer (*cis*-1,4-polyisoprene) from isoprene units derived from isopentenyl diphosphate (IPP) (Figure 1). The rubber transferase also requires an allylic diphosphate (allylic-PP) cosubstrate (produced by soluble *trans*-prenyl transferases) to initiate polymer formation and a divalent cation, such as Mg^{2+} or Mn^{2+}, as cofactor (Archer & Audley, 1987; Madhavan et al., 1989; Cornish & Backhaus, 1990; Cornish, 1993; Tanaka et al., 1996). Rubber transferases exhibit similar kinetic constants and are able to accept a similar range of allylic-PP's as initiating substrate (Berndt, 1963; Lynen, 1969; Archer & Audley, 1987; Madhavan et al., 1989; Cornish & Backhaus, 1990; Cornish, 1993; Cornish & Siler, 1995; Cornish, Castillón & Chapman, 1998). Also, studies have shown that the longer the allylic-PP, up to C_{15} or C_{20}, the faster the rate of rubber biosynthesis in vitro (Archer &

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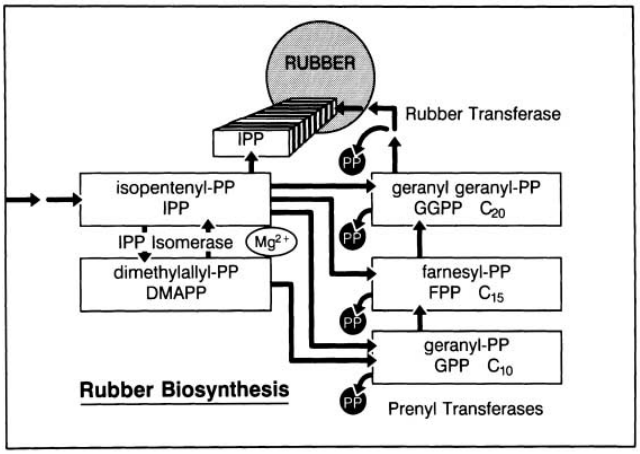

Fig. 1. Rubber biosynthesis from isopentenyl diphosphate (IPP). The rubber particle membrane-bound rubber transferase (*cis*-prenyl transferase) binds an allylic diphosphate (allylic-PP) to initiate a new rubber molecule and then elongates the polymer by *cis*-1,4-polymerization of isopentenyl units derived from IPP. Allylic-PP's larger than DMAPP are synthesized by soluble *trans* prenyl transferases. All the allylic diphosphates shown can be used as initiators by rubber transferase, not only GGPP as shown. All reactions shown require a divalent cation cofactor such as magnesium.

Audley, 1987; Cornish and Siler, 1995; Cornish et al., 1998). However, very little is known about the regulation of polymer molecular weight in rubber-producing species. A single report from this laboratory, using enzymatically-active rubber particles isolated from *P. argentatum* (guayule), indicated that, under nonlimiting substrate concentrations in vitro, geranyl diphosphate (GPP) and farnesyl diphosphate (FPP) initiated the formation of rubber of different mean polymer molecular weight (Cornish & Siler, 1995).

P. argentatum is a rubber-producing, woody, perennial shrub native to the Chihuahuan desert of Texas and northern Mexico (Whitworth & Whitehead, 1991). It is currently under investigation as a new industrial crop since the rubber it produces in vivo is primarily long chain rubber ($M_r \geq 10^6$ Da) comparable in quality to that obtained from *Hevea brasiliensis*, presently the only commercial source of natural rubber (d'Auzac, Jacob, & Chrestin, 1989; Whitworth & Whitehead, 1991). Recent reports of increasing type I latex allergies in response to the use of *H. brasiliensis* latex products, and of the hypoallergenicity of guayule rubber, with respect to *H. brasiliensis* latex allergy, have provided new prospects for its commercialization (Siler & Cornish, 1994; Nakayama, Cornish, & Schloman, 1996; Siler, Cornish, & Hamilton, 1996; Cornish & Siler, 1996a; Cornish, 1996, 1998). Advances in plant transformation techniques have made it feasible to increase yield through genetic engineering of cultivated guayule plants (Pan, Ho, Feng, Huang, & Backhaus, 1996). However, increasing yield will only be beneficial if the rubber produced still consists of high molecular weight polymers. In order to design effective strategies for the development of *P. argentatum* lines capable of greater yields of high quality (high molecular weight) rubber, it is essential to gain a better understanding of the regulation of rubber biosynthesis and polymer molecular weight.

In this paper, we investigate the effect of cosubstrate type and concentration on the initiation and molecular weight of rubber polymers synthesized in vitro by enzymatically-active rubber particles of *P. argentatum*.

