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Magnesium ion regulation of in vitro rubber biosynthesis by *Parthenium argentatum* Gray

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This paper is dedicated to Professor R. Croteau on the occasion of his 60th birthday.

Abstract

Natural rubber is produced by a rubber transferase (a *cis*-prenyltransferase). Rubber transferase uses allylic pyrophosphate to initiate the rubber molecule and isopentenyl pyrophosphate (IPP) to form the polymer. Rubber biosynthesis also requires a divalent metal cation. Understanding how molecular weight is regulated is important because high molecular weight is required for high quality rubber. We characterized the in vitro effects of Mg^{2+} on the biosynthetic rate of rubber produced by an alternative natural rubber crop, *Parthenium argentatum* (guayule). The affinity of the rubber transferase from *P. argentatum* for IPP · Mg was shown to depend on the Mg^{2+} concentration in a similar fashion to the *H. brasiliensis* rubber transferase, although to a less extreme degree. Also, in vitro Mg^{2+} concentration significantly affects rubber molecular weight of both species, but molecular weight is less sensitive to Mg^{2+} concentration in *P. argentatum* than in *H. brasiliensis*.

Keywords: *Parthenium argentatum*; Compositae; *Hevea brasiliensis* Muell. Arg.; Euphorbiaceae; Regulation; Rubber transferase; *cis*-Prenyltransferase; Magnesium; FPP; IPP

1. Introduction

Although there are over 2500 plant species that produce natural rubber (Bealing, 1969; Bonner, 1991), there are only a few species that produce the high molecular weight rubber necessary for high product quality and performance (Swanson et al., 1979). Currently, the main commercial source of natural rubber is *Hevea brasiliensis* Muell. Arg. (Brazilian or Para rubber tree), which produces high molecular weight rubber obtained by tapping the laticifers in the tree bark (D'auzac et al., 1989). However, *H. brasiliensis* commercial production is limited to tropical regions of the world. Also, it is one of the most genetically uniform crops under cultivation, as it has a narrow breeding ancestry, and depends almost entirely on plantation-grown clonal trees (clonal scions grafted onto seedling root stocks), which makes it prone to pathogenic attack (Davis, 1997). *Parthenium argentatum* Gray (guayule), a native of the Chihuahuan desert of Mexico and Texas, is another plant species that produces high molecular weight rubber (Ray, 1993), and is an alternative source of natural rubber for commercial use. *P. argentatum* is being commercially developed as a source of latex for medical products because its rubber particle-associated proteins do not cross react with IgE (Type I latex allergy) and IgG antibodies to *H. brasiliensis* latex proteins (Siler and Cornish, 1994; Carey et al., 1995; Siler et al., 1996; Cornish et al., 2005).

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Natural rubber is synthesized by rubber transferase (EC 2.5.1.20), a membrane bound *cis*-prenyltransferase (Archer and Audley, 1987; Madhavan et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Cornish and Siler, 1995; Cornish et al., 1999), which requires an allylic pyrophosphate (APP) to initiate the rubber molecule, isopentenyl pyrophosphate (IPP) as the source of monomer used to elongate the polymer (Archer and Audley, 1987; Madhavan et al., 1989; Benedict et al., 1990; Cornish and Backhaus, 1990; Cornish, 1993; Tanaka et al., 1996; Scott et al., 2003; da Costa et al., 2005) and a divalent cation, such as Mg^{2+} or Mn^{2+}, as cofactor (Archer and Audley, 1987; Madhavan et al., 1989; Cornish and Backhaus, 1990; Cornish, 1993; Scott et al., 2003; da Costa et al., 2005).

In vitro, a number of APPs can be used by rubber transferases from *P. argentatum*, *H. brasiliensis* and *Ficus elastica* Roxb. (Indian rubber tree) to initiate the rubber molecule (Archer and Audley, 1987; Madhavan et al., 1989; Cornish and Siler, 1995). In vivo, farnesyl pyrophosphate (FPP) is thought to be the main initiator of rubber molecules as it has a lower binding constant than other APPs (Cornish, 2001), is produced in the cytosol of laticifers, which is the same compartment as rubber transferase, and NMR data indicates that at least in *H. brasiliensis* the rubber molecule has a *trans* double bond at the initiation site (Tanaka et al., 1996). In vitro, changes in IPP and APP concentrations affect the rubber molecular weight of the rubber produced by *H. brasiliensis*, *P. argentatum* and *F. elastica* (Castillón and Cornish, 1999; Cornish et al., 2000). Under identical [IPP] and [FPP] (IPP and FPP concentration, respectively) in vitro conditions, *H. brasiliensis* and *P. argentatum* produce rubber with half the molecular weight of that made by *F. elastica* (Cornish et al., 2000), while, in vivo, *F. elastica* produces rubber with a lower molecular weight than *H. brasiliensis* or *P. argentatum*. This discrepancy suggests that the rubber transferases themselves are not the primary determinators of rubber molecular weight and was attributed to the higher affinity of *F. elastica* rubber transferase for IPP in the presence of FPP (Cornish et al., 2000). This result also suggests that plants regulate their rubber molecular weight in vivo by a mechanism other than, or in addition to, regulation of [IPP] and [FPP]. Possible mechanisms could involve regulation of polymer termination or regulation of cofactor availability.

