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*Full Length Research Paper*

# Detection of a rapidly accumulating 50 kDa polypeptide and increased rubber accumulation in guayule under low temperatures

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**Effects of low temperature on rubber particle proteins and rubber transferase activity were investigated in stem bark tissue of guayule (*Parthenium argentatum*, cv. 11591), a desert shrub of commercial interest as an additional source of natural rubber. Three-year-old guayule plants were subjected to 60 cycles of 15/10°C day/night temperature regime with a 12-h photoperiod in a controlled environment growth chamber. Rubber particles were extracted and the associated proteins were characterized in stem tissues of guayule. SDS-PAGE protein profile of the rubber particles showed an abundant rubber particle protein (RPP) with a molecular mass of 50 kDa accumulating at higher concentrations under low temperatures. The higher levels of this polypeptide persisted for 60 cycles when the low temperature stress was continued in the growth chamber. Low temperatures also induced rubber transferase activity, estimated from the rubber particle fractions. The correlation between rubber production and net photosynthetic rates was examined in low temperature-treated guayule. There was a two-fold increase in rubber content associated with a high photosynthetic rate. The results indicate that rubber biosynthesis in plants treated with 15/10°C low temperature for 60 cycles was superior to that of those grown under natural photoperiod and the increased activity of rubber transferase could possibly result from enhanced RPP synthesis. Further characterization of this polypeptide will be vital for understanding why enhanced rubber production is restricted to the winter months in field-grown guayule.**

**Key words:** Guayule, low temperature, *Parthenium argentatum*, rubber, rubber particle proteins, rubber transferase.

## INTRODUCTION

About 2500 plant species produce polymeric natural rubber (*cis*-1,4-polyisoprene) from isoprenoid monomers ($C_5H_8$) derived from isopentenyl pyrophosphate (Backhaus, 1998). All natural rubber currently used is obtained from a single tropical species, *Hevea brasiliensis.* Nonetheless, a woody shrub guayule (*Parthenium argentatum* Gray) is currently being exploited as a domestic source of natural rubber. Guayule is native to the Chihuahuan desert of northeastern Mexico and southern Texas (Whiteworth and Whitehead, 1991). It is a bushy perennial shrub and has narrow leaves covered in a white wax, which alternate along the stem. One of the major factors delaying the economical commercialization of guayule is its low rubber yield (Estilai and Waines, 1987). Hence the primary emphasis on the guayule breeding programs has been to increase the rubber content and quality. Unlike the laticiferous species, guayule produces rubber in the

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in the parenchyma cells of root and stem. Rubber formation in guayule is cyclic (Bonner, 1943). Most rubber is produced during the cool temperatures of fall and winter as the plant is exposed to low night temperatures which may increase the expression of genes coding for enzymes involved in rubber synthesis (Bonner, 1975). Although the variations in rubber forma-tion have been due to low temperature stimulation in the cold and winter months, the precise biochemistry of the rubber particle proteins and rubber transferase activity under low temperature is still not completely understood. Rubber transferase catalyses the *cis*-1,4-polymerization of isoprene monomers derived from isopentenyl pyrophosphate (IPP), an allylic diphosphate initiator (Archer and Audley, 1987; Cornish et al., 2000). Each rubber transferase is capable of producing many rubber polymers (Cornish and Backhaus, 2003). Rubber transferase has been reported to increase in guayule from the months of October to December (Madhavan et al., 1989). However, a comparative study of changes in rubber transferase activity with the poly-peptide profile of rubber particles has not been studied till now.

In a wide range of plant species, experimental manipulations that enhanced photosynthesis increased the pace of secondary metabolite production (Gershenzon and Croteau, 1993). Photosynthetically fixed carbon is actively incorporated into isoprenoids (Goodwin, 1965; Heintze et al., 1994). The role of photosynthetic and photorespiratory metabolites in providing precursors for rubber biosynthesis in guayule has been shown previously (Reddy and Das, 1987; Reddy et al., 1987). However, little is known about the relationship of rubber productivity with photosynthetic capacity in guayule.

