Molecular Analysis of Methylerythritol Phosphate (MEP) Pathway in *Hevea* Latex

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Thesis Title	Molecular Analysis of Methylerythritol Phosphate (MEP) Pathway in *Hevea* Latex
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ชื่อวิทยานิพนธ์	การศึกษาในระดับโมเลกุลของวิถีเมทิลอิริทริทอลฟอสเฟตในน้ำยางพารา
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บทคัดย่อ

การสังเคราะห์ยางธรรมชาติที่ได้จากยางพารานั้นเกิดขึ้นโดยปฏิกิริยาพอลิเมอไรเซชันของหน่วยย่อยไอโซเพนทีนิลไดฟอสเฟต ปฏิกิริยาดังกล่าวคาดว่าน่าจะเกิดขึ้นภายในท่อน้ำยางตรงบริเวณโครงสร้างที่มีลักษณะคล้ายเครือข่ายเส้นด้ายที่เกิดจากท่อเรติคิวลัมเชื่อมต่อกับออกาแนลล์ที่มีเมมเบรนห่อหุ้ม ออกาแนลล์ดังกล่าวคืออนุภาคเฟรวิสลิงซึ่งมีเมมเบรนห่อหุ้มสองชั้นและลูทอยด์ซึ่งมีเมมเบรนชั้นเดียว ในเบื้องต้นได้ทดลองใช้สารสกัดจากอนุภาคเฟรวิสลิงทำปฏิกิริยากับกลูโคสที่ติดฉลากด้วยสารกัมมันตรังสี (คาร์บอน 14) พบว่าสารสกัดจากอนุภาคเฟรวิสลิงสามารถเปลี่ยนกลูโคสไปเป็นสารประกอบพรีนิลได้ทั้งในกรณีที่มีหรือไม่มีการใช้ตัวยับยั้งปฏิกิริยาในวิถีเมทิลอิริทริทอลฟอสเฟตและวิถีเมวาโลเนต โดยผลการทดลองพบว่าสารฟอสมิโดมัยซินซึ่งมีฤทธิ์ยับยั้งวิถีเมทิลอิริทริทอลฟอสเฟตสามารถออกฤทธิ์การยับยั้งการสร้างสารประกอบพรีนิลได้มากกว่าสารเมวิโนลินซึ่งเป็นตัวยับยั้งวิถีเมวาโลเนต ผลการทดลองดังกล่าวแสดงให้เห็นว่าในน้ำยางพารามีวิถีเมทิลอิริทริทอลฟอสเฟตสำหรับเป็นอีกทางเลือกหนึ่งในการสังเคราะห์ไอโซเพนทีนิลไดฟอสเฟต จึงได้ทำการโคลนยีนของเอนไซม์ดีออกซีไซลูโลสฟอสเฟตรีดักโทไอโซเมอเรสซึ่งเป็นเอ็นไซม์ควบคุมตัวหนึ่งของวิถีเมทิลอิริทริทอลฟอสเฟต ยีนของเอนไซม์ที่โคลนได้จากน้ำยางพารานี้ประกอบด้วยนิวคลีโอไทด์จำนวน 1,413 นิวคลีโอไทด์ สามารถแปลไปเป็นเอนไซม์ที่มีน้ำหนักโมเลกุลประมาณ 51 กิโลดาลตัน และมีความเหมือนมากกว่า 80 เปอร์เซ็นต์กับเอนไซม์ดังกล่าวในพืชชนิดอื่น มีลำดับกรดอะมิโนที่ทำหน้าที่จับกับสารนิโคตินาไมด์อะดีนีนไดนิวคลีโอไทด์ฟอสเฟต และที่ปลายด้านหมู่อะมิโนมีลำดับที่เชื่อว่าเป็นตำแหน่งตัดสำหรับทรานสิทเปปไทด์ และได้มีการทดลองเชื่อมต่อยีนที่ได้กับยีนโปรตีนเรืองแสงกรีนฟลูออเรสเซนต์และส่งถ่ายยีนเข้าสู่เซลล์อะราบิดอบซีส พบว่ายีนดีออกซีไซลูโลสฟอสเฟตรีดักโทไอโซเมอเรสนี้สามารถแสดงออกในคลอโรพลาสต์ของเซลล์พืชทดลอง ดังนั้นในยางพารายีนนี้น่าจะแสดงออกในอนุภาคเฟรวิสลิง นอกจากนี้ยังพบว่ายีนนี้มีปริมาณการแสดงออกในเนื้อเยื่อจากลำต้นอ่อน ดอก และน้ำยางพาราจากบริเวณลำต้นได้ดีกว่าในใบยางพารา ดังนั้นจึงเป็นไปได้ว่าอนุภาคเฟรวิสลิงน่าจะมีบทบาทร่วมในการสังเคราะห์หน่วยย่อยไอโซเพนทีนิลไดฟอสเฟตโดยวิถีเมทิลอิริทริออลฟอสเฟต เพื่อใช้เป็นสารตั้งต้นสำหรับการสังเคราะห์ยางธรรมชาติ

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Abstract

Natural rubber from *Hevea brasiliensis* is synthesized by enzymic polymerization of isopentenyl diphosphate (IDP) units. This has been proposed to occur inside the latex vessel in a thread-like tubular reticulum, connecting rubber particles to single- (lutoid) and double- (Frey-Wyssling, FW) membrane-bound organelles. We show that a membrane free preparation from FW particles converted [^{14}C] glucose into radio labeled prenyl products and this was more efficiently inhibited by fosmidomycin, the MEP pathway inhibitor, than mevilonin, the inhibitor used to block the MVA pathway. This implicated the alternative plastid associated MEP pathway for IDP synthesis. We then identified a cDNA clone (*Hbdxr*) from a *Hevea* rubber latex cDNA library, encoding for 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), a key enzyme of the MEP pathway for the IDP biosynthesis. Sequence analysis and the deduced amino acid sequence had >80% homology to other plant DXR enzymes with an ORF consisting of 1413 bp capable of encoding a 51 kDa polypeptide. A highly conserved binding site for NADPH was identified and an N-terminal transit peptide with a putative conserved cleavage site. The *Hbdxr-gfp* gene, transformed into *Arabidopsis* cells was located in the chloroplasts, thus *Hbdxr* may be expressed and localized in the FW plastids. The levels of *Hbdxr* mRNA detected in young latex containing tissues, inflorescence and seedling stems, were higher than those found in the latex from the tree and were barely detectable in the mature leaves. We therefore suggest that one function of the FW particles could be to supply IDP for rubber biosynthesis through the tubular thread-like reticulum.

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Krueawan Yoonram

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List of Abbreviations and Symbols

A^o	= angstrom
ATP	= adenosine triphosphate
bp	= base pair
^oC	= degree of Celcius
cDNA	= complementary deoxynucleic acid
CDP-ME	= 4-diphosphocytidyl-2-C-mthyl-D-erythritol
CDP-ME2P	= 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate
Ci	= Curie
CIP	= calf intestinal phosphatase
cm	= centimeter
cMEPP	= 2-C-methylerytriol-2,4-cyclodiphosphate
CMP	= cytidine monophosphate
CTP	= cytidine triphosphate
Da	= Dalton
DEPC	= diethylpyrocarbonate
DMADP	= dimethylallyl diphosphate
dNTP	= deoxynuclotide triphosphate
DTT	= dithiothreitol
DXR	= 1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	= 1-deoxy-D-xylulose-5-phosphate synthase
EDTA	= ethylamine diamine tetraacetic acid
FDP	= farnesyl diphosphate
FW	= Frey-Wyssling particles
g	= gram
× g	= gravitation acceleration
GAP	= glyceraldehydes-3- phosphate
GGDP	= geranylgeranyl diphosphate
GDP	= geranyl diphosphate

List of Abbreviations and Symbols (continued)

HbDXR	= *Hevea brasiliensis* 1-deoxy-D-xylulose-5-phosphate reductoisomerase
HbSDS	= *Hevea brasiliensis* solanesyl diphosphate synthase
HMEPP	= 1-hydroxy-2-methyl-2-enyl diphosphate
HMG	= 3-hydroxy-3-methylglutaryl
HMGS	= 3-hydroxy-3-methylglutaryl CoA synthase
HMGR	= 3-hydroxy-3-methylglutaryl CoA reductase
HRT	= *Hevea brasiliensis* rubber transferase
IDP	= isopentenyl diphosphate
IPTG	= isopropyl-β-D-thiogalactopyranoside
kb	= kilobase
kDa	= kilodalton
KF	= potassium fluoride
LB	= Luria Bertani
M	= molar
ME	= 2-*C*-methylerythritol
MECDP	= 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate
MEP	= 2-*C*-methyl-D-erythritol 4-phosphate
MEOP	= 2-*C*-methyl-D-erythrose 4-phosphate
mA	= milliampere
min	= minute
ml	= milliliter
mM	= millimolar
mRNA	= messenger ribonucleic acid
NADH	= nicotinamide adenine dinucleotide
NADPH	= nicotinamide adenine dinucleotide phosphate
nM	= nanomolar
NMR	= Nuclear magnetic resonance

List of Abbreviations and Symbols (continued)

NPP	= neryl diphosphate
MVA	= mevalonate
ORF	= open reading frame
O.D.	= optical density
PAGE	= polyacrylamide gel electrophoresis
PCR	= polymerase chain reaction
ppm	= part per million
PQ	= plastoquinone
RACE	= rapid amplification of cDNA ends
RB	= rubber biosynthesis
REF	= rubber elongation factor
RT-PCR	= reverse transcription-polymerase chain reaction
rRNA	= ribosomal ribonucleic acid
sec	= second
SDP	= solanesyl diphosphate
SDS	= sodium dodecyl sulfate
TAP	= Tobacco acid pyrophosphatase
TEMED	= N, N, N', N' tetramethylethylenediamine
TLC	= thin layer chromatography
U	= unit
WRP	= washed rubber particles
X-gal	= 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
μg	= microgram
μl	= microliter
μM	= micromolar
%	= percentage

CHAPTER 1

Introduction

Introduction

Rubbers in the latex from various plants are polyisoprenoides of the high molecular weight hydrocarbon polymers consisting almost entirely of the five-carbon isoprene (C_5H_8) units. The polyisoprene rubber is major component of latex synthesized by special differentiated cells of the plants. They are synthesizd by series of enzymes-catalyzed polymerization (Lynen, 1969) of isoprene units to various diferrent degrees, resulting in a wide range of molecular weight (MW). Although over 2,000 species of higher plants belonging to several different families can synthesize rubber latex (Backhaus, 1985), *Hevea brasiliensis* has been established as only one commercial rubber source due to its good yield of rubber and excellent physical properties of rubber product (Archer and Audley, 1973). The latex composition may vary to a large extent among the different plant species. The polymers in the suspension of which it is formed may contain varying proportions of rubber and of different compounds in addition. Of some 12,500 species of laticiferous plants, about 7,000 are found to produce polyisoprenes. In most case the polyisoprene is mixed with resin, making it difficult to use when the content of the resin is high. A limited number of rubber-producing plants can be suitably utilized, and only a few species are cultivated and have economic importance.

Hevea rubber latex is obtained as the white-colored fluid that exudes from the trunk of rubber trees after tapping. The tapping severs a number of latex vessel rings, allowing the latex to flow out. All the components present in the vessel ring, at their different stages of development, can be found in the tapped latex. The latex contains particles the majority of which are rubber, lutoids, and Frey-Wyssling (Hamzah and Gomez, 1982; Hamzah and Gomez, 1983; Wititsuwannakul and Wititsuwannakul, 2001).The rubber particles consist mainly of a highly hydrophobic rubber core surrounded by a lipid monolayer containing a few embedded proteins (Cornish *et al*., 1999).The lutoids, the most abundant

non-rubber particles, are vacuoles enclosed by a single membrane. Within the lutoids there is an aqueous environment containing dissolved substances such as acids, minerals, proteins and sugars. Acid phosphatase and lysozyme have been detected in these particles, and in addition the lutoids contain mainly characteristic acid hydrolase (Dupont *et al*., 1976) suggesting that the particles are analogous to lysosomes (Pujarniscle, 1968). Lutoids possess one of the factors that lead to cessation of latex flow, because the dilution reaction that occurs inside the latex vessel on tapping causes swelling of the osmotically sensitive lutoids remaining in the tube (Boatman, 1966; Sounthorn, 1969; Subramaniam, 1972). The Frey-Wyssling (FW) particles are surrounded by a double membrane and contain many membranes or tubular structures. They are specialized chromoplasts containing plastochromonol, plastoquinone and β-carotene with characteristic orange to yellow colors and assumed to be modified plastids (Dickenson, 1965; 1969; Moir, 1969; Archer *et al*., 1969; Ohya, and Koyama, 2001; Phatthiya *et al*., 2007). The highly complicated structure of FW particles indicates that they have important functions in the metabolism of *Hevea* latex (Dickenson, 1969).

When viewed with a phase contrast microscope, these three major latex particles seen in fresh latex after being collected at a low temperature, seem to be attached to a network of threads or a thread-like reticulum,. The yellow FW particles were found in grape-like clusters enclosed within a bag-like membrane. The lutoids were strung along the reticulum thread, like beads, and the rubber particles were attached through their stalks (Figure 1, Southorn, 1961). The different particulate materials in fresh latex can be separated by centrifugation with the rubber particles in the top layer, the middle fraction composed of the aqueous C serum, and the sedimented bottom fraction of lutoid and FW particles. It has been suggested that the thread-like structures, commonly observed throughout the centrifuged fresh latex, represent the remnants of an extensive reticulum of hollow thread-like processes, within the latex vessel to which the various particles attach and through which they interact. It has been proposed that this thread-like reticulum could serve as the site for the biosynthesis of the natural rubber hydrocarbon (Southorn, 1961). It is possible that the tube -like reticulum act as a conduit between these particles.

Although natural rubber is synthesized and made almost entirely of *cis*-isoprene units derived from IDP, an allylic diphosphate is also required as the priming co-

substrate to initiate the subsequent extensive prenyl chain elongation process for the formation of the rubber molecule (Archer and Audley, 1987; Madhavan *et al*., 1989; Cornish and Backhaus, 1990). Synthesis of the allylic prenyl diphosphates are catalyzed by *trans*-prenyl transferase enzymes, that were found in both the bottom fraction and the cytosol C serum of centrifuged fresh *Hevea* latex (Wititsuwannakul and Wititsuwannakul, 2001; Tangpakdee *et al*., 1997a). The allylic diphosphates of chain length C_5- C_{20} have been shown to stimulate *in vitro* rubber formation, the efficiency of which increases with the increasing chain length of the allylic diphosphates, i.e. $C_{20} > C_{15} > C_{10} > C_5$ (Archer and Audley, 1987). A structural analysis study to characterize the initiating species of *Hevea* rubber has indicated that farnesyl diphosphate (FDP) is the preferred starting molecule for rubber biosynthesis (Tanaka *et al*., 1996). Recently, the expression of *Hevea* FDP synthase in rubber latex was shown to be much higher than that of the geranylgeranyl diphosphate (GGDP) synthase (Takaya *et al*., 2003). The rubber transferase enzymes responsible for the *cis*-1,4-polymerization of isoprene units onto the allylic primer have until now been thought to be firmly bound to the surface of rubber particles (Light and Dennis, 1989; Cornish, 1993) and requires IDP as the elongation substrate, an allylic diposphate as the acceptor and wash rubber particle. By successively adding isoprene moieties derived from IDP, rubber synthase catalyzes the elongation of pre-existing polyisoprene molecules by five carbon atoms in each step.

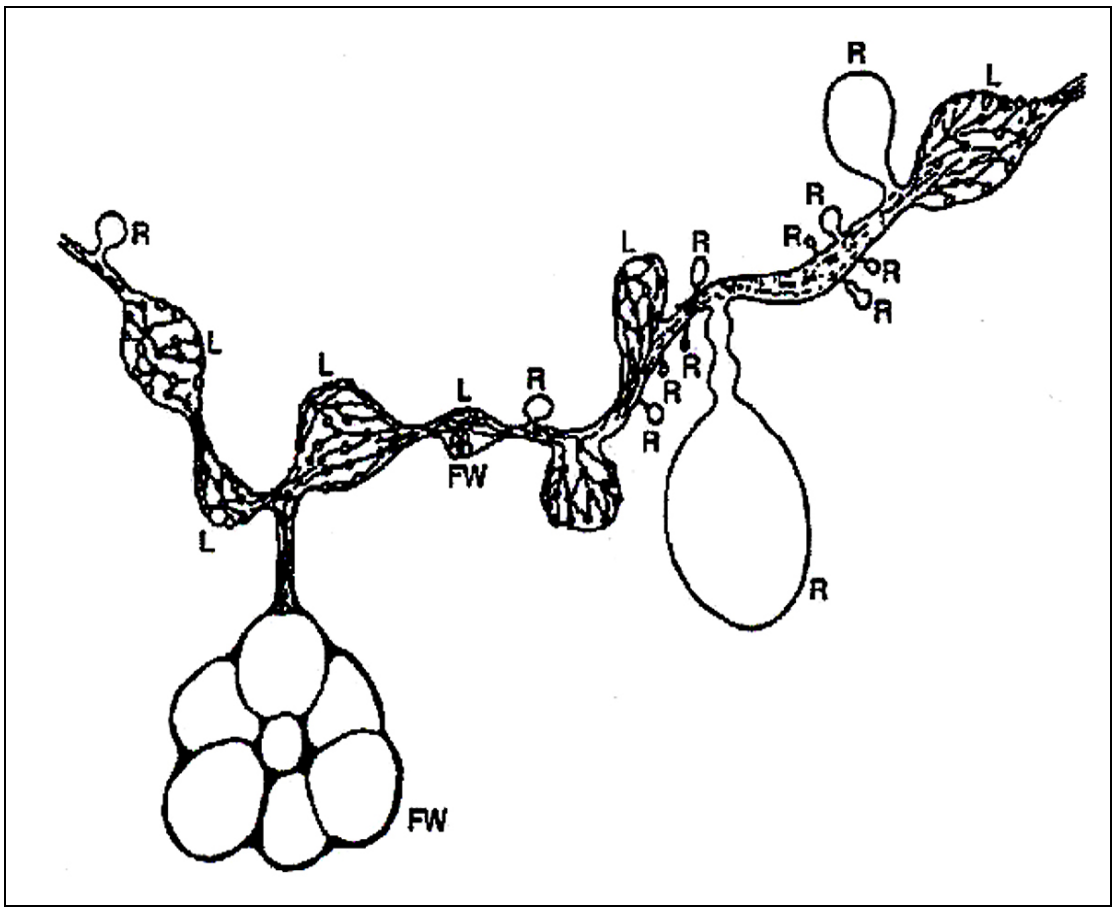
Hevea latex was shown to be active (Lynen, 1967; Archer *et al*, 1963) in the synthesis of rubber for quite some time. Study on rubber biosynthesis (RB) process is of must interest as appeard in several reviews (Archer and Audley, 1987; Ohya and Koyama, 2001). Most of studies focused mainly on the surface of rubber particles (RP) and was already reviewed (Archer and Audly, 1987; Audly and Archer, 1988; Kekwick, 1989; Kush, 1994; Tanaka *et al*., 1996; Ohya and Koyama, 2001) as the only prerequisite site required for synthesis of rubber molecules. This might seem a paradox to address the question on how and where the original RP was form if the new rubber has to be synthesized on its preexisting surface. According to the complex nature of *Hevea* latex and its myriad compositions, it might be possible that the RB can take place at certain specific site other than the RB surface. This has been earlier postulated (Dickenson, 1969; Moir, 1959) but has received little

attention and no careful investigation was carried out to substantiate this suggestion. Study with the condition that is free of RP should be attempted which served as ideal system to solve this query. In support of this possibility we have recently provided evidence for an active involvement of the latex bottom fraction, containing the sedimented lutoid and FW particles, in rubber biosynthesis (Tangpakdee *et al*., 1997a; Wititsuwannakul *et al*., 2003). The results might thus suggest that the actual RB site could be localized on theses particles membrane other than the RP surface. As stated in most recent report (Wititsuwannakul *et al*., 2003), this was in contrast with the previous numerous studies, in witch the RP surface was implicated as the one and only prerequisite site for the in vitro RB process (Archer *et al*, 1963; Archer *et al*, 1982). Moreover, rubber transferase (termed HRT) was successfully cloned (Asawatreratnakul *et al*., 2003) that was RB active with bottom fraction membranes, strongly support bottom fraction role. Subsequently, further studies on RB activities of the membrane proteins from the bottom fraction particles were carried out (Wititsuwannakul *et al*., 2004). The results thus strongly suggested that the RB enzyme in the membrane is not only capable of catalysis for rubber biosynthesis, but it also contains a controlling element in regulatory mechanism for the rubber biosynthesis and the biogenesis control.

Since a laticiferous vessel is specialized in synthesizing the rubber polymer, it must have a means for a sufficient and efficient supply of IDP as this is the most important precursor required for the biosynthesis of rubber (Cornish, 2001). In higher plants, IDP is formed by two pathways that operate in different subcellular compartments (Eisenreich *et al*., 1998; Rohmer, 1999; Lichtenthaler, 2000). The well-known mevalonate (MVA) pathway is present in the cytosol-endoplasmic reticulum while the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway is localized in the plastid. The two pathways start from different central intermediates: the MVA pathway starts from acetyl-CoA whereas the MEP pathway starts from pyruvate and glyceraldehydes 3-phosphate.Within the MEP pathway, the initial enzymatic step involves the condensation of glyceraldehyde 3-phosphate and pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DXP), catalysed by DXP synthase (DXS), the product of the *dxs* gene. The next reaction, the conversion of DXP to MEP, is catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), encoded by the *dxr* gene. The DXP is an intermediate precursor of not only the IDP but is also a common precursor for thiamine

pyrophosphate and pyridoxol phosphate (Julliard and Douce, 1991; Hill *et al*., 1996). Thus, the reaction catalyzed by DXR is thought to represent the first committed step in the MEP pathway. Ko *et al.* (2003) have previously reported the expression of a gene encoding DXS in the *Hevea* latex, thus the MEP pathway is initiated in the latex. The high level of latex *dxs* gene expression reported indicates a possible involvement of the MEP pathway in supplying the isoprene precursor for the synthesis of the major rubber (polyisoprene) product, in addition to other minor isoprenoids, in the *Hevea* latex. A specialized plastid of *Hevea* latex, FW particles, is likely to contain MEP pathway metabolism and have been suggested to perform important metabolic functions including rubber biosynthesis. This concurs with reports on the biosynthesis capacity on non-rubber particles of latex bottom fraction which contains predominantly FW particles and lutoids (Tangpakdee *et al*., 1997b; 1997c; Wititsuwannakul *et al*., 2003). IDP generate by FW particle were suggested that it may be transport out into the cytosol as a substrate for further IDP condensation (Chow *et al*., 2007).

To gain further insights into the MEP pathway in a non photosynthetic latex vessel, with the possibility of implicating it in rubber biosynthesis, we decided to attempt to clone the *dxr* gene from the latex of the rubber tree. Our previous detection of radio labeled prenyl products in the isolated FW particles after incubation with [^{14}C] glucose gave us some confidence that this might be possible. At the present time, the detailed biological function of these particles in latex, besides their possible involvement in carotenoid biosynthesis, is still largely unknown (Phatthiya *et al*., 2007). In this study we describe the isolation and characterization of latex cDNA encoding the *dxr* gene.



(Southorn, 1961)

Figure 1 Illustration possible relationship between the reticulum and the various particulate phase in unperturbed latex vessels R = rubber particle; L = lutoid; FW = Frey-Wyssling particle

Review of literatures

1. Rubber latex

Rubber (*cis*-1,4-polyisoprene) is produced in varying quantities and qualities by about 2,000 plant species. This isoprenoid polymer has no identified physiological function in plants, but it has many important industrial uses due to its elasticity, flexibility, and resilience. The diminishing acreage of rubber plantation and life-threatening latex allergy to *Hevea* rubber, coupled with increasing demand, have prompted research interests in the study of rubber biosynthesis and development of an alternative rubber source. Although natural is present in many species but only that from the tree *Hevea brasiliensis* is used commercially. This is due to its combination of high quality, high yield, and ease of harvest (Allen and Jones, 1988)

2. *Hevea brasiliensis*: the rubber tree

The *Hevea* rubber tree is a tropical tree native to the Amazon Basin in Brazil and adjoining countries. However that area has no longer play any significant part in the world natural rubber tread. *Hevea* was taken from the Amazon to South Asia (Sri-Lanka) and South East Asia (Singapore and Malaysia) by British Colonial Office where it was grown experimentally and later on plantation. Cultivation spread to Vietnam and Cambodia, Indonesia and Thailand, and subsequently to Africa. Nowadays, the most world wild natural rubber is provided by some South East Asia countries (Thailand, Indonesia, Malaysia and Sri-Lanka). Among these countries, Thailand is the first producer and exporter. A majority of rubber plantations are located in southern part of the country due to the suitable climatic factors. *Hevea* rubber grows best at temperature of 20-28 ^{o}C with well-distributed annual rainfall of 1,800-2,000 mm. The tree is the tallest species of the genus, in the wild these may grow to over 40 meters and live of over 100 years. But in plantations, they rarely exceed 20

meters because the growth is reduced by tapping. Commercially, they are usually replanted after 23-25 years when yields fall to an uneconomic level.

The rubber tree, *Hevea brasiliensis*, synonym Siphonia brasiliensis is a member of the Euphorbiaceae family. There are many common names associated with this plant. Some of the names are: Rubber tree, Jebe, Para rubber, Arbre de Para, Parakautschukbaum, Cauchotero de Pará, Árbol del Caucho, Seringueira, seringueira-branca, siringa, etc. The classification chart of the *Hevea* rubber tree is shown below.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euhobiaceae
Genus	Hevea
Species	Brasiliensis

3. Latex of *Hevea Brasiliensis*

The *Hevea* latex is accumulated in specialized cells or vessels known as laticifers. In *Hevea*, the rubber is formed and sorted in the rings of laticifers in the bark. Anatomoses between adjacent vessels in the rings allow the latex from a large area of the cortex to drain upon tapping. The opening of the latex vessels from tapping cuts cause the latex to flow out due to the high turgor pressure inside. The latex flow will continue for a certain length of time and subsequently stop due to rubber coagulation and flocs formation leading to plugging of the vessels ends. The latex is specialized cytoplasm containing several different organelles in addition to rubber particles. Organelles include nucleus, mitochondria,

fragments of endoplasmic reticulum and ribosome. In addition to these minor components, there are two major specialized particles which are unique characteristic of *Hevea* latex, namely the lutoid and Frey-Wyssling particles.