In vitro, the metal ion cofactor concentration was shown to affect the IPP incorporation rate by the rubber transferases from *F. benghalensis* (Kang et al., 2000b) and *F. carica* (Kang et al., 2000a), *F. elastica* (Scott et al., 2003), *H. brasiliensis* (Kang et al., 2000a,b; Scott et al., 2003; da Costa et al., 2005) and *P. argentatum* (Scott et al., 2003). The rubber transferase from *F. elastica*, *H. brasiliensis* and *P. argentatum* requires the metal ion as a cofactor and as an activator, such that, at low metal ion concentrations, the metal ion deinhibits the rubber transferase activity, whereas at high concentrations, it inhibits the rubber transferase activity (Scott et al., 2003). Therefore, there is a metal ion concentration, $[A]_{max}$, that gives a maximal IPP incorporation rate, V_{max} (Scott et al., 2003). Throughout the remainder of this paper we shall refer to the metal ion as a cofactor even though it also acts as an activator. It also has been shown that the rubber transferase from *F. elastica*, *H. brasiliensis* and *P. argentatum* can bind FPP, FPP · metal or metal ion alone, whereas it can bind IPP · metal or metal ion alone, but not IPP alone, and that Mg^{2+} is the in vivo cofactor (Scott et al., 2003).

In vitro, the concentration of Mg^{2+} radically affects the affinity of the *H. brasiliensis* rubber transferase for IPP · Mg, which suggests that the Mg^{2+} concentration may have a regulatory role in rubber biosynthesis (da Costa et al., 2005). The metal ion cofactor concentration also affects the molecular weight of the rubber produced by *H. brasiliensis* (da Costa et al., 2005).

Here, we characterize the role of Mg^{2+} concentration in rubber biosynthesis by enzymatically active rubber particles purified from *P. argentatum*. The effect of $[Mg^{2+}]$ on initiation, biosynthetic rate, molecular weight and substrate affinity was determined and then compared to rubber biosynthesis in *H. brasiliensis*.

2. Results and discussion

2.1. IPP incorporation rate dependence on $[Mg^{2+}]$

In vitro, the IPP incorporation rate by *P. argentatum* was $[Mg^{2+}]$ dependent (Fig. 1). At low levels of $[Mg^{2+}]$,

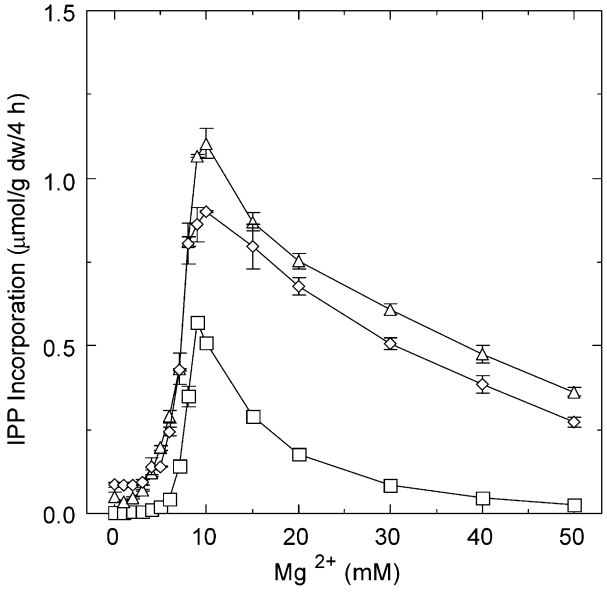

Fig. 1. Dependence of [^{14}C]-IPP incorporation on magnesium ion concentration by rubber transferases of *Parthenium argentatum* purified rubber particles. Incorporation rates of [^{14}C]-IPP were measured in 15 µM FPP, 7 mM EDTA, varying $[Mg^{2+}]$ and different [IPP]: 37.5 µM, □; 375 µM, △; 1000 µM, ◇. The error bars in the figure represent the standard deviation of triplicates.