The aim of the present investigation was to closely follow the changes in proteins associated with the guayule rubber particles, focusing in particular on the abundant polypeptide in guayule treated to 60 cycles of low temperatures (15/10ºC). This study reports a novel polypeptide of 50 kDa, which is rapidly and predominantly accumulated in guayule rubber particles. We also describe the rubber transferase activity in rubber particles from low temperature-treated guayule. Additionally, we show the close relationship of rubber production with the net photosynthetic capacity in guayule under low temperatures.

## MATERIALS AND METHODS

### Biochemicals

Radiochemical (1-$^{14}$C) isopentenyl pyrophosphate (IPP, specific activity 2035 GBq mol$^{-1}$) was obtained from Amersham Pharmacia Biotech Intl. (UK) and NaH$^{14}CO_3$ (specific activity 1942.5 GBq mol$^{-1}$) was obtained from Bhabha Atomic Research Centre (BARC),Mumbai, India. All other biochemicals were purchased from Sigma (St. Louis, MO, USA).

### Plant material

Guayule (*P. argentatum* Gray cv.11591) was grown in 50-cm pots under natural photoperiod in the botanical garden. The plants received full solar irradiance for most of the day in a 12-h photoperiod. The maximum light intensity (PAR, 400-700 nm) available at the top of the canopy was 1600 to 1800 µmol m$^{-2}$s$^{-1}$ on a clear day. The average daily maximum and minimum air temperatures were 34 and 28ºC. Plants were well watered and periodically fertilized with nutrient solution. Three-year-old plants were used for this study.

### Controlled-environment growth chamber experiments

Temperature treatments were given by transferring three-year-old guayule plants to a controlled-environment growth cabinet (Labline, Illinois, USA). The control plants received 12-h light (1600 µmol m$^{-2}$s$^{-1}$)/12-h dark at 34/28°C temperature. The low temperature treatment was given at 15ºC day/10ºC night and with the same light/dark regime mentioned above for 60 days.

### Protein extraction and gel electrophoresis

Rubber particles were isolated from 40 g of guayule stem bark as described previously (Cornish and Backhaus, 1990). The washed rubber particles were suspended in 100 mmol/L Tris-HCl (pH 7.5) containing 2 mmol/L $MgSO_4$ and 5 mmol/L DTT. Analytical PAGE with SDS was performed using the method of Laemmli (1970). Rubber particle samples were solubilized in 2X-SDS sample buffer containing 63 mmol/L Tris-HCl (pH 6.8), 5% (w/v) SDS, 1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), 2% (w/v) 2-mercaptoethanol, 10% glycerol and 0.01% (w/v) bromophenol blue and heated to 70°C for 3 min. Aliquots of the denatured washed rubber particles (WRP) samples in SDS sample buffer were then resolved by SDS-PAGE. The resolving gel was made up of 12.5% T (total acrylamide concentration) and 2.6% cross linker using methyulenebis (acrylamide; %CBIS). The apparent molecular mass of proteins was estimated by comparison with mobility of standard proteins. After electrophoresis, the gels were stained with Coomassie Brilliant Blue following the standard protocol (Sambrook et al., 1989).

### Extraction and assay of rubber transferase

Stem portions were rinsed with distilled water and homogenized in a pre-cooled blender with 100 mmol/L Tris-HCl buffer (pH 7.5) containing 2 mmol/L $MnSO_4$ and 0.1mmol/L GSH. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 30,000 g for 45 min at 2°C. The supernatant was fractionated with solid ammonium sulphate, and centrifuging at 25,000 g collected the protein that precipitated between 40 and 60% saturation. The protein was dissolved in a small volume of extraction buffer and desalted by passing through a column of Sephadex G-25, pre-equilibrated with the extraction buffer. The fractions were pooled and assayed for rubber transferase.