2. Results

2.1. Rubber biosynthesis

When kinetic parameters were determined for the *P. argentatum* rubber transferase (Table 1), it was clear, as indicated earlier (Cornish & Siler, 1995) that the affinity of the enzyme is greater for the allylic-PP than for IPP. In addition, v_{max} was 1.44 times higher with FPP than GPP, but apparent K_m was 29 times lower for FPP than GPP.

2.2. Initiation of rubber molecules

When GPP was used as the initiator, it was found that GPP incorporation rates increased with increasing GPP concentration at all IPP concentrations investigated

Table 1
Binding constants and maximum reaction velocity of the *Parthenium argentatum* rubber transferase

Substrate	Size (No. of carbons)	Apparent K_m (μM)	v_{max} (μmol IPP·gdw rubber^{-1}·4 h^{-1})
IPP	5	300	–
DMAPP	5	1.6	0.32
GPP	10	0.49	1.10
FPP	15	0.017	1.82

Kinetic values for the allylic diphosphates were determined at 16°C in the presence of 5 mM [^{14}C]isopentenyl diphosphate (IPP). DMAPP represents dimethyl allyl diphosphate, GPP, geranyl diphosphate and FPP, *E,E*-farnesyl diphosphate. v_{max} was determined at the same time, for all allylic diphosphates, using the same rubber particle preparation.

(Figure 2a). Incorporation rates ranged from 0.006 to 23.0 nmol GPP·g dry weight (gdw) rubber particles^{-1}·4 h^{-1} depending on the substrate concentration. GPP incorporation rates also increased with increasing IPP concentration (Figure 2a) at all GPP concentrations, with maximal GPP incorporation rates achieved in 5 mM IPP, indicating that the rubber transferase was saturated with IPP at this level, which agrees with earlier reports (Cornish & Backhaus, 1990; Cornish & Siler, 1995). Similar patterns of incorporation were observed when FPP was used as the initiator, with the FPP incorporation rate increasing in response to both FPP and IPP concentration (Figure 2b). Incorporation rates of FPP ranged from 0.047 to 65.8 nmol FPP·gdw rubber particle^{-1}·4 h^{-1}, 2–10 times higher than the rates observed for GPP. At all IPP concentrations, initiator incorporation rates were slightly higher in 50 μM allylic-PP than at 25 μM, suggesting that the rubber transferase was not completely saturated by 25 μM allylic-PP. Earlier

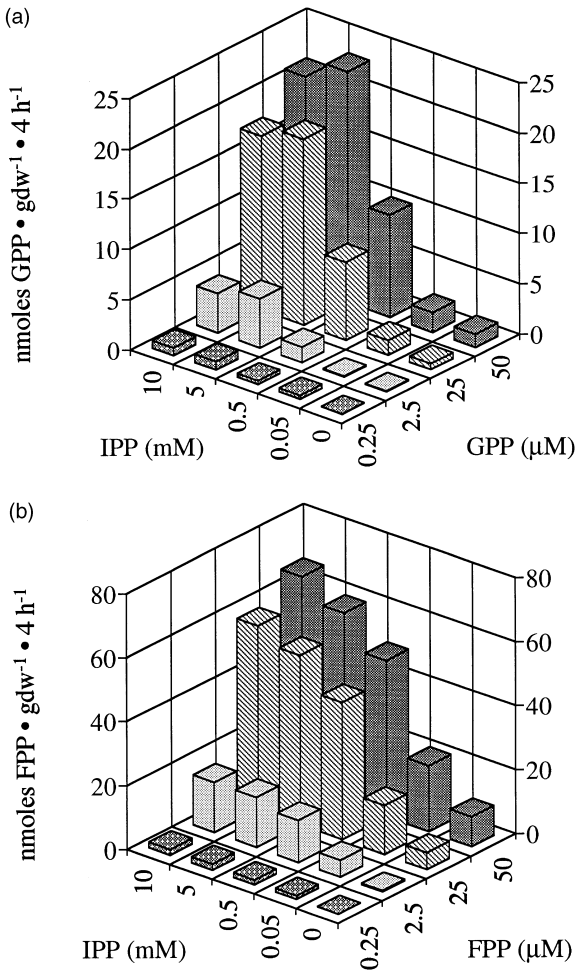

Fig. 2. Initiation rate of rubber molecules using (a) [^{3}H]GPP or (b) [^{3}H]FPP at various IPP and initiator concentrations. Values are given as initiator incorporation rate (nmol initiator·gdw rubber particles^{-1}·4 h^{-1}). Each value is the mean of three replicates. Mean coefficient of variation is 8.4% for GPP and 5.9% for FPP.

reports had found that 20 μM allylic-PP was sufficient to saturate the enzyme when IPP incorporation rates were determined at saturating levels of IPP (Cornish & Backhaus, 1990; Cornish & Siler, 1995), as would be expected with apparent K_m's of less than 1 μM for FPP and GPP (Table 1). As expected, maximum allylic-PP depletion rates from the assay medium were observed when the lowest allylic-PP concentrations were coupled with the 5 and 10 mM IPP (Table 2). However, even here, depletion rates did not exceed 15.5% of the initial FPP concentration or 7.25% of the GPP (over the 4-h incubation period).