an increase in $[Mg^{2+}]$ increased the IPP incorporation rate. The increase in $[Mg^{2+}]$ led to an increase in $[IPP \cdot Mg]$, which were calculated using the [IPP], $[Mg^{2+}]$ and $K_d^{IPP \cdot Mg} = 520$ μM (King and Rilling, 1977) as previously described (da Costa et al., 2005), as well as to the activation of the rubber transferase by the Mg^{2+}. At $[A]_{max}$, the IPP incorporation rate was maximal, and occurred at a slightly higher $[Mg^{2+}]$ at 375 and 1000 μM IPP than at 37.5 μM IPP. Increasing $[Mg^{2+}]$ beyond $[A]_{max}$ decreased the IPP incorporation rate, as the Mg^{2+} inhibited the rubber transferase. Thus far, $[Mg^{2+}]$ dependence of IPP incorporation by *P. argentatum* is very similar to that previously reported for *H. brasiliensis* (da Costa et al., 2005). Although the dependence is similar in both species, some differences also are apparent. In *P. argentatum*, for $[Mg^{2+}] \geqslant 1/2\ [A]_{max}$, the maximum IPP incorporation rate occurred when $[IPP] = K_m^{IPP}$ (375 μM) (Fig. 1). In contrast, in *H. brasiliensis*, the IPP incorporation rate is considerably more stimulated by 375 μM IPP and $[Mg^{2+}] \geqslant 1/2\ [A]_{max}$ than seen in *P. argentatum*, but only remains at a maximum over a narrow range of $[Mg^{2+}]$ (6–10 mM) (da Costa et al., 2005). By 20 mM Mg^{2+}, in *H. brasiliensis*, the incorporation rate in 375 μM IPP falls below that in 1000 μM and the rates remain strictly dependent upon [IPP] out to 50 mM Mg^{2+} as rates become progressively inhibited by Mg^{2+} (da Costa et al., 2005). In *P. argentatum*, incorporation rates were not strictly dependent upon [IPP] and remained highest in 375 μM IPP at $[Mg^{2+}] \geqslant 1/2\ [A]_{max}$ (Fig. 1).

2.2. Rubber transferase affinity for IPP · Mg dependence on $[Mg^{2+}]$

The rubber transferases from *H. brasiliensis*, *P. argentatum* and *F. elastica* can interact with Mg^{2+} or $IPP \cdot Mg$, but not with IPP alone (Scott et al., 2003). This implies that either $IPP \cdot Mg$ is the substrate for the rubber transferase or that Mg^{2+} binds to a different site and allows interaction of IPP and/or $IPP \cdot Mg$ with the active site. There are three possible ways that the enzyme · IPP · Mg (E · IPP · Mg) may be formed:

$$Mg + IPP \leftrightarrow IPP \cdot Mg + E \leftrightarrow E \cdot IPP \cdot Mg \quad (1)$$

$$E + IPP \leftrightarrow E \cdot IPP + Mg \leftrightarrow E \cdot IPP \cdot Mg \quad (2)$$

$$E + Mg \leftrightarrow E \cdot Mg + IPP \leftrightarrow E \cdot IPP \cdot Mg \quad (3)$$

In reaction (1), $IPP \cdot Mg$ is formed when Mg^{2+} and IPP are present in solution, $IPP \cdot Mg$ then interacts with the enzyme to form the E · IPP · Mg complex. In reaction (2), free IPP interacts with the enzyme forming an E · IPP complex, which then interacts with a Mg^{2+} in solution to form the E · IPP · Mg complex. In reaction (3), a Mg^{2+} in solution interacts with the enzyme forming an E · Mg complex, which then interacts with free IPP forming the E · IPP · Mg complex. It has been shown that, at physiological levels of IPP, undecaprenyl pyrophosphate synthase (UPPS), a *cis*-prenyltransferase (as is rubber transferase), requires Mg^{2+} for IPP binding (Chen et al., 2002; Guo et al., 2005), suggesting that reaction (2) is irrelevant in vivo. Similarly to UPPS, IPP alone does not interact with rubber transferase from *F. elastica*, *H. brasiliensis*, and *P. argentatum* (Scott et al., 2003; da Costa et al., 2005), and so it is unlikely that formation of the E · IPP · Mg complex proceeds through reaction (2) in any of these species. In *P. argentatum*, as in *H. brasiliensis* (da Costa et al., 2005), when a large excess of Mg^{2+} was used, such that there was no free IPP, the reaction still proceeded (Fig. 2), supporting the idea that neither reaction (2) or (3) are relevant under physiological conditions. Therefore, the most likely rubber elongation pathway in *P. argentatum* is through reaction (1) and $IPP \cdot Mg$ is the true substrate for the enzyme. This agrees with previous observations of rubber transferase from *H. brasiliensis*, which also likely follows reaction (1) for rubber elongation and uses $IPP \cdot Mg$ as its true substrate (da Costa et al., 2005).

