EXPRESSION, PURIFICATION AND CRYSTALLIZATION TRIALS OF SMALL RUBBER PARTICLE PROTEIN (SRPP) FROM *Hevea brasiliensis*

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by

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LIST OF ABBREVIATIONS

Acetyl-CoA	Acetyl Coenzyme A
CPT	*Cis*-prenyl tranferase
CD	Circular dichroism
DMAPP	Dimethylallyl diphosphate
DXS	1-deoxy-D-xylulose 5-phosphate synthase
DLS	Dynamic light scattering
EDTA	Ethylenediaminetetraacetic acid
FPP	Farnesyl pyrophosphate
GGPP	Geranyl-geranyl pyrophosphate
GPP	Geranyl pyrophosphate
HLL	*Hevea* latex lectin-like protein
HMG-CoA	3-hydroxy-3-methyglutaryl Coenzyme A
IPP	Isopentenyl diphosphate
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-bertani
LRP	Large rubber particle
MEP	Methylerythritol 4-phosphate
MVA	Mevalonate
NMR	Nuclear magnetic resonance
NusA	N-utilization substance A

NR	Natural Rubber
PDB	Protein databank
pI	Isoelectric point
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulfonyl fluoride
RBIP	Patatin-like inhibitor protein of Rubber biosynthesis
RBSP	Rubber biosynthesis stimulator protein
REF	Rubber elongation factor
RER	Rough endoplasmic reticulum
RP	Rubber particle
SALB	South American Leaf's Blight
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SRP	Small rubber particle
SRPP	Small rubber particle protein
SUMO	Small ubiquitin like modifier
TBE	Tris-borate-EDTA
TPT	*trans*-prenyltransferase
WBP	Washed bottom fraction particles

PENGEKSPRESAN, PENULENAN DAN PERCUBAAN PENGKRISTALAN PROTEIN SMALL RUBBER PARTICLE (SRPP) DARIPADA *Hevea brasiliensis*

ABSTRAK

Getah asli daripada *Hevea brasiliensis* yang diperbuat daripada *cis*-1,4-poliisopren, merupakan komoditi perusahaan yang penuh dengan ciri-ciri keunikan. Biosintesis getah asli daripada *H. brasiliensis* berlaku pada permukaan partikel getah yang dimangkinkan oleh '*small rubber particle protein'* (SRPP) dan '*rubber elongation factor'* (REF). Protein SRPP ini dipercayai menggabungkan isopentenil difosfat (IPP) pada permukaan partikel getah. Tambahan pula, protein SRPP juga merupakan salah satu alergen getah utama dalam *H. brasiliensis.* Walaupun terdapat banyak bukti yang menunjukkan penglibatan protein ini dalam biosintesis getah dan alergen, namun, masih terdapat kekurangan penyelidikan yang mengaitkan mekanisme SRPP dalam sintesis getah dan mekanisme alahan. Oleh demikian, kajian ini bertujuan untuk mengeklon, mengekspres, menulenkan, dan secara langsung membuat percubaan pengkristalan protein SRPP. Secara ringkas, kodon gen *srpp* telah dioptimumkan dan dilabelkan sebagai SRPP_His lalu diekspreskan dalam sel *E. coli* BL21 (DE3). Hasil pengekspresan SRPP_His dalam bentuk rangkuman jasad telah dinyahasli dan ditulenkan melalui keadah '*immobilized metal affinity chromatography'* (IMAC). Protein tersebut yang bersaiz 23 kDakemudiannya dilipatkan dengan menggunakan 1 M arginina hidroklorida. Seterusnya, dengan menggunakan kromatografi saiz pengecualian (SEC) bersertakan '*dynamic light scattering*' (DLS), SRPP_His didapati membentuk oligomer bersaiz

tinggi dengan polidispersiti tinggi (~35%), disebabkan oleh interaksi hidrofobik tidak tertentu. Oleh itu, beberapa set detergen telah diuji dengan harapan untuk mendapatkan SRPP_His dalam bentuk yang seragam. Di samping itu, gabungan NusA_SRPP turut direka bagi mengekspres protein SRPP tersebut dalam keadaan terlarut. Dengan menggunakan 0.2% (w/v) sarkosil, SRPP_His telah dilarutkan dan seterusnya ditulenkan dengan menggunakan kaedah IMAC dan SEC. Namun begitu, kehadiran sarkosil masih mengekalkan SRPP_His sebagai oligomer terlarut. Siasatan lanjut menggunakan spektroskopi *'far-UV circular dichroism'* (CD) telah mendedahkan bahawa SRPP_His terlarut dalam sarkosil dalam keadaan sebahagiannya terbentang dan tidak berlipat. Bagi NusA_SRPP pula, protein tersebut kelihatan dalam bentuk dimer dan tetramer. Selanjutnya, semasa pemeriksaan pengkristalan, kristal jarum berkelompok kecil telah diperhatikan dalam kondisi #45 Crystal screen 1 dan kondisi #27 Crystal screen 2 bagi SRPP_His terlarut dalam sarkosil. Walau bagaimanapun, dengan menggunakan eksperimen kawalan, didapati bahawa kristal berbentuk jarum adalah semata-mata kristal garam, dan bukannya kristal protein. Bagi SRPP_His yang terlarut dalam arginine hidroklorida dan NusA_SRPP, ujian pengkristalan awal menyebabkan pemendakan protein. Pendekatan baru harus diperkenalkan melalui pendekatan '*lipidic cubic phase'* (LCP) supaya kristal protein SRPP_His dapat diperoleh. Kajian struktur protein SRPP akan memberi kefahaman mekanisme khusus yang terlibat dalam biosintesis getah dan alergen.