The *Hevea* latex collected by regular tapping consists of the cytoplasm expelled from latex vessels and is similar to the latex in situ. The cytoplasmic nature of tapped latex was firmly established by electron microscope studies (Dickenson, 1969). Latex is the cytoplasm of an anatomosed cell system which is specialized in the synthesis of *cis*-polyisoprenes. The latex usually contains 25 to 50% dry matter, 90% of which is made of rubber. Tapping severes a number of latex vessels rings and the latex which flows out comprises the contents of vessels at different stage of development. All the organelles in the latex vessels can be found in the tapped latex. The major particles most common in latex are rubber particles, the lutoids and Frey-Wyssling particles which are less numerous than the other two. The composition of latex is about 30-40% rubber, 10-20% lutoids and 2-3% other substances. The structure and composition of fresh latex has been elucidated by high-speed centrifugation (Moir, 1959). Generally, the latex can be fractionated into 3 distinct zones. The top fraction consists almost entirely of rubber, the middle fraction is the metabolic active aqueous phase of latex called C serum, and the relatively heavy bottom fraction consists mainly of lutoids. The yellow, lipid containing Frey-Wyssling complexes are normally found at the upper border of the bottom fraction (Figure 2).

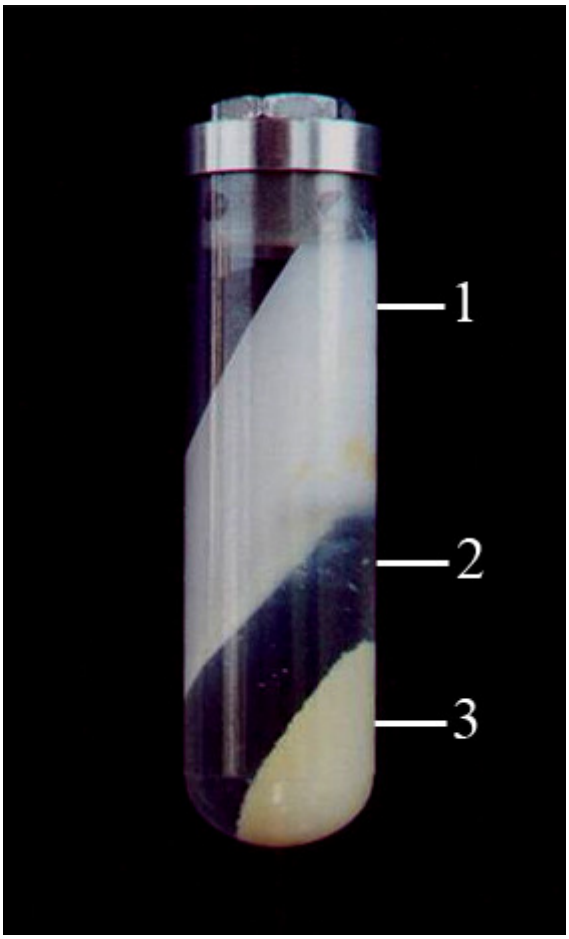


Figure 2 Fractionation of fresh *Hevea* rubber latex by ultracentrifugation into three major zones. (1: rubber layer, 2: C-serum, 3: bottom fraction)

4. Rubber latex composition

The fresh latex can be separated into three major zones by ultracentrifugation as shown in Figure 2. The top rubber fraction contains, in addition to the rubber hydrocarbon, the proteins and lipids associated with the rubber particles. The serum phase contains most of the soluble substances normally found in the cytosol of plant cells. The bottom fraction can be studied by repeated freezing and thawing of the lutoids. In this manner the membranes of the lutoids are ruptured and their liquid content, B serum, can be analyzed. B serum has been found to contain proteins and other nitrogen compounds as well as metal ions. It can be visualized that the latex is a cytoplasmic system consisting of particles of rubber hydrocarbon dispersed in an aqueous serum phase. There are also numerous nonrubber particles called lutoids. The rubber particles are made up of rubber hydrocarbon surrounded by a protective membrane layer consisting of proteins and lipids. Besides from the rubber hydrocarbon which is the major component of the latex, various other components (protein, lipids, carbohydrates and inorganic substances) are also present which play important roles in the latex metabolism, include rubber biosynthesis.

5. Nonrubber constituents of *Hevea* latex

The latex contains numerous nonrubber constituents besides the rubber as were recently reviewed (Subramaniam, 1995). The nonrubber constituents are present and being distributed in all the three latex fractions. Proteins and lipids are found associated with the rubber particles. C serum contains substances normally found in the cytosol (carbohydrates, proteins, amino acids, inositols, enzymes and intermediates of various biochemical processes, including rubber biosynthesis). Lutoids contain specific substances unique to its functions.

5.1 Proteins

Apart from rubber hydrocarbon and water, proteins and carbohydrates are present in highest proportion in latex. The proteins content in latex shows clonal variations

and can range from 1% to more than 1.8% in different samples of latex. About 25-30% of the proteins are found in the rubber phase and 45-50% in the serum phase, and about 25% in the bottom fraction in a typical latex sample. The amount of proteins in the rubber phase is less variable than the total amount of proteins in different samples of latex. The serum proteins consist of around 19 anionic and 5 cationic proteins (Tata and Moir, 1964). The major protein is α-globulin with an isoelectric point (pI) of 4.8. There are 7 anionic and 6 cationic proteins in the bottom fraction. The major proteins are hevein (>50%) of pI 4 and hevamine (≈30%) of pI 9. The amino acid sequences of hevein (Walujono *et al*., 1975) and rubber elongation factor (REF), a 14 kDa protein in the rubber phase (Dennis and Light, 1989), have been established. Recent report showed more than 200 different proteins are present in the latex (Alenius *et al*., 1994), suggesting that the proteins composition in the latex is very complex.

5.2 Carbohydrates

Sucrose supply and utilization for latex production plays a very important role in the metabolism of latex and has been reviewed (Tupy, 1989). Of important note and quite unique to the latex is the presence of quebrachitol, an inositol derivative. Quebrachitol (1-methyl inositol) is the most abundant and makes up about 75% to 95% of the total carbohydrates present in latex. It is found mainly in the serum phase. The large amount and ubiquitous presence of this compound is unique characteristic of the *Hevea* latex. The reason for its accumulation and its physiological function in latex is not known but has been postulated to serve active role in rubber biosynthesis (Bealing, 1975). About 6 to 7 other carbohydrates components are found in small amounts. These are mostly common sugars for various metabolic processes in the latex.

5.3 Lipids

Lipids in the latex play important role in the stability of rubber particles. They not only are found associated with the rubber particles, but are also found throughout the latex fractions. The lipids content of latex also shows clonal variations (Ho *et al*., 1976). The neutral lipids constitute more than 59% of the total lipids. The other components are mainly phospholipids and glycolipids. The presence of proteolipids has also been reported.

The lipids are mainly in the rubber particles and the bottom fraction. Several neutral lipids found are triglycerides (also mono- and diglycerides), free fatty acids and esters, sterols, and lipid soluble vitamins (carotenoids and tocotrienols). Phosphatidyl choline is the major phospholipid while phosphatidyl ethanolamine and phosphatidyl inositol are found in a smaller amounts. Phosphatidic acids are also reported as the important components of the lutoid membrane (Dupont *et al*., 1976). Fatty acids in the latex occur mostly in the esterified form. The major fatty acids are C-16, C-18, and C-20 (palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, and arachidic acids). Also present is a rare furanoic acids (10,13-epoxy-11-methyloctadeca-10,12-dienoic acid) which is found mainly in the triglycerides fraction. The rubber latex is only the second known plant source of this furanoic acid (Hasma and Subramaniam, 1978). It is a bit unusual to find this fatty acid in the latex and its physiological function is not yet known. This is somewhat like the presence of quebrachitol in large quantity of which functions have yet to be found for their presence.

5.4 Inorganic substances

In addition to the above major nonrubber constituents, several inorganic components are also present. Of these, potassium is the most abundant element in the latex and its concentration is of the order of a few thousand part per million (ppm). The next most common element with a few hundred ppm is magnesium which is mainly contained in the lutoids. The effect of magnesium was found to decrease mechanical stability of the latex (Philpott and Westgarth, 1953). These two elements also show clonal variations. Others occurring in much smaller concentrations are the common cellular elements (sodium, calcium, iron, copper, manganese and zinc). Also present is rubidium, but the function of this element in latex is not yet known.

6. Rubber particles

Rubber hydrocarbon is the major component of *Hevea* latex. The rubber content may vary from 25 to 45% as dry content of latex. The number average molecular weight ranges from 200 to 600 kDa. The rubber molecules are found as particles in the latex.

The particles consist mainly of rubber (90%) associated with lipophilic molecules, mainly lipids and proteins, forming the film enclosing rubber particles (Ho *et al*., 1976). This film carries negative charges and is responsible for the stability of rubber particles suspended in aqueous serum. The particles size ranges from 5 nm to 3 μm with spherical shape. They also show plasticity as having polygonal shape in mature laticifers where the particles are numerous. The size distribution as determined by the electron microscope showed maximum distribution of 0.1 μm particles (Gomez and Moir, 1979). The 0.1 μm particles may contain several hundred of rubber molecules. Molecular weight analyses using gel permeation chromatography showed the bimodal distribution of rubber for low and high molecular weights, with the average values of 100-200 kDa and 1,000-2,500 kDa, respectively (Subramaniam, 1976). The other main component of rubber particles is the enclosing membrane consisting of lipids, proteins, and enzymes. These components contribute colloidal charge to the rubber particle and their stability in the latex.

The rubber particles are commonly found associated with lipids which are thought to be of membrane nature. The particles appeared to have a uniform structure of the rubber molecules enclosing by a thin film seen under the microscope (Sountorn, 1961). When examined under electron microscope, the rubber particles have homogeneous and the uniform internal structure but are surrounded by a film that is more opaque than the polyisoprene inside (Lau *et al*., 1986) Analyses of the nature of the film enclosing the rubber particles show the presence of phospholipids and proteins together with natural lipids similar to the membrane structure. Analyses of the rubber particle purified by ultracentrifugation revealed that it contained up to 3.2% lipids of which about 2.1% are neutral lipids express as rubber weight (Ho *et al*., 1976). Separation of natural lipids showed that it was composed of at least 14 different substances. Triglycerides are the most abundant for almost 45% of neutral lipids. Next to triglycerides are sterols, sterol esters and fatty acid eaters which constitute about 40%. Other neutral lipids present in trace amount are diglycerides, monoglyceride and free fatty acids. Tocotrienols and some phenolic substances are also found associated with rubber particles (Ho *et al*., 1976). Phospholipids are important components of the rubber particles. Analyses of the phospholipids always consistently show the three spots on TLC separation. It was found to be a considerable amount of phosphatidyl choline and smaller amount of

phosphatidyl ethanolamine and phophatidyl glycerol. In addition, the presences of spingolipids and glycolipids have also been reported. The stability of rubber particles suspension in latex is dependent on the negative charges film of protein and phospholipids (Philpott and Weagarth, 1953). The pI of these proteins found as indigenous component of the film enclosing rubber particle ranges from 3.0-5.0 which is characteristic value of the surface proteins. Particles in electric field will move toward the anode, indicating they have net negative charge on the surface (Verhaar, 1959). These proteins can be considered as intrinsic or peripheral depending on their binding and affinity with rubber particles.

Proteins are found as indigenous component of the film enclosing rubber particle. Together with lipids, these proteins form the membrane of particles which contribute to their stability. The pI of these proteins ranges from 3.0 to 5.0 which is characteristic value of the surface proteins. Particles in electric field will move toward the anode, indicating they have net negative charge on the surface (Verhaar, 1959). Anionic soaps do not affect the particles colloidal stability but cationic soaps cause the flocculation, probably due to neutralization of the surface charge. These proteins can be considered as intrinsic or peripheral depending on their binding and affinity with rubber particles. One of the most plentiful proteins in latex is α-globulin with pI of 4.5. It was found both in cytosol and adsorbed on particles surface and might contribute to their colloidal stability in latex (Archer *et al*., 1963). Proteins group of hydrophobic nature was also found in rubber particles. Proteolipids have been isolated and characterized (Hasma, 1987). This protein was suggested to be the component of polar lipid backbone which is part of the membrane of rubber particles. The proteins content of rubber particles has been recently refined for a more accurate quantitative analysis (Yeang *et al*., 1996) in light of the concern over rubber protein allergens. Other proteins with enzyme activity have been described. One of the interesting and well characterized enzymes is rubber transferase on the washed rubber particle surface (Audley and Archer, 1988). This enzyme involves in synthesis and formation of rubber molecules on the particles surface. It was found to be distributed between cytosol and the rubber particle. It has been isolated from latex C serum but was active only when absorbed onto particle for chain elongation process of the rubber molecules (Light and Dennis. 1989). Another important protein activity involved in rubber biosynthesis was a 14

kDa protein referred to as rubber elongation factor (REF). Moreover, molecular cloning of a protein tightly bound on the small rubber particles has been reported (Oh *et al*., 1999). The cloned cDNA encodes a 24 kDa protein which is tightly bound on rubber particles. This protein was suggested to be active in synthesis of rubber together with the REF. The 24 kDa has previously been reported as always found together with the 14 kDa proteins as the tightly bound proteins and remain with the particles even after extensive washing.

Washed rubber particles and the bound rubber synthesis enzymes have been studied and reviewed (Audley and Archer, 1988). The formation of rubber molecules, at least in elongation steps, occurs at the particles surface (Archer *et al*., 1982). Rubber transferase is the enzyme responsible for this process. The enzyme was also found in the C serum and probably being distributed between the two fractions (McMullen and McSweeney, 1966). It has been isolated from the C serum and purified for enzyme characterization (Archer and Cockbain, 1969). The enzyme was without activity in the absence of the washed rubber particles. It remains inactive so long as the enzyme has not been adsorbed onto the particles, even when the particles have been purified by gel filtration and repeated washings. The reaction catalyzed by this enzyme appears to be essentially the chain extension or elongation of the preexisting rubber molecules even though the formation of new rubber molecules has been suggested (Lynen, 1969). However, the formation of new rubber particles is still not known at present.

So far nobody has been able to demonstrate the polymerization of IDP *in vitro* except on the preexisting rubber particles. The reaction as occurring in vivo must be at other sites as a prelude to formation of the new rubber molecules. Some findings strongly suggest that membrane phospholipids might have a key role in enabling the combined functions of different transferase enzymes to operate in vivo for new rubber formation (Keenan and Allen, 1974; Baba and Allen, 1980). It appears that some phospholipids, membranes, or other amphipathic micelles are essential for the initiation and formation of new rubber molecules. The rubber particles surface was also suggested as the site of IDP isomerase (Lynen, 1969), the enzyme catalyzing conversion of IDP into DMADP. The IDP isomerase is essential for the formation of DMADP and the chain initiation of new rubber

molecules. It has long been suggested to be present in latex but only the indirect evidence was given for its detection. More recently, the direct detection and characterization of this enzyme was reported (Koyama *et al*., 1996). The isomerase was located in the cytosol or C serum of latex, not with the rubber particles as previously suspected. The enzyme was activated in the presence of reducing agents and detergents (Koyama *et al*., 1996). The findings might provide a partial support for the initiation of rubber chain probably at the site other than the rubber particles.

7. C-serum of *Hevea* latex

C serum fraction of centrifuged latex is the aqueous phase of laticiferous cytoplasm which can be considered as latex cytosol. This cytosol is not fundamentally different from normal cytosol of plant cells. Analyses of the latex cytosol fraction obtained by ultracentrifugation show that it contains various different organelles and particles. All glycolytic enzymes (d'Auzac and Jacob, 1969) and other common cytosolic enzymes including isoprenoid pathway (Suvachittanont and Wititsuwannakul, 1995; Koyama *et al*., 1996) have been detected, indicating that it is active in various metabolic processes.

The involvement of C serum in rubber biosynthesis was noted (Tangpakdee *et al.,* 1997a). The importance of calcium binding protein (calmodulin) in contolling many different metabolic processes was investigated. It was found to activate HMG CoA reductase in the bottom fraction. Purification and characterization of calmodulin from C serum (Wititsuwannakul *et al*., 1990b) suggested that it had important role in the regulation of latex metabolism. Highly positive correlation between calmodulin level and latex yield was also of important note (Wititsuwannakul *et al*, 1990b). Composition of latex cytosol was reviewed (d'Auzac and Jacob, 1989).

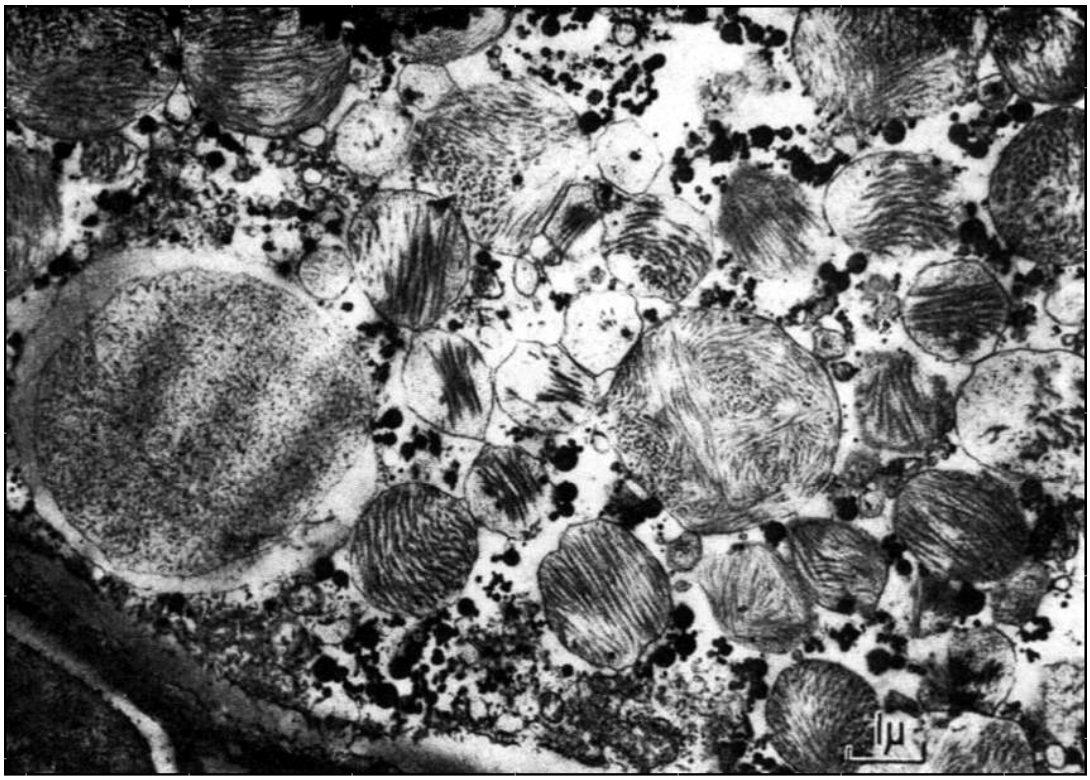
8. Lutoid particles

Lutoids are membrane bound particles sedimented in bottom fraction of centrifuged fresh latex (Moir, 1959). They can be considered as polydispersed lysosomal vacuoles and have been extensively discussed (d'Auzac and Jacob, 1989). The lutoids content of latex is quite considerable and found to play many important functions in the latex. They constitute about 20% by volume of fresh latex whereas the rubber phase forms approximately average of 30-40%. They are spherical in shape and larger in diameter than the rubber particles. Lutoids are enclosed by single layer membrane and the lipids composition has been determined. They play an important role in colloidal stability of latex due to negatively charged membrane which is very rich in the phosphatidic acids content (Dupont *et al*., 1976). They are considered as similar to the lysosomes with the high content of acid hydrolases (Jacob *et al*., 1976). Lutoids are found to play a major role in coagulation of rubber particles in colloidal suspension of latex (Southorn and Edwin, 1968). The mechanism of coagulant effect is due to the release of cations and proteins from ruptured lutoids (Pakianathan and Milford, 1973). The storage function of lutoids was shown in the accumulation of several proteins, enzymes and solutes with active membrane transport activity (Chrestin and Gidrol, 1986). Lutoids are important latex component, both as regard to volume and the functions of their chemicals and enzymes (d'Auzac and Jacob, 1989). Lutoids composition can be considered as two distinct components, lutoids membrane and the internal contents called B serum.

Electron microscopic study showed the micellar nature of the lutoids membrane structure (Gomez and Southorn, 1969). It is very osmosensitive single layer membrane of 8-10 nm thickness. Chemical composition showed the phospholipids content forming 37.5% of the weight of proteins. It has been found to be very rich in phosphatidic acids which accounted for 82% of the total phospholipids fraction. Fatty acids composition analyzed by methanolysis of phospholipids shows predominance of saturated fatty acids as palmitic and stearic acids. The unsaturated ones are oleic and linoleic acid (Dupont *et al*., 1976). There are practically equivalent quantities of saturated and unsaturated fatty acids in the membrane. The exceptionally high phosphatidic acids content may explain the negative

surface charge of lutoid (Southorn and Yip, 1968). The relative abundance of saturated fatty acids in lutoids stand out clearly from the membranes of other plant organelles. The relative rigidity and fragility of lutoids membrane to osmotic shocks and low resistance to mechanical stress (Pakianathan *et al*., 1966) can partly be explained by its fatty acids composition. The analysis of rubber particles membrane carried out under the same condition shows that it is totally free of phosphatidic acids but contains mainly phosphatidyl choline and phosphatidyl ethanolamine (Ho *et al*., 1976). The other major lutoids membrane components are the proteins, several of which are enzymes important in the lutoids function. The membrane of lutoids thus plays essential and very complex role in latex as has been shown in numerous reports and reviews (Paardekooper, 1989; d'Auzac and Jacob, 1989). Several proteins are present in lutoid membrane and many of which are active enzymes. One of the well characterized membrane enzymes is ATPase (Moreau *et al*., 1975). The operation of the lutoid membrane ATPase as an electrogenis proton pump has been demonstrated (Chrestin and Gidrol., 1986) and extensive reviewed (Chrestin *et al*., 1989). Other membrane enzyme included NADH-Cytochrome C reductase that function in an outward proton pumping redox system which tends to reduce the concentration of protons in lutoids and hence acidify the cytosol (Moreau *et al*., 1975). NADH-quinone reductase has also been described as responsible for production of superoxide ions.

Recently, the enzyme HMG CoA reductase has been purified from lutoids membrane by solubilization with mild detergent (Wititsuwannakul *et al*., 1990a). The native enzyme was tetrameric of 44 kDa subunits similar to the finding in other plants specimen as membrane bound enzyme (Bach, 1986). It was found to be activated by reducing agent for maximum activity. Regulation of this enzyme by calmodulin in C serum was also studied in detail (Wititsuwannakul *et al*., 1990b). The findings suggest the important interactions of C serum contents for optimum lutoids membrane activities. Rubber formation by the bottom fraction was recently noted (Tangpakdee *et al*., 1997a; 1997b). It might thus demonstrate the participation of lutoids in rubber biosynthesis process.



(Dickenson, 1969)

Figure 3 Lutoid particles of the fresh *Hevea* latex (EM). The picture shows the spherical shape lutoids which have larger diameter than the rubber particles, with full development of the enclosed proteinaceous microfibrils. The unit membrane surrounding the particles is contorted because of slight plasmolysis as the lutoid are osmosensitive.

9. Frey-Wyssling particles

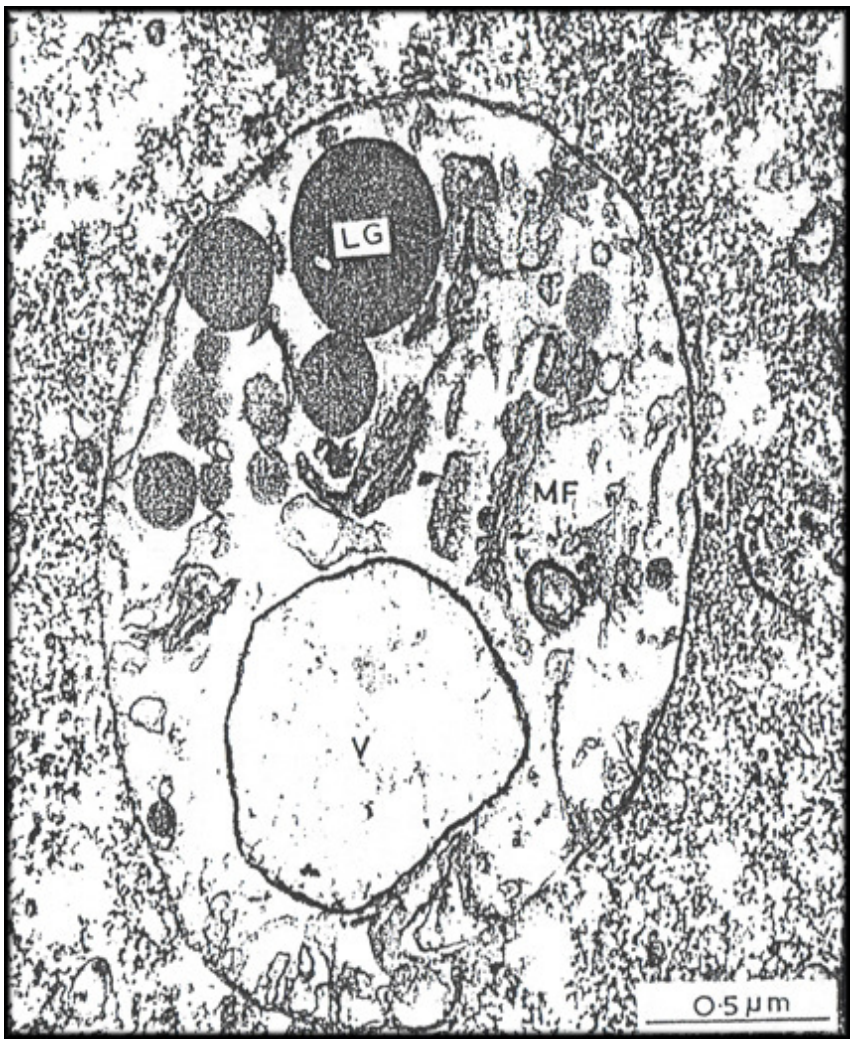
Frey-Wyssling (FW) particles represent a minor component of latex as compared to lutoids. These particles were discovered in latex as yellow-orange globules which contained carotenoid pigments (Frey-Wyssling, 1929). The name of organelle "Lutoids" describe above (=yellow) turned out to be a misnomer, because as originally isolated they were contaminated with the yellow FW particles.