In vitro, when all the IPP was present as $IPP \cdot Mg$, the rubber transferase from *P. argentatum* was inhibited by increasing levels of Mg^{2+} (Fig. 2), as has also been shown for *H. brasiliensis* (da Costa et al., 2005), thus suggesting that Mg^{2+} has a regulatory effect on rubber biosynthesis. At high $IPP \cdot Mg$ concentrations, regardless of the actual $[Mg^{2+}]$, the IPP incorporation rate in *P. argentatum* decreased while $[IPP \cdot Mg]$ increased (Fig. 2), an effect not observed in *H. brasiliensis* (da Costa et al., 2005). Although the precise mechanism for this difference is not yet clear, there are strong kinetic differences between the active sites of the two species – for example, the *P.*

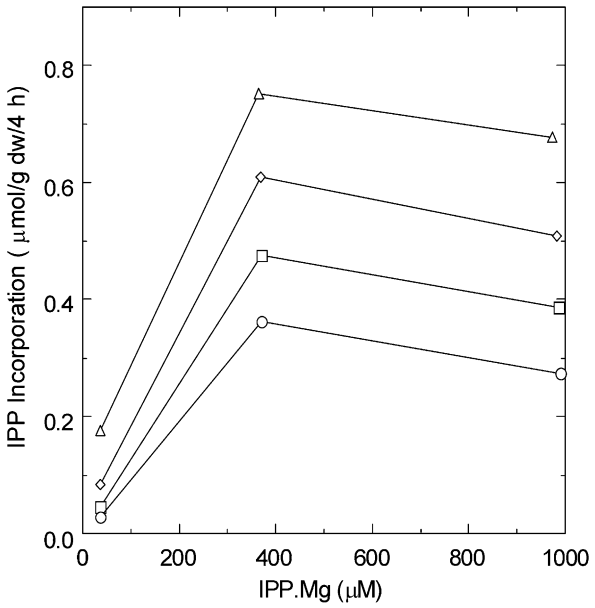

Fig. 2. Dependence of [^{14}C]-IPP incorporation by rubber transferases on $IPP \cdot Mg$ concentration of *Parthenium argentatum* purified rubber particles in excess $[Mg^{2+}]$. Incorporation rates of [^{14}C]-IPP were measured in 15 μM FPP, 7 mM EDTA, varying [IPP] and different $[Mg^{2+}]$: 20 mM Mg^{2+}, △; 30 mM Mg^{2+}, ◇; 40 mM Mg^{2+}, □; 50 mM Mg^{2+}, ○.

argentatum rubber transferase demonstrates negative cooperativity for FPP binding over a much wider range of [FPP] than seen in *H. brasiliensis* (Cornish et al., 2000). Such kinetic differences must have a structural basis, which also could lead to other kinetic differences such as the inhibition at high [IPP · Mg] in *P. argentatum*.

In UPPS, an excess of Mg^{2+} can interact with the active site and inhibit further binding of IPP · Mg (Guo et al., 2005). The affinity of the rubber transferase from *P. argentatum* for IPP · Mg depends on the [Mg^{2+}] (Fig. 3) as has previously been shown for *H. brasiliensis* (da Costa et al., 2005). In both plant species, when [Mg^{2+}] is increased from a limiting value to $[A]_{max}$, the affinity is significantly increased as demonstrated by a decrease in $K_m^{IPP \cdot Mg}$ of two orders of magnitude for *H. brasiliensis* and one order of magnitude for *P. argentatum* (Table 1). When the [Mg^{2+}] increased from $[A]_{max}$ to an excess, the affinity of both the rubber transferases for IPP · Mg decreased, as shown by an increase in $K_m^{IPP \cdot Mg}$ (Table 1). However, this decrease in affinity is much larger in *H. brasiliensis* than in *P. argentatum*. Thus, [Mg^{2+}] has a greater impact on rubber biosynthesis in *H. brasiliensis* than in *P. argentatum*.