2.3. Rubber biosynthetic rate

The total amount of rubber synthesized was determined by following the incorporation of [^{14}C]IPP into the new rubber polymers. IPP incorporation rates increased with IPP concentration at all concentrations of GPP (Figure 3a) and FPP (Figure 3b). Increasing initiator concentrations, up to 25 μM, also resulted in higher rates of IPP incorporation (Figure 3a and b) with FPP supporting higher incorporation rates than GPP, as reported earlier (Cornish & Siler, 1995). IPP incorporation rates ranged from 0.248 to 7.86 μmol IPP·gdw rubber particles^{-1}·4 h^{-1} (Figure 3a) when GPP was used as the initiator, and from 0.253 to 12.96 μmol IPP·gdw rubber particles^{-1}·4 h^{-1} (Figure 3b) when FPP was used as the initiator. As expected, maximum rates of IPP depletion from the assay medium (calculated by subtracting the amount of IPP incorporated into rubber from the initial IPP concentration) occurred when the allylic-PP concentrations were not rate-limiting, and at the lowest IPP concentration, up to 60% of the IPP was polymerized (Table 2).

2.4. Molecular weight of newly synthesized rubber polymers

The mean M_r's of rubber molecules synthesized were highly dependent upon the concentration of IPP and allylic-PP initiator. Mean M_r's were highest at high IPP and low initiator concentrations (Figure 4a and b). When FPP was used as the initiator (Figure 4b), mean M_r increased with increasing IPP concentration at all FPP concentrations. This trend was only clear at the lowest GPP concentration when GPP was used as the initiator (Figure 4a). On the other hand, increasing GPP and FPP concentrations decreased mean M_r of the rubber molecules at all IPP concentrations (Figure 4a and b). The mean M_r was also higher for all IPP/GPP combinations than for the corresponding IPP/FPP combinations (Figure 5). This difference was most marked at the lowest IPP and highest initiator combination, where the mean M_r for the newly synthesized rubber was 8.8 times higher with GPP than with FPP. However, under nonlimiting IPP concentrations ($\geq$5 mM), the mean M_r

Table 2
Substrate consumption during incubation of assays in dual-labeled experiments

Initial substrate concentrations		Substrate mixture in assay			
		IPP + FPP		IPP + GPP	
[IPP]	[Allylic-PP]	IPP	FPP	IPP	GPP
(*mM*)	(*μM*)		(*% used*)		
0	0.25	–	0.39	–	0.05
	2.50	–	0.34	–	0.06
	25.0	–	0.85	–	0.05
	50.0	–	0.39	–	0.05
0.05	0.25	28.5	9.74	23.5	3.14
	2.50	52.1	4.45	31.9	0.08
	25.0	60.0	1.28	37.9	0.12
	50.0	55.0	0.86	37.5	0.08
0.50	0.25	6.41	12.2	6.54	3.03
	2.50	20.8	10.8	9.82	1.31
	25.0	23.8	3.59	14.2	0.64
	50.0	22.5	2.05	13.5	0.42
5.0	0.25	1.27	15.5	1.19	7.25
	2.50	2.24	13.1	1.53	4.05
	25.0	4.28	4.44	2.80	1.55
	50.0	3.79	2.47	2.44	0.96
10.0	0.25	0.78	14.3	0.67	6.36
	2.50	1.27	13.1	0.71	3.25
	25.0	2.70	4.82	1.64	1.45
	50.0	2.35	2.76	1.35	0.88

Each value is the mean of three. IPP represents isopentenyl diphosphate, FPP, *E,E*-farnesyl diphosphate and GPP, geranyl diphosphate.

with GPP consistently remained about twice as large as the mean M_r with FPP.

In an additional comparison, mean M_r's were compared under increasing substrate concentrations but identical substrate ratios of 200 IPP: 1 allylic-PP (Figure 6). With FPP, rubber mean M_r steadily decreased with increasing concentration. However, with GPP, rubber mean M_r only decreased at concentrations above 0.5 mM IPP with 2.5 μM GPP.