The FW particles were originally noted to occur in clusters. There are often appeared to be grouped in two or three globules. These data were subsequently confirmed by phase contrast microscopy (Southorn, 1961). It was shown that small numbers of these globules were enclosed in larger structure which is complex in character (Dickenson, 1965). The particles were named after Frey-Wyssling. FW particles were studied in particular by Dickenson (1965; 1969). FW particles are regularly present in sections of trunk laticifers and abundant in latex. These particles are spherical organelles 3 to 6 μm in diameter bounded by a double membrane and containing one or more voluminous osmiophilic globules which consist of lipids and carotenoids, together with complex membrane systems (Figure 4). He observed a ramifying system of tubules, each of which is approximately 75 nm in diameter, with fibrils arranged in spiral on their internal surface; the whole was enclosed in a matrix bounded by unit membrane. He noted a series of two, three, or four concentric lamellae formed from the double unit membrane of the particle that associated with the system of tubules. He also described a structure made up of a minutely and elaborately folded invagination of the inner membrane of the organelle envelope, and finally, nearby, a group of particles enclosed in a membrane.

The highly complicated structure of FW complex suggests that it has an important function in the metabolism of *Hevea* latex. Since the particles contain plastoquinone and plastochromanol, in which is β-carotene synthesized, they are assumed to be modified plastids (Ohya and Koyama, 2001). Recently, the prenylquinones in the lipids extracted from FW particles were identified and compared to those of leaf chloroplast and mitochondria. Their chemical structures were confirmed as plastoquinone-9 (PQ-9). Moreover, the *Hevea* solanesyl diphosphate synthase (HbSDS) gene, solanesyl diphosphate

synthase involved in the synthesis of the C_{45} prenyl side chain of the PQ-9, was isolated and characterized from the rubber latex (Phatthiya *et al*., 2007). These findings indicated a possible involvement of FW particles in carotenoid biosynthesis and suggested that FW particles has an important function in the laticiferous vessels were rubber biosynthesis is highly active.

Threadlike structures have also been observed throughout centrifuged rubber latex (Figure 1). It has been suggested that these may be the remnants of an extensive reticulum of hollow threadlike processes which originally existed within the latex vessels, to which the various particulate phase of the latex were initially connected. Possibly such a reticulum could be the site for the biosynthesis of natural rubber hydrocarbon (Southorn, 1961)



(Gomez and Hamzah, 1989)

Figure 4 Frey-Wyssling particle showing few lipid globules (LG), vesicles (V) and membrane fragments. Magnification 42,000x.

10. *Hevea* latex metabolism

Hevea latex is a very unique system as consisting of specialized cytoplasm (Dickenson, 1965; Gomez and Moir, 1979). Organization and composition of this cytoplasm reflect biological functions of *Hevea* specialized laticiferous tissue. It has long been shown that fresh latex can synthesize rubber in vitro from labeled precursors acetate or mevalonate (Kekwick *et al*., 1959; Park and Bonner, 1958). The enzymes activity and their location with substrates and effectors make it possible to study metabolism and the biochemical function of latex as "rubber factory". Latex as obtained by tapping is not so destructive compared to the general method for preparing cell cytoplasm, making it very suitable to study latex metabolic functions. Latex metabolism has been studied in various different aspects, especially the rubber biosynthesis and other related metabolic processes (Jacob *et al*., 1989). Tapping of rubber trees for latex makes it necessary for laticiferous tissue to make up for the lost materials between successive tappings. Regeneration of cell materials is increase along with latex production. If it is not sufficiently effective, it can be limiting factor for latex production (Jacob *et al*., 1986). It requires intense metabolic activity and numerous enzymes to be involved in at least four important processes for the latex regeneration: (1) the catabolism which provide energy and reducing capacity for the anabolic processes; (2) the activity of anabolic pathways for various syntheses including isoprenoids; (3) the mechanisms associated with regulatory systems and homeostasis; and (4) the supply of nutrients to the zones or subcellular components in which cell materials are regenerated. The specificity of laticiferous tissue is so organized that its particular major function is directed to the production of rubber in the latex.

The formation of rubber in *Hevea* laticifers seems to be a very complex control system. Several questions regarding the regulation of rubber biosynthesis have been raised and investigated. The continuing requirement of carbon, NADPH, and the need of ATP for rubber biosynthesis must place a high degree of demands on the metabolic economy of the tissues. It has been calculated that the required rate of regeneration of rubber in alternate daily tapping is of the order of 1 μmole isoprene unit per ml latex per minute

(Bealing, 1975). The capacity of latex to incorporate acetate and mevalonate has been found to fluctuate markedly with the season (Bealing, 1975). The control mechanism in the formation of rubber appears to be a complex and intricate process. The interesting feature of *Hevea* metabolism is not only that for rubber formation but also that it can be stimulated to produce rubber and other many components of latex by repeated tapping. This replenishment is not called for in the untapped tree and although a few terpenoids have been shown to suffer catabolism in plants, there is no evidence for the breakdown of rubber in vivo. The enhance rubber yield by ethylene, a hormone associated with response to wounding in plants, has been extensively studied and reviewed (d'Auzac, 1989a; 1989b). Specific genes activation may shed some light on the mode of action of the tapping stimulus and ethylene activation. This will be the aspect to be described.

11. Rubber and isoprenoids biosynthesis in *Hevea brasiliensis*

Hevea rubber biosynthesis and its control has been often reviewed (Archer and Audly, 1987; Kekwick, 1989; Kush, 1994; Tanaka *et al*., 1996) and so it is only briefly described here as to the function of specialized laticifers, together with a discussion on the key regulatory enzymes. An interest aspect of *Hevea* laticifers is the fine-tuning in term of compartmentalization o function or the division of labor for rubber biosynthesis pathway. It was shown that the laticifers have a differential gene expression profile (Kush *et al*., 1990; Kush, 1994). The genes involved in rubber synthesis are highly expressed in latex as compared to those in the leaves. The specialized differential expression serves a two-fold function. First, that the desired enzymes for rubber synthesis are expressed in the very tissues where formation is taking place. Localization the rubber synthesis activity in laticifers allows other, different, metabolic process in other tissues to operate at their optimum and to be well-balanced with the whole of the plant's functions. Second, specialized functions and well-coordinated divisions of labor thus appear to be well organized for specific channeling of precursor and metabolites for different metabolic pathway. The tissue and cell differentiations destined to perform certain functions to best fit the metabolic distribution can thus clearly be

seen in *Hevea brasiliensis*. This is some different for other rubber-producing plants such as guayule.

Hevea brasiliensis is unlike in some plants like guayule (*Parthenium argentatum*) where rubber synthesis is taking place in cytosol of the parenchyma tissues along with the rest of other metabolic processes needed for orderly parenchyma cell functions. Extraction of rubber from guayule plants is difficult because of the relatively low abundance of rubber particles in the cell and limited by the cell volume (Backhaus and Walsh, 1983). Procedure of obtaining rubber from guayule is very destructive because of subcellular localization of rubber. Guayule rubber can only be obtained by crushing of the stems and extracted together with all other cellular materials and impurities of all cell types. This is one of the prime reasons that the *Hevea* rubber tree is the only commercially viable source of natural rubber in the world. Even though about two thousand species of plants producing rubber of varying types and amounts are known (Backhaus, 1985; Mahlberg, 1993), none is used or found comparable to *Hevea* rubber superior quality. This is in contrast to the *Hevea* laticifers containing high content of latex and gushed out after opening the latex vessels by small excision of the bark or tapping which is not so destructive to the plant tissues. The flow of latex is due to the high turgor pressure inside the laticifers compared to the outside. After some time of the latex flow out of the cut open vessels, the flow will stop due to coagulation of rubber particles forming the latex plugs at the vessel ends or the wounding site resulted from tapping. A lectin- like small protein, hevein, which is localized in lutoids (Gidrol *et al*., 1994) has been shown to play an important role in latex vessels plugging. Lutoids have been shown to have very fragile membranes which burst in response to tapping because of difference in the turgor pressure as earlier discussed on the lutoid and its properties. The tapped latex contains vast number of intact organelles which makes it an excellent specimen for the study of differential and specialized metabolic functions in plants. As *Hevea* laticifers are anastomosed system, the latex in essence represents the cytoplasm of a single cell type. The rubber yields are the results of two contributing factors, the latex flow properties and regenerating synthetic capacity.

12. Isoprenoids biosynthesis

Besides the rubber, *Hevea* laticifers also synthesize several diverse isoprenoids (Kush, 1994). Different plants have capacity in synthesizing certain isoprenoid compounds for specific functions, from simple isoprenoids to the more complicated ones like natural rubber. They produce this wide range of isoprenoids in different amounts in specific organelles at different stages of growth and development. Since the diverse isoprenoid compounds are produced by a more or less conserved biosynthetic pathway (Randall *et al*., 1993), plants must execute control mechanism to ensure the synthesis of the necessary isoprenoids in the right place at the right time. Such control is very likely mediated through some of regulatory enzymes, attempts have been made to understand the regulatory mechanism of the isoprenoids and polyisoprenoids biosynthesis as well as their interrelationship (Kekwick, 1989; Mahlberg, 1993). The pathway used for the formation of isoprenoids in plants is similar to the sterol biosynthetic pathway that was worked out in animals and yeast (Clausen *et al*., 1974; Taylor and Parks, 1978).

Recently, the oligoprenoid and polyprenoid in *Hevea* latex were examined (Koyama *et al*., 1996; Tangpakdee *et al*., 1997a). The chain length of B serum isoprenoids showed several components of C_{15}- C_{60} which are more or less of equal proportion. However, the C serum isoprenoids was quite different. The major chain length in the C serum isoprenoids was the C_{20}-GGDP as analyzed by autoradiogram. Only a few isoprenoids were detected in the C serum as compared to several in the B serum. The C_{15}-FDP in the C serum was much less than the C_{20}. The differences between the two are presently still unclear. It can be assumed that the component in B serum may be the intermediates of the rubber formation as it was recently reported the rubber formation in the fresh bottom fraction of centrifuged latex (Tangpakdee *et al*., 1997b). The major C serum of C_{20}-GGDP and to a lesser extent C_{15}-FDP might be the substrates for the prenylation of proteins as the presence of prenylated proteins has been increasingly reported to occur in the plant cells. However, the exact role of thes isoprenoids in the two latex sera is still needed to be elucidated. More recently, the polyprenoids of the dolichols group and other group in

Hevea latex were analyzed by two dimention TLC (Tateyama *et al*., 1999). It was found that the chain length of dolichols in *Hevea* ranges from C_{65}-C_{105}. The analysis on dolichols of the *Hevea* seeds, root, shoots, and leaves of different ages were also carried out. Comparisons on the differences were examined to understand the changes associated with growth and development. The function of dolichols is commonly known to associate with glycosylation process and it is assumed that the presence of dolichols in Hevea is no exception. The role of glycoproteins has received much attention in light of the reports on the presence of lectin in the *Hevea* latex (Wititsuwannakul *et al*., 1997a; 1997b) which were found to have important role in latex metabolism and colloidal stability.

The enzymes HMG CoA synthase (HMGS) and HMG CoA reductase (HMGR) have been implicated as the essential regulatory enzymes in the biosynthesis of IDP (Brown and Goldstein, 1980; Bach, 1986; Goldstein and Brown, 1990). Similar roles have been implicated in plants as well, although conclusive evidence for the regulatory role of HMGS is not yet available. Downstream of IDP in formation of specific isoprenoids varies, depending on the end products and the subcellular compartmentation. The role of HMGR in regulation of isoprenoids and rubber biosynthesis in *Hevea brasiliensis* has been well documented (Wititsuwannakul, 1986). Diurnal variations of HMGR levels and dry rubber contents of latex were conclusively shown with high corresponding and positive correlations (Wititsuwannakul, 1986), the enzyme was located as membrane bound and purified from the membrane of lutoids (Wititsuwannakul *et al*., 1990a). The purified enzyme was analyzed and characterized which was found to be similar to the HMGR from other plant specimens. *Hevea* HMGR was activated by calmodulin, the calcium binding protein involved in myriad regulatory processes, located in the latex C serum (Wititsuwannakul *et al*., 1990b). Positive correlation between the HMGR activity and calmodulin levels were demonstrated corresponding with the levels of dry rubber contents. Comparison between the high-yielding and low-yielding clones also showed the clonal variations of the calmodulin level in the same direction and correspondingly. In the rubber tree, this compartmentation is highly specialized for the syntheses of the rubber and isoprenoids as demonstrated by the regulatory mechanism of lutoid HMGR by C serum

calmodulin in the control of rubber biosynthesis (Wititsuwannakul, 1986; Wititsuwannakul et al., 1990a; 1990b) and the difference in isoprenoids distribution in B serum and C serum.

13. Rubber biosynthesis pathway

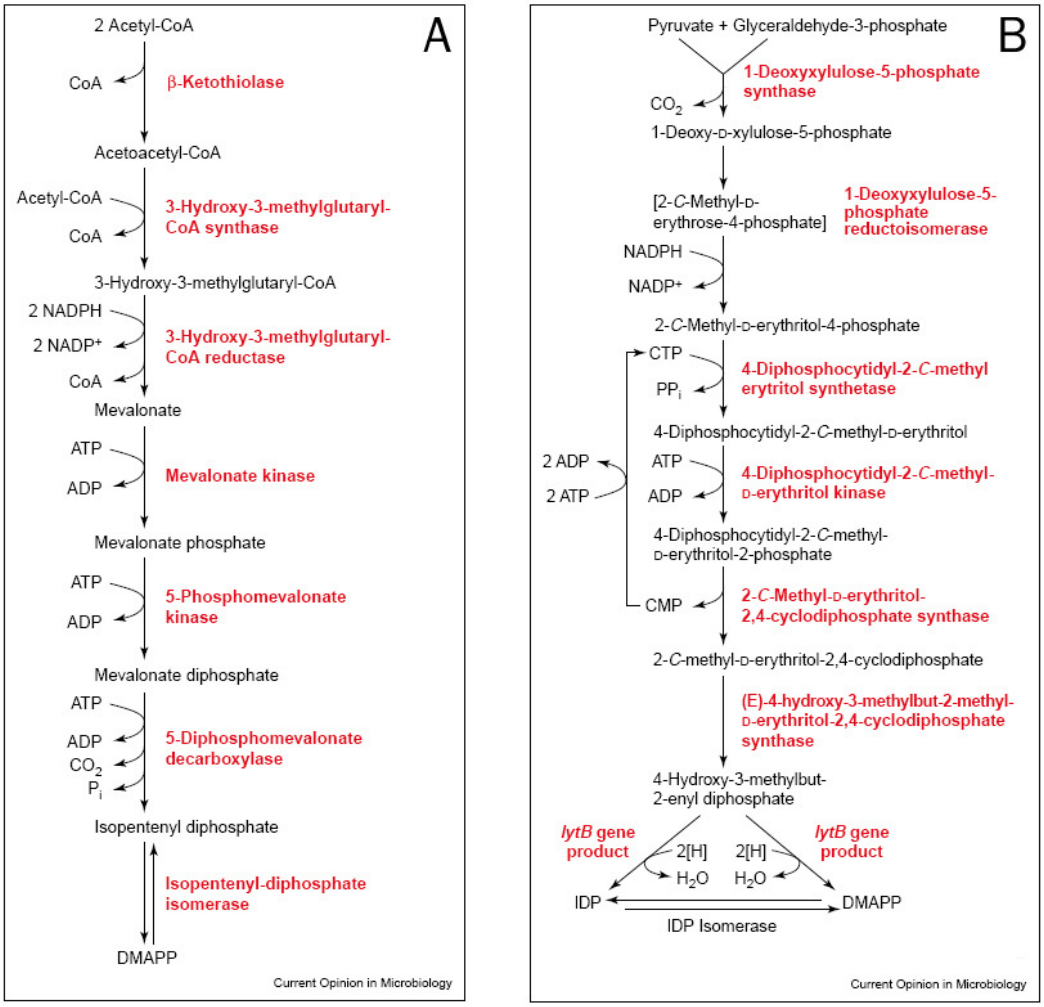
The pathway of rubber biosynthesis can be divided into two major sections: first, isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMAPP) are synthesized from central intermediates, second, theses two components are polymerized to a polyisoprene molecule of high molecular weight.

13.1 Formation of IDP and DMAPP

Since the initial discovery of the mevalonate (MVA) pathway in yeast and animals in the 1950s, it was widely accepted that IDP was only formed by condensation of acetyl- CoA through the ubiquitous MVA pathway in all organisms (Charpell, 1995; McGarvey and Croteau, 1995). In the MVA pathway (Figure 5A), the condensation of acetyl-CoA produces 3-hydroxy-3-methylglutaryl CoA (HMG-CoA), after which one of the carboxyl groups of HMG-CoA is reduced to a hydroxyl group to form MVA. Two sequential phosphorylations of MVA, followed by a phospharylation-assisted decarboxylation, yield IDP (Newman and Chapell, 1990). However, it has been disclosed recently that many organisms including several bacteria, green algae, and chloroplasts of higher plants use an alternative mevalonate-independent pathway for formation of IDP (Rohmer *et al*., 2001; Lichenthaler, 1999). The recently discovered 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway (Figure 5B) starts with a condensation of pyruvate and glyceraldehydes-3- phosphate (GAP) to form 1-deoxy-D-xylulose-5-phosphate (DXP), followed by rearrangement and reduction of DXP to MEP, formation of the diphodphocytidyl derivative , phosphorylation at C2, and cyclization to 2-C-methylerytriol-2,4-cyclodiphosphate (cMEPP), the reductive ring opening to 1-hydroxy-2-methyl-2-enyl diphosphate (HMEPP). IDP and DMAPP are produced as final products. Plants possess both pathways, the MEP pathway is expressed in the plastids and the MVA pathway is expressed in the cytoplasm. Some exchange of IDP or a common downstream intermediate does also appear to take place between the plastid and the

cytoplasm (Eisenreich *et al*., 1998; 2001; Lichtenthalar *et al*., 1997; Lichtenthalar, 1999; Rohmer, 1999).

The role of HMG CoA reductase (HMGR), catalyzes the first committed step of MVA pathway, in regulation of rubber biosynthesis in *Hevea brasiliensis* has been well documented (Wititsuwannakul, 1986). Recently, Ko *et al.* (2003) have reported the expression of a gene encoding DXP synthase in the *Hevea* latex, thus the MEP pathway is existes in the latex. The high level of latex *dxs* gene expression reported indicates a possible involvement of the MEP pathway in supplying the isoprene precursor for the synthesis of rubber molecule. IDP generate by MEP pathway in FW particles , *Hevea* putative plastids, may be transport out into the cytosol as a substrate for further IDP condensation (Chow *et al*., 2007).



(Steinbüchel, 2003)

Figure 5 The mevalonate (A) and methylerythriol phosphate (B) pathway of IDP and DMAPP biosynthesis and enzyme catalyzing the individual steps**.**

13.2 Rubber molecule formation

Natural rubber is made almost entirely of isoprene units derived from IDP. The polymerization of the rubber catalyzed by rubber transferase, requires divalent cations such as Mg^{2+} or Mn^{2+} for activity, but rubber transferase, IDP and Mg^{2+} together is not sufficient conditions for rubber biosynthesis (Archer and Audley, 1987; Madavan *et al*., 1989; Cornish and Backhaus, 1990). An allylic diphosphate is required as substrate to initiate the polymerization process. Rubber formation requires three distinct biochemical processes: (1) Initiation, i.e., synthesis of allylic diphosphate molecule catalyzed by transferase enzymes. (2) Chain elongation, i.e., cis-1,4-polymerization of isoprene-units from IDP catalyzed by rubber transferase. (3) Termination i.e., the release of resulting polymer from rubber transferase (Cornish, 1993)

The steps before polymerization probably involve the isomerization of IDP to DMAPP, which is a five-carbon compound acting as the initiator of condensation reaction. This is analogous with the biosynthesis mechanism of acrylic terpenes such as geraniol, farnisol and geranylgeraniol. IDP isomerase [EC 5.3.3.2], which catalyzed the reversible isomerization of IDP to DMAPP has been presume to be present in *Hevea* latex for the initiation of rubber biosynthesis (Audley and Archer, 1988). More recently, the direct detection and characterization of this enzyme was reported (Koyama *et al*., 1996). The isomerase was located in the cytosol or C serum.

In the biosynthesis of isoprenoid compounds, the compounds are derived from linear prenyl diphosphate synthesized by sequential condensation of IDP with allylic prenyl diphosphates. Theses condensations are catalyzed by a family of prenyltransferases, which can be classified into four groups (Koyama, 1999) according to the mode of requirement for enzymatic activity as listed in Table 1. Short-chain *trans*-prenyl diphosphate synthases such as FDP and GGDP synthases require no cofactor except divalent metal ions such as Mg^{2+} or Mn^{2+}, which are commonly required by all prenyltransferases. The products, short-chain transprenyl diphosphate, are used as precursors for other three types of prenyltransferases. The enzymes that catalyze the formation of *cis*-polyprenyl chains require phospholipids or detergent. The *trans*-prenyl transferase enzymes which catalyzed the synthesis of the allylic prenyl diphosphates were found in both the bottom fraction and the

cytosol C-serum of centrifuged fresh *Hevea* latex (Wititsuwannakul and Wititsuwannakul, 2001; Tangpakdee *et al*., 1997b).

The prenyl chain elongation catalyzed by prenyltransferases proceeds consecutively, and terminates precisely at discrete chain lengths according to the specificities of individual enzyme (Koyama and Ogura, 1999). Since natural rubber is made almost entirely of cis-isoprene units derived from IDP, the polymerizayion enzymes is thought to have similar characteristics to *cis*-prenyltansferases. The conversion of IDP to rubber was found to take place only on the surface of pre-existing rubber particles (Archer *et al*., 1963; McMullen and McSweeney, 1966; Lynen, 1967). This indicates that rubber transferase is bound to rubber particles and it still present even after the particles are washed repeatedly. The particle-bound rubber transferase activities were demonstrated in various rubber producing plants; guayule (Madhavan *et al*., 1989; Cornish and Backaus, 1990), *Ficus elastica* (Siler and Cornish, 1993), *Ficus carica* (Kang *et al*., 2000) and *Hevea brasiliensis* (Archer and Audley, 1987; Light and Dennis, 1989; Cornish, 1993; Asawatreratanakul *et al*., 2003).

According to the earlier study on the structure of rubber, geranyl diphosphate (GDP, *trans*-C_{10}) synthase should not be utilized for rubber formation. However, the incorporation of [1-^{3}H]GDP into rubber in the presence of IDP and washed rubber particles (WRP) was observed as well as the case of [1-^{3}H]neryl diphosphate (NPP, *cis*- C_{10}) (Archer and Audley, 1987). These finding suggest that the rubber formation system has a comparable affinity for *cis*- and *trans*- initiator compounds. It was also confirmed that FDP and GGDP are powerfull stimulators of rubber synthesis in a system containing [^{14}C]IDP and WRP. These finding suggest that the direct initiator is FDP or GGDP. The efficiency of allylic diphosphate was found to increase with increasing chain length, and that little effect was due to geometric isomerism of isoprene. Although biochemical studies indicate the possibility of utilizing short-chain allylic diphosphate as the initiating species, they give no direct evidence as to the true initiating molecules. The occurrence of certain special chain termination reaction is expected, but no information is available on the process, which is the most important step in controlling the molecular weight. In addition, the site where new rubber molecules are formed has not been established, thought Dickenson has suggested that FW or

other latex particles with a specialized structure might act as a site other than the surface of rubber particle (Gomez and Moir, 1979)

Table 1 Classification of prenyltransferases

Group name	Structure	Products
Short-chain *trans*-prenyl diphosphate synthase	Homodimer	C_{15}, C_{20}
Medium-chain *trans*- prenyl diphosphate synthase	Heterodimer	C_{30}, C_{35}
(*trans*)-Polyprenyl diphosphate synthase	Homodimer	C_{40}, C_{45}, C_{50}
(*cis*)-Polyprenyl diphosphate synthase	Homodimer	C_{45}, C_{50}

14. Rubber biosynthesis site in *Hevea brasiliensis*

The study of rubber biosynthesis process focused mainly on the surface of rubber particle and was always reviewed (Archer and Audley, 1987; Audley and Archer, 1988; Kekwick, 1989; Kush, 1994; Tanaka *et al*., 1996; Ohya and Koyama, 2001) as the only prerequisite site required for synthesis of rubber molecules. The reaction catalyzed by rubber transferase appears to be essentially elongation of pre-existing rubber molecules. However, the mechanism by which new rubber particles are formed is still unknown at present. It has not been possible to demonstrate the polymerization of IDP in vitro except on the pre-existing rubber particles. The reactions that occur in vivo must do so at some other site as prerequisite on initiation that preludes the formation of new rubber molecules. Some reports have suggested vehemently that membrane phospholipids may have a key role in enabling the combined functions of different transferase enzymes to operate in vivo for new rubber formation (Keenan and Allen, 1974; Baba and Allen, 1980) It appear that some phospholipids membranes or other amphipathic micelles are essential for initiation and formation of new rubber molecules.