A similar change in affinity of porphobilinogen synthase (PBGS) for its substrate, 5-aminolevulinic acid, was observed in the presence and absence of Mg^{2+} (Breinig et al., 2003). In the presence of Mg^{2+}, PBGS exists as an octamer and it has a high affinity for its substrate, while in the absence of Mg^{2+} PBGS exists as a hexamer and it has a low affinity for its substrates (Breinig et al., 2003). Thus, it seems possible that the observed affinity changes may be mediated by Mg^{2+} causing a similar conformational effect on the rubber transferase, although this is not yet proven. Allosteric effects and other biochemical mechanisms are also possible.

Unlike *H. brasiliensis*, in *P. argentatum* a decrease in IPP incorporation rate occurred around 250 μM IPP · Mg (Fig. 3), and was repeated in two more experiments (not shown). The underlying cause of the lower activity is unknown but may be related to a structural effect on the active site caused by a combination of IPP · Mg and Mg^{2+}. We also are uncertain why, in the experiment data shown in Fig. 3, that 40 mM Mg^{2+} supported the highest rate of rubber biosynthesis. In a similar experiments using 6, 8, 10 and 20 mM Mg^{2+}, the first three concentrations supported similar rates whereas 20 mM was inhibitory.

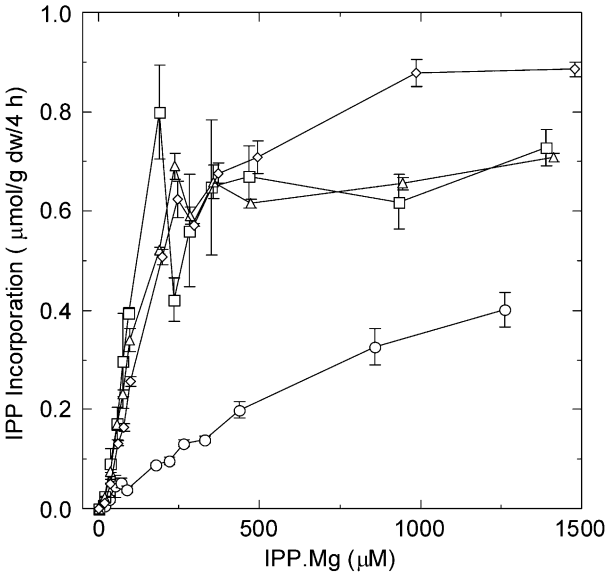

Fig. 3. Dependence of [^{14}C]-IPP incorporation by rubber transferases on IPP · Mg concentration of *Parthenium argentatum* purified rubber particles in different [Mg^{2+}]. Incorporation rates of [^{14}C]-IPP were measured in 15 μM FPP, 7 mM EDTA, varying [IPP] and different [Mg^{2+}]: 4 mM Mg^{2+}, ○;; 8 mM Mg^{2+}, □; 10 mM Mg^{2+}, △; 40 mM Mg^{2+}, ◇. The error bars in the figure represent the standard deviation of triplicates.

Table 1
Variation of $K_m^{IPP \cdot Mg}$ for rubber transferase in *Hevea brasiliensis* and *Parthenium argentatum* purified rubber particles with magnesium ion concentration

Mg (mM)	*Hevea brasiliensis*		*Parthenium argentatum*	
	$K_m^{IPP \cdot Mg}$ (μM)	SE[a]	$K_m^{IPP \cdot Mg}$ (μM)	SE[a]
4	8000	600	1700	250
8	68	10	120	45
10	–	–	140	35
30	970	70	–	–
40	–	–	290	50

[a] SE = Standard error.

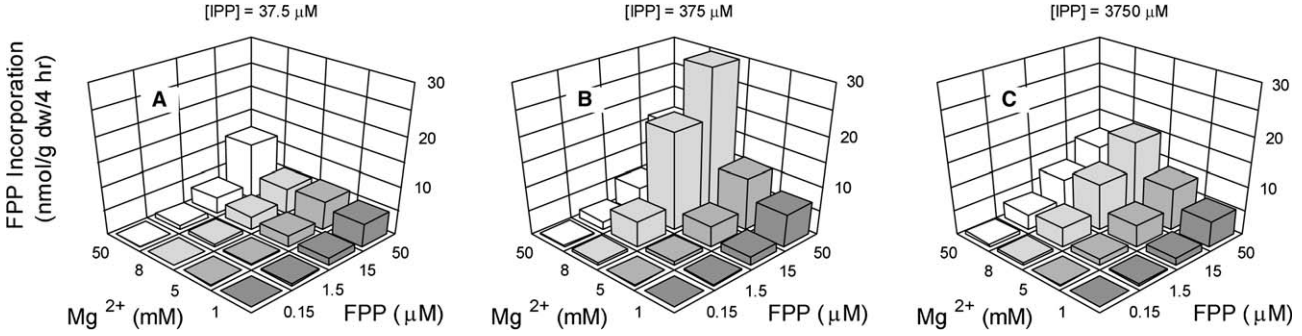

Fig. 4. FPP and metal ion concentration dependence of FPP incorporation rate of *Parthenium argentatum* purified rubber particles. Incorporation rates of [^{3}H]-FPP were measured in 7 mM EDTA and one of the following [IPP]: 37.5 μM (A); 375 μM (B); 3750 μM (C) and varying [Mg^{2+}] and [FPP].