3. Discussion

It has been shown previously (Cornish & Siler, 1995) that the rate of rubber biosynthesis increases with the chain length of the allylic diphosphate initiator and that, under nonlimiting substrate concentrations, IPP and allylic-PP are incorporated at constant rates over time. Also, under these conditions, the mean molecular weight of polymer produced remained constant.

The results reported here, are entirely consistent with the earlier data, in that the rate of rubber biosynthesis was both concentration dependent and higher in the presence of the C_{15} FPP than in the C_{10} GPP (Figures 2–3). At all IPP concentrations, IPP incorporation rates reached maximal values by 25 μM allylic-PP (Fig. 2), which agrees with the earlier reports that 20 μM allylic-PP is not rate-limiting so far as IPP incorporation rates are concerned (Cornish & Backhaus, 1990; Cornish & Siler, 1995). Maximal IPP-incorporation rates were also largely achieved by 5 mM IPP at any allylic-PP concentration (Fig. 3) again supporting earlier determinations made in the presence of non-rate-limiting allylic-PP concentrations. Since the rubber transferase apparent K_m is nearly 30 times larger for GPP than for FPP (Table 1), we propose that the higher affinity of the rubber transferase for FPP leads to the higher overall IPP-incorporation rate since molecules are initiated more rapidly with FPP than GPP and provide more steady state locations for IPP incorporation.

Similarly, the rate of rubber molecule initiation increased with both increasing WRP and allylic-PP concentration. Since rubber molecule initiation requires an allylic-PP, it is reasonable to expect the rate of initiation to increase with initiator concentration until all available rubber transferases had initiated a rubber molecule; a normal enzyme-substrate equilibrium is unlikely since the enzyme product is not soluble and is certainly not fully

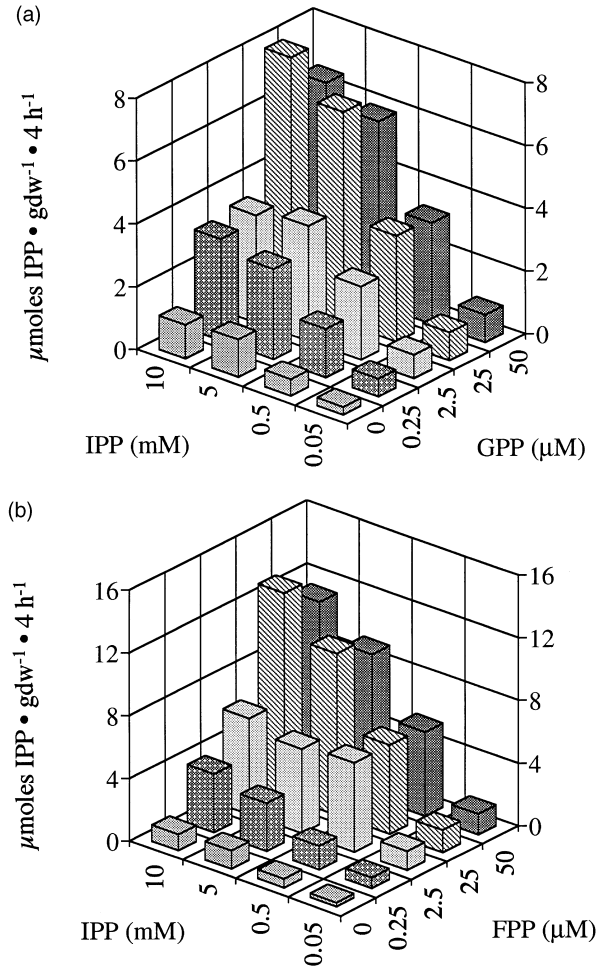

Fig. 3. Total amount of rubber synthesized using (a) [^{3}H]GPP or (b) [^{3}H]FPP at various IPP and initiator concentrations. Values are given as [^{14}C]IPP incorporation rate (μmol IPP·gdw^{-1}·4 h^{-1}). Each value is the mean of three replicates. Mean coefficient of variation is 3.6% in GPP and 2.9% in FPP.