EXPRESSION, PURIFICATION AND CRYSTALLIZATION TRIALS OF SMALL RUBBER PARTICLE PROTEIN (SRPP) FROM *Hevea brasiliensis*

ABSTRACT

Natural rubber (NR) of *Hevea brasiliensis* predominantly made up of *cis*-1,4-polyisoprene, is an essential industrial commodity with unique characteristics. The biosynthesis of NR from *H. brasiliensis* is catalyzed on the surface of rubber particles by a set of integrated proteins, namely the small rubber particle protein (SRPP), and rubber elongation factor (REF). The SRPP protein has been speculated to incorporate isopentenyl diphosphate (IPP) monomers in rubber particles. Furthermore, the SRPP protein has also been implicated as a major latex allergen. Though evidence suggests the involvement of these proteins in rubber biosynthesis and as an allergen, there is still a dearth of research pertaining to the mechanism that supports the association of this protein onto the rubber particles and mechanism of allergenic disease. Hence, this research aims to clone, express, purify and thereafter to perform crystallization trials on the purified SRPP protein. Briefly, the codon optimized vector designated as SRPP_His was expressed in *E. coli* BL21 (DE3) cells. The SRPP_His protein of 23 kDa expressed in the form of inclusion bodies was then denatured, purified by immobilized metal affinity chromatography (IMAC) and refolded in the presence of 1 M arginine hydrochloride. Using size-exclusion chromatography (SEC) coupled with Dynamic Light Scattering (DLS), SRPP_His was seen to form higher order oligomers with high polydispersity (~35%), which could be a result of unspecific hydrophobic interactions. Hence, several sets of

detergents were tested in the hope to obtain SRPP_His in a homogenous form. In addition, NusA_SRPP fusion construct was designed parallelly to readily express the SRPP protein in a soluble form. Using 0.2% (w/v) of sarkosyl, the SRPP_His was solubilized and purified by IMAC and SEC. Surprisingly, even in the presence of sarkosyl, the SRPP_His remained as a soluble oligomer. Further investigations by far-UV circular dichroism (CD) spectroscopy revealed that SRPP_His solubilized in sarkosyl is partially unfolded. As for NusA_SRPP, the protein was observed to elute as tetramers and a dimers. During crystallization screening, tiny clustered needle crystals were formed in solution #45 of Crystal screen 1 and solution #27 of Crystal screen 2 for SRPP_His solubilized in sarkosyl. However, using control experiments, it was shown that the needle-like crystals were merely salt crystals, and not protein crystals. For SRPP_His in arginine hydrochloride and NusA_SRPP, preliminary crystallization trials resulted in protein precipitation. Different approaches are necessary such as Lipidic Cubic Phase (LCP) method in order to crystallize SRPP_His. Structural studies of SRPP will provide an understanding on the specific mechanism that is involved in the rubber biosynthesis and allerginicity.

CHAPTER 1

INTRODUCTION

1.1 Background of Research

Natural rubber (NR), the white blood of the world economy, is an essential industrial commodity that possesses unique characteristics such as high elasticity, resilience and resistance to high temperature. NR is made up of poly *cis*-1,4-polyisoprene, the high molecular mass polymer formed from isopentenyl diphosphate (IPP) units linked in *cis*-configuration, with many other minor additional components such as proteins, minerals, and lipids (Nor and Ebdon, 1998; Wititsuwaannakul *et al.,* 2003; Bushman *et al.,* 2006). *Hevea brasiliensis* is presently the sole crop exploited for commercial production of high quality natural rubber.

The biosynthesis of natural rubber is carried out by a set of complex machinery proteins involving *cis*-prenyl tranferase (CPT), and other rubber particle associated proteins. Even though the biochemical pathways involving rubber biosynthesis is now fully understood, the factors affecting chain elongation and termination is poorly understood. Proteins other than rubber polymerase also could be involved in the process of rubber biosynthesis. It is reported that small rubber particle protein (SRPP) (Oh *et al.,* 1999) and rubber elongation factor (REF) (Dennis and Light, 1989), rubber biosynthesis stimulator protein (RBSP) (Yusof *et al.,* 2000), and a patatin-like inhibitor protein of rubber biosynthesis (RBIP) (Yusof *et al.,* 1998) are also involved in rubber biosynthesis.

However, due to the lack of crystal structure of the SRPP and REF proteins, the actual role played by these proteins is not yet determined. It is hoped that the information gained from the crystal structure of SRPP protein, will be utilized to genetically manipulate the plant to improve and increase the rubber yield. In addition, the structure of SRPP will also serve as a basis for designing vaccines or drugs targeting allergenic reaction caused by SRPP protein.

1.2 Research Objectives

I. To clone and express Small Rubber Particle Protein (SRPP) from *Hevea brasiliensis* in a heterologous system

II. To purify the SRPP protein

III. To perform crystallization trials on the purified SRPP protein

CHAPTER 2

LITERATURE REVIEW

2.1 Scientific Classification, Cultivars and Historical Outline of *Hevea brasiliensis*

The rubber tree (*Hevea brasiliensis*), a fast-growing upright tropical tree crop, is predominantly cultivated for its production of latex (a milky-white plant liquid), which serves as a primary source of natural rubber (NR). The genus *Hevea* belongs to a large family of Euphorbiaceae with about 280 genera and 8,000 species. In total, there are nine species recognized under the genus of *Hevea* other than *H. brasiliensis* (Table 2.1), ranging from large forest tree to little more than shrubs. All of them contain latex in their parts, but with little economic value, except for *H. brasiliensis* (Verheye, n.d.).

Table 2.1: Nine cultivars of *Hevea* and their respective characteristics of latex (Verheye, n.d.)

***Hevea* Species**	**Characteristics of Latex**
H. benthamaina	Pure white latex; lower yield than *H. brasiliensis*
H. camporum	Not known
H. guianensis	Yellowish latex; yields inferior rubber
H. microphylla	White watery latex; lacks rubber
H. nitida	White latex; act as an anti-coagulant
H. pauciflora	White latex; low rubber; high resin content
H. rigidifolia	Cream-colored latex; poor rubber quality; high resin content
H. spruceana	Watery latex; lack of rubber
H. brasiliensis	White latex; highest yield

The real success story of rubber as a modern commodity started in the year 1876 when Sir Henry Nicholas Ridley collected some 70,000 seeds from Amazon, Brazil and cultivated them in Kew Gardens, London and later in Sri Lanka (Ceylon) and Singapore (1877). In Malaysia, however, rubber plantations were established as early as 1890. Sir H. Ridley, was the first to identify *H. brasiliensis* as being one of the supreme rubber-producing plants as opposed to other rubber-producing plants due to its unique properties, which include resilience, abrasion, elasticity and impact resistance (Cataldo, 2000 and Cornish, 2001a). Until today, more than 99% of the world production of NR comes from *H. brasiliensis*.

2.2 Global Distribution of *H. brasiliensis* Natural Rubber and its Role as an Indispensible Biopolymer

H. brasiliensis tree is indigenous to Brazil, Columbia, Bolivia, Peru, Venezuela and the Guianas (Figure 2.1, shaded with green), but most of the world's rubber (almost 90%) comes from plantations in an exotic regions such as Indonesia, Thailand and Malaysia (Challen, n.d. and Van Beilen and Poirier, 2007). This is because, at present, a fungal (*Microcyclus ulei*) disease known as South American Leaf's Blight (SALB) has hampered the production of *Hevea* rubber in the South America.