2.3. Dependence of FPP incorporation rate on [Mg^{2+}]

FPP incorporation rate increased with increasing [FPP] at all [IPP] and [Mg^{2+}] used (Fig. 4). In *P. argentatum* the FPP incorporation rate was dependent on [Mg^{2+}] when [IPP] $\geqslant K_m^{IPP}$ (375 and 3750 μM), but not when [IPP] = 37.5 μM, whereas in *H. brasiliensis* the FPP incorporation rate was dependent upon [Mg^{2+}] when [IPP] $\leqslant K_m^{IPP}$ (37.5 and 375 μM), but not when [IPP] = 3750 μM (da Costa et al., 2005). The rubber transferase stability might be affected by IPP and Mg^{2+}, which could, in turn, affect FPP incorporation rate. In *P. argentatum*, IPP and Mg^{2+} together could stabilize the enzyme, thus enhancing FPP incorporation rate at non-limiting [IPP], as at limiting [IPP] the Mg^{2+} alone would not be able to stabilize the enzyme and affect FPP incorporation rate. The contrasting picture in *H. brasiliensis* might occur if, at saturating levels of IPP, the enzyme is not affected by [Mg^{2+}], leading to an FPP incorporation rate independent of [Mg^{2+}].

2.4. Effect of [Mg^{2+}] on rubber molecular weight

In vitro, [IPP] and [FPP] have been shown to affect rubber biosynthesis and rubber molecular weight in *F. elastica*, *H. brasiliensis* and *P. argentatum* (Castillón and Cornish, 1999; Cornish et al., 2000). Thus, their regulation in vivo would provide a mechanism for plants to control rubber biosynthesis. It is unlikely that this mechanism is the sole source of regulation, as under the same conditions, in vitro, *F. elastica* produces a higher molecular weight rubber than *H. brasiliensis* or *P. argentatum*, while in vivo, *F. elastica* produces rubber of lower molecular weight than the other species (Cornish et al., 2000). In vitro, Mg^{2+} affects the affinity of the rubber transferases from *H. brasiliensis* and *P. argentatum* for IPP · Mg, and so in vivo regulation of [Mg^{2+}] would provide an additional mechanism for the plant to control rubber biosynthesis.

Our molecular weight results are in agreement with previous reports that increasing [FPP] leads to a reduction in molecular weight, whereas increasing [IPP] leads to an increase in molecular weight (Castillón and Cornish, 1999; Cornish et al., 2000). The Mg^{2+} also affects the molecular weight of the rubber produced by the rubber transferases of *P. argentatum* (Fig. 5) and *H. brasiliensis* (da Costa et al., 2005). When [IPP] $\leqslant K_m^{IPP}$, the molecular weight of rubber is significantly affected by [Mg^{2+}] in both species. However, when saturating levels of IPP (3750 μM) were used, the molecular weight in *P. argentatum* was not strongly dependent on [Mg^{2+}], while it was still highly dependent in *H. brasiliensis*. This again indicates that [Mg^{2+}] is a more important regulatory factor in *H. brasiliensis* rubber biosynthesis than in *P. argentatum*. It seems possible that the greater sensitivity to [Mg^{2+}] in *H. brasiliensis* is related to the laticiferous production of rubber in this species. It has previously been noted that the rubber transferase from *P. argentatum* (which produces rubber in generalized bark parenchyma cells) appears to exert much more direct control over rubber molecular weight than the rubber transferases of either *H. brasiliensis* or *F. elastica*, both of which produce rubber in laticifers (Cornish and Scott, 2005). Regulation of [Mg^{2+}] may be a factor that contributes to the control of rubber biosynthesis exerted by the laticifer rather than directly by the rubber transferases in *H. brasiliensis* and *F. elastica* (and probably in other laticiferous species).