Rubber transferase was assayed according to Cornish and Backhaus (1990) with certain modifications and by measuring the incorporation of $^{14}$C-IPP into newly synthesized rubber molecules in the presence of an allylic diphosphate and cofactor $Mg^{2+}$. The reaction mixture (1 ml) contained Tris-HCl buffer (100 µmoles; pH 8.2), $MgSO_4$ (5 µmol), GSH (6 µmol), FPP (0.23 µmol), 0.8 µCi (1-$^{14}$C) IPP (50 nmol, specific activity 2035 GBq mol$^{-1}$), washed rubber particles (WRP, 50 mg), and the enzyme protein (100 µg). Incuba-

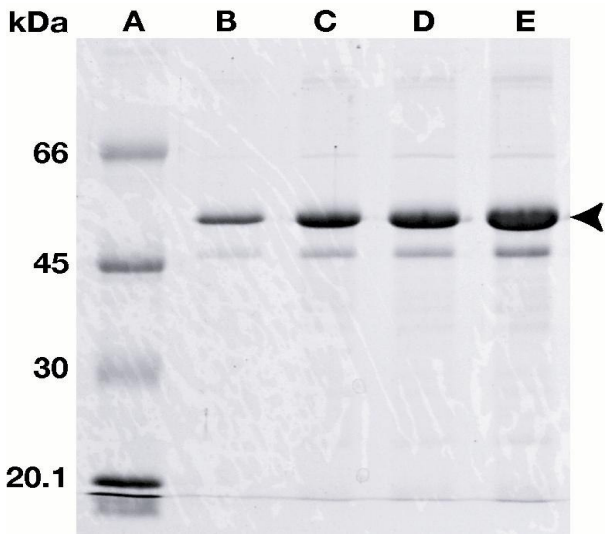


**Figure 1**. Effect of low temperature on polypeptide profile of guayule rubber particles. The polypeptides were resolved on 12.5% polyacrylamide gel; the amount of protein loaded in "lane B" from control samples was two times (~ 40 µg) the amount loaded on other "lanes C-E" (~ 20 µg). Lane A: molecular weight markers; Lane B: rubber particle protein (RPP) in control plants; Lane C: RPP after 20 cycles of low temperature treatment (15/10°C); Lane D: RPP after 40 cycles and Lane E: RPP after 60 cycles at 15/10°C. The molecular weights (kDa) of standard proteins are shown on the left side of the panel. The accumulating 50 kDa polypeptide is shown by the arrowhead.

bations were carried out for 1-h at 30°C and terminated by the addition of 0.5 ml of 0.2 mol/L EDTA. The contents in the reaction tubes were dried in a stream of air at 70°C. The rubber films were saponified and coagulated as described by Madhavan and Benedict (1984). The rubber coagulate was dissolved in 2 ml of 1% TCA in toluene. Scintillant was added to give a total volume of 6 ml, and the solutions were counted for radioactivity in a scintillation counter. Estimation of protein concentration was carried out following Coomassie blue bye binding assay of Bradford (1976) using BSA as the standard protein.

#### Estimation of rubber content

Stem portions of guayule were cut into pieces and dried in an oven at 70°C until constant weights were obtained. The dried tissue was ground in a mill through 40-mesh screen, the powder was thoroughly mixed, and samples were placed in cellulose extraction thimbles (Whatman) and extracted with acetone for 16 h in a Soxhlet apparatus to remove resins. The extraction thimbles were then dried and further extracted for 16-h in the Soxhlet apparatus.

Hexane extracts containing the dissolved rubber were prepared and were made up to 25 ml in a volumetric flask. A 2 ml aliquot was taken into a cuvette and 6 ml of acidified ethanol was added as a precipitating agent. The cuvettes were agitated and allowed to stand for 20 min. The percent transmittances of the samples were measured at 750 mm (Naqvi et al., 1984). Transmittance values were compared with a standard curve prepared with pure natural rubber.

#### Photosynthesis

Rates of net photosynthesis ($P_N$) were measured in low temperature treated guayule leaves. Leaves were enclosed in an 18 x 18 x 6.7 $cm^3$ perspex chamber inside which the air was stirred by a miniature electric fan. The leaves were illuminated by quartz halide lamps. $CO_2$ exchange was monitored using an infra red gas analyzer (Grub Parsons & Co., UK) by measuring the difference between the $CO_2$ content of an air stream that has passed over the leaves in an open circuit, and one that had not. $P_N$ measurement for individual leaves was repeated four times on different individual shoots.