Figure 2.1: Global distribution of *H. brasiliensis*, both in native and exotic regions (Orwa *et al.*, 2009).

NR is of strategic importance because it cannot be replaced by any other synthetic alternatives in many of its most noteworthy applications (Van Beilen and Poirier, 2007). The plant-based commodity is essential for the manufacture of more than 40,000 consumer products including aircraft tires, footwear, medical devices (latex surgical gloves) and innumerable engineering products (Davis, 1997; Mooibroek and Cornish, 2000; and Hagel *et al.*, 2008). The market share of *H. brasiliensis* natural rubber has increased from close to 30% in the 1970s and 1980s and almost 40% in the year 2007 (Van Beilen and Poirier, 2007).

2.3 Composition of *Hevea* Latex

The latex from *H. brasiliensis* is composed of about 36% rubber fraction and 5% non-rubber substances such as proteins, carbohydrates, fatty acids and lipids, which give rise to the extraordinary characteristic of natural rubber (Nor and Ebdon, 1998; Sakdapipanich, 2007) than the other rubber-producing plants. The remaining is water, which accounts for 59% (Sansatsadeekul *et al.*, 2011).

Of the 5% of non-rubber components in *Hevea*'s latex, the composition of protein is 1-1.5%, while phospholipids and tocotrienols account for 0.6% and 0.09%, respectively. In addition, *Hevea* rubber molecules have also been reported to contain esters, aldehydes and epoxides (Ohya and Koyama, 2001). Recently, Sansatsadeekul *et al.,* (2011) have successfully characterized phospholipids and proteins that are associated to the rubber-chain. Their study showed a wide variety of fatty acid components associated with phospholipids such as palmitic acid, stearic acid, lauric acid, myristic acid, linoleic acid and linolenic acid. As for the proteins, it was found that the serum phase of natural rubber latex contains proteins with molecular weights different from those of rubber particles (ranging from 6 kDa to more than 200 kDa) (Sansatsadeekul *et al.,* 2011).

When ultra-centrifuged, the *Hevea* latex is segregated into four distinct fraction (Figure 2.2 A): (1) Rubber cream of rubber particles, which is the top layer; (2) the yellow layer, which contains the intermediate Frey-Wyssling particles and the cytoplasm or the C-serum; and finally (3) the bottom layer containing predominantly the lutoids. However, the investigations of Moir (1959) using a specific stain characterized 11 distinct fractions (Figure 2.2 B) in centrifuged latex (known as Moir's zones). Additionally, ribosomes, mitochondria and nuclei are present as well (Dickenson, 1969).

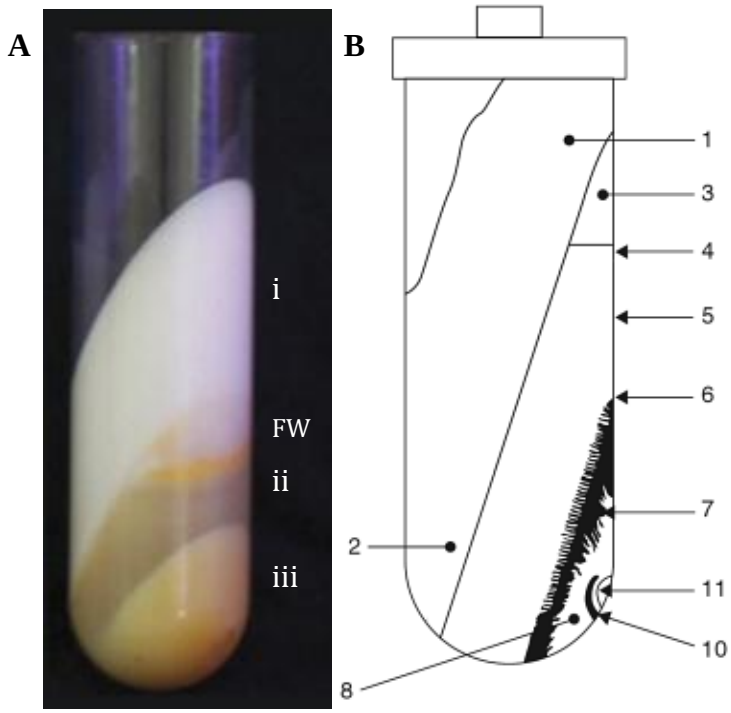

Figure 2.2: Fractions of Hevea's latex**.** (A) represents fractionation of freshly tapped *Hevea* latex (clone PB235) by ultra-centrifugation. The top layer (i): rubber cream that mainly constitutes rubber particles; intermediate layer (FW): Frey-Wyssling particles; (ii): C-serum; and (iii) is the bottom fraction. (B) 11 fractions of latex obtained by Moir (Priyadarshan, 2011; Chow *et al.*, 2012).

2.3.1 *Hevea* Frey-Wyssling Complexes

The presence of yellow globules (in clusters) in tapped latex was first noted by Frey-Wyssling in the year 1929 (Priyadarshan, 2011). Using phase contrast microscope, Southorn (1969) observed that these particles were associated with a vacuolar group and that a double layer membrane covered them. The Frey-Wyssling complexes are spherical in shape ranging from 3-6 μm in diameter.

There are two types of particles enclosed within the membrane 1) large osmiophilic globules and 2) a system of rope-like tubules (Figure 2.3) (Dickenson, 1969). The yellow globules observed by Frey-Wyssling are due to the presence of carotenoid pigments. It has been reported previously that these Frey-Wyssling complexes contains plastoquinone and plastochromanol (in which β-carotene is synthesized) and are assumed to be modified plastids (Dickenson, 1969). Also, it is believed that the highly complicated structure of a Frey-Wyssling complex may play a functional role in the metabolism of *Hevea* latex (Ohya and Koyama, 2001).