Recently, the surface of membrane-bound organelles, the lutoids and FW particles, were shown that was quite active in the synthesis of new rubber molecules (Tangpakdee *et al*., 1997b, Wititsuwannakul *et al*., 2003). A kinetic study on radio labeled IDP incorporation into the rubber and the product analyse showed new appearance of low molecular weight rubber molecules (Tangpakdee *et al*., 1997b). These results suggested that the synthesis of new rubber molecules being initiated and formed by these particles enzymes. Subsequently, further careful studies on RB activities of the membrane (Wititsuwannakul *et al*., 2003) from bottom fraction particles were carried out and detailed properties were characterized. Parameters affecting the membrane functions were investigated on the RB process. Effect of detergents and heat treatments of the bottom fraction membranes before being subjected to the optimum RB assays conditions were characterized in details (Wititsuwannakul *et al*., 2003). The RB stimulation results thus suggest a possible increase of active surface area by formation of micelle caused by these parameters. The active membrane RB assays clearly indicated that the synthesis of new rubber could effectively occur with no requirement of the RP prerequisite site.

15. Plants plastid

Plastids are dynamic organelles of prokaryotic origin within the plant cells. In addition to photosynthesis, other important metabolic activities take place within plastids including the production of starch, certain amino acids and lipids, some of the colorful pigments in flowers, vitamins and several key aspects of sulfur and nitrogen metabolism. Plastids possess their own genome and a full complement of transcriptional and translation machinery to express their genetic information. Plastids are thought to have arisen as a result of an endosymbiotic event in which an early photosynthetic prokaryote invaded a primitive eukaryotic host (Margulis, 1970; Gray, 1992). They developed around 1500 million years ago (Hedges *et al.*, 2004). The gradual conversion of the endosymbiont into a cell organelle was accompanied by a dramatic reduction in genome size. Whereas the cyanobacterium *Synechocystis* has >3,100 genes (and ORFs) in a genome of 3.57 Mbp (Kaneko *et al*., 1996; Kaneko and Tabata, 1997; Kotani and Tabata, 1998), higher plant plastid genomes harbor

only about 130 genes in 150 kbp (Wakasugi *et al*., 2001). Of the estimated 3,000 or so distinct proteins found in higher plant plastid (Richly and Leister, 2004) only a small fraction are encoded by the plastid genome (Shimada and Sugiura, 1991). The bulk of plastid proteome is nuclear encoded, translated on cytosolic ribosomes and subsequently translocated across the plastid envelopes (Zerges, 2000). The circular plastid genome is divided into four regions: large single copy, small single copy and inverted repeat which is present in exact duplicate separated by the two single copy regions. Although overall gene content and order are highly conserved among land plants, this same conversation is not observed in non-coding sequences such as introns and intergenic spacers, which along with the untranslated regions of genes, comprise about 50% of the plastid genome (Jansen *et al*., 2006; Lee *et al*., 2006; Daniell *et al*., 2002; Kim *et al*., 2004). Plastid genomes are well characterized (Shimada and Sugiura, 1991; Maier *et al*., 1995).

Recently, partial genome sequences of a putative plastidic genome from *Hevea* FW particles were identified. (Phatthiya, 2007) The plastidic genome was found to have gene contents very similar to those previously reported in unrearranged angiosperm chloroplast genomes. Besides providing a supportive evidence for confirming FW particle as one of the modified plastid, the partial genome sequences reveal valuable information on spacer regions for integration of transgenes at optimal sites via homologous recombination, as well as endogenous regulatory sequences for optimal expression of transgenes and should help in extending this technology to other useful plants. The availability of FW particle genome sequence should pave the way for genetic manipulation of *Hevea* and other members of rubber producing plants.

16. Methylerythritol phosphate (MEP) pathway

Since the discovery of MVA pathway, it was widely accept that IDP and DMAPP were formed only through this pathway in all living organism. However, several results inconsistent with the operation of the MVA pathway in certain bacteria had been reported. For example, [^{13}C]actate, a precursor of MVA pathway, was not incorporated into ubiquinone of *E.coli* (Zhou and White, 1991). Feeding experiments with [U-^{13}C]glucose and

pentalenolaceone-producing *Streptomycess* exfoliates showed labeling patterns inconsistent with the MVA pathway (Cane *et al*., 1979; 1981). Furthermore, mevinolin, a highly specific inhibitor of HMG CoA reductase, did not inhibit the growth of *E.coli* (Zhou and White, 1991). These result suggested the existence of an alternative pathway for isoprenoid biosynthesis, which was not identified for sometime.

The alternative, non-mevalonate, pathway was first discovered in bacteria by Rohmer *et al*. (1993), and is best characterized in *Escherichia coli*. In 1996, Rohmer discovered the first reaction step of this pathway in *E.coli*. This pathway was original named non-mevalonate pathway or Rohmer pathway. After the identification of the first steps of the pathway, its name was changed to indicate the substrate (pyruvate/glyceraldehydes 3-phosphate [GAP] pathway) or the first intermediate, deoxyxylulose 5-phosphate (DXP) pathway. However, it is becoming more accepted to name the pathway after what is currently considered its first committed precursor, methylerythriol 4-phosphate (MEP), following the same rule used to name MVA pathway.

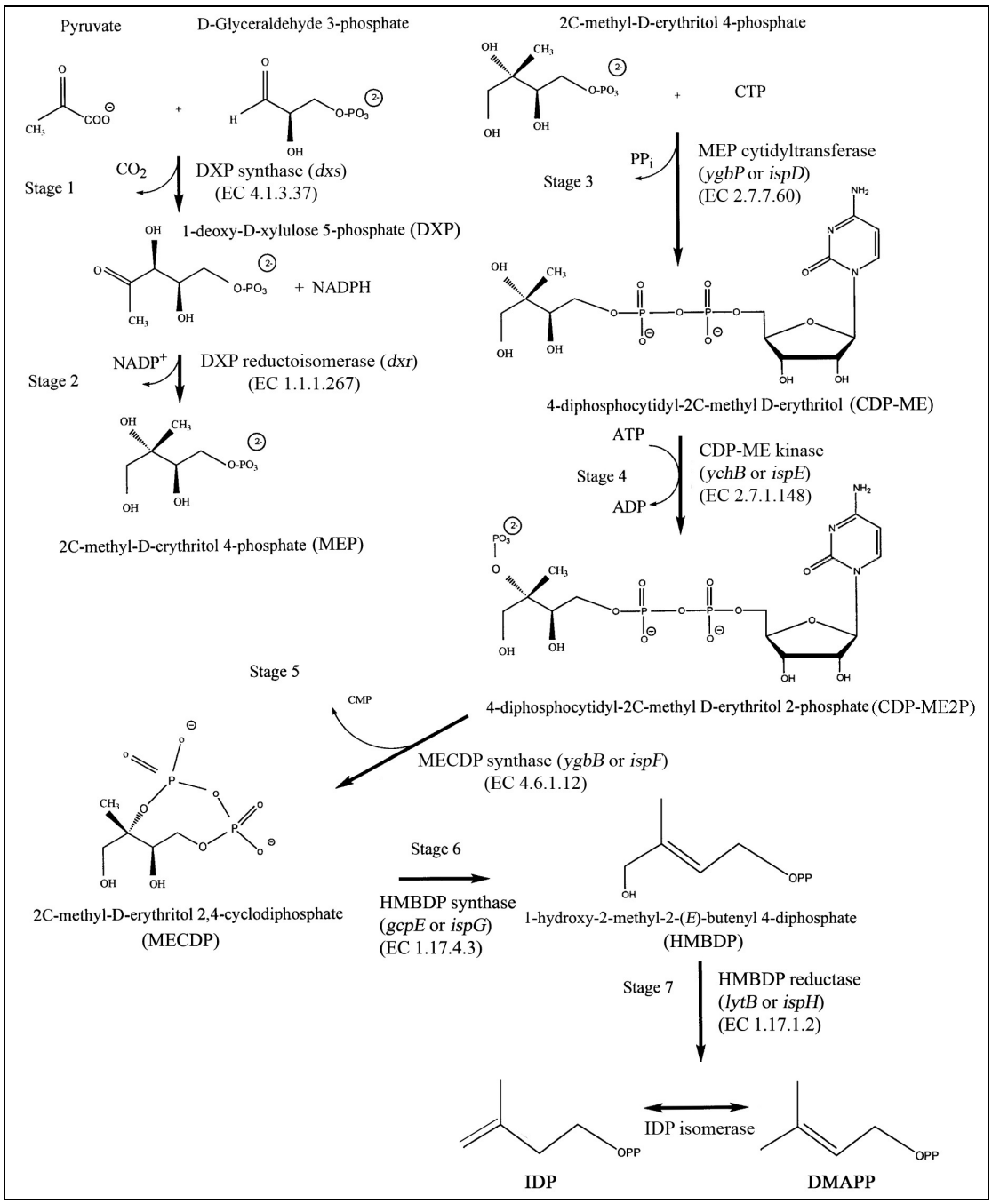
In plant cells, isoprenoids like carotenoids and phytol, the side chain of chlorophylls, are synthesized and accumulated inside the plastid compartment, whereas sterols are formed in the cytoplasm/endoplasmic reticulum (Lichtenthaler, 1977; 1993; Goodwin, 1977; Gray, 1987; Kleinig, 1989). Mevinolin strongly inhibits sterol biosynthesis in higher plants (Bach and Lichtenthaler, 1982; 1983; Doll *et al*., 1984). However, the biosynthesis of chlorophylls, carotenoids and plastoquinone-9 was unaffected by mevinolin (Bach and Lichtenthaler, 1982; 1983; Doll *et al*., 1984). Therefore the question arises if in higher plants mevalonate is involved in the synthesis of plastidic isoprenoids or if plastids possess a separate IDP biosynthesis pathway. Recent finding on a novel IDP biosynthesis in bacteria (Rohmer *et al*., 1993) and a green alga (Schwender *et al*., 1996) suggested that plastids, being of prokaryotic endosymbiotic origin, might posses their own mevalonate-independent IDP biosynthesis. From the evidences report by labeling experiments using ^{13}C-labeled carbon sources, appears that plants possess MEP pathway in chloroplasts (Lichtenthaler *et al*., 1997).

This MEP pathway, comprised of seven enzymatic steps, begins with the condensation of pyruvate and glyceraldehydes 3-phospate to form 1-deoxy-D-xylulose-5-

phosphate (DXP) catalysed by DXP synthase (DXS) and ends with the formation of the isoprenoid precursor IDP and DMAPP (Figure 6). The starting enzyme of this pathway is DXS. The *dxs* gene has been cloned from several higher plants (Lange *et al*., 1998; Bovier *et al*., 1998), *Escherichia coli* (Lois *et al*., 1998; Sprenger *et al*., 1997), green algae (Lichtenthaler, 1999) and from a strain of *Streptomyces* (Kuzuyama *et al*., 2000b). The enzyme requires thiamine diphosphate and divalent cations suc as Mg^{2+} or Mn^{2+} for its activity (Sprenger *et al*., 1997; Bovier *et al*., 1998). Based on sequence data from nucleic acid data bases it can be conclude that the DXS-like sequences are highly conserved in evolution. DXS-like gene (*CLA1*) found in *Arabidopsis thaliana* (Mandel *et al*., 1996) is supposed to be single copy gene regulated by light. The mutation of the *CLA1* impairs the proper development of chloroplast, arresting these organelles at an early stage of development. In addition, DXP is also an intermediate in the biosynthesis of coenzyme thiamine and pyridoxal phosphate (Julliard and Douce, 1991). The second enzymatic step, a C-C-skeleton rearrangement and reduction of DXP to 2-C-methyl-D-erythtitol 4-phosphate (MEP), is catalyzed by *dxr* gene product, namely 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) in the presence of NADPH and Mn^{2+}. The DXR enzyme has been characterized in *E.coli* (Kuzuyama *et al*., 2000c). A cloning strategy was also developed for isolation of the gene encoding a plant homolog of this enzyme from peppermint (Lange and Croteau, 1999), *A. thaliana* (Schwender *et al*., 1999), blue green algae *Synechocystis* (Proteau, 1998) and the parasite *Plasmodium falciparum* (Jomaa *et al*., 1999). Unlike the microbial reductoisomerase, the plant ortholog encodes preprotein bearing an N-terminal plastidial transit peptide that direct the enzyme to the plastids. It was found that DXR activity was strongly and specifically inhibited by fosmidomycin, an antibiotic possessing formyl and phosphonate groups in the molecule (Kuzuyama *et al*., 1998a). It was also shown that fosmidomycin inhibits the biosynthesis of carotenoids and chlorophylls in greening leaves as well as isoprene emission (Zeidler *et al*., 1998).

The further biosynthetic step consists in the conversion of MEP to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) in a CTP-dependent reaction by MEP cytidyltransferase enzyme. This enzyme is active in the presence of Mg^{2+} or Mn^{2+} and has been cloned in *E.coli* (Rodich *et al*., 1999) and *A.thaliana* (Rodich *et al*., 2000). The

following step is catalyzed by an ATP-dependent 4-(cytidine 5´-diphospho)-2C-methyl-erythritol kinase (Kuzuyama *et al*., 2000a) phosphorylates CDP-ME, producing 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate (CDP-ME2P). In the fifth step CDP-ME2P is converted to 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MECDP) and CMP by MECDP synthase (Rodich *et al*., 2001a; 2001b; Herz *et al*., 2000; Takagi *et al*., 2000). The cyclic diphosphate product MECDP is reduced to 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate by a reductase encoded by the *ispG* (formally *gcpE*) gene in *E coli*. Next, the *ispH* gene (or *lytB*) gene product converts the butenyl diphosphate to IDP and also DMAPP (Adam *et al*., 2002), so seemingly duplicating IDP isomerase activity.



(Hunter *et al.*, 2003)

Figure 6 Schematic plan of the MEP pathway of IDP/DMAPP biosynthesis.

17. Enzyme 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)

Within the MEP pathway, the initial enzymatic step is the condensation of glyceraldahyde 3-phosphate and pyruvate to form DXP, catalyzed by thiamine diphosphate-dependent DXP synthase (DXS). The second reaction in the pathway (Figure 7) is the conversion of DXP to MEP, catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267), encoded by the *dxr* gene. Because DXP is an intermediate precursor of not only the IDP but also of a common precursor for thiamine pyrophosphate and pyridoxol phosphate (Julliard and Douce, 1991; Hill *et al*., 1996), thus, the second reaction catalyzed by DXR is thought to represent the first committed step in the MEP pathway.

The *Escherichia coli dxr* gene and the successful expression of the recombinant protein were first reported in 1998 (Kuzuyama *et al*., 1998b; Takahashi *et al*., 1998). This discovery was aided by an earlier biosynthetic study that established that 2-C-methyl-D-erythritol, but not the enantiomer 2-C-methyl-L-erythritol, was incorporated into *E. coli* isoprenoids (Duvold *et al*., 1997b). In order to identify the gene(s) necessary to convert DXP into the putative intermediate, MEP, *E. coli* mutants were generated and screened for those that could survive only in the presence of added 2-C-methylerythritol (ME). Complementation studies with the surviving mutants using a genomic library from *E. coli* resulted in the identification of the *yaeM* gene as the single gene that allowed growth of the mutants. This gene was overexpressed in *E. coli* and incubation of the recombinant enzyme with DXP resulted in a product that was identified as MEP, revealing that a single enzyme promoted isomerization and reduction steps (Kuzuyama *et al*., 1998b). An enzyme involved in branched chain amino acid biosynthesis, ketol acid reductoisomerase (EC 1.1.1.86), also combines rearrangement and reduction steps, so the name DXP reductoisomerase was proposed for this new enzyme. The corresponding gene was renamed *dxr*.

The general characteristics of the DXR enzymes are that they are homodimers with monomer molecular weights of 42-45 kDa. The cofactor NADPH and a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}) are required for activity. The enzymes typically have a pH optimum in the range 7-8, with a maximum rate at 50–60°C. In plants, the gene product typically has a plastidial targeting sequence that is subsequently cleaved to provide the active

enzyme (Lange and Croteau, 1999; Carretero-Paulet *et al*., 2002), although there are examples of the full-length recombinant enzyme being active (Lange and Croteau, 1999; Rodríguez-Concepción *et al*., 2001).

In addition to the gene from *E. coli*, the *dxr* genes from a variety of sources have been cloned: *Plasmodium falciparum* ((Jomaa *et al*., 1999), *Arabidopsis thaliana* (Schwender *et al*., 1999), *Mentha* x *piperita* (Lange and Croteau, 1999), *Zymomonas mobilis* (Grolle *et al*., 2000), *Synechococcus leopoliensis*(Miller *et al*., 2000), *Catharanthus roseus* (Veau *et al*., 2000), *Pseudomonas aeruginosa* (Altincicek *et al*., 2000), *Streptomyces coelicolor* (Cane *et al*., 2001), *Lycopersicon esculen*tum (Rodríguez-Concepción *et al*., 2001), *Synechocystis sp. PCC6803* (Yin and Proteau, 2003), *Mycobacterium tuberculosis* (Argyrou and Blanchard, 2004), and *Zea mays* (Hans *et al*., 2004).

The metal ion requirement for DXR was established in initial studies to be Mg^{2+}, Mn^{2+}, or Co^{2+}. Numerous other divalent metal ions were assayed, but all of the others resulted in minimal activity or no activity at all (Takahashi *et al*., 1998; Grolle *et al*., 2000; Yin and Proteau, 2003; Argyrou and Blanchard, 2004). The K_m values for the metal ions have been determined for the *Synechocystis* and *Mycobacterium* enzymes (Yin and Proteau, 2003; Argyrou and Blanchard, 2004). DXR has higher affinity for Co^{2+} (*Synechocystis* $K_{m(Co)}$ = 10 μM; *M. tuberculosis*, 1.2 μM) and Mn^{2+} ($K_{m(Mn)}$ = 15 μM; 21 μM) ions, than for Mg^{2+} ($K_{m(Mg)}$ = 2400 μM; 1200 μM). Although the greatest k_{cat}s are often achieved with Mn^{2+}, the lowest $K_{m(DXP)}$s are seen with Co^{2+}, and the affinity of DXR for Mg^{2+} is comparatively low, it is likely that Mg^{2+} is the relevant divalent cation in vivo (Yin and Proteau, 2003; Argyrou and Blanchard, 2004; Koppisch *et al*., 2002). This is due to the more abundant levels of Mg^{2+} ions available relative to Mn^{2+} or Co^{2+} in vivo.

As mentioned above, DXR utilizes NADPH as a cofactor, in preference to NADH. The *E. coli* DXR was reported to use NADH to an extent of 1% relative to NADPH (Takahashi *et al*., 1998), while no use of NADH was reported for the *Z. mobilis* (Grolle *et al*., 2000) and *Synechocystis* (Yin and Proteau, 2003) enzymes. Slightly less discrimination was observed with partially purified *S. leopoliensis* DXR, where a drop in activity to 16% was found (Miller *et al*., 2000). Recent experiments with the *M. tuberculosis* DXR have provided more detailed information on the utilization of NADH as a cofactor (Argyrou and Blanchard,

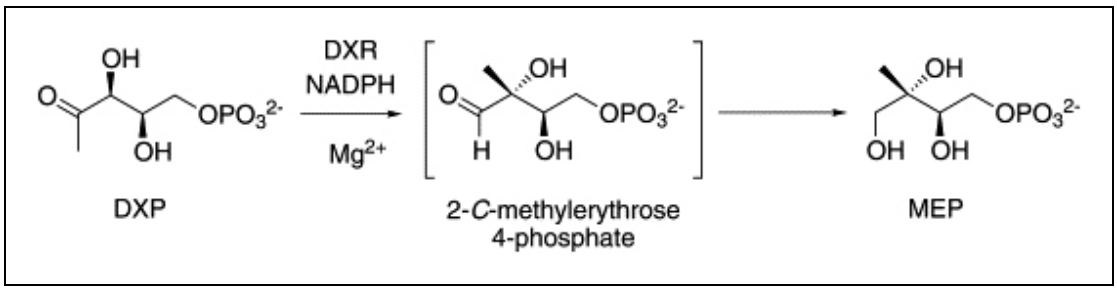
2004). With this enzyme, similar k_{cat} values were obtained with both cofactors, but with NADH, the $K_{m(NADH)}$ was 80-120-fold higher than the corresponding $K_{m(NADPH)}$ depending on the metal ion present. For example, when Mg^{2+} was the divalent ion, $K_{m(NADPH)}$ was 5 μM, while $K_{m(NADH)}$ was 410 μM and the $K_{m(DXP)}$ increased from 42 to 210 μM with the change to NADH (Argyrou and Blanchard, 2004). These results suggest that the lower activity with NADH observed with DXR from other sources was likely due to insufficient concentrations of NADH and DXP. Even though NADH can be utilized as a cofactor in vitro, the higher k_{cat}/K_m values with NADPH support NADPH as the relevant in vivo cofactor

The compound 2-*C*-methyl-D-erythrose 4-phosphate (MEOP), which was first proposed as a potential intermediate in the MEP pathway (Duvold *et al*., 1997a), was subsequently proposed as an intermediate in the DXR reaction (Figure 7). Attempts to identify this intermediate from a standard reaction mixture were unsuccessful (Takahashi *et al*., 1998), as were attempts to detect the intermediate by NMR while using dihydro-NADPH, an unreactive NADPH analog (Koppisch *et al*., 2002) and (Hoeffler *et al*., 2002). Finally, MEOP was synthesized and demonstrated to be kinetically competent for the reduction step, supporting its role as an intermediate for the enzymatic reaction (Hoeffler *et al*., 2002). Not only was the conversion of MEOP into MEP demonstrated, but when DXR was incubated with $NADP^+$ and MEOP, a low level conversion to DXP was detected. These experiments strongly suggest that MEOP is an intermediate in the enzymatic transformation even though efforts to directly detect its formation have failed.

Three general mechanisms have been proposed for the rearrangement of DXP to the methylerythrose intermediate (Figure 8): (1) an *α*-ketol rearrangement (Hoeffler *et al*., 2002), (2) a retro-aldol/aldol reaction sequence (Hoeffler *et al*., 2002), and (3) a hydride/methyl shift mechanism (Argyrou and Blanchard, 2004). There is precedence for the retro-aldol/aldol-type sequence in the mechanism for the enzyme L-ribulose 5-phosphate 4-epimerase (Johnson and Tanner, 1998). If the retro-aldol reaction occurs, an enolate of hydroxyacetone and glycolaldehyde phosphate would be generated. Attempts have been made to mimic the aldol condensation part of this mechanism by incubating DXR with hydroxyacetone and glycolaldehyde phosphate, but no production of MEP was observed (Hoeffler *et al*., 2002). The mechanism was further probed with 3*S*-hydroxypentan-2-one 5-

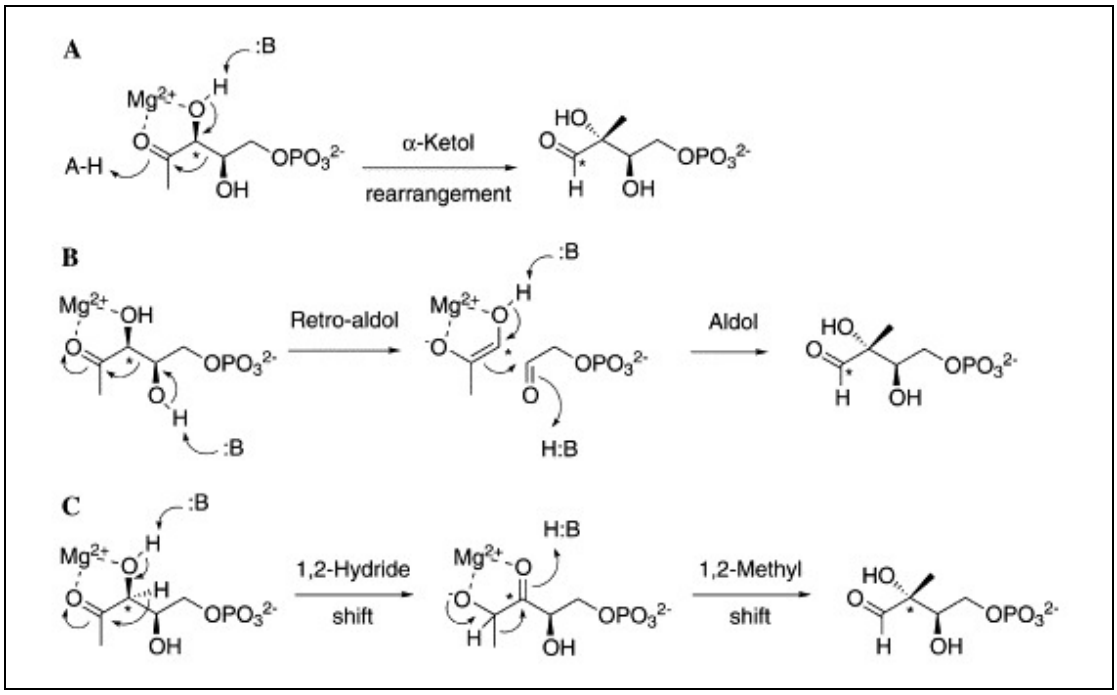
phosphate, which could potentially undergo the rearrangement by the α-ketol mechanism, but would be incapable of participating in the retro-aldol/aldol mechanism. This compound, however, acts as an inhibitor of the enzyme and is not an alternate substrate (Hoeffler *et al*., 2002). The hydride/methyl shift mechanism is not consistent with labeling studies (Rohmer *et al*., 1996; Arigoni *et al*., 1997) and can be ruled out. At the current time, either of the first two mechanisms is still possible for DXR.

Shortly after the discovery of DXR, it was reported that the natural product fosmidomycin was a potent inhibitor for this enzyme (Kuzuyama *et al*., 1998a). Fosmidomycin, an antibacterial compound, was determined to be a mixed inhibitor of DXR with a K_i of 38 nM. When fosmidomycin was tested as an inhibitor of the *Z. mobilis* DXR, it was determined to be a competitive inhibitor with a K_i of 600 nM (Grolle *et al*., 2000). Recent studies have more accurately defined fosmidomycin as a slow, tight-binding inhibitor that displays two inhibition modes, an initial step competitive with DXP and another non-competitive with DXP (Koppisch *et al*., 2002). A related natural product, FR900098, is also known as an effective inhibitor of DXR and has shown greater antimalarial activity than fosmidomycin in a mouse model system (Jomaa *et al*., 1999). Although fosmidomycin was initially proposed to inhibit DXR by binding in a similar fashion to the intermediate aldehyde, the crystal structure of *E. coli* DXR with Mn^{2+} and fosmidomycin bound provided evidence that fosmidomycin binds in a fashion more similar to the substrate DXP (Steinbascher *et al*., 2003).