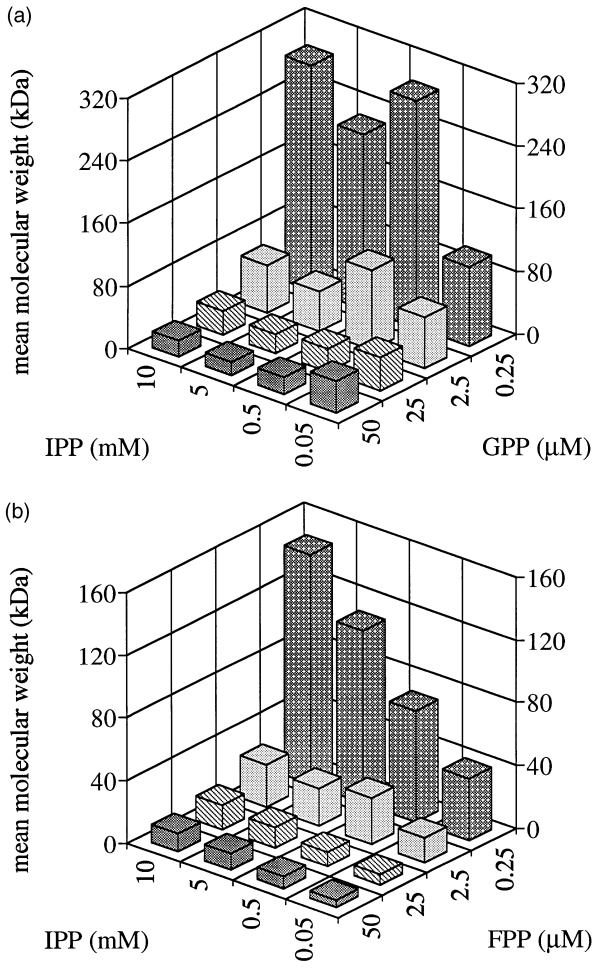

Fig. 4. Mean molecular weight (kDa) of rubber molecules synthesized using (a) [^{3}H]GPP or (b) [^{3}H]FPP at various IPP and initiator concentrations. Values were calculated as given in Section 4. Each value is the mean of three replicates. Mean coefficient of variation is 6.9% in GPP and 3.6% in FPP.

released from the active site during the polymerization reaction. We propose that once allylic-PP binds to the active site, the polymer that begins to form restricts access to the allylic-PP binding site by free allylic-PP. This allows IPP polymerization to proceed even though the apparent K_m for IPP is much higher than for allylic-PP (Table 1). However, the dominant effects of allylic-PP type and concentration on polymer molecular weight support there being some competition between free allylic-PP and the allylic-PP end of the growing polymer for the allylic-PP binding site. The site binds the original allylic-PP initiator and all the allylic-PP intermediates formed as the rubber polymer grows (the terminal five-carbon unit always includes the pyrophosphate and allylic carbon). Hence, the higher the allylic-PP concentration, the more likely that free allylic-PP could displace the polymer and initiate a new rubber molecule, and we propose that this competition accounts for the shortening of

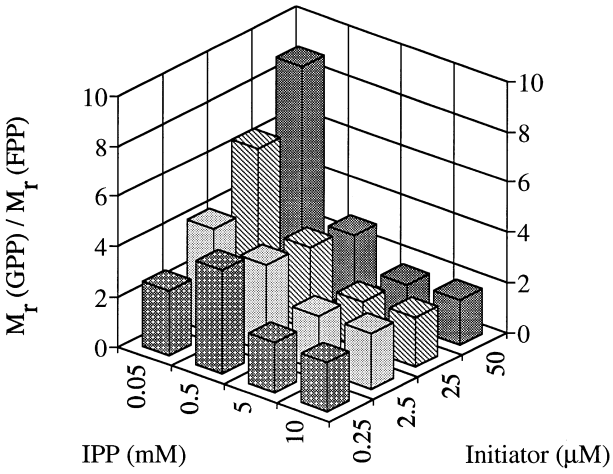

Fig. 5. Comparison of mean molecular weights (M_r) of rubber molecules synthesized using GPP to those synthesized using FPP at the same IPP and initiator concentrations. Values represent the ratio $M_{r(GPP)}/M_{r(FPP)}$.

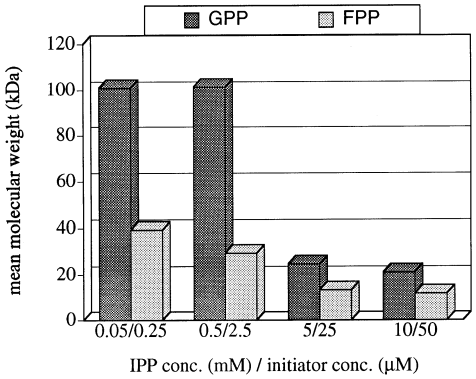

Fig. 6. Effect of increasing substrate concentration on molecular weight (M_r) of rubber molecules synthesized using either GPP or FPP as the allylic-PP initiator, while maintaining a constant IPP:allylic-PP ratio of 200:1.