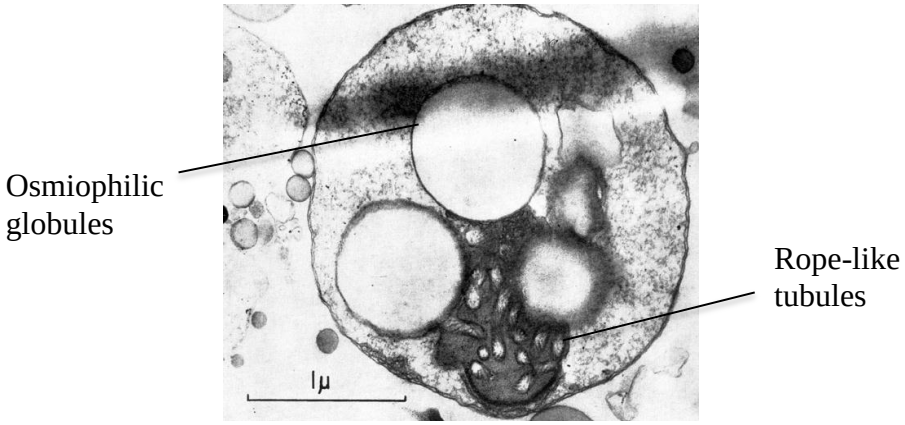

Figure 2.3: A Frey-Wyssling complex depicting the double membrane envelope. Two types of particles are observed to be present within the membrane. Bar=1.0 μm (Dickenson, 1969).

2.3.2 *Hevea* C-Serum

The C-serum represents the aqueous phase of the laticiferous cytoplasmic content and contains about 47.5% of whole latex proteins (Tata, 1980). It is the metabolic active fraction of the latex cytosol, where the glycolytic enzymes and other common cytosolic enzymes, including those of isoprenoid pathway have been detected in this fraction (d'Auzac and Jacob, 1969; Suvachitanont and Wititsuwannakul, 1995 and Li *et al.*, 2009).

The first protein to be isolated from latex was from C-serum, known as α-globulin, which is a major component of the C-serum (Priyadarshan, 2011). Using polyacrylamide gel electrophoresis, Yeang *et al.,* (1977) reported 26 other protein bands from C-serum at alkaline pH and 15 protein bands at acidic pH.

2.3.3 *Hevea* Bottom Fraction

The fresh *Hevea* latex bottom fraction contains predominantly lutoids. Lutoids were first described by Homans *et al.,* (1948) as membrane bound vacuoles, with the single layer membrane rich in phosphatidic acids (Dupont *et al.*, 1976), thus rendering them as negative charged vesicles. The intra-lutoids contents (also called B-serum) are enzymes, proteins (such as cathepsin, lysozymes and acid hydrolases), some divalent cations (Mg^{2+} and Ca^{2+}) and a wide range of metabolites, considered as a type of phytolysosomes (Pujarniscle, 1968; Wititsuwaannakul *et al.*, 2004).

The intra-lutoids have been known to play an essential role as a coagulant. Lutoids are able to destabilize the negatively charged colloidal suspension of rubber particles. The negative charges of rubber particles can be neutralized with the attributes such as the acidic pH, divalent cations and entrapped positively charged proteins that are available in lutoids. In addition, the acid hydrolases trapped in lutoids can attack the protective coating (phospholipoproteins) of rubber particles, during the breakdown of lutoids before or after tapping (Priyadarshan, 2011).

Wititsuwaanakul *et al.,* (2008) have demonstrated that a *Hevea* latex lectin-like protein (HLL) is present on the lutoid membrane, responsible for rubber particle aggregation. A binding protein (BP) ligand counterpart for HLL was also identified along with, which was confirmed to be the SRPP (or RP-HLLBP) by peptide mass fingerprinting. Hence, Wititsuwaanakul *et al.,* (2008) postulated that a rubber particle glycoprotein might be the key component in the formation of rubber latex coagulum.

Additionally, recent evidence suggests that bottom fractions of *Hevea* might play a vital role in the synthesis of new rubber molecules (Wititsuwaannakul *et al.,* 2003 and 2004), other than rubber particles. The findings of their report claims that the rubber biosynthesis on the rubber particles surface as reported by other researchers, (Archer and Audley, 1987; Audley and Archer 1988; Kush, 1994; and Ohya and Koyama, 2001) might be due to the associated membrane fragments (proteins from bottom fraction) of the ruptured bottom fraction particles.

2.3.4 *Hevea* Rubber Particles

Rubber particles (RP) are colloidal components present in the latex, which comprise about 30-45% of the whole latex volume. To date, the *de novo* formation and development of RP in the laticifers remains unresolvable and controversial. Recent investigations by Chrispeels and Herman, (2000) and Dai *et al.,* (2013) have postulated that RP may originate from the rough endoplasmic reticulum (RER). However, there is still dearth of *in vitro* evidences pertaining the actual origination of rubber particles.

The size of rubber particles varies over a wide range (0.02-3 μm) (Southorn and Yip, 1968; Gomez and Hamzah, 1989; and Wititsuwannakul *et al.,* 2008) in the latex of *Hevea brasiliensis*. The particles are usually spherical, but the larger ones in latex are often pear-shaped (Dickenson, 1969 and Singh *et al.,* 2003). The largest RPs is found on the top layer (Moir's zone 1), comprising of RPs as large as 3-6 μm. In Moir's zone 2, the size of RP varies from 0.05-0.25 μm and those in Moir's zone 3 are of lower average sizes (0.035-0.2 μm) (Hamzah and Gomez, 1982). According to Singh *et al.,* (2003) and Xiang *et al.,* (2012), the rubber particles in *H. brasiliensis* can be classified into large rubber particles (LRPs) and the small rubber particles (SRPs), as shown in Figure 2.4 below.