(Proteau, 2004)

Figure 7 Conversion of DXP to MEP mediated by DXR.



(Proteau, 2004)

Figure 8 Proposed mechanisms for the rearrangement of DXP to the 2-*C*-methylerythrose 4-phosphate intermediate: (A) α-ketol rearrangement, (B) sequential retro-aldol/aldol reactions, (C) sequential 1,2-hydride and 1,2-methyl shifts. The asterisk marks the position of the carbon originally at C3 of DXP.

Objectives

1. To demonstrate the presence of the methylerythritol (MEP) pathway in *Hevea* latex

2. To isolate the gene encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) from *Hevea* latex and study to expression in *E. coli* cells

3. To study the mRNA expression of DXR in *Hevea* tissues and latex

4. To reconstruct phylogenetic tree and compare the relationships among various DXR

CHAPTER 2

Materials and Methods

Materials

1. Chemicals

Chemicals were from Sigma-Aldrich (St Louis, MO). Solvents were from Lab-Scan. [1-^{14}C] Glucose was from Amersham Biosciences (Amersham, UK). DXP was purchased from Echelon Research Labs (Salt Lake City, UT, USA). Fosmidomycin was from Invitrogen, USA.

2. Thin layer chromatographic materials

The RP-18 TLC and silica gel 60 GF_{254} were from Merck (Darmstadt, Germany). The imaging plate was Fuji film BAS-IIIs and bioimage analyzer was Fuji BAS 1000 Mac from Fuji Photo Film Co (Tokyo, Japan).

3. Plasmid Vector

pBluescript SK vectors were product of Stratagene, USA. pGEMT-Easy vectors were from Promega, USA. pCR4-TOPO, pENTR/TEV/D-TOPO vectors and pDEST (Gateway system) were from Invitrogen, USA. pET-32(a+) vectors were from Novagen, USA. pGWB5 and pGWB2 vectors were kindly provided by Dr. Tsuyoshi Nakagawa (Shimane University, Japan)

4. Bacterial strain

E. coli strain TOP10 [F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] and *E. coli* strain BL21(DE3) [F$^-$ *ompT hsdS*$_B$ (rB$^-$mB$^-$) *gal dcm* (DE3)] were from Invitrogen, USA.

5. Primers

All nucleotide primers in this study were from BioService Unit, BIOTEC and Qiagen, Germany and are shown in Table 2.

6. Enzymes

Restriction endonucleases and DNA polymerases were from TOYOBO, Japan, TAKARA, Japan and New England Biolabs, USA.

Table 2 Primers and nucleotide sequence of primers for PCR amplification

Set	Name of primer	Size (bases)	Sequence 5' to 3'	Application
1	M13 For	19	CACGACGTTGTAAAACGAC	Sequencing
1	M13 Rev	20	GGATAACAATTTCACACAGG	Sequencing
1	T7	20	TAATACGACTCACTATAGGG	Sequencing
1	SP6	19	TATTTAGGTGACACTATAG	Sequencing
2	F1	23	GCNCTNGCNGCNGGNTCNAAYGT	DXR amplification
2	F2	23	GTNGTNACNGGNATHGTNGGNTG	DXR amplification
2	F3	23	GTNGCTGCNATHGAAGCNGGNAA	DXR amplification
2	R1	23	CATATCNGGCCANCCNARYTGNGC	DXR amplification
2	R2	23	GCANAGRYCNAGNCKNGGCCANG	DXR amplification
2	R3	23	GCNGCRCTNAGA ACTCCNGTCAT	DXR amplification
3	GSP1	23	TCCAGCTTCTATTGCAGCCACCG	RACE
3	GSP2	23	CTACACTGACAGCATCTGGATGG	RACE
3	GSP3	23	TGGAGCTTTCAGGGATTGGCCTG	RACE
3	GSP4	22	CAGTGGACTCCGCTACCCTTTC	RACE, RT-PCR

Table 2 (Continued)

Set	Name of primer	Size (bases)	Sequence 5' to 3'	Application
4	GeneRacer 5' Primer	23	CGACTGGAGCACGAGGACACTGA	RACE
4	GeneRacer 5' Nested Primer	26	GGACACTGACATGGACTGAAGGAGTA	RACE
4	GeneRacer 3' Primer	25	GCTGTCAACGATACGCTACGTAACG	RACE
4	GeneRacer 3' Nested Primer	23	CGCTACGTAACGGCATGACAGTG	RACE
4	GeneRacer RNA Oligo	44	CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAAGUAGAAA	RACE
4	GeneRacer Oligo dT	60	GCTGTCAACGATACGCTACGTAACGGCATGACAGTGT_{24}	RACE
5	FDR1	27	GAATTCATGGCGCTCAATTTGCTTTCC	Full length
5	FDR2	27	GAAATGGCAGTGTTCGGCCCAGCCTCC	Full length
5	RDR1	26	TCATGCAAGAACAGGGCTTAGACCAG	Full length, RACE
5	RDR2	27	GTCGACTCATGCAAGAACAGGGCTTAG	Full length

R is G or A, K is G or T, S is G or C, W is A or T, M is A or C, Y is T or C, D is G or A or T, V is G or A or C, B is G or T or C, H is A or T or C, and N is G or A or T or C

Methods

1. Preparation of materials from fresh latex

1.1 Collection of fresh latex

Fresh latex was obtained from regular tapped *Hevea* trees under age of 25 years old, grown at the Songkhla Rubber Research Center, Hat-Yai, Songkhla. These trees were tapped every other day at 7.00 a.m., in a half-spiral fashion with V-shape knife by stripping the bark to make cuts across the latex vessels. The latex was collected in ice-chilled plastic bag. The pooled latex was kept in an ice container, brought to the library and separated latex fractions at the same day. The chilled latex was filtrated through 4 layer of cheesecloth to remove the particulate materials and tissue debris. The filtrate was collected and centrifuged to separate FW particles.

1.2 Isolation of Frey-Wyssling particle

FW particle isolation was carried out as described by Phatthiya *et al*. (2007). Briefly, fresh latex was subjected to a series of centrifugations. First, a low speed centrifugation (1700 × *g* at 10°C for 30 min), using swing-out bucket rotors, was employed to minimize inter-particle collisions, especially between FW and lutoid particles, to allow all the particles to travel down the centrifuge tube through the viscous latex medium according to their density gradients. The upper half of the centrifuged latex, containing the less dense rubber particles, was removed whereas the lower half was further separated, by using a fixed angle rotor centrifuge (7000 × *g* at 4°C for 15 min), into three distinct layers: a minor uppermost rubber fraction with yellowish FW particles lying beneath, a middle aqueous fraction and a bottom fraction containing the most dense lutoid particles. After careful removal of the uppermost rubber layer, the floating yellowish-orange FW particles were collected from the top layer of the aqueous phase and collected as a pellet by ultracentrifugation (59,000 × *g* at 4 °C for 20 min). The pellet was washed by resuspending in 2 vol. of washing buffer (50 mM Tris-HCl, pH 7.4) and centrifuged. The washed FW particles were kept at -20 ^{o}C until used.

2. Assay of radioactivity incorporation from [^{14}C] glucose into prenyl products

The washed FW particles were sonicated with 10 short 10 sec bursts interspersed by intervals of 10 sec for cooling. FW membranes were removed by centrifugation at 10,000 × g at 4 ^{o}C for 10 min. An aliquot (100 µl containing 250 µg of protein) of supernatant was added to a 500 µl reaction mixture containing 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 30 mM KF, 0.15 NADPH, 100 and 1000 µM fosmidomycin or 10 and 100 µM mevinolin (as indicated) and 1 mM [^{14}C]glucose (5 Ci/mol). After the reactions were incubated at 37^{o}C for 6 h, radioactive prenyl diphosphate products were extracted with 1-butanol saturated with water and hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously (Fuji *et al*., 1982). The hydrolyzed products were extracted with pentane and analyzed by TLC on a reverse LKC-18 plate (Whatman) with acetone/water (19:1, v/v) as the developing solvent system (Ohnuma *et al*., 1988). The RP-TLC plates were then exposed overnight on Fuji film BAS-III imaging plate at room temperature. The distribution of radioactive products on TLC plate was analyzed with a Fuji BAS-100 Mac Bioimage analyzer and spots of marker prenols were visualized with iodine vapor.

3. Cloning of *Hevea* DXR

The DXR gene from *Hevea* was isolated by amplification method using PCR. First total RNAs were isolated and reversed into cDNA. Then cDNAs were ligated into vector to construct the cDNA library. The DXR gene fragment was amplified using degenerated primers designing from highly conserved amino acid regions of plants DXRs. Next, 5' and 3' cDNA fragments were amplified by rapid amplification of cDNA ends (RACE) method using specific primers designing from DXR gene fragment. The final step, full length cDNA was obtained using specific primers designing based on the information of 5' and 3'-RACE cDNA fragments.

3.1 Designing PCR primers for gene fragment amplification

Gene fragment of *Hevea* DXR were obtained from 6 degenerate oligonucleotide primers, sense primers F1 - F3 and antisense primers R1 - R3 (Table2), based on the amino acid sequences of highly conserved domains of *Arabidopsis thaliana* (Carretero-Paulet *et al*., 2002), *Lycopersicon esculentum* (Rodríguez-Concepcion *et al*., 2001), *Mentha x piperita* (Lange and Croteau, 1999), *Catharantus roseus* (Veau *et al*., 2000) and *Stevia rebaudiana* (Totte *et al*., 2003).

3.2 Amplification of DXR gene fragment

PCR was performed in a final volume of 20 µl containing 1x *Taq* DNA polymerase buffer, 2.5 mM dNTPs, 7.5 mM forward and reverse primers , 2 µl of preheat (5 min at 95^{o}C) phage cDNA library template, and 0.25 U of KOD-plus DNA polymerase. The PCR was performed for 30 cycles of 30 s at 94^{o}C, 30 s at 50^{o}C and 1 min at 68^{o}C with a 2 min preheat at 94^{o}C. DNA fragments amplified by this PCR were subjected to electrophoresis, purified and subcloned into pGEMT-easy vector. The recombinant vector were isolated and subjected to sequencing analysis. The sequences of DNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software.

3.3 Electrophoresis of PCR products on agarose gel

Agarose gel (1%) was melted in TAE buffer (0.4 M Tris-HCl, 0.4 M glacial acetic acid and 0.1 mM EDTA, pH 8.0). After agarose gel was cooled to 50^{o}C a drop of ethidium bromide solution was added to the agarose gel solution to yield 0.5 µg/ml ethidium bromide gel. The gel was transfer to electrophoresis chamber and DNA sample was applied to the bottom of the wells. The electrophoresis was carried out for 20 min at 100 volts and DNA bands were visualized under UV transilluminator.

3.4 Purification of DNA bands from agarose gel

QIAquick Gel Extraction Kit was used for purification of DNA bands from agarose gel. Gel slide containing interesting band was applied in buffer QG and incubated at 50^{o}C for 10 min to dissolved gel. DNA in the solution was applied to the membrane of QIAquick column and centrifuged at 18,000 × g for 1 min at room temperature. QIAquick column was washed with 0.75 ml of buffer PE and centrifuged at 18,000 × g for 1 min at room temperature. The residual of ethanol from buffer PE was completely remove by an

additional centrifugation at 18,000 × g for 1 min at room temperature. To elute DNA from QIAquick column membrane, 50 µl of 10 mM Tris-HCl (pH 8.5) was added and centrifuged at 18,000 × g for 1 min at room temperature.

3.5 Subcloning of DNA fragments into pGEMT-easy vector

Each PCR DNA fragments (3 µl) were mixed with 1 µl of pGEMT-easy vector, 5 µl of ligation buffer and 1 µl of T4 DNA ligase, and the ligation mixture was incubated 1 h at room temperature. After the reaction was stopped on ice for 5 min, the ligated plasmids were mixed with 50 µl of *E. coli* competent cells and incubated on ice for 20 min. The ligated plasmids were transformed into *E. coli* cells using heat-shock method (45 s at 42^{o}C) and stand on ice for 2 min. To recover the membrane permeability of *E. coli* cells, LB medium (250 µl) was added and the *E. coli* cells were incubated in shaking incubation at 37^{o}C for 1 h (200 rpm).

3.6 Performing of Nested PCR

PCR was performed as method describe above with first PCR product as a template. The resulting products were applied to electrophoresis, purified from agarose gel and subcloned to pGEMT-easy vector. The sequences of DNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software.

3.7 RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE)

3.7.1 Designing PCR primers for RACE

Four specific primers (GSP1, GSP2, GSP3 and GSP4) were designed from the sequence obtained from the first cDNA cloning and paired with GeneRacer primer (GeneRacer 5' primer', GeneRacer 5' nested primer, GeneRacer 3' primer, and GeneRacer 3' nested primer).

3.7.2 Isolation of RNA

Young *Hevea* plants (3 months) were grinded with pestle and mortar under liquid nitrogen and lysed with guanidine isothiocynate containing buffer. The homogenized sample was transfer into QIAshredder column and centrifuge at 18,000 × g for 2 min at room temperature. 0.5 volumes of ethanol (95-100%) were added to the clear lysate and mix immediately. The supernatant was applied to RNeasy spin column and centrifuged at 8,000 ×

g for 15 s. The RNeasy spin column was washed with 700 µl of buffer RW1 and 500 µl of buffer RPE, respectively. The second wash with 500 µl of buffer RPE was performed. This step was centrifuged at 8,000 × g for 2 min ensuring that no ethanol was carried over during RNA elution. RNA was eluted from RNeasy spin column membrane by incubating the membrane with 30 µl of RNase-free water and centrifuged at 8,000 × g for 1 min.

3.7.3 Dephosphorylation of RNA

To eliminate truncated mRNA and non-mRNA from subsequent ligation with GeneRacer RNA Oligo, total RNA was treated with calf intestinal phosphatase (CIP) to remove the 5' phosphates. The reaction mixture was performed in a final volume of 10 µl containing 5 µg total RNA, 1 µl CIP buffer, 40 U RNase inhibitors, and 10 U CIP. The dephosphorylation reaction was mixed, incubated at 50^{o}C for 1 h and placed on ice.

3.7.4 Precipitation of RNA

To precipitate RNA, 90 µl DEPC water and 100 µl phenol:chloroform were added, vortex vigorously and centrifuge at 18,000xg for 5 min at room temperature. The aqueous phase containing RNA was transfer to a new tube and 2 µl 10 mg/ml mussel glycogen, 10 µl 3 M sodium acetate, pH 5.2, and 220 µl 95% ethanol were added. After reaction mixture was freeze on dry ice for 10 min, RNA was precipitated by centrifugation at 18,000 × g for 20 min at 4^{o}C. RNA pellet was washed with 500 µl 70% ethanol, centrifuged at 18,000 × g for 2 min at 4^{o}C and resuspended in 7 µl DEPC water.

3.7.5 Removal of mRNA cap structure

The decapping reaction was performed in a final volume 10 µl containing 7 µl dephosphorylated RNA, 1 µl Tobacco acid pyrophosphatase (TAP) buffer, 40 U RNase inhibitors, 0.5 U TAP. The decapping reaction was mixed, incubated at 37^{o}C for 1 h, placed on ice and precipitated as described above.

3.7.6 Ligation of GeneRacer RNA Oligo to decapped mRNA

Dephosphorylated, decapped RNA (7 µl) was mixed with 0.25 µg GeneRacer RNA oligo, incubated at 65^{o}C for 5 min and placed on ice for 2 min. After briefly centrifugation, the relax RNA was added with 1 µl ligase buffer, 1 µl 10 mM ATP, 40 U

RNase inhibitors, 5 U T4 RNA ligase, incubated at 37°C for 1 h, placed on ice, and precipitated as described above.

3.7.7 Reverse transcription of mRNA

Ligated RNA was mixed with 1 µl sterile distilled water, 1 µl dNTP mix and 1 µl GeneRacer Oligo dT, incubated at 65°C for 5 min and chilled on ice for 2 min. After briefly centrifugation, ligated RNA and primer mixture were added with 4 µl first strand buffer, 1 µl 0.1 M DTT, 40 U RNase inhibitors, and 200 U SuperScript III reverse transcriptase, and the reaction mixture was incubated at 50°C for 60 min. The reverse transcriptase activity was stopped by incubation the reaction at 70°C for 15 min and chilled on ice for 2 min. The remaining RNA template was removed from the reaction by an additional of 2 U of RNase H and incubation at 37°C for 20 min. The obtained cDNAs were store at -20°C until use.

3.7.8 Amplification of cDNA ends

To amplify the 5' end and 3' end, PCR were set up in a final volume of 50 µl containing 0.6 µM GeneRacer 5' or 3' primer, 0.6 µM gene specific primer (GSP1 for 5', GSP3 for 3'), 1 µl cDNAs template, 5 µl High Fidelity PCR buffer, 0.2 µM dNTP, 2.5 U Platinum *Taq* DNA polymerase High Fidelity, 2 mM $MgSO_4$ and sterile water. The PCR was performed for 5 cycles of 30 s at 94°C and 2 min at 72°C, 5 cycles of 30 s at 94°C and 2 min at 70°C, and 25 cycles of 30 s at 94°C, 30 s at 60°C and 2 min at 72°C with a 2 min preheat at 94°C and a 10 min final extension at 72°C. The resulting products were applied as template for nested PCR.

3.7.9 Performing of nested PCR

To eliminate the possibility of artifacts of multiple bands obtained in 5' or 3' RACE PCR, nested PCR were performed in a final volume of 50 µl containing 0.2 µM GeneRacer 5' or 3' Nested primer, 0.2 µM gene specific primer (GSP2 for 5', GSP4 for 3'), 1 µl first PCR template, 5 µl High Fidelity PCR buffer, 0.2 µM dNTP, 2.5 U Platinum *Taq* DNA polymerase High Fidelity, 2 mM $MgSO_4$ and sterile water. The PCR was performed for 25 cycles of 30 s at 94°C, 30 s at 65°C and 2 min at 72°C with a 1 min preheat at 94°C and a 10 min final extension at 72°C. DNA fragments amplified by the PCR were subjected to

electrophoresis, purified and subcloned into pCR4-TOPO vector. The sequences of DNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software.

3.7.10 Amplification of Full length cDNA

Based on the sequence information obtained from the 5'- and 3'-RACE reaction, 5' full length primers (FDR1 and FDR2) and 3' full length primers (RDR1 and RDR2) were designed. PCR were set up in a final volume of 50 µl containing 0.6 µM FDR1 primer, 0.6 µM RDR1 primer, 1 µl cDNAs template, 5 µl High Fidelity PCR buffer, 0.2 µM dNTP, 2.5 U Platinum *Taq* DNA polymerase High Fidelity, 2 mM $MgSO_4$ and sterile water. The PCR was performed for 5 cycles of 30 s at 94^{o}C and 2 min at 72^{o}C, 5 cycles of 30 s at 94^{o}C and 2 min at 70^{o}C, and 25 cycles of 30 s at 94^{o}C, 30 s at 64^{o}C and 2 min at 72^{o}C with a 2 min preheat at 94^{o}C and a 10 min final extension at 72^{o}C. Nested PCR were performed in a final volume of 50 µl containing 0.2 µM FDR2 primer, 0.2 µM RDR2 primer, 1 µl first PCR template, 5 µl High Fidelity PCR buffer, 0.2 µM dNTP, 2.5 U Platinum *Taq* DNA polymerase High Fidelity, 2 mM $MgSO_4$ and sterile water. The PCR was performed for 25 cycles of 30 s at 94^{o}C, 30 s at 64^{o}C and 2 min at 72^{o}C with a 1 min preheat at 94^{o}C and a 10 min final extension at 72^{o}C. DNA fragments amplified by the PCR were subjected to electrophoresis, purified and subcloned into pCR4-TOPO vector. The sequences of DNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software.

4. Construction of expression vector and overproduction of recombinant pET-HbDXR

Expression vector system for the HbDXR was constructed using pET-32(a+) vector which designed to express the gene product as a thioredoxin- and His-tagged fusion protein, suitable for production of soluble protein in *E. coli* cytoplasm and rapid purification. The pET-32 (a+) vector (30 µl) was mixed with 1 µl *BamH*I, 1 1 µl *Sal*I4 µl NEBuffer 1 (New England Biolab) and 4 µl BSA. After incubation at 37^{o}C for 2 hrs, the reaction mixture

was transfer to electrophoresis chamber. The corresponding *BamH*I and *Sal*I cut vector was purified from gel and kept at -20^{o}C until use.

To construct the expression vector system in *E. coli*, the restriction enzyme recognition site for *BamH*I and *Sal*I was introduced at the 5'-end and 3'-end of the coding regions of pCR4-TOPO-full length cDNA. The *BamH*I and *Sal*I site was introduce into FDR1 and RDR2 primer, respectively. PCR were performed in a final volume of 50 µl containing 0.2 µM N3 primer, 0.2 µM C3 primer, 3 µl pCR4-TOPO-full length cDNA, 5 µl High Fidelity PCR buffer, 0.2 µM dNTP, 2.5 U Platinum *Taq* DNA polymerase High Fidelity, 2 mM $MgSO_4$ and sterile water. The PCR was performed for 35 cycles of 30 s at 94^{o}C, 30 s at 60^{o}C and 2 min at 72^{o}C with a 1 min preheat at 94^{o}C and a 10 min final extension at 72^{o}C. DNA fragments amplified by the PCR were subjected to electrophoresis, purified and ligated into *BamH*I and *Sal*I cut pET-32(a+) vector yielding the expression plasmid pET-HbDXR. The expressed plasmid was used for transformation of *E. coli* BL21(DE3), and 1 ml of an overnight culture of the transformant in Luria-Bertani medium containing 100 µg/ml ampicillin was inoculated into 200 ml of the same medium. The cells were grown at 37^{o}C to reach an A_{600} value of 0.8. Isopropyl thio-β-D-galactoside was added to a final concentration of 0.1 mM, and the culture medium was further incubated at 30^{o}C for 5 h. Overproduction of the proteins was confirmed by SDS/PAGE according to the standard method of Laemmli (1970). The cells were harvested by centrifugation (5000 × g, 10 min) and then disrupted by sonication. The cell homogenates were separated into soluble and insoluble protein (inclusion body) fractions by centrifugation at 8000 × g for 10 min. The expressed proteins were purified essentially according to the protocol of Xpress Protein Purification System (Invitrogen), using a Ni^{2+} nitrilotriacetic acid-agarose column. The insoluble proteins were extracted with buffer A (20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 8 M urea) at 37^{o}C for 1 h. The protein extract was collected by centrifugation at 8000 × g for 10 min, filtered through a filter membrane, and applied to a Ni^{2+} resin column. The column was washed with buffer A and the tagged protein was eluted with buffer B (20 mM phosphate buffer, pH 7.4, containing 0.5 M NaCl and 0.3 M imidazole). The purified protein was

renatured by dialysis against buffer C (10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100).

5. Construction of expression vector and overproduction of recombinant pDEST-HbDXR

To overexpress HbDXR in *E. coli*, various expression vectors harboring coding sequences of *Hbdxr* were constructed using Gateway system. The *Hbdxr* sequences were amplified by PCR using a sense primer, 5'-CCACATGGCGCTCAATTTGCTTTCC-3' and antisense primers, 5'-TCATGCAAGAACAGGGCTTAG-3' (containing stop codon) or 5'-TGCAAGAACAGGGCTTAGACC-3' (without stop codon), which were then subcloned into pENTR/TEV/D-TOPO entry vector The entry clones were applied for the recombination reaction with Gateway destination vectors, pDEST15 or pDEST17, which is constructed by our collaborator, using LR Clonase to construct plasmids for the expression of HbDXR fused with glutathione S-transferase (GST)-tag (pDEST15) or Histidine-tag (pDEST17).

These plasmids were introduced into *E. coli* BL21(DE3) or BL21-AI. *E. coli* hosts harboring each expression vector were grown to mid-log phase (OD_{600} ~0.6) in LB broth at 37ºC, and then, grown in LB broth supplemented with inducers, 1 mM IPTG for BL21(DE3) or 0.2% arabinose for BL21-AI, at 18ºC for 24 h. Collected cells were lysed by treatment with 1 µg/ml lysozyme, followed by sonication. After the centrifugation at 20,000 x g for 30 min, the supernatants were applied for affinity purification with HisTrap HP column or GSTrap column (GE Healthcare, NJ, USA) according to the manufacture's instructions.

6. Analysis of DXR activity

DXR activity was monitored by the oxidation of NADPH in the reaction solution containing DXP and enzyme solution as described by Dhiman *et al*. (2005).The reaction mixture contained, in final volume of 500 µl, 100 mM Tris-HCl pH8.0, 1 mM $MgCl_2$, 0.3 mM DXP and 0.15 mM NADPH. The reaction was initiated by adding the enzyme solution to the complete assay mixture. The reaction mixture was incubated at 37 ºC

for 30 minute. The oxidation of NADPH was monitored at 340 nm (DU 650i spectrophotometer, BECKMEN). One unit of DXR activity is defined as the amount of the enzyme that cause oxidation of 1 μmol of NADPH per minute.

7. Analysis of gene expression

The first-strand DNA was synthesized from the total RNA (1 μg) by using Omniscript reverse transcriptase (Qiagen) and Oligo-dT. A 4 units Omniscript reverse transcriptase (RT), 1x RT buffer, 0.5 mM dNTP, 1 μM Oligo-dT primer, and 10 units RNase inhibitor were added to the preheated total RNA (5 min at 65^{o}C). After transcription for 1 h at 37^{o}C, the first-strand DNA was used as template for PCR performed in standard condition: 35 cycles of 15 s at 94^{o}C, 30 s at 54^{o}C, and 1 min at 68^{o}C with a 2 min preheat at 94^{o}C using sense primer GSP4 and antisense primer RDR1. As control, 18S rRNA primers were used. The PCR products were analyzed by agarose gel electrophoresis with ethidium bromide staining.