the rubber polymer with increasing allylic-PP concentration. Furthermore, since the rubber transferase apparent K_m is nearly 30 times larger for GPP than for FPP, FPP may be able to compete more effectively with the allylic-PP end of the elongating rubber polymer than can GPP, and so can more often displace the polymer from the allylic-PP binding site. This would lead to a shorter time in which an existing rubber molecule could elongate. In this way, the larger initiator, FPP (C_{15}), leads to shorter rubber than the shorter GPP (C_{10}) even though the overall rate of biosynthesis was enhanced. These trends are supported by preliminary dual-labeling experiments performed using nonlimiting concentrations of dimethyl allyl diphosphate (DMAPP) as the initiating cosubstrate. The rubber transferase apparent K_m for DMAPP is over three times larger than that for GPP and over 90 times that for FPP (Table 1), whereas v_{max} is the lowest of the three cosubstrates. As expected, the mean molecular weight of the polymers produced with DMAPP appeared the largest of the three cosubstrates. However, the data are not definitive using the techniques employed in this paper: a small amount of IPP isomerase is present in the rubber particle preparations which causes interconversion of the ^{14}C-IPP and the ^{3}H-DMAPP. Although, when inhibited by a site-directed isomerase inhibitor (3,4-oxido-3-methyl-1-butyl diphosphate (Muehlbacher & Poulter, 1988)), most of the spurious tritium is eliminated, and the trends described in the paper are supported, it is difficult to prove that inhibition is complete.

Our data also indicate that there may be competition for the IPP binding site by IPP and allylic-PP, although catalysis only occurs if IPP is bound. If such competition occurs, increasing nonlimiting concentrations of allylic-PP should inhibit IPP incorporation rates. This is, in fact, what we observe (Fig. 3), with less IPP incorporated into rubber in 50 μM allylic-PP than in 25 μM.

In contrast, substrate activation between IPP and allylic-PP for the allylic-PP binding site may explain why the rate of initiation, at all allylic-PP concentrations investigated, was enhanced by increasing IPP concentration (Fig. 2). Competition between IPP and allylic-PP for the allylic-PP binding site is not likely since allylic-PP incorporation rate does not decline at high IPP concentrations (Fig. 2) as would be expected (and as we saw in Fig. 3 for the IPP incorporation rate at the highest allylic-PP concentrations). Nonetheless, an increasing initiation rate means that there must either be more rubber molecules being synthesized at any one time, and therefore, more enzyme molecules actively engaged in polymerization, or that the rate of release and reinitiation (the chain transfer reaction) is increased. The maximum allylic-PP incorporation rates in the absence of IPP (Fig. 2) may reflect the number of rubber transferase molecules available, although the equilibrium between free and bound allylic-PP may differ from the equilibrium between the free allylic-PP and the allylic-PP end of the elongating rubber molecule. Nevertheless, in the presence of IPP and nonlimiting concentrations of allylic-PP, it would be expected that rubber molecules would be initiated by all active rubber transferases. Thus, the large IPP concentration dependence still apparent, even at nonlimiting allylic-PP concentrations (Fig. 2), is unlikely to reflect a parallel increase in the number of occupied enzymes. We suggest that some sort of cosubstrate activation is occurring in which IPP enhances the rate of allylic-PP binding. However, polymer molecular weight seems to be primarily regulated by the allylic-PP type and concentration, with increasing IPP concentrations causing longer rubber to be formed only at rate-limiting allylic-PP concentrations (Fig. 4).

Our results clearly demonstrate that both IPP and initiator concentration affect molecular weight of in vitro synthesized rubber, although allylic-PP has the greatest effect. Previous work reported mean M_r's + S.D. of 6787 ± 624 and 5353 ± 457 Da for rubber molecules initiated by GPP and FPP, respectively (Cornish & Siler, 1995). However, this was under nonlimiting substrate concentrations (5 mM IPP and 20 μM GPP or FPP). The results presented here show a range of molecular weights from 17,600 to 290,000 for GPP and 4700 to 148,000 for FPP, with the largest polymers being produced at the lowest initiator concentrations (Fig. 4). These higher values are closer to those found in vivo ($\geq 10^6$ Da; Cornish et al., 1993) and indicate that polymer molecular weight in vivo may be regulated, at least in part, by different endogenous substrate pool sizes, rather than by radically different rubber transferase enzymes in different species. For example, a 'low molecular weight' species such as *Ficus elastica* (Cornish et al., 1993) might have much higher endogenous levels of allylic-PP than 'high molecular weight' species such as *P. argentatum* or *H. brasiliensis*, and perhaps different levels of the prenyl transferases that catalyze the synthesis of these molecules.