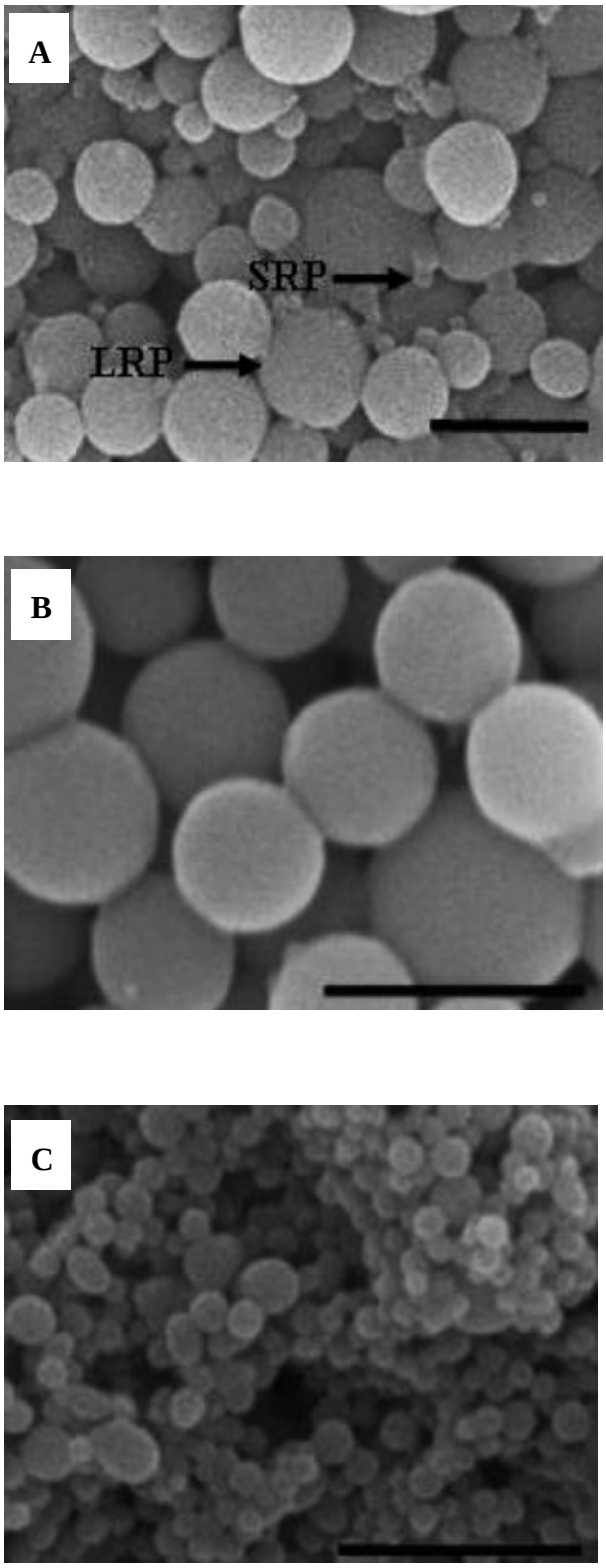

Figure 2.4: Rubber particles from the latex of *H. brasiliensis*. (A) total rubber particles (TLP), (B) large rubber particles (LRP) and (C) small rubber particles (SRP). Bar =1.0 μm (Xiang *et al.,* 2012).

Rubber particles of 0.1 μm size contain several hundreds of *cis*-polyisoprene molecules, which are hydrophobic in nature. These hydrophobic rubber polymers are protected from the hydrophilic medium by a complex film of proteins and phospholipids (Ho *et al.,* 1975). Additionally, triglycerides, sterols, sterol esters, tocotrienols and other lipids are also associated to the rubber particles. As illustrated in Figure 2.5, the rubber particles, consisting of hydrophobic rubber polymers are surrounded by spherical shells that contain phospholipids and proteins (Gomez and Moir, 1979).

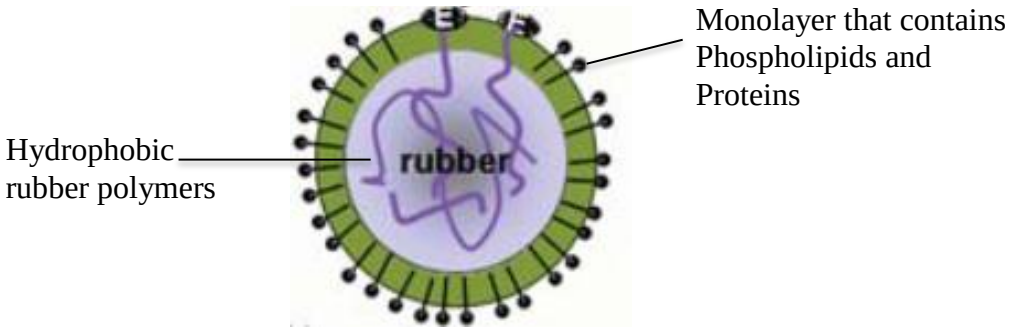

Figure 2.5: Schematic drawing of the rubber molecule surface. The natural rubber is packed within the rubber particle membrane, which is surrounded by proteins and phospholipids (Gronover *et al.,* 2009).

The existences of negative charge protein films coating the rubber particle membrane are believed to contribute to the integrity and the stability of the rubber particles. According to Bowler, (1953), proteins embedded on the *Hevea'*s fresh latex rubber particles have isoelectric points ranging from pH 4.0 to 4.6, depending on the rubber clones, hence indicating that more than one kind of protein is adsorbed on the rubber particle.

In addition, a considerable amount of literature has been published suggesting that the surface of rubber particles contains proteins, enzymes or factors necessary for rubber biosynthesis, and is the place where rubber biosynthesis occurs (Benedict *et al.,* 1990; Cornish and Backhaus, 1990; Cornish, 2001a; and Singh *et al.,* 2003). These proteins are: 1) the 14.6 kDa or rubber elongation factor (REF) believed to be embedded on the LRP; 2) the 22 kDa or small rubber particle protein (SRPP) believed to be associated on SRP and 3) Isopentenyl pyrophosphate polymerase. Also, a key enzyme known as rubber transferase or *cis*-prenyltransferease (CPT) that is responsible for the polymerization of polyisoprene is believed to be associated to the monolayer membrane of the rubber particle (Cornish, 2001b).

Subsequently, even more recently, Dai *et al.,* (2013) have successfully identified a total of 186 rubber particle proteins, in addition to REF, SRPP, and CPT with a wide molecular range of 3.9-194.2 kDa and with isoelectric point values of 4.0-11.2. These proteins include cytochrome P450, phospholipase D, clathirin, Rab GTPases, TUA3, cylophilin, ubiquitin, polyubiquitin and many others.

2.4 Structure of Natural Rubber and Rubber Biosynthesis

2.4.1 Structure of Natural Rubber

The chemical composition of NR from *Hevea* is *cis*-1,4-polyisoprene formed as a result of progressive condensation of isopentenyl diphosphate (IPP). However, the exact structure of NR remains unknown till today. The *cis*-configuration of the isoprene repeats was discovered in a study led by Nyburg, (1954) using X-ray diffraction. It was Tanaka *et al.,* (1989) who later showed that the second and the third units of *Hevea* rubber are trans, followed by repetitive *cis* enchainment (Figure 2.6)

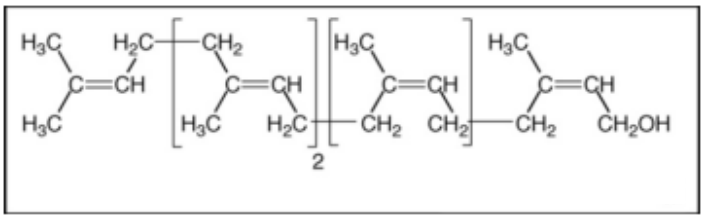

Figure 2.6: Image depicting the microstructure of *Hevea*'s natural rubber (Beilen and Poirier, 2007).