8. Subcellular localization of HbDXR

The gateway entry clone harboring *Hbdxr* without stop codon was applied for recombination with a Gateway destination vector. The pGWB5, kindly provided by Dr. Tsuyoshi Nakagawa (Shimane University, Japan), was used for construction of a binary vector to express HbDXR fused with sGFP at C-terminal region in plant cells. The resulting plasmid was introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) by triparental mating. *Arabidopsis* T87 cultured cells (Axelos *et al*., 1992) were transformed by co-cultivation with GV3101 (pMP90) carrying the resulting construct as reported (Suzuki *et al*., 2006). A stationary phase culture of *A. tumefaciens* was washed and resuspended with the same amount of JPL medium (Jouanneau and Péaud-Lenoël, 1967). Seven-day-old T87 cultured cells grown in JPL medium under continuous illumination at 22°C rotating at 120 rpm were filtered through a 0.5-mm nylon sieve. The filtrated culture was diluted 5 times in

the fresh JPL medium and cultured to grow until 20-40 mg fresh weight/ml medium. Then 5 ml of the cultured cells were incubated with 50 µl of the *Agrobacterium* suspended with JPL medium in a 100 ml Erlenmeyer flask by gentle agitation (120 rpm) under continuous illumination at 22°C. After 48 h, the cells were collected, washed three times in the same amount of JPL medium supplemented with 200 mg/l claforan (Hoechst Marion Roussel, Tokyo, Japan). The cells were resuspended with 5 ml of JPL medium supplemented with 200 µg/ml claforan and cultured for 3-4 days in a 100 ml Erlenmeyer flask as mentioned above. The co-cultivated cells were plated on cell growth medium, containing 0.5 g/l MES (pH 5.7), 3.3 g/l Gamborg's B5 medium salt mixture (Wako Pure Chemical Industries, Osaka, Japan), B5 vitamin (SIGMA, MI, USA), 3% (w/v) sucrose, 0.2 mg/l a-NAA, 200 mg/l claforan and 0.6% Bact-Agar, on which a nylon membrane was over-laid, and cultured under continuous illumination at 22°C for three days. The nylon membranes were transferred on new cell growth medium supplemented with 200 mg/l claforan and 10 µg/ml hygromycin and stored until green calli formation (for about two-weeks). Small cell aggregates of calli were transferred to JPL medium containing 200 mg/l claforan. The suspension was kept under continuous illumination at 22°C rotating at 120 rpm. After 7-10 days, an aliquot of cell suspensions was transferred to fresh medium containing 200 mg/l claforan and 10 µg/ml hygromycin and maintained as an independent cell line. For GFP imaging, T87 cells expressing GFP-fused HbDXR were observed under BX50 microscope equipped with a FLUOVIEW confocal scanning system (OLYMPUS, Tokyo, Japan). The green (GFP) and red (chlorophyll autofluorescence) emissions were detected with filters BA510IF-BA550RIF and BA585IF, respectively. The images were then pseudo-colored and combined into one image.

9. Measurement of pigments in *Arabidopsis* cultured cells expressing of HbDXR

To prepare the pCaMV35S-HbDXR binary vector, the Gateway entry clone harboring HbDXR with stop codon was applied for recombination with a Gateway destination vector, pGWB2. The resulting binary vector was introduced into *A. tumefaciens* GV3101 (pMP90) by triparental mating. Arabidopsis T87 cultured cells (Axelos *et al*., 1992) were

transformed by co-cultivation with GV3101 (pMP90) carrying the resulting construct. Extraction of chlorophylls and carotenoids was basically conducted under the dim-light as described by Fraser et al. (2000). About 100 mg (fresh weight) T87 cells grown 7 days after subculture were collected and frozen by liquid N_2 in 1.5 ml microtube, and then ground to a fine powder in Liquid N_2. Cells were extracted with 300 µl ice-cold methanol by shaking for 5 min at 4 ºC, followed by the addition of 300 µl Tris-HCl (pH 7.5) containing 1 M NaCl and mixture of the tubes for 5 min at 4 ºC. Plastidial pigments were extracted with 800 µl chloroform by the mixing for 10 min at 4ºC. After centrifugation at 3,000 x g for 5min, 500 µl of lower phase was transferred to a new tube, and solvent was evaporated. Residual pigments were dissolved with 500 µl acetone, which were measured by spectrophotometer (Genespec, Hitachi High-Technologies, Tokyo, Japan) at 470, 645 and 662 nm. Total amount of carotenoids and chlorophylls were calculated as described by Lichtenthaler (1987).

10. Construction of phylogenetic tree

Phylogenetic tree were generated by MEGA3 program based on degree of similarity between plants DXRs. Sequence analysis was performed using CLUSTAL W. The branches were validated by bootstrap analysis from 500 replications, which are represented by percentages in branch nodes. The 22 cDNA sequences are DXRs of *Salvia. Miltiorrhiza* (Wu *et al*., 2008), *Menha* x *piperita* (Lange and Croteau, 1999), *Plectranthus barbatus* (Engprasert *et al*., 2005), *Antirrhinum majus* (Dudareva *et al*., 2005), *Picrohiza kurrooa* (Singh *et al*., unpublished result), *Catharanthus roseus* (Veau *et al*., 2000), *Nicotiana tabacum* (Ma *et al*., unplublished result), *Lycopersicon esculentum* (Rodríguez-Concepción *et al*., 2001), *Stevia rebaudiana* (Totte *et al*., 2003), *Artemisia annua* (Souret *et al*., 2003), *Chrysanthemum* x *morifolium* (Kishimoto and Ohmiya, 2006), *Arabidopsis thaliana* (Carretero-Paulet *et al*., 2002), *Pueraria Montana var lobata* (Sharky *et al*., 2005), *Cistus creticus* (Pateraki *et al*., 2007), *Populus tremula* x *P. alba* (Mayrhofer *et al*., 2005), *Croton Stellatopilosus* (Wungsistaweekul *et al*., unpublished result), *Hordeum vulgare* (Hans *et al*., 2004), *Oryza sativa* (Carretero-Paulet *et al*., 2002), *Ginkgo biloba* (Kim *et al*., 2006), and *Taxus cuspidate* (Jennewein *et al*., 2004). Upon extraction of Blast hits, a series of files containing

homologous sequences from these species in EMBL format (Genetyx program) was created. Each set of files containing homologous sequences was automatically aligned using the Genetyx program and manually edited. The nucleotides at the first and at the end of each set of files were filled to correct the homologous position of each sequences using Geneatsq program. A final set of files containing homologous sequences was concatenated in one file and EMBL format was replaced with DNASIS format using SeqVerter program. A set of concatenated sequences was aligned using ClustalW program implemented in the MEGA3 program (Kumar *et al*., 2004). In general, alignment of the DNA sequences was straightforward and simply involved removing gaps included in the data set. In some case, small in frame insertions or deletions were required for correct alignment.

11. Determination of proteins

11.1 Protein assay by Lowry method (Lowry *et al*., 1951)

Protein determination by this method was calibrated using bovine serum albumin (20-80 μg) as a standard. Protein samples (30 μl) were mixed with alkaline copper solution [1% (w/v) copper sulfate/ 2% (w/v) potassium-sodium tartrate/ 2% (w/v) sodium carbonate in 0.1 M NaOH, 1:1:100 (v/v)] and the reactions were stand at room temperature for 10 min. In this step, the peptide bonds of proteins were reacted with copper under alkaline conditions producing Cu^{+} and called Biuret chromophore. Then the Folin reagent [Folin-Ciocalteu/water, 1:1 (v/v)] 90 μl was added to the first reaction and stand at room temperature for 30 min. The phosphomolybdate and phosphotungstate in Folin reagent is reduced by the copper-catalyzed oxidation of aromatic acids (tyrosine and tryptophan residues). The reduced Folin-Ciocalteu is blue and detectable with a spectrophotometer in the range of 500-750 nm. After left the reaction mixture for 30 min, the absorbance of the solution was measured at λ_{650} nm.

11.2 Protein assay by Bradford's method

In order to avoid the interference from Tris buffer, proteins were determined by using commercial Protein Assay reagent (from Bio-Rad). The principle of this reagent is according to the modified Bradford's method (Bradford, 1976). The analyzing procedures were according to the product leaflet.

11.3 SDS-polyacrylamide-gel electrophoresis (PAGE)

Samples were boiled for 5 min in SDS-sample buffer [0.063 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol (v/v), 0.1 M DTT and 0.01% bromophenol blue] and then subjected to SDS-PAGE in 12% (w/v) polyacrylamide gels (Laemmli, 1970). The acrylamide gel preparation recipe was according to Table 4. The SDS-PAGE was carried out with electrophoresis buffer (25 mM Tris-HCl, pH 8.3) at 20 mA constant current per slab gel until the bromophenol blue dye reached the bottom of the separating gel. The separating gel was then fixed and stain with staining solution [50% (v/v) methanol, 7% (v/v) acetic acid and 0.2% (w/v) Coomassie brilliant blue R-25] for 2 h. The excess dye was removed using destaining solution [20% (v/v) methanol and 10% (v/v) acetic acid] until the gel background was clear.

Table 3 Recipes for polyacrylamide separating and stacking gels

Stock solution	Stacking gel (µl)	Separating gel (µl)
	(3%)	**(12%)**
Distilled water	1,745	1,880
1.5 M Tris-HCl, pH 8.9	-	1,500
0.5 M Tris-HCl, pH 6.8	750	-
*30% acrylamide solution	300	2,400
1% (w/v) ammonium persulfate	150	150
10% (w/v) SDS	30	60
0.5 M EDTA, pH 7.0	20	-
TEMED	5	10
Total volume	3,000	6,000

*29.2% (w/v) acrylamide+0.8% (w/v) bisacrylamide

12. DNA sequencing

12.1 Preparation of polyacrylamide gel for electrophoresis

After polyacrylamide in gel solution (6 ml 50% Long Ranger Gel Solution; Cambrex Bio Science, 6 ml 10xTBE, 21 g urea, H_20 added to 45 ml) dissolved completely, the solution was filtered and degassed. Then 0.04 g of ammonium persulfate and 40 μl of TEMED were added to the solution and the gel solution was applied to the gel assembly immediately.

12.2 Preparation of DNA samples

The template/primer/enzyme mix (1 μl ThermoSequenase buffer, 0.75 μl IRD41 labeled primer, 1 μl ThermoSequenase DNA polymerase, 100-250 fmol DNA template, H_2O added to 9 μl) was divided into 4 tubes (A, T, G, T) and PCR was performed for 30 cycles of 30 s at 95°C, 30 s at 50°C and 1 min at 70°C. After the cycling was completed, 2 μl of Stop Solution was injected into each of the reaction mixtures. The samples were denatured by heating at 95°C for 3 min, and then chilled on ice. 1.6 μl of each samples were applied to each lane of gel. The sequences of the DNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by GENETYX software

CHAPTER 3

Results

1. [^{14}C]-labeled prenyl products derived from [^{14}C] glucose

In order to evaluate the presence of the MEP pathway in the putative FW plastids, a membrane-free FW extract was prepared and incubated with [^{14}C] glucose. The expected [^{14}C] prenyl products derived from IDP synthesized from [^{14}C] glucose via the MEP pathway was analyzed. The washed FW particles were bursted by sonication and after the removal of FW membranes by centrifugation, an aliquot of supernatant containing 250 μg of protein was isolated and incubated in a 500 μl reaction mixture containing [^{14}C] glucose at 37^{o}C for 6 h. The 500 μl saturated NaCl solution was then added, mixed thoroughly and treated twice with 1000 μl 1-butanol saturated with water in order to extract the radiolabeled prenyl diphosphate products. The radioactive products were hydrolyzed to the corresponding alcohols with acid phosphatase. The hydrolyzed products were extracted with pentane and analyzed by RP-TLC. The distribution of radiolabeled products on TLC plate was analyzed with a radioactive image analyzer.

An autoradiogram of the total acid phosphatase treated prenyl products derived from [^{14}C] IDP, revealed a major component, [^{14}C] C_{15}-OH (Figure 9, lane 1). This result may indicate the presence of the MEP pathway in the FW particles. Fosmidomycin was added to the incubation mixture to inhibit DXR, the second enzyme of the MEP pathway (Kuzuyama *et al*., 1998a). An autoradiogram of the prenyl alcohol products produced from [^{14}C] glucose gave a similar pattern to the original without fosmidomycin but the intensity was significantly reduced (Figure 9, lane 2 and lane 3). Similarly, the mevilonin was added to the incubation mixture to inhibit HMG CoA reductase, a rate-limiting enzyme of the MVA pathway that might be present due to contamination by residual latex cytosol and the thread-like reticulum in the original isolated FW particles. The concentration of mevinolin (100 μM) employed was about four times higher than that reported to completely inhibit the HMG CoA reductase in *Parthenium argentatum* bark (Ji *et al*., 1993). The blocking effect of mevilonin on prenyl alcohol products produced from [^{14}C] glucose was significantly lower than that of

fosmidomycin (Figure 9, lane 4 and lane 5). This result confirmed that the MEP pathway was present in the FW particle preparation.

A short-chain allylic diphosphate, especially the C_{15}-diphosphate (FDP), had been identified as a species necessary for initiating rubber formation (Takaya *et al*., 2003). The presence of [^{14}C] C_{15}-OH in the autoradiogram indicated that the FW particles may be also involved in providing the initiating primer for the rubber biosynthesis. This warranted an attempt to detect and isolate the gene encoding for *Hevea* 1-deoxy-D-xylulose-5-phosphate reductoisomerase (HbDXR), a first committed step enzyme in the MEP pathway. This assists in establishing a biological function for the FW particles in *Hevea* latex biosynthesis.

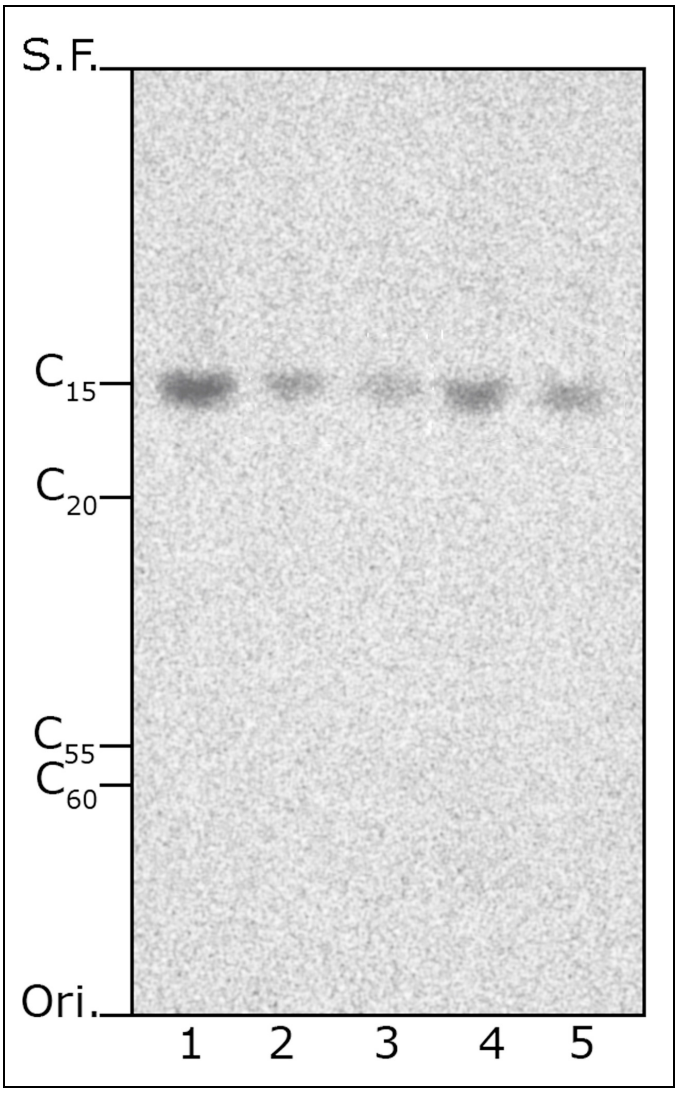


Figure 9 TLC autoradiogram of the prenyl alcohols obtained by enzymatic hydrolysis of products formed by the reaction mixture containing [^{14}C] glucose and FW particles. The products (lane 1-5), obtained from reaction mixtures containing no inhibitors (lane 1), 100 μM and 1000 μM fosmidomycin (lane 2 and 3, respectively) and 10 μM and 100 μM mevinolin (lane 4 and 5, respectively), were analyzed by TLC on a reversed-phase LKC-18 plate with a solvent system of acetone/water (19:1, v/v). S.F.: solvent front; Ori: origin.

2. Isolation of *Hevea* DXR gene

To isolate *Hevea* DXR gene (*dxr*), the methods were carried out as follow. First, the total RNAs from *Hevea* latex were isolated and constructed to latex cDNA library. Next, the amplification of *dxr* cDNA fragment was performed with PCR method using the latex cDNA library as a template and using degenerated PCR primers which synthesized from the highly conserved amino acid region of selected plant DXRs. After the *dxr* cDNA fragment was obtained, this sequence information was used to designed specific PCR primers for amplification of 5' and 3'-cDNA ends using RACE methods. The sequence information of both 5' and 3'-cDNA ends was used to design *Hevea dxr* specific PCR primers for amplification of full length *dxr*. The full length cDNA amplification of *Hevea dxr* was summarized in Figure 10.

2.1 Amplification of cDNA fragment

The sequence database of DXR homologues were searched using the BLAST program from NCBI database and aligned using Genetyx program. According to the conserved amino acid regions of DXRs, six degenerate oligonucleotide primers were designed (Figure 11). PCR was performed with nine pairs of primer (F1 and R3, F1 and R2, F1 and R1, F2 and R3, F2 and R2, F2 and R1, F3 and R3, F3 and R2, and F3 and R3) at annealing temperature 55^{o}C and 60^{o}C. Between nine reactions, only two reactions performed with primer pair of F1-R1 and F2-R1 contained the expected size cDNA fragments, 800 and 700 bp, respectively. To confirm which reactions contained the cDNA fragment corresponding to DXR gene, two nested PCRs amplified with internal PCR primers were carried out. The PCR products of nested reaction, performed with F2-R1 primer pair and the products of first PCR reaction were used as a template, were subjected to electrophoresis (Figure 12). The PCR products that showed the size as expectation, 700 bp, were purified and ligated into pGEMT-easy vector. The sequences of cDNA fragment were determined by ABI PRISM 310 Genetic Analyzer and analyzed by Genetyx software. The sequence of this cDNA was subsequently verified by using the BLAST program in a search of the NCBI database. This showed that it had a high sequence homology to the other plant DXRs (Figure 13). This

cDNA fragment was termed HDR705 and used as a template to design the *Hevea dxr* specific primer for amplification of 5’ and 3’-cDNA ends.

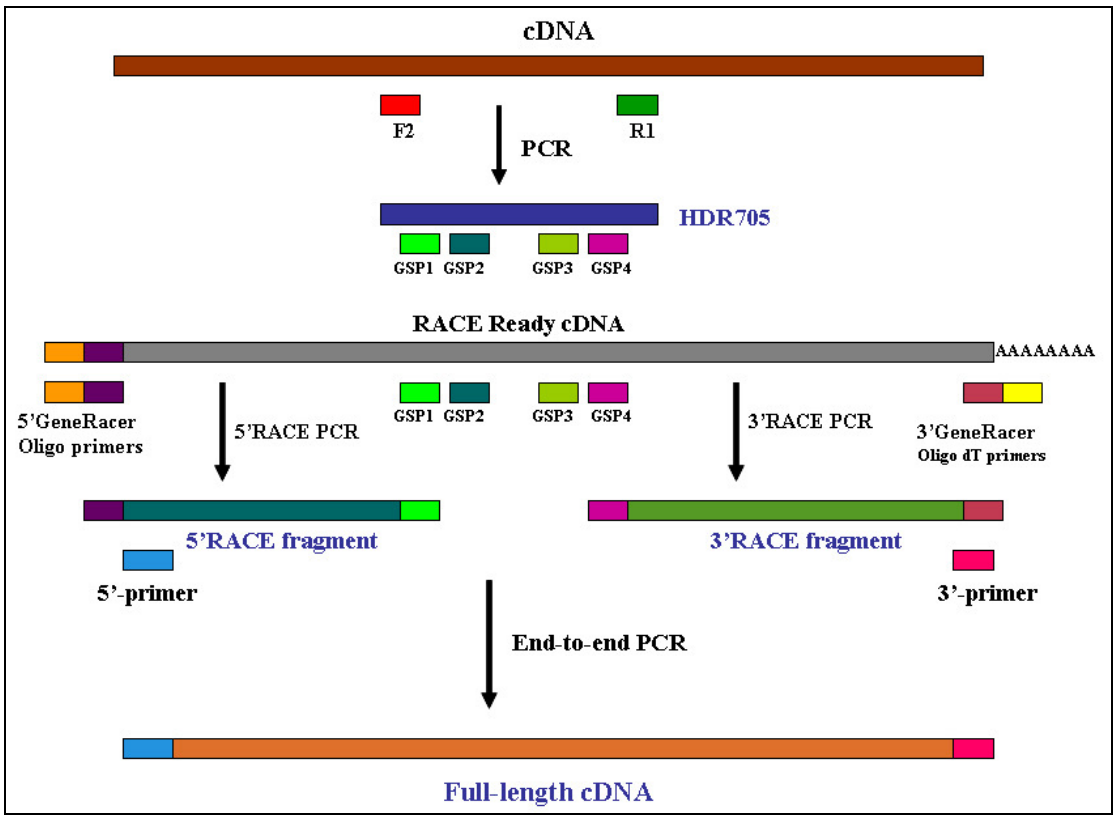


Figure 10 Schematic representation of the *Hevea dxr* cDNA cloning.

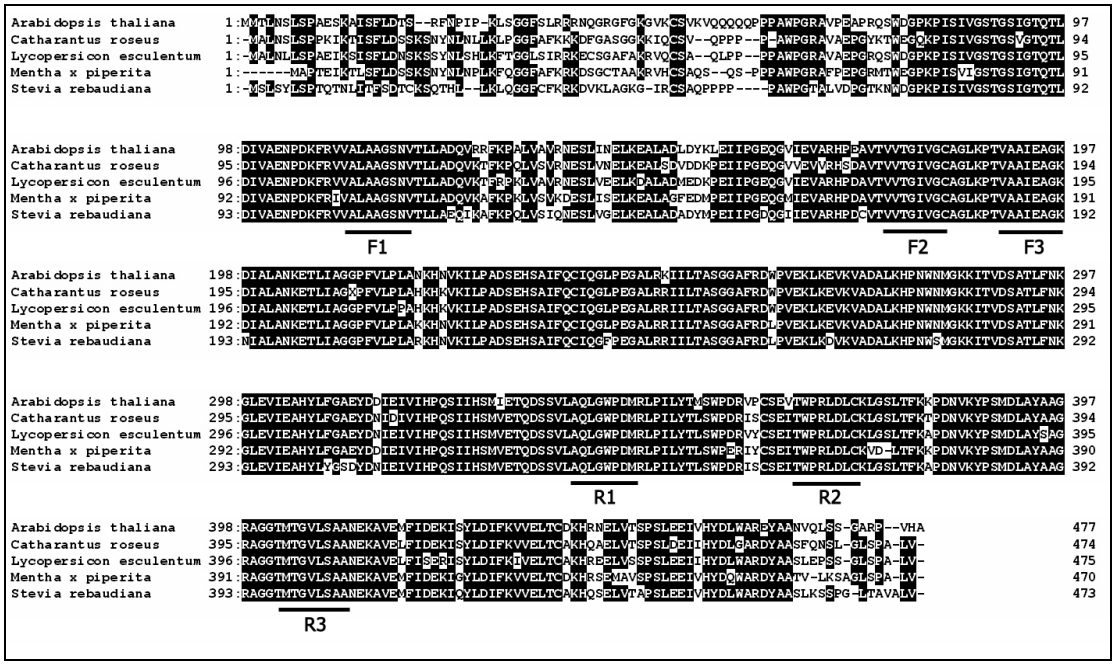


Figure 11 Multiple alignment of plant DXRs. F1-F3 and R1-R3 indicated the position of primer site. The black shades indicate the identical amino acid of the same position.