## RESULTS

### Rubber particle proteins

The effect of low temperatures (15/10°C) on proteins associated with guayule rubber particles are shown in Figure 1. Two polypeptides of 50 and 46 kDa were observed, amongst which the 50 kDa polypeptide was the most prominent. The 50 kDa polypeptide was very intense in staining when shoot rubber particle proteins from low temperature-treated guayule was investigated by SDS-gel electrophoresis. Rubber particles from control shoot samples showed a rather low level of 50 kDa polypeptide which was easily detectable only when the protein amount loaded on the gel was two times (that is, ~40 µg) the standard amount (that is, ~20 µg) loaded. The 50 kDa polypeptide could be clearly seen in Coomassie blue-stained polypeptide profile of guayule stem rubber particles.

### Rubber transferase activity

Low night temperature treatment significantly enhanced the activity of rubber transferase in guayule stem extracts. The enzyme extracts from 60 cycle-treated guayule plants showed approximately two-fold increase in the activity (Figure 2) which were well correlated with the rubber accumulation (Figure 3).

### Correlation between rubber production and net photosynthetic rates ($P_N$)

$^{14}C$-incorporation rates into newly formed rubber molecules were followed in washed rubber particles obtained from low temperature-treated guayule shoots. Also, rates of net photosynthesis ($P_N$) were measured in low temperature treated guayule leaves. A strikingly positive correlation was obtained between the rubber accumulation and the net $CO_2$ assimilation rates of guayule (Figure 3). Guayule plants exposed to low temperatures possessed significantly higher rubber content (14% dry weight) after

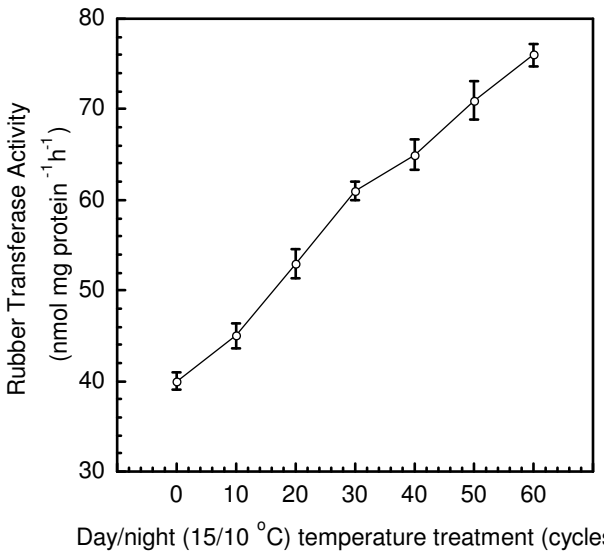


**Figure 2.** Effect of low temperature (15/10°C) treatment on rubber transferase activity assayed in three-year-old guayule plants. The values are expressed as means ± SD for four plants per treatment.

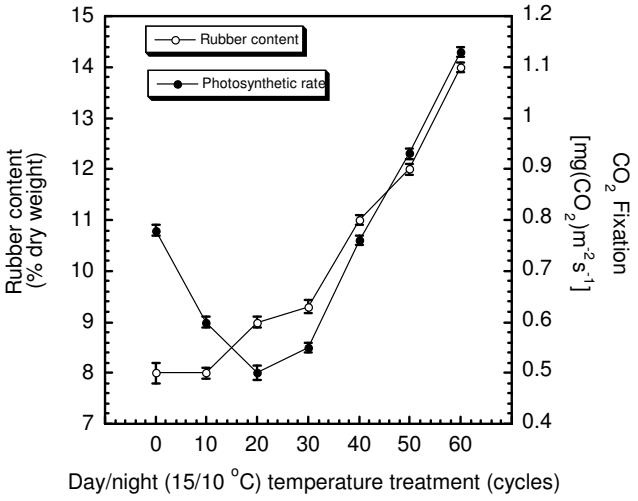


**Figure 3.** Effect of low temperature (15/10°C) treatment on rubber accumulation and net photosynthetic rate ($P_N$). The rubber content was estimated in young stem slices of three-year-old guayule plants and is expressed on percent dry weight basis. $P_N$ was measured in guayule leaves. The values are expressed as means ± SD for four plants per treatment