Studies of rubber transferases from these short and long chain rubber-producing species have shown the transferases to be 'indeterminant' enzymes, which have no predetermined product size, with similar substrate requirements and kinetic constants (Cornish et al., 1993, 1998; Cornish & Siler, 1996b). These similarities suggest that the rubber produced in vitro by the *H. brasiliensis* and *F. elastica* rubber transferases may differ in size under changing substrate concentrations in a similar way to that seen here for *P. argentatum*. It has also been noted that the rubber produced by greenhouse-grown *F. elastica* plants includes a small amount (ca. 2%) of high molecular weight rubber (Cornish et al., 1993). It seems reasonable to propose that these large rubber polymers may have been synthesized during a period of increased demand for allylic-PP by other enzymes of the isoprenoid pathway, such as might be created by a spurt of rapid growth or development. Our in vitro results suggest that the inevitable, but transient lowering in endogenous allylic-PP concentration would result in much higher molecular weight polymer being synthesized while those conditions prevailed. Nevertheless, our findings do not rule out the possibility that specific polymer termination events occur in vivo. Very little is known about rubber polymer termination, apart from some rubber structural studies indicating different polymer end-groups in different species (Tanaka et al., 1996). Also, it should be noted that the allylic-PP's we have examined separately coexist in plant cytoplasm — the effect of such mixtures on the rate of rubber biosynthesis is poorly understood, and nothing is yet known about how molecular weight may be affected.

It is apparent that the substrate effects observed in our rubber transferase investigation cannot be explained by the rubber being formed as a 'living polymer', such as occurs in the bacterial polyhydroxyalkanoate synthase from *Alcaligenes eutrophus* (Martin, Zhang, Su, & Lenz, 1998). In this system, polymer molecular weight is dependent upon substrate concentration but directly results from the ratio of enzyme to monomer, with chain extension only halting with total depletion of free monomer. The polymer molecular weights produced by the *P. argentatum* rubber transferase vary without exhaustion of free substrate, and in many cases without large changes in substrate concentration (Table 2). Also, in the *A. eutrophus* PHA-synthase in vitro system, the polymer and enzyme do not dissociate (Martin et al., 1998). In contrast, the *P. argentatum* rubber transferase system in vitro does appear to be able to terminate the polymers and initiate new molecules. For example, the incorporation rate of allylic-PP, under saturating concentrations of IPP and either FPP or GPP was linear over 6 h (Cornish & Siler, 1995), and so the rate of termination and reinitiation (the chain transfer reaction rate), remained constant under these conditions.

In conclusion, we have shown that rubber biosynthetic rate and polymer molecular weight in vitro are dependent upon substrate concentration, the ratio of IPP and allylic-PP and the size of the allylic-PP initiator. Thus, the higher the IPP concentration, the higher the biosynthetic rate, the greater the number of rubber molecules produced and, when allylic-PP levels are limiting, the larger the polymer molecular weight produced. The higher the allylic-PP concentration, the higher the overall biosynthetic rate, the greater the number of rubber molecules, but the smaller the polymer molecular weight produced. Also, the larger the allylic-PP initiator, the greater the overall rubber biosynthetic rate, the faster the rate of rubber molecule initiation, but the shorter the rubber polymer length. Thus, strategies to enhance the yield of high molecular weight rubber in *P. argentatum*, through genetic manipulation of endogenous substrate concentrations, must be carefully crafted, and will be absolutely dependent upon in vivo testing of regenerated transformants.

4. Experimental

4.1. Materials

Mature, field-grown *P. argentatum* Gray (line 11591) plants were grown at the US Water Conservation Laboratory, Phoenix, AZ. Unlabeled IPP, GPP and *E,E*-FPP as well as [^{14}C]IPP (55 mCi/mmol), [^{3}H]GPP (15 Ci/mmol) and [^{3}H]*E,E*-FPP (60 Ci/mmol) were obtained from American Radiolabeled Chemical Inc., St. Louis, MO.

4.2. Preparation of washed rubber particles

Enzymatically-active rubber particles were purified using a method similar to that previously described (Cornish & Backhaus, 1990; Cornish & Siler, 1995), maintaining a temperature of 4°C throughout. Stem bark was removed from mature stem tissue which had been shipped on ice overnight to the Albany, CA, laboratory. Approximately 350 g of bark were then homogenized in a 1 gallon Waring blender in 1L of extraction buffer (EB): pH 7.5, 100 mM Tris–HCl, 5 mM $MgSO_4$, 50 mM KF, 0.1 mM phenylmethanesulfonyl fluoride (PMSF, Boehringer Mannheim Corp., Indianapolis, IN), 1% ascorbic acid, 5 mM 2-mercaptoethanol, 0.05 μL/ml antifoam A emulsion (Sigma Chemical Co., St Louis, MO), and 0.07 g/ml polyvinylpolypyrrolidone (PVPP). The homogenate was filtered through eight layers of cheese cloth, and the filtrate was centrifuged at $2500g_n$, for 12 min. The floated rubber particles were collected and resuspended in wash buffer 1 (WB1), pH 8, containing 100 mM Tris–HCl, 5 mM $MgSO_4$, 5 mM dithiothreitol (DTT, Sigma Chemical Co., St Louis, MO) and 0.1 mM PMSF. The particles were then recentrifuged at $2000g_n$ for 12 min. The par-