We are not aware of any studies, as yet, that correlate [Mg^{2+}] in the latex with the quality or quantity of rubber produced in vivo. However, the strong effects of [Mg^{2+}] we have observed in vitro suggest that it might be possible to affect or regulate in vivo rubber biosynthesis in *H. brasiliensis* and *P. argentatum* by adjusting the levels of magnesium in the soil to an optimal level. However, these optimal levels must be determined: if the soil has a limiting level of Mg^{2+}, a cation essential to the energy metabolism of the plant, the plant could both grow slower and reduce the amount of rubber biosynthesized; on the other hand, if

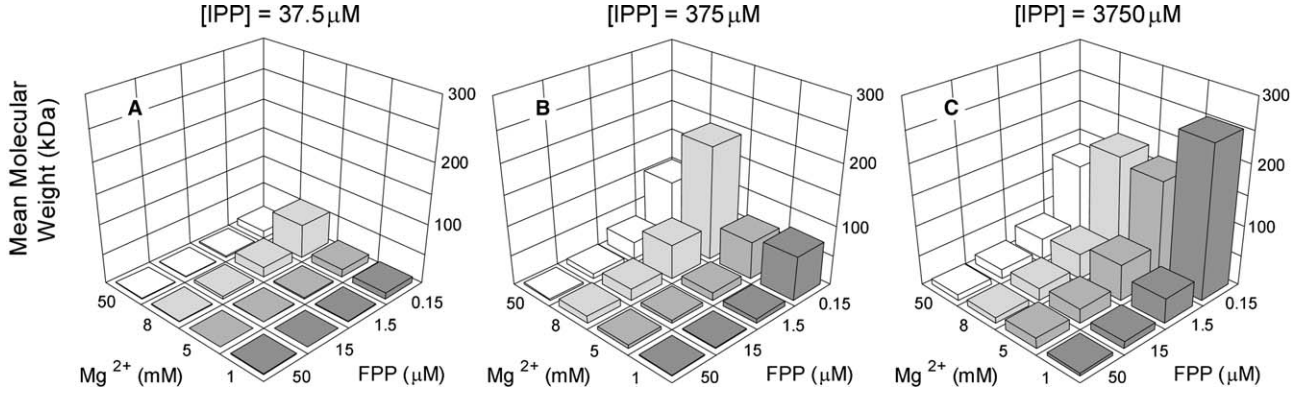

Fig. 5. FPP and metal ion concentration dependence of rubber molecular weight produced by rubber transferases in *Parthenium argentatum* purified rubber particles. Mean molecular weight were estimated based on IPP and FPP incorporation rates measured in 7 mM EDTA and one of the following [IPP]: 37.5 μM (A); 375 μM (B); 3750 μM (C) and varying [Mg^{2+}] and [FPP].

the soil has an excessive Mg^{2+} level, plant growth and rubber biosynthesis might be inhibited by negative interactions of excess Mg^{2+} with the uptake of other cations, such as calcium or potassium.

Although, in vitro, [IPP] and [FPP] have been shown to affect rubber molecular weight of *F. elastica*, *H. brasiliensis* and *P. argentatum* (Castillón and Cornish, 1999; Cornish et al., 2000), their regulation in vivo is unlikely to be easily achieved by altering edaphic factors. One alternative is to genetically engineer the plants to control the cytosolic [IPP] and [FPP] (Veatch et al., 2005) but development of a robust plant line could take years. As another alternative, the plants could be physically or chemically stimulated to increase rubber production: it is known that low temperatures and 2-(3,4-dichlorophenoxy)triethylamine (DCPTA) stimulate rubber biosynthesis in *P. argentatum* (Madhavan et al., 1989). However, neither alternative is very attractive for a commercial plantation as it is unrealistic to control temperatures, while the costs of DCPTA are high and results inconsistent across locations. In *H. brasiliensis* plantations, it is common to stimulate latex production by application of a stimulating paste containing ethephon. It is used despite disadvantages, such as the increase in production costs due to handling and the risk of toxicity to the tree by overdosing plant with stimulants (Derouet et al., 2003). Control of soil Mg^{2+} levels, if proven efficacious, would provide a simple means of regulation.

3. Conclusions

Our studies indicate that in vitro rubber biosynthesis and rubber molecular weight in *P. argentatum* are affected by $[Mg^{2+}]$ although less so than in *H. brasiliensis*. The affinity of the rubber transferase for IPP · Mg is highly dependent on $[Mg^{2+}]$ for both species. The control of endogenous Mg^{2+} levels by the plant may be one mechanism by which rubber biosynthesis is regulated.

4. Experimental section

4.1. General experimental procedures

P. argentatum (line 11591) plants were grown at the US Water Conservation Laboratory, Phoenix, AZ. Unlabelled IPP and FPP were obtained from Echelon Biosciences Incorporated (Salt Lake City, UT, USA), [^{14}C]-IPP (55 mCi/mmol) and [^{3}H]-FPP (60 Ci/mmol) were obtained from American Radiolabeled Chemical Inc., St. Louis, MO.