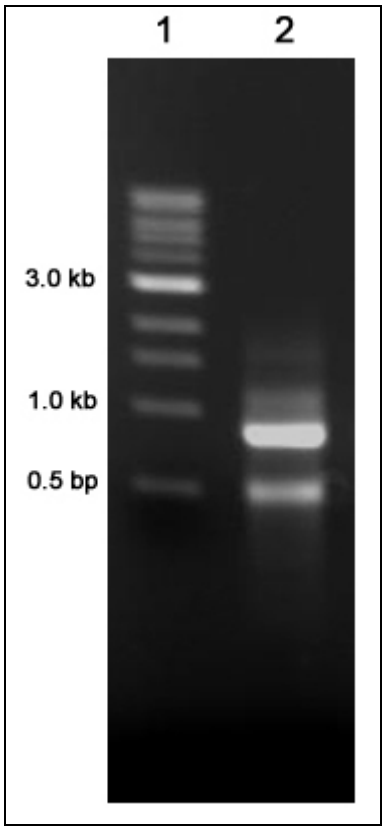


Figure 12 Amplification of cDNA fragment. Lane 1, 1 kb DNA marker; lane 2: PCR products using degenerate primers F2 and R1, electrophoresis on 1% agarose gel

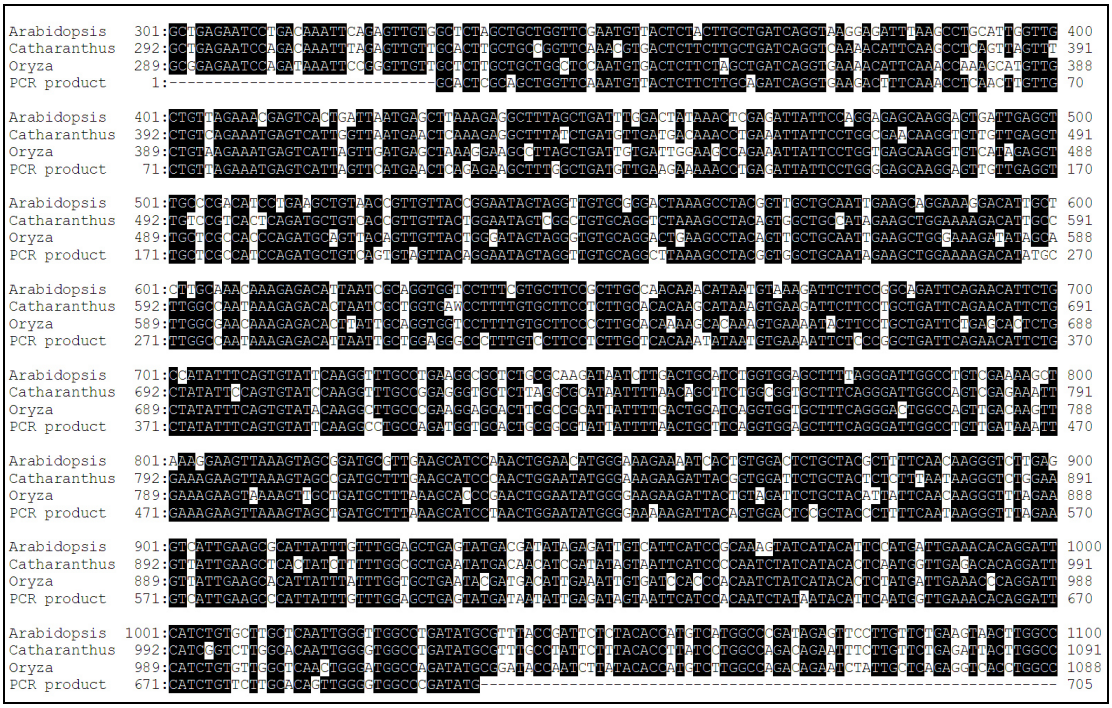


Figure 13 Multiple alignment of nucleotide sequence of *dxr* cDNA fragments. Nucleotide sequence of PCR products obtained from primer F2 and R1.

2.2 Amplification of 5'- and 3'-cDNA ends

According to the nucleotide sequence of the HDR705, the gene specific primers, GSP1-GSP4 (Table2), were designed and used to amplify 5' and 3'-cDNA ends by rapid amplification of cDNA ends (RACE) methods. The PCR products that obtained from nested PCR of 5'- and 3'-cDNA ends of RACE amplification revealed the PCR product band which might be contained DNA sequences corresponded to plant *dxr* (Figure 14). These PCR products were then purified from agarose gel, ligated to pCR4-TOPO and transformed to *E.coli* cells. The plasmid were extracted and purified from host cells. The purified plasmids were then sequenced and analyzed by Genetyx software. After sequence analysis, PCR products obtained from 5'- and 3'-cDNA ends of RACE amplification showed that they had a high sequence homology to the other plant DXR genes. These sequence information were used to design primer for full length amplification.

2.3 Amplification of full length cDNA of *Hevea* DXR gene

According to the nucleotide sequence of the 5'- and 3'-cDNA ends, the specific PCR primers, FDR1, FDR2, RDR1 and RDR2, were designed. The PCR products (approximately 1.4 kb) that amplify by these specific PCR primers were purified and ligated into pCR4-TOPO vector. DNA fragments was obtained after full length cDNA was cut with restriction enzyme *Eco*RI indicating that this DNA fragment contained one *Eco*RI site. This result was revealed by the determination of DNA sequence using ABI PRISM 310 Genetic Analyzer and GENETYX software. The sequence revealed the presence of an ORF consisting of 1,413 bp capable of encoding a 471 amino acid protein with a predicted molecular mass of 51 kDa. We termed this clone HbDXR (Figure 15). The GenBank accession number of this cDNA had been assigned as DQ437520. The amino acid sequence deduced from the nucleotide sequence was compared with the NCBI databases. It had a very high homology throughout the entire ORF with the plant DXRs: *L.esculentum* (86%), *A.thaliana* (82%), *S.rebaudiana*, (82%) and *O.sativa* (80%) (Figure 16).The highly conserved GSTGSIG motif, proposed to constitute the NADPH binding site of a ketol acid reductoisomerase (Kuzuyama *et al*., 2000c), was present near the N-terminal of HbDXR (positions 81-87).

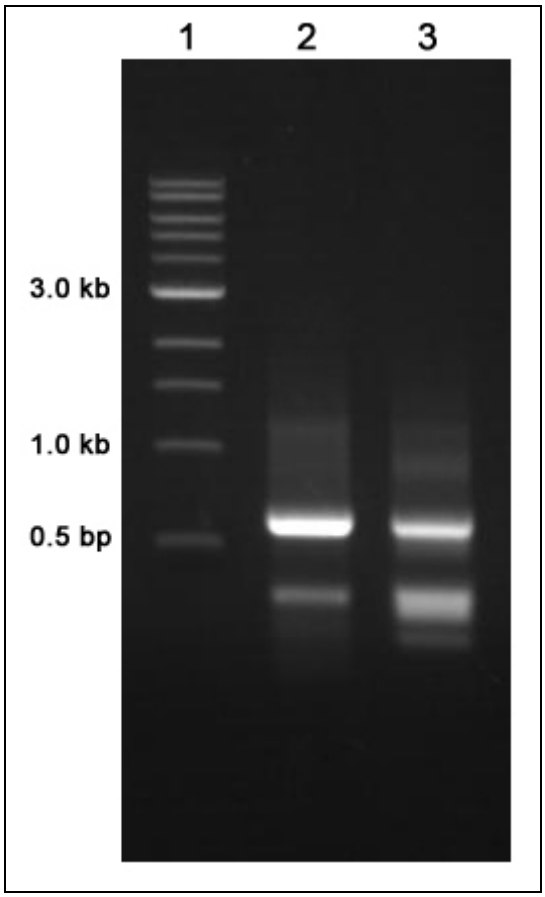


Figure 14 PCR products of 5'- and 3'-RACE method electrophoresis on 1% agarose gel. Lane 1, 1 kb DNA marker; lane 2: 5'-RACE PCR products (First PCR using 5'-RACE primer and GSP1, nested PCR using nested primer and GSP2; lane 3: 3'-RACE PCR products (First PCR using 3'-RACE primer and GSP3, nested PCR using nested primer and GSP4).

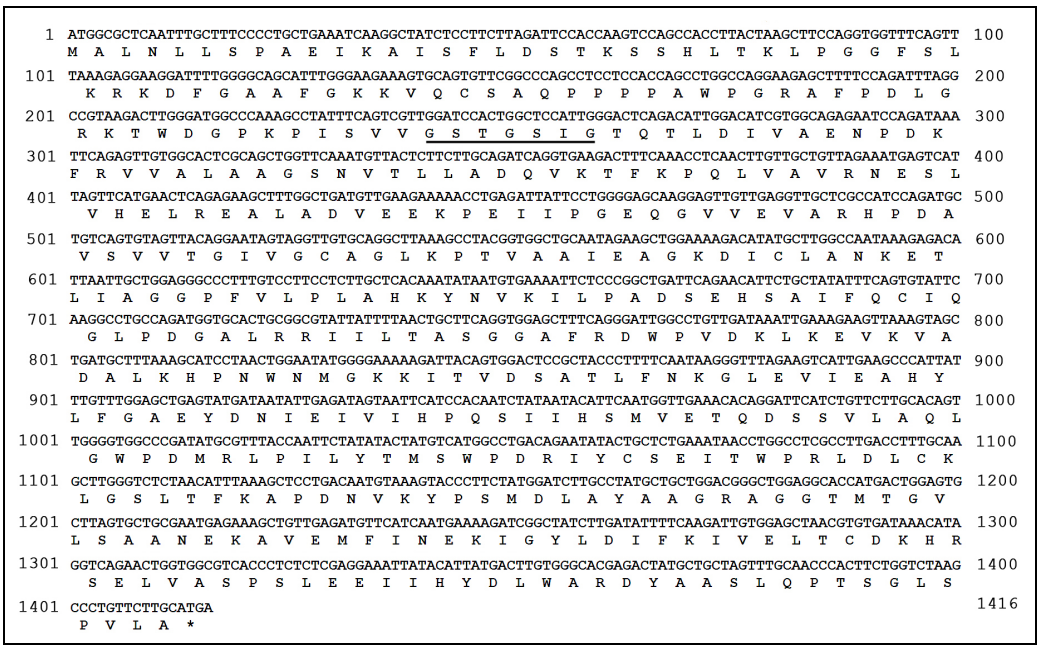


Figure 15 Nucleotide and deduced amino acid sequence of HbDXR. The highly conserved GSTGSIG motif, proposed to constitute the NADPH binding site of a ketol acid reductoisomerase, is underlined.

3. Comparison of the primary structure of *Hevea* DXR with other plant DXRs and prokaryotic DXRs

To characterize the sequence of the N-terminal region, two prokaryotic DXRs; *E.coli* (Kuzuyama *et al*., 2000c) and *Synechococcu sleopoliensis* (Miller *et al*., 2000) DXRs, were aligned with the plant DXRs. (Figure 16).The plant enzyme contains an extension of about 80 residues that is not present in the prokaryotic sequences. The extended region at the N-terminal of the plant DXRs indicated that it was likely to be a transit peptide for a plastid protein and contained a conserved cleavage site (Carretero-Paulet *et al*., 2002). A CS motif, putative conserved processing site, was present at about position 50 of plant proteins (Cys-Ser-Ala residues at positions 49-51 of the HbDXR sequence). These results suggested that the N-terminus of the mature *Hevea* and other plant enzymes is located significantly upstream in comparison with bacterial enzymes. Accordingly, an approximate length of only about 50 amino acid residues would be predicted for the plastid-targeting sequences of plant DXR proteins and the length of the mature protein would exceed that of the bacterial enzymes by about 30 amino acid residues. The extended region at the C-terminal side of the putative cleavage site contains a consensus motif PPPPAWPGRA (positions 53-62 of HbDXR), defined as the Pro-rich region at the N-terminus of the mature plant DXR (Kuzuyama *et al*., 2000c).The biological function of this Pro-rich region in plants is now not fully understand.

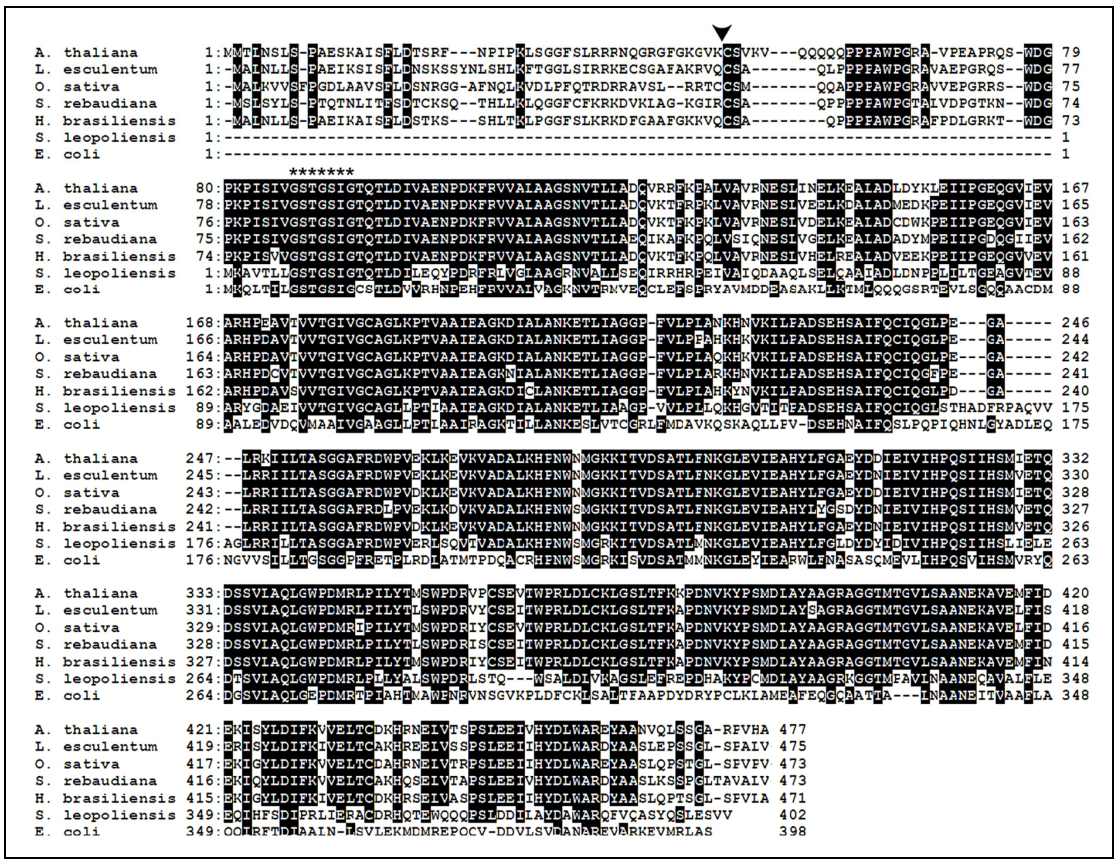


Figure 16 Multiple sequence alignment of the deduced amino acids of HbDXR and DXRs from four other plants and two from bacteria. The residues boxed in black indicate the positional identity for at least four of six compared sequences. Dashes indicate gaps introduced in order to optimize the alignment. Asterisks indicate the NADPH-binding motif. The putative cleavage site is indicated with an arrowhead. The cDNA accession numbers are *A. thaliana*; AF148852, *L. esculentum*; AF331705_1, *O. sativa*; AF367205, *S. rebaudiana*; AJ429233, *H. brasiliensis*; DQ437520, *S. leopoliensis*; AJ250721 and *E. coli*; AB013300.

4. Phylogenetic tree analysis of HbDXR and other DXRs

Phylogenetic analysis, based on the alignment of 22 plant *DXR*s, which is deduced by the CLUSTAL W program (as implemented in MEGA3 program), revealed a relationship between the phylogeny of *DXR* sequences and phylogenic distances (Figure 17). The cDNA accession number are Salvia (*S. miltiorrhiza*); DQ991431, Mentha (*M.* x *piperita*); AF116825, Plectranthus (*P. barbatus*); AY515699, Antirrhinum (*A. majus*); AY770406, Picrorhiza (*P. kurroa*); DQ347963, Catharanthus (*C. roseus*); AF250235, Nicotiana (*N. tabacum*); DQ839130, Lycopersicon (*L. esculentum*); AF331705, Stevia (*S. rebaudiana*); AY834755, Artemisia (*A. annua*); AF182287, Chrysanthemum (*C.* x *morifolium*); AB205048, Arabidopsis (*A. thaliana*); AF148852, Pueraria (*P. Montana var lobata*); AY315651, Cistus (*C. creticus*); AY297794, Populus (*P. tremula* x *P. alba*); AJ574852, Croton (*C. Stellatopilosus*); EF451544, Hordeum (*H. vulgare*); AJ583446, Oryza (O. sativa); AF367205, Ginkgo (*G. biloba*); AY494186 and Taxus (*T. cuspidata*); AY575140. There appeared to be three major lineages of these genes; gymnosperms, monocots, and eudicots. The eudicot genes formed two major clades, rosids and asterids. The *Hevea* gene was most closely related to the one from *Croton stellatopilosus* which is in the same Euphorbiaceae family as the rubber tree.

Protein sequences were used instead of the nucleotide sequences for the preliminary consensus analysis (Figure 18). The simplified tree was constructed by neighbor-joining method from an alignment of DXR amino acid sequences from plants, cyanobacteria, and proteobacteria species. All plant sequences group together, plant enzymes are highly conserved in evolution, and the cluster of cyanobacterial sequences is relatively close to that of the plants, whereas the distances between bacterial proteins and the plant/cyanobacterial cluster are much larger. The similarities of sequences considered that **dxr** have evolved directly from the plastid ancestor. Since the plastids are thought to have arisen as a result of an endosymbiotic event in which a cyanobacteria invaded a primitive eukaryotic host.

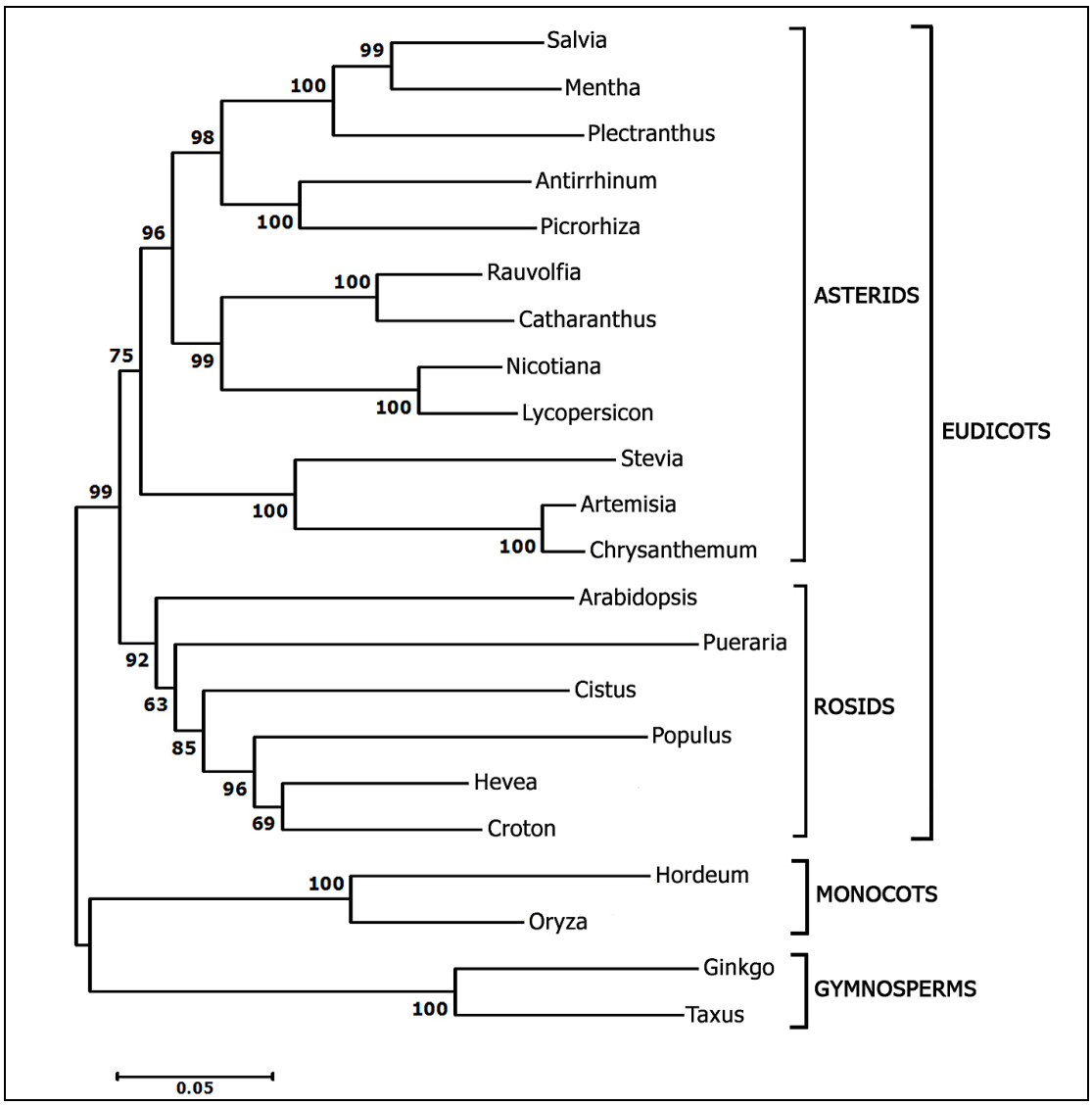


Figure 17 A phylogenetic tree generated by MEGA3 program based on the degree of similarity between plants *dxr*s. Sequence analysis was performed using CLUSTAL W. The branches were validated by bootstrap analysis from 500 replications, which are represented by percentages in the branch nodes.

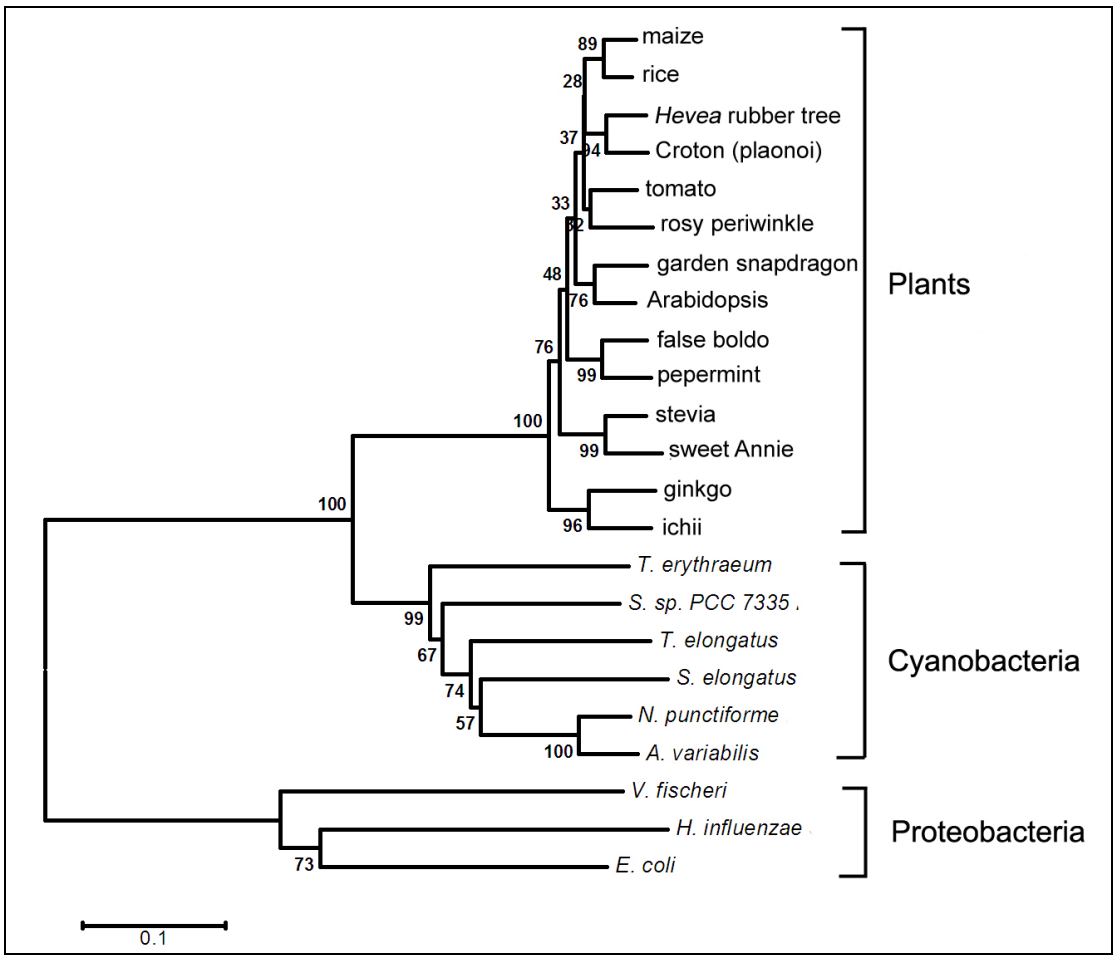


Figure 18 A phylogenetic tree generated by MEGA3 program, neighbor-joining analysis, from an alignment of DXR amino acid sequence from 14 plant species, 6 cyanobacteria species, and 3 proteobacteria. Sequence analysis was performed using CLUSTAL W. The branches were validated by bootstrap analysis from 500 replications, which are represented by percentages in the branch nodes.

5. Overproduction of the recombinant HbDXR protein in *E. coli* cells

In order to overexpress HbDXR in *E. coli*, various expression vectors harboring coding sequences of *Hbdxr* were constructed using Gateway system. The *Hbdxr* sequences were amplified by PCR using a primer pair for full length amplification, which were then subcloned into pENTR/TEV/D-TOPO entry vector The entry clones were applied for the recombination reaction with Gateway destination vectors, pDEST15 or pDEST17 using LR Clonase to construct plasmids for the expression of HbDXR fused with glutathione S-transferase (GST)-tag (pDEST15) or Histidine-tag (pDEST17).

Because overexpression of exogenous DXR in *E. coli* was expected to affect the viability of the host cells causing perturbation of the endogenous IDP biosynthetic pathway, the expression plasmids were introduced not only into BL21(DE3) but also BL21-AI, which can regulate the arabinose-inducible expression of recombinant proteins strictly. As results, we succeeded in the overexpression of HbDXR both in BL21-AI and BL21(DE3) (Figure 19). By induction with IPTG or arabinose for BL21(DE3) and BL21-AI, respectively, the *E. coli* cells harboring pDEST17-HbDXR produced recombinant proteins at 54 kDa, 51 kDa HbDXR with 2.7 kDa His-tagged, but non-induced *E. coli* cells harboring pDEST17-HbDXR did not show detectable expression proteins. However, most of the recombinant proteins, His-tagged-HbDXR and GST-tagged-HbDXR, were included in insoluble pellet fractions as inclusion body. We then tried to obtain the soluble protein by affinity purification from insoluble fractions in denaturing condition, followed by renaturation by step-wise dialysis. Although we could obtain purified HbDXR (Figure 20), the recombinant protein did not show distinct DXR activity. These result suggested that the predicted transit peptide at the N-terminal region may affect the protein folding or solubility of HbDXR in *E. coli* as the case of the recombinant DXR protein from *A. thaliana* (Rohdich *et al*., 2006).