2.4.2 Rubber Biosynthesis

Sucrose is the main carbon and energy source for the rubber biosynthesis to occur in the *Hevea* tree (Silpi *et al.,* 2007; and Rahman *et al.,* 2013). The formation of the high molecular weight *cis*-1,4-polyisoprene requires four distinctive biochemical processes: i) biosynthesis of a precursor molecule, known as isopentenyl diphosphate (IPP); ii) initiation; iii) elongation by *cis*-prenyltransferase (CPT) and iv) termination. Each of the processes will be discussed in a greater detail in the following sub-section:

2.4.2.1 Biosynthesis of Isopentenyl Diphosphate (IPP): a precursor molecule

IPP is the monomeric unit for the huge linear rubber biopolymer as well for all isoprenoids producing plants. In *Hevea,* IPP is produced via two biosynthetic pathways; 1) the well-described mevalonate (MVA) pathway (Gronover *et al.,* 2009) compartmentalized in the cytosol and 2) and the recently discovered 1-deoxy-D-xylulose-5-phosphate/2-C-methyl-D-erythritol-4-phosphate (DOXP/MEP) pathway, which is localized in the plastids (Ko *et al.,* 2003). Both pathways are thought to utilize a simple sugar as the main source of carbon and are naturally dependent on enzymes (Ohya and Koyama, 2001).

Evidence for the MVA pathway route to rubber biosynthesis emerged based on experiments involving incubation of latex with ^{14}C-labelled intermediates (Keckwick, 1989). The MVA mechanistic pathway involves the formation of acetyl Coenzyme A (acetyl-CoA) and the six-carbon intermediate, mevalonate. This mevalonate arises from the sequential condensation of three acetyl-CoA molecules that produces 3-hydroxy-3-methyglutaryl Coenzyme A (HMG-CoA), which are then converted to MVA in an irreversible reaction catalyzed by HMG-CoA reductase. Consequently, MVA is then sequentially phosphorylated and decarboxylated to generate IPP and its isomer, dimethylallyl diphosphate (DMAPP) by a set of enzymes (Figure 2.7) (Goldstein and Brown, 1990; and Dubey and Bhalla, 2003).

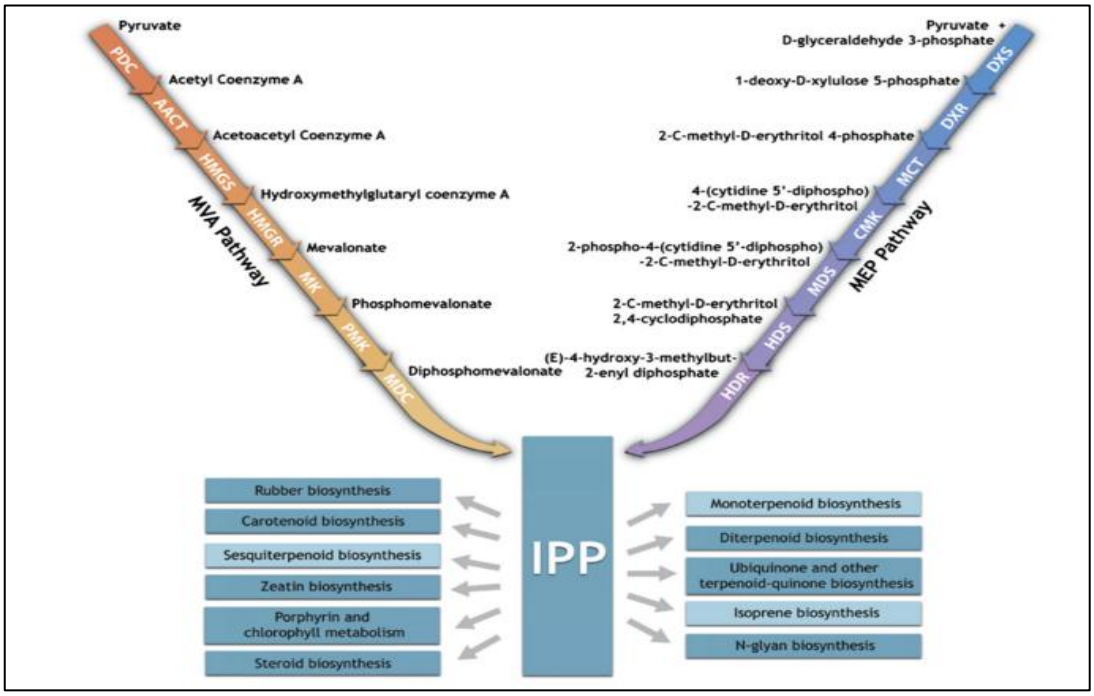

Figure 2.7: Illustration of isoprenoid biosynthesis. IPP is a common intermediate of numerous isoprenoids-producing organisms and may be synthesized via the cytosolic MVA pathway or the plastidic MEP pathway. Isoprenoids end products, including rubber (*cis*-1,4-polyisoprene), are indicated within darker blue boxes (Chow *et al.*, 2012).

In the *Hevea* rubber tree, the existence of MEP pathway was supported by the identification of an enzyme sequence, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) from the latex transcriptome sequencing (Ko *et al.*, 2003 and Chow *et al.*, 2007). Hence, this led to the idea that the MEP pathway synthesizes IPP for carotenoids in Frey-Wyssling particles but could, in addition, provide IPP for *cis*-polyisoprene synthesis (Chow *et al.*, 2012). Surprisingly, in feeding experiments using the [1-^{13}C] 1-deoxy-D-xylulose triacetate, (an intermediate of MEP pathway) no rubber molecules could be detected that carry an isotope label (Sando *et al.*, 2009).

However, in another important investigation performed by Chow *et al.*, (2012), two schemes of IPP partitioning and utilization within one species has been proposed, whereby the supply of IPP for *cis*-polyisoprene from MEP pathway is related to the carotenoid production in latex. In another words, in clones, which do not produce large amount of carotenoids (for instance, RRIM 600 clone), the MEP pathway is an alternative provider of IPP for *cis*-polyisoprene synthesis, where as in clones that produces higher carotenoid content (for example, PB235 clone), partitioning of IPP exists between carotenoid biosynthesis in Frey-Wyssling particles and *cis*-polyisoprene synthesis in the rubber particles.