60 cycles of 15/10°C compared to that of control (34/28°C) plants (8% dry weight). Under low temperature treatment, the guayule leaves showed a decline in photosynthetic rate in the initial stages and later a gradual increase. Plants exposed to 60 nights of low temperatures exhibited higher $P_N$ (1.1 mg $m^{-2}$ $s^{-1}$) than the control plants (0.80 mg $m^{-2}$ $s^{-1}$).

## DISCUSSION

Guayule produces more rubber during winter and low night temperatures favor more rubber formation (Madhavan and Benedict, 1984; Benedict et al., 1989). However, the mechanism of stimulation of rubber forma-0tion in guayule by low night temperature is still elusive. Guayule plants accumulate large quantities of rubber within parenchyma cells of their stem bark tissues. Rubber is packaged within discrete organelles called rubber particles (Cornish and Siler, 1995; Pan et al., 1995).

Rubber particles contain rubber transferase, which is a crucial enzyme for the biosynthesis of rubber from isoprene monomers. Rubber formation in guayule has been reported to show seasonal variations (Bonner and Galston, 1947; Benedict, 1983). Rubber particles are believed to contain key proteins that are responsible for polymerization of isoprene units to form rubber. Here we report that an abundant 50 kDa polypeptide accumulates during low temperatures. Previous studies from our laboratory and others have shown the low temperature served as a stimulus to induce rubber transferase activity in guayule (Sundar and Reddy, 2001; Cornish and Backhaus, 2003). It is probable that the increased levels of rubber transferase activity observed during low temperature conditions could arise from higher accumulation of rubber particle protein, which could be due to a shift in the *de novo* synthesis of the enzyme protein, resulting in the altered patterns of rubber accumulation in guayule (Bonner, 1975). It was earlier believed that an abundant rubber particle protein may be rubber trans-ferase and rubber particles with high rubber transferase activity contain an abundant rubber particle protein (Cornish and Backhaus, 1990). Further investigations on characterizing the accumulating 50 kDa polypeptide detected in this study, could reveal its function in rubber formation.

In a wide range of plant species, experimental manipulations that enhanced photosynthesis increased the pace of secondary metabolite production (Gershenzon and Croteau, 1993). Photosynthetically fixed carbon is actively incorporated into isoprenoids (Goodwin, 1965, Heintze et al., 1994). Therefore, the role of photosynthetically fixed carbon and the availability of photosynthates are very significant for regulating rubber formation and accumulation in guayule. Acetyl Co-A is used as the initial substrate and ATP and NADPH as cofactors during rubber biosynthesis in guayule (Reddy and Das, 1987). The availability of these three metabolites would provide a mechanism for regulating the secondary metabolite production superimposed upon controls at the enzyme and cellular level. Acetyl Co-A, ATP and NADPH

are ubiquitous cellular metabolites derived either from stroma saccharides or arising directly from photosynthesis (Reddy and Das, 1995). We found that low temperature treatment resulted in a relative increase in $P_N$ after an apparent acclimation of about 20 days. Previous studies from our laboratory have shown the ability of guayule to acclimate the key photo-synthetic enzymes with low night temperature treatment of 15°C in a controlled-growth environment that may contribute to an increase in the carbon flow from the leaves to the stem for enhanced rubber production (Sundar and Reddy, 2000). This study provides an evidence for the implication of low temperature treatment for carbon gain required for rubber biosynthesis.

## Concluding remarks

The present investigation indicates that rubber biosynthesis in plants treated with 15/10°C low temperature for 60 cycles was superior to that of those grown under natural photoperiod and the increased activity of rubber transferase could possibly result from enhanced 50 kDa rubber particle protein synthesis. Further characterization of this polypeptide will be vital for understanding why enhanced rubber production is restricted to the winter months in field-grown guayule. In this study, we also show that photosynthetic carbon assimilation is crucial for rubber formation in guayule.

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