Multiwell plates used in this study were MultiScreen® R1 plates (MultiScreen – R1; 1 mm Hydrophilic PTFE Membrane; Glass-Filled PP Plate; Non-Sterile with Lid, Millipore, Bedford, MA, USA; catalogue number MAR1N1010). A vacuum manifold (Millipore catalogue number MAVM0960R) was used for *P. argentatum* rubber transferase assays. Siliconized 1.5 mL tubes were supplied by USA Scientific (Ocala, FL, USA). ScintiVerse BD Cocktail was purchased from Fisher Scientific (Santa Clara, CA, USA). Chemicals, unless otherwise noted, were purchased from Sigma (St. Louis, MO, USA).

4.2. In vitro assay of rubber synthesis

Enzymatically active washed rubber particles from *P. argentatum* were purified and stored as previously described (Cornish and Backhaus, 1990; Cornish and Bartlett, 1997). IPP incorporation rates were assayed in *P. argentatum* purified rubber particles (or washed rubber particles – WRP) using a modification of a previously described method (Mau et al., 2000). The reaction took place in individual wells of a 96-well plate. The wells were siliconized with Sigmacote (Sigma–Aldrich, Corp., St Louis, MO, USA, #SL-2) for 2 min, rinsed with deionized water and with 95% ethanol, and dried at room temperature overnight. The reaction volume was 50 μL (100 mM Tris–HCl pH 7.5; 5 mM DTT; IPP; FPP; [^{14}C]-IPP; [^{3}H]-FPP and $MgSO_4$ as indicated). To study the effect of metal cations on rubber transferase activity, it was first necessary to chelate the pre-existing Mg^{2+} pool. If this is not done, there is sufficient Mg^{2+} to support an activity rate near the maximum, which then prevents kinetic characterization. Thus, all experiments with varying Mg^{2+} concentrations included pretreatment of the WRP with 7 mM ethylenediamintetra-acetic acid (EDTA), to chelate the pre-existing Mg^{2+} before the remaining ingredients were added. The reaction was begun by the addition of 0.25 mg WRP of *P. argentatum* into each well, the filter plate was placed on a ceramic cooling plate (Amershan Biosciences, Piscataway, NJ, USA) equipped with a circulating water bath to control the temperature. After 4 h at 16 °C, the reaction was stopped by addition of 25 μL of 500 mM EDTA. For experiments using [^{14}C]-IPP only, the filters were washed using the Millipore vacuum manifold with 100 μL 95% ethanol, 3 × 150 μL 95% ethanol, 2 × 150 μL deionized H_2O and 2 × 150 μL 95% ethanol to each well. For experiments using [^{14}C]-IPP and [^{3}H]-FPP, the filters were washed using the Millipore vacuum manifold with 100 μL 95% ethanol, 3 × 150 μL 95% ethanol, 2 × 150 μL 0.1% 3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate-hydrate (CHAPS), 2 × 150 μL 95% ethanol, 2 × 150 μL 0.1% CHAPS, 2 × 150 μL 95% ethanol, 2 × 150 μL 0.1% CHAPS and 2 × 150 μL 95% ethanol to each well. Filter plates were oven-dried at 37 °C for 30 min, the filters were removed from the plate and placed individually into vials with 2.5 mL ScintiVerse BD Cocktail. The amounts of [^{14}C]-IPP and [^{3}H]-FPP were determined by liquid scintillation spectroscopy using Beckman LS6500 (Beckman Coulter, Fullerton, CA, USA). Each value is the average of three replicates. The mean molecular weights (MW_{rubber}) were calculated based on the IPP incorporation rate (IPP Inc) and the FPP incorporation rate (FPP Inc), as shown by Eq. (4). Each value is the average of three replicates:

$$MW_{rubber} = \frac{IPPInc + 3 \times FPPInc}{FPPInc} \times MW_{Isopentenyl} + MW_{PP} \quad (4)$$

where $MW_{Isopentenyl}$ is the molecular weight of the isoprene monomer (68) and MW_{PP} is the molecular weight of the pyrophosphate group (176).

In order to calculate the concentration of IPP · Mg and FPP · Mg in solution, we used $K_d^{IPP \cdot Mg} = 520$ μM (King and Rilling, 1977) and $K_d^{FPP \cdot Mg} = 120$ μM (Pickett et al., 2003). The calculations were performed using Matlab (version 5.3.0.10183 (R11), MathWorks, Natick, MA) as previously described (da Costa et al., 2005).

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