2.4.2.2 Initiation

For the initiation of rubber biosynthesis to occur, an initiator molecule known as allylic diphosphate is needed (Cornish, 2001a). IPP is isomerized to DMAPP by IPP-isomerase and is used as a substrate by *trans*-prenyltransferase (TPT) or also known as (E)-prenyl diphosphate synthases to generate an allylic initiator molecule (Priya *et al., 2*006; and Gronover *et al.,* 2009). TPT catalyzes the condensation of an allylic diphosphate with IPP or DMAPP molecule. Both, IPP-isomerase and TPT are found in the bottom fraction and the supernatant cytosol (also known as C-serum) of the centrifuged fresh *Hevea* latex (Koyama *et al.,* 1996; Tangpakdee *et al.,* 1997; and Asawatreratanakul *et al.*, 2003).

It has been shown through *in vitro* experiments in wide variety of rubber producing plants such as *Ficus elastica, Parthenium argentatum* and *H. brasiliensis* that the initiation of rubber biosynthesis is most efficient with the C-15 farnesyl pyrophosphate (FPP) (Xie *et al.,* 2008). This is because FPP has a lower binding constant than other allylic initiators (Cornish, 2001 and Cornish, 2006), such as the C-10 geranyl pyrophosphate (GPP) and the C-20 geranyl-geranyl pyrophosphate (GGPP) (Cornish, 1993 and Tanaka *et al.,* 1996). Figure 2.8 below exemplifies the mechanism catalyzed by TPT to produce allylic diphosphates.

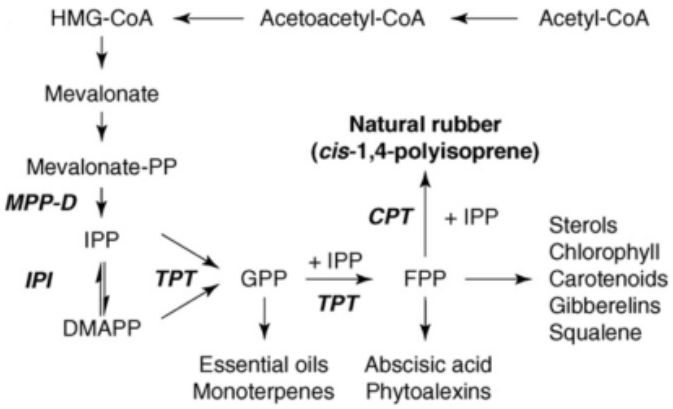

Figure 2.8: Image depicting the production of IPP molecule by the mevalonate pathway. IPP molecule is isomerized to DMAPP by IPP-isomerase. IPP is further condensed in several steps with IPP or the DMAPP molecule to produce geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP) and gernyl-geranyl pyrophosphate (GGPP), by the action of *trans*-prenyltransferase (TPT). The polymerization of *cis*-1,4-polyisoprene is catalyzed by rubber transferase or the *cis*-prenyltransferase (CPT), which uses non-allylic IPP as a substrate (Beilen and Poirier, 2007).

2.4.2.3 Elongation by *cis*-prenyltransferase (CPT)

The enzyme, *cis*-prenyltransferase (CPT) or (Z)-prenyl diphosphate synthases, responsible for the *cis*-1,4-polymerization of isoprene units from IPP or DMAPP onto the allylic initiator molecular (FPP in this case) from *trans* to *cis* manner has been identified as a particle-bound rubber transferase (EC 2.5.1.20) (Archer and Audley, 1987; Light and Dennis, 1989; Cornish, 1993 and Asawatreratanakul *et al.*, 2003).

Previous investigations carried out by Dennis and Light, (1989) suggested that the association of a soluble *trans*-prenyltransferase mediates the rubber biosynthesis in *H. brasiliensis* together with the rubber elongation factor (REF) protein, which is tightly bound to the large rubber particles in the latex. However, the findings of Cornish, (1993) contradicts and does not support the results exhibited by Dennis and Light, (1989) and have demonstrated that the soluble *trans*-prenyltransferase functions as farnesyl diphosphate synthase, and does not play a role in the *cis*-1,4-polymerization elongation.

In 2003, Asawatreratanakul *et al.*, isolated two cDNA clones (designated as HRT1 and HRT2) that possibly encodes the CPT of the rubber tree *H. brasiliensis*. In addition, they also showed that one of the two identified CPTs (HRT2) found highly expressed in the laticifers of *Hevea* could exhibit IPP-condensation activity with a high molecular mass when heterologously expressed in *Escherichia coli* and co-incubated with *H. brasiliensis* latex. Subsequently, it was shown that the rubber transferase activity of HRT2 increased proportionally by the addition of washed bottom fraction particles (WBP) thus, suggesting the involvement of certain

activating factors residing in the *Hevea*'s latex bottom fraction (Asawatreratanakul *et al.,* 2003) that promotes the elongation of the rubber molecule.

However, the rubber transferase activity of the former (HRT1) clone could not be detected, although high sequence homology in the five highly conserved region to other *cis*-prenyl chain-elongating enzyme were observed. This led to a postulation that the HRT1 might possibly encode a *cis*-prenyl chain-elongating enzyme of short chain C-5 isoprene such as the dehydrodolichyl diphosphate synthase, whose function in the rubber tree remains unknown.

Even more recently, Rahman *et al.,* (2013) have identified eight CPT coding genes from the recently published draft genome of *Hevea* rubber. They found that five out of eight of the newly discovered CPTs are homologous to other plant CPTs, such as the undecaprenyl pyrophosphate synthase and dehydrodolichyl diphosphate synthase. This finding is persistent to the postulation laid by Asawatreratanakul *et al.,* (2003) pertaining HRT1 as a short chain-elongating enzyme (dehydrodolichyl diphosphate synthase). Only three CPTs were observed to be specific to *H. brasiliensis* in the findings reported by Rahman *et al.,* (2013).

Apart from CPT, a number of other proteins have also been shown to take part in the biosynthesis of *cis*-1,4-polyisoprene. These proteins includes, Rubber Elongation Factor (REF) (Dennis and Light, 1989), Small Rubber Particle Protein (SRPP) (Oh *et al.,* 1999), Rubber Biosynthesis Stimulator Protein (eIF-5A) (Yusof *et al.,* 2000; Chow *et al.,* 2003; and Chow *et al.,* 2007) and Patatin-like inhibitor protein (Yusof *et al.,* 1998). Of all the rubber biosynthesis proteins, SRPP and REF

are of particular interest, as these two recombinant proteins has been previously demonstrated to enhance the incorporation of IPP molecules based on an *in vitro* assays containing isolated rubber particles (Oh *et al.,* 1999; and Chow *et al.,* 2007). The subsequent section of this chapter will focus more on the aspects of SRPP and REF protein.