ticles were then collected, resuspended in WB1 and recentrifuged as before two more times with the particles being resuspended in wash buffer 2 (WB2) for the final centrifugation. WB2 was the same as WB1 except it contained 10 mM DTT and 0.1 mM Pefabloc (4-(2-aminoethyl)-benzenesulphonyl fluoride), hydrochloride powder (Boehringer Mannheim Corp., Indianapolis, IN). The three times washed rubber particles (WRP) were then resuspended in a small amount of WB2, adjusted to 10% glycerol, frozen as droplets in liquid N_2 and stored in liquid N_2 until used.

4.3. Assay of in vitro rubber biosynthesis

In vitro rubber biosynthesis was carried out in siliconized 1.5-cm^3 microfuge tubes containing 50 μL total reaction volume (40 mM Tris–HCl, pH 7.8, 0.5 mM $MgSO_4$, 1.15 mg WRP (dry mass), concentrations of IPP, GPP and FPP were varied as given in Section 2). Unlabeled substrates were added as 10× stocks to provide the required final concentrations for each reaction, taking into consideration the amounts contributed by the labeled substrates. Labeled substrate stocks were adjusted to provide, per reaction, 3.33 kBq of [^{14}C]IPP, 16.67 kBq of [^{3}H]GPP or [^{3}H]FPP for reactions requiring 0.25 or 2.5 μM GPP or FPP and 83.33 kBq of [^{3}H]GPP or [^{3}H]FPP for reactions requiring 25 or 50 μM GPP or FPP. Some reactions were carried out in the presence of 20 mM EDTA to determine the background levels of radiolabel passively adhering to the rubber particles (EDTA chelates the Mg^{2+} cofactor required for activity). Siliconized tubes are used to prevent loss of substrate by binding to the plastic. No binding of newly synthesized rubber to the siliconized tubes occurs since this polymeric endproduct is compartmentalized inside the rubber particles. Reaction mixtures were incubated for 4 h at 16°C, and then the reactions were halted by the addition of 2 μL of 0.5 M EDTA. The rubber particles were harvested by filtration using 0.25-μm cellulose acetate/cellulose nitrate filters; the assay tubes were rinsed with 200 μL of ddH_2O, and the rinse also filtered. Filters were dried overnight at 37°C, washed to remove unincorporated radiolabel (Cornish & Backhaus, 1990), and the radioactivity on the filters measured by liquid scintillation spectroscopy.

4.4. Determination of rubber molecular weight

Since only one initiator molecule is used for each rubber molecule, the number of newly synthesized rubber molecules can be calculated from the incorporation rate of [^{3}H]allylic-PP initiator. Also, virtually all IPP incorporated by *P. argentatum* WRP has been shown to be via newly initiated polymers; it is not incorporated by extension of the chains present in the rubber particles before incubation (Cornish & Backhaus, 1990; Cornish & Siler, 1995). Thus, the ratio of the rate of [^{14}C]IPP incorporation to [^{3}H]-labeled initiator incorporation reflects the mean molecular weight of newly synthesized polymer. Mean molecular weights (mean M_r) were calculated using the following formula:

$$\frac{(^{14}\text{C-IPP incorporation rate}^{(1)} + a(^{3}\text{H-allylic-PP incorporation rate}^{(1)})) \times 68^{(2)}}{^{3}\text{H-allylic-PP incorporation rate}} + 176^{(3)},$$

where (1) is nmol tracer·gdw rubber^{-1}·4h^{-1}, (2) is the molecular weight of isoprene, (3) is the diphosphate end-group (177 is molecular weight of -$P_2O_7H_3$) and $a=2$ for GPP and 3 for FPP. It should be noted that adjusting for the size of the initiator has only a small effect on the calculated molecular weights of the newly synthesized rubber. Also, we do not know whether terminated rubber molecules are, or are not, phosphorylated; if they are, the diphosphate would be ionized to some degree and (3) could be 174–176. Whatever the actual number, the impact on the calculated weight is negligible.

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