2.5 Small Rubber Particle Protein and Rubber Elongation Factor (SRPP & REF)

The latex of *Hevea* consists of many proteins, particularly the SRPP (GenBank accession no: O82803) and REF (GenBank accession no: P15252). SRPP (22.3 kDa) and REF (14.7 kDa) proteins are two water insoluble acidic proteins with isoelectric points (pI) of 4.80 and 5.04, respectively. Intriguingly, these two proteins share a significant sequence homology of amino acids at the N-terminal (Berthelot *et al.,* 2012; and Berthelot *et al.,* 2014), but differ in their C-terminal part (Figure 2.9). It has been postulated, that the extra C-terminal part present on the SRPP protein may play an important role in sustaining the stability of the protein. In addition, the sequences of SRPP and REF do not contain cysteine residues and they have been exhibited to be non-glycosylated proteins in the *Hevea* latex (Goyvaerts *et al.,* 1991; Wagner *et al.,* 1999; Arif *et al.,* 2004; and Berthelot *et al.,* 2014).

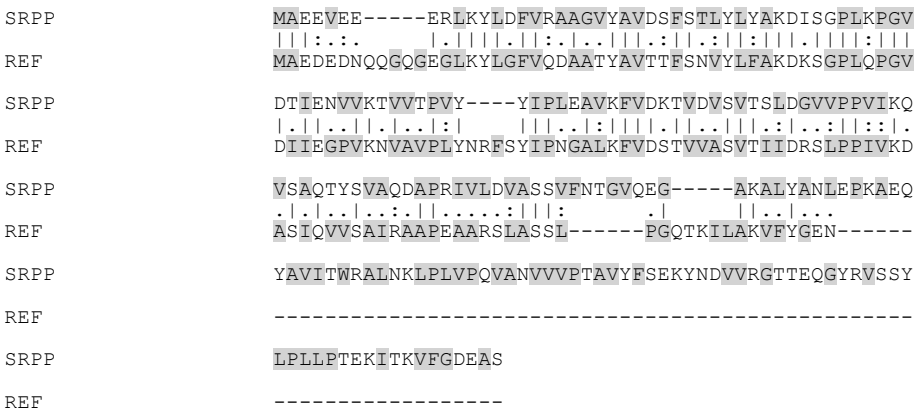

Figure 2.9: Sequence homology of SRPP protein to REF from *H. brasiliensis.* Both the proteins share sequence homology of amino acids at the N-terminal. Sequences that are similar are denoted as (|). Hydrophobic sequences are shaded in grey. Sequences were aligned by using CLUSTAL OMEGA.

Furthermore, evolutionary analysis conducted by Berthelot *et al.,* (2012) clearly show that both the SRPP and REF are homologous proteins originating from a common ancestor gene, belonging to plant stress-related protein family, such as *Parthenium argentatum, Capsicum annuum, Arabidopsis lyrata, Medicago truncatula* and many other (see Figure 2.10). They are small proteins ranging from 14-28 kDa in size and can be found in plant kingdom.

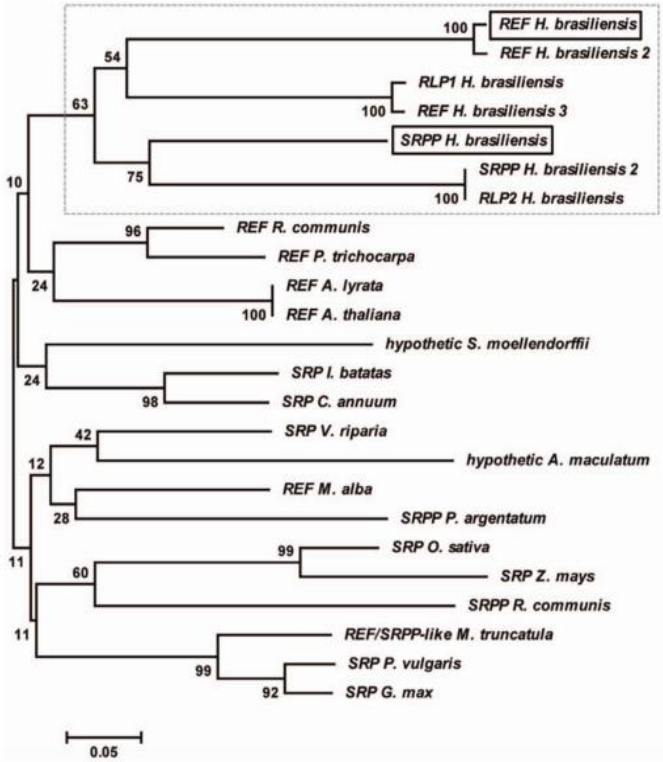

Figure 2.10: Phylogenetic analysis of SRPP and REF protein family. SRPP and REF proteins are related to the larger plant stress-related protein family. REF *Hevea brasiliensis* (Genbank accession no: P15252), REF *Hevea brasiliensis* (Genbank accession no: AEH05970), REF *Hevea brasiliensis* (Genbank accession no: AAR11448), SRPP *Hevea brasiliensis* (Genbank accession no: O82803), SRPP *Hevea brasiliensis* (Genbank accession no: AAO66432), RLP1 *Hevea brasiliensis* (Genbank accession no: AAP46159), RLP2 *Hevea brasiliensis* (Genbank accession no: AAP46160), REF *Ricinus communis* (Genbank accession no: XP_002512427), REF *Arabidopsis thaliana* (Genbank accession no: NP_187201), SRP *Vitis riparia* (Genbank accession no: Q9SW70), REF *Morus alba* (Genbank accession no: ACV90044), REF *Amblyomma maculatum* (Genbank accession no: AEO33677), SRP *Ipomoea batatas* (Genbank accession no: ABP35522), SRP *Oryza sativa* (Genbank accession no: AAO72547), SRP *Zea mays* (Genbank accession no: ACG39345), REF *Selaginella moellendorffii* (Genbank accession no: XP_002969776), SRPP *Parthenium argentatum* (Genbank accession no: AAQ11374), SRP *Capsicum annuum* (Genbank accession no: ADI60300), SRPP *Ricinus communis* (Genbank accession no: XP_002514917), REF *Populus trichocarpa* (Genbank accession no: XP_002319520), REF *Arabidopsis lyrata* (Genbank accession no: XP_002882419), SRP *Glycine max* (Genbank accession no: XP_003543052), REF/SRPP-like protein *Medicago truncatula* (Genbank accession no: XP_003593563). Phylogenetic tree adapted from Berthelot et al., ()