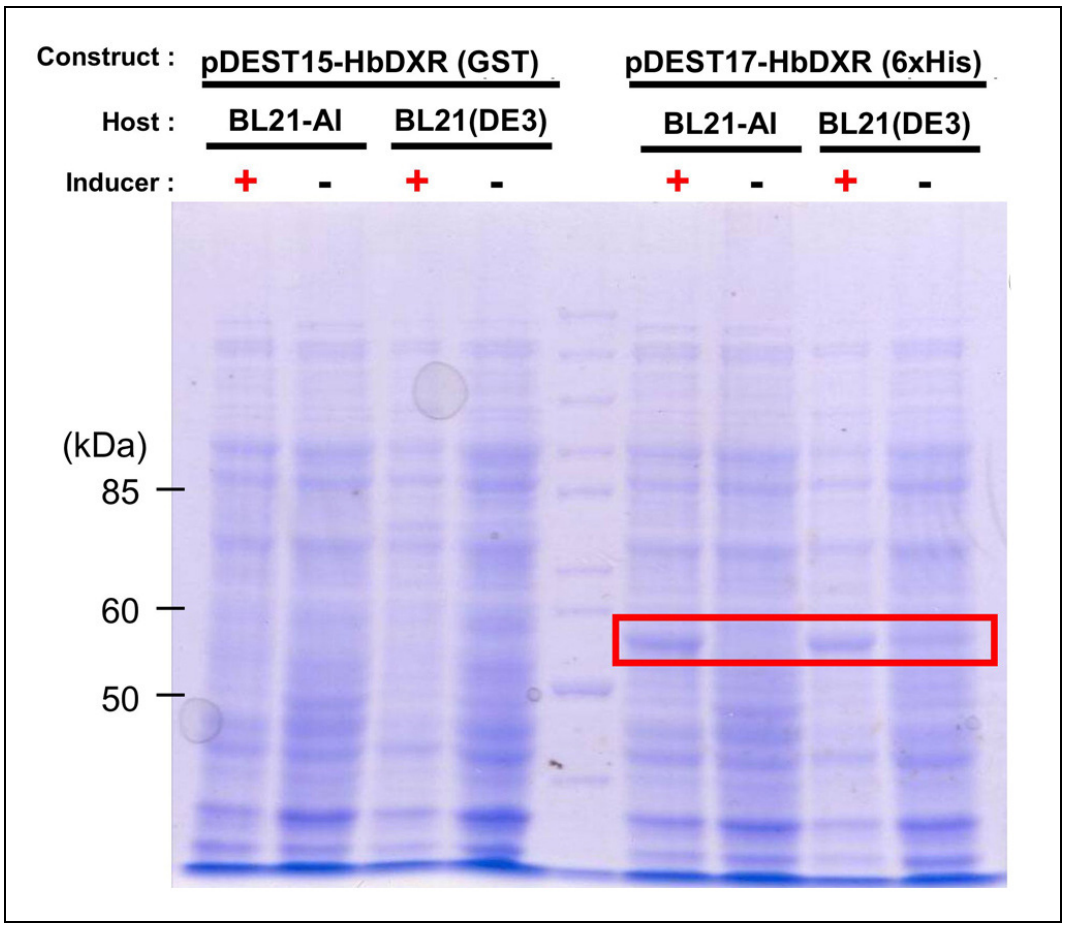


Figure 19 Overexpression of HbDXR in *E.coli*. Inducers for BL21-AI and BL21(DE3) were arabinose and IPTG, respectively. Calculated molecular weight of each is as follow; HbDXR: 51 kDa, GST: 27.7 kDA, 6xHis: 2.7 kDa.

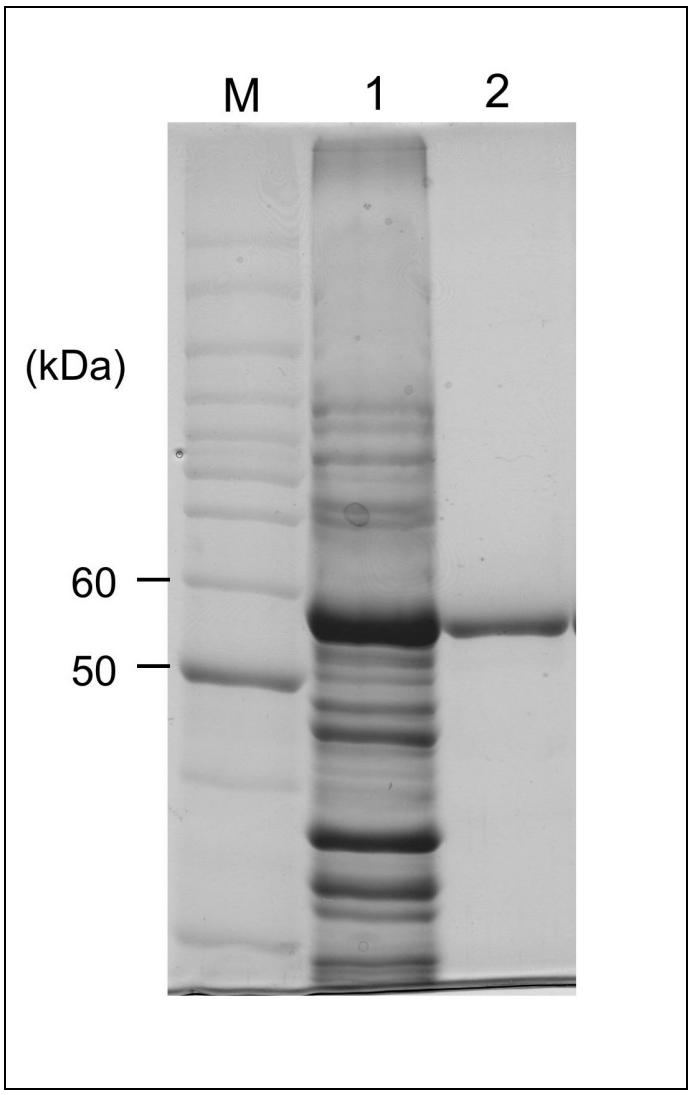


Figure 20 Affinity purification of recombinant HbDXR from pDEST17-HbDXR (6xHis) expression vector. M: molecular weight marker, lane 1: *E.coli* crude protein denaturated by guanidinium, lane 2: recombinant protein after affinity chromatography.

6. Expression analysis of HbDXR mRNA

To examine the level of mRNA expression for DXR in latex and compare with various *Hevea* tissues, RT-PCRs using total RNA extracted from young stems, inflorescences, leaves and latex were performed. As shown in Figure 21, the levels of PCR products in the inflorescences and young stem were higher than that of the latex tapped from the tree. The PCR product was, however, barely detected in the mature leaves. These results indicate that HbDXR function especially in young actively dividing tissues and latex of *H. brasiliensis*. The MEP pathway having roles in growth and tissues development and that may also exist to supply IDP in latex for rubber biosynthesis, although other enzymes of the MEP pathway in *Heve*a latex remain to be elucidated.

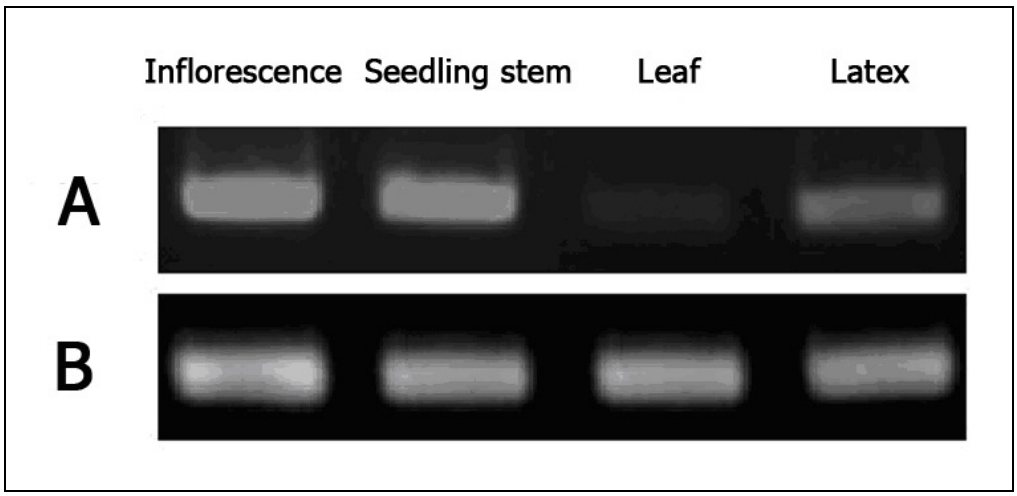


Figure 21 Analysis of mRNA expression patterns by RT-PCR. (A) mRNA expression of HbDXR (28 cycles), (B) Control (18S rRNA).

7. Subcellular localization of HbDXR

HbDXR was predicted by the ChloroP algorithm to have a putative plastid transit peptide in the 80 amino acid region at the N-terminus thus indicating the function of HbDXR in the plastid. Green fluorescence protein (GFP) was used to test if the putative transit peptide is functional. GFP, a novel marker protein isolated from the jellyfish *Aequorea victoria*, permits direct observation of the localization and intracellular trafficking of fusion proteins *in vivo*. The GFPs emit green light (509 nm) when excited by either blue (475 nm) or UV (395 nm) light. Chlorophyll can emit red autofluorescence when excited, can be used to indicate the location of chloroplast. The gateway entry clone harboring *Hbdxr* without stop codon was applied for recombination with a Gateway destination vector. The pGWB5 was used for construction of a binary vector to express HbDXR fused with sGFP, at C-terminal region, in plant cells. The resulting plasmid was introduced into *Agrobacterium tumefaciens* GV3101 (pMP90). *Arabidopsis* T87 cultured cells were transformed by co-cultivation with GV3101 (pMP90) carrying the resulting construct. The transformed T87 cells were cultured in conditions as described in chapter 2 until calli were formed. Small cell aggregates of calli were then transferred to medium and the suspension was kept under continuous illumination. After 7-10 days, an aliquot of cell suspensions was transferred to fresh medium and maintained as an independent cell line. For GFP imaging, T87 cells expressing GFP-fused HbDXR were observed under BX50 microscope equipped with a FLUOVIEW confocal scanning system.The green (GFP) and red (chlorophyll autofluorescence) emissions were detected with filters BA510IF-BA550RIF and BA585IF, respectively. The images were then pseudo-colored and combined into one image (Figure 22). As expected, fluorescence of HbDXR fused with GFP at the C-terminal region was detected in chloroplasts of *Arabidopsis* cultured cells, and this was confirmed by an overlapping of the autofluorescence of chloroplasts as shown in Figure 22. These results not only confirm the *Arabidopsis* chloroplastic localization of the HbDXR but also suggest its possible localization in *Hevea* latex plastids.

In higher plant, the MEP pathway is known to localize in plastid, such as chloroplast. On the other hands, latex has been known to contain plastid-like organelles

termed Frey-Wyssling particle, which contains carotenoids. Localization of HbDXR in chloroplast of *Arabidopsis* cells indicate that HbDXR has distinct transit peptide and that the transit peptide may function in translocation of HbDXR to the plastid-like organelles, Frey-Wyssling particle, in latex.

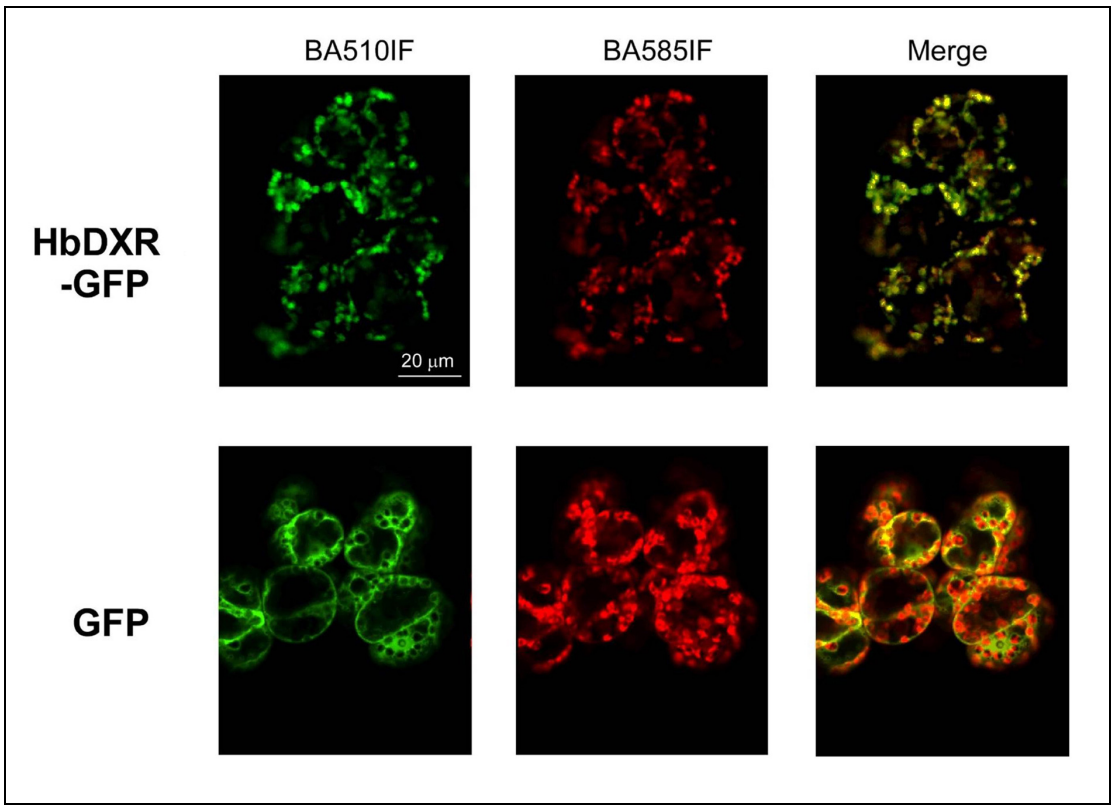


Figure 22 Subcellular localization of HbDXR in Arabidopsis T87 cells. Cells expressing HbDXR, fused with GFP at the C-terminus and GFP, were observed by laser confocal microscopy. The panels indicated as BA510IF, BA585IF, and Merge show fluorescence images of GFP, the autofluorescence of chlorophylls, and the merged image of green and red fluorescence, respectively.

8. Measurement of pigments in *Arabidopsis* cultured cells expressing of HbDXR

In the MEP pathway of higher plant, enzymes catalyzing the first two steps, DXP synthase (DXS) and DXP reductoisomerase (DXR), were considered as key enzymes controlling IDP biosynthesis from MEP pathway. DXS is considered to be one of rate-limiting enzymes in the MEP pathway, which was shown by change in the levels of plastidial pigments in transgenic plants manipulating the expression level of DXS (Estévez *et al*., 2001; Botella-Pavía *et al.*, 2004). On the other hand, DXR is also thought as a key enzyme of MEP pathway, because DXR catalyzes the first committed step of the MEP pathway. There are several reports showing the positive correlation between the expression levels of DXR and the accumulation of isoprenoids biosynthesized using IDP from the MEP pathway (Walter *et al.*, 2000; Mayrhofer *et al*., 2005; Bede *et al*., 2006). Moreover, some reports showed direct evidences for the contribution of DXR in the regulation of isoprenoid biosynthesis by monitoring the accumulation of isoprenoids in the transgenic plants which were enhanced flux of the MEP pathway by constitutive expression of DXR (Mahmoud and Croteau 2001; Carretero-Paulet *et al.*, 2006). To investigate effect of HbDXR on the flux of MEP pathway, HbDXR was expressed constitutively under the control of cauliflower mosaic virus 35S promoter in *Arabidopsis* T87 cells. The expression levels of HbDXR in independent transgenic T87 lines were analyzed by semi-quantitative RT-PCR. All of transgenic lines tested showed distinct expression of HbDXR (Figure 23A). Because the pair of primers used for RT-PCR could anneal to the sequence of *Arabidopsis* DXR, we could detect endogenous *Arabidopsis* DXR expression, showing the relatively high expression levels of HbDXR in each transgenic T87 line. Total amounts of chlorophylls calculated from absorbance values of pigments were slightly varied among the transgenic cell lines (Figure 23B). Total chlorophylls were increased in line-2, but decreased in line-1. In addition, we could not find significant difference in total carotenoids among the transgenic lines (Figure 23C). In conclusion, significant correlation between the expression level of *HbDXR* and the accumulations of plastidial pigments could not observed in this study. These results have been suggested to be implicated in post-transcriptional and post-translational regulations of plastidial MEP pathway proteins. Post-transcriptional and post-translational regulations of the

enzymes in MEP pathway have been described in several reports (Guevara-García *et al*., 2005; Sauret-Güeto *et al*., 2006; Xia *et al*., 2008) but the mechanism by which plastid-encoded proteins directly or indirectly affect the accumulation of certain plastid-targeted proteins remains unknown.

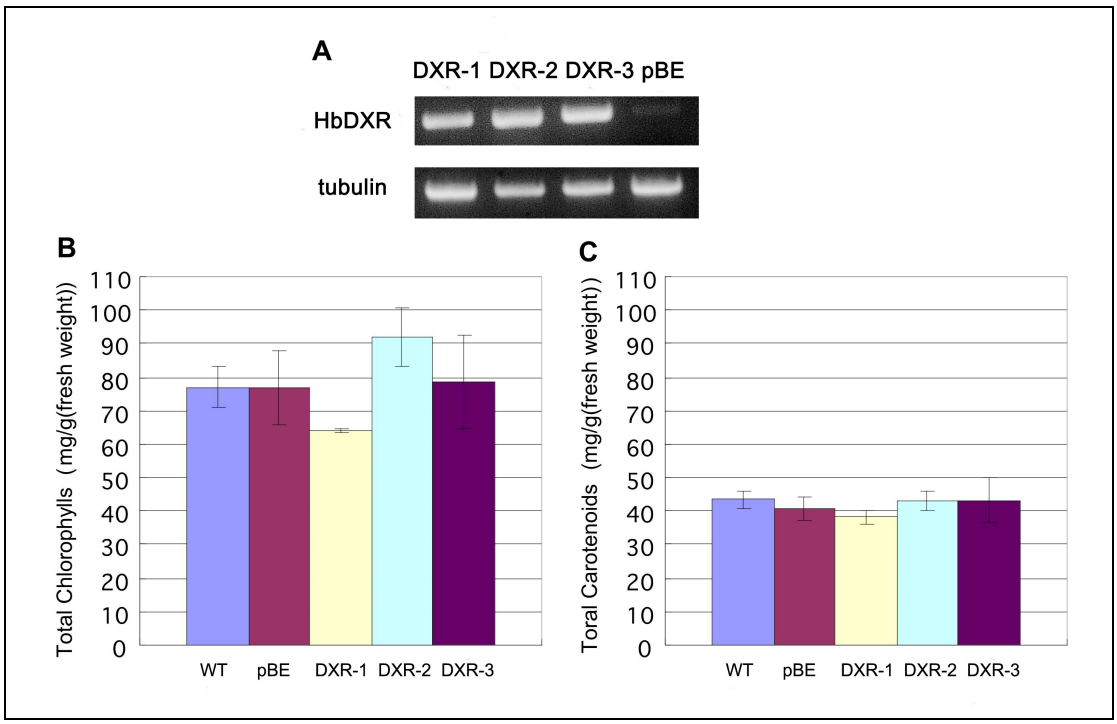


Figure 23 Level of platidial isoprenoid in HbDXR-overexpressing T87 cell lines. A. Expression levels of HbDXR in the transgenic T87 cell lines. pBE: vector control harboring pB2113Not, DXR 1-3 independent three lines of the transgenic T87 cell lines harboring pBE-HbDXR. B. Amount of total chlorophylls in the transgenic cell lines and T87 cell (WT). C. Amount of total carotenoids in the transgenic cell lines and T87 cell (WT). Error bars show standard deviations from three independent experiments.

CHAPTER 4

Discussion

1. [^{14}C]-labeled prenyl products derived from [^{14}C] glucose

An autoradiogram of the prenyl alcohol products produced from [^{14}C] glucose incorporation into FW particles revealed a major [^{14}C] C_{15} prenyl products. The incubation of similar reaction mixture with fosmidomycin, inhibitor of DXR, gave a similar pattern to the original without fosmidomycin but the intensity was significantly reduced. This result may indicate the existing of MEP pathway in the FW particles. Mevilonin was also added to the incubation mixture to inhibit HMG CoA reductase, a rate-limiting enzyme of the MVA pathway that might be present due to contamination by residual latex cytosol and the thread-like reticulum in the original isolated FW particles. The blocking effect of mevilonin on prenyl alcohol products produced from [^{14}C] glucose was significantly lower than that of fosmidomycin. This result confirmed the presence of the MEP pathway in the FW particle preparation.

The presence of MEP pathway was in agreement with their suggestive role in isoprenoids, such as carotenoids and plastoquinone biosynthesis. The highly complicated structure of FW complex suggests that it has an important function in the metabolism of *Hevea* latex. Since the particles contain plastoquinone and plastochromanol, in which is β-carotene synthesized, they are assumed to be modified plastids (Ohya and Koyama, 2001). Recently, the prenylquinones in the lipids extracted from FW particles were identified and compared to those of leaf chloroplast and mitochondria. Their chemical structures were confirmed as plastoquinone-9 (PQ-9). Moreover, the *Hevea* solanesyl diphosphate synthase (HbSDS) gene encoding solanesyl diphosphate synthase which involved in the synthesis of the C_{45} prenyl side chain of the PQ-9, was isolated and characterized from the rubber latex (Phatthiya *et al*., 2007). These findings, together with our results presented in this radioactive glucose incorporation experiment, indicated that FW particles indeed possess the MEP pathway. Since the FW particles have an important function in the laticiferous vessels where

rubber biosynthesis is highly active. In addition to supplying IDP for other minor isoprenoids biosynthesis, this MEP pathway might, therefore, provide an alternative means of generating IDP for the biosynthesis of *cis*-polyisoprene or rubber, the major synthesized isoprenoid compounds, in *Hevea* laticifer through the tubular thread-like reticulum. According to the enzymatic studies and stereochemical configuration of natural rubber molecule, C_{15}-farnesyl diphosphate (FDP) seems to be the preferred starting molecule for rubber biosynthesis (Steinbüchel, 2003). The presence of [^{14}C] C_{15}-OH in the autoradiogram indicated that the FW particles may be also involved in providing the initiating primer for the rubber biosynthesis in the *Hevea* tree.

2. Isolation of *Hevea* DXR

Our previous detection of radio labeled prenyl products in the isolated putative FW plastids after incubation with [^{14}C] glucose, confirmed the existing of MEP in FW particles, warranted an attempt to study and isolate the gene encoding for 1-deoxy-D-xylulose-5-phosphate reductase (DXR), the first step specific to isoprene biosynthesis by this pathway, in *Hevea* latex.

The *dxr* genes from a variety of sources have been cloned: *E.coli* (Kuzuyama *et al*., 1998b)*Plasmodium falciparum* ((Jomaa *et al*., 1999), *Arabidopsis thaliana* (Schwender *et al*., 1999), *Mentha* x *piperita* (Lange and Croteau, 1999), *Zymomonas mobilis* (Grolle *et al*., 2000), *Synechococcus leopoliensis*(Miller *et al*., 2000), *Catharanthus roseus* (Veau *et al*., 2000), *Pseudomonas aeruginosa* (Altincicek *et al*., 2000), *Streptomyces coelicolor* (Cane *et al*., 2001), *Lycopersicon esculen*tum (Rodríguez-Concepción *et al*., 2001), *Synechocystis sp. PCC6803* (Yin and Proteau, 2003), *Mycobacterium tuberculosis* (Argyrou and Blanchard, 2004), and *Zea mays* (Hans *et al*., 2004). The general characteristics of the DXR enzymes are that they are homodimers with monomer molecular weights of 42-45 kDa. The cofactor NADPH and a divalent cation (Mg^{2+}, Mn^{2+}, or Co^{2+}) are required for activity. In plants, the gene product typically has a plastidial targeting sequence that is subsequently cleaved to provide the active enzyme (Lange and Croteau, 1999; Carretero-Paulet *et al*., 2002), although

there are examples of the fully active full-length recombinant enzymes. (Lange and Croteau, 1999; Rodríguez-Concepción *et al*., 2001).

The *dxr* from *Hevea* was isolated by PCR amplification method using latex cDNA library as a template. First, in order to obtain a *dxr* cDNA internal fragment, six degenerate oligonucleotide primers were designed from the amino acid sequences that are highly conserved in various plants DXRs. The expected size PCR products obtained by these set of primers were directly subcloned into pGEMT-easy vector, and their nucleotide sequences were determined. The clone containing 700 bp PCR products was found to have a sequence that resembled to plants DXR sequences. The sequence information of putative *Hevea dxr* (HDR705) was used to design primers for 5'- and 3'-cDNA ends amplification. Two RACE strategies were employed to obtain the sequence information necessary to isolate a full-length cDNA using two sets of primers. The ORF of *Hevea dxr* consists of 1,413 capable of encoding a 471 amino acid protein with a predicted molecular mass of 51 kDa. The amino acid sequence deduced from the nucleotide sequence was compared with the NCBI databases. It had a very high homology throughout the entire ORF with the plant DXRs. The highly conserved GSTGSIG motif, proposed to constitute the NADPH binding site of a ketol acid reductoisomerase (Kuzuyama *et al*., 2000c), was present near the N-terminal of HbDXR (positions 81-87).

3. Comparison of the primary structure of *Hevea* DXR with other plant DXRs and prokaryotic DXRs

A detailed sequence comparison, including all putative DXR amino acid sequences from plants that are available in public databases, revealed, among other distinctive features, a proline-rich block of seven contiguous amino acids residues (PPPAWPG) located upstream of the N-terminus of bacterial orthologs that was absolutely conserved in all plant sequences available in the databases, biological function in plants is now unclear. Moreover, a CS motif, hypothetical cleavage sites, and a tryptophan residue (residues 49 and 71 of the *Hevea* DXR protein, respectively) were absolutely conserved within the N-termini of the plant proteins. These findings suggested that the N-terminus of the mature plant enzymes is

located significantly upstream in comparison with bacterial enzymes. Consequently, an approximate length of only about 50 amino acid residues would be predicted for the plastid-targeting sequences of plant DXR proteins and the length of the mature protein would exceed that of the bacterial enzymes by about 30 amino acid residues. The estimated length and molecular mass of HbDXR mature protein are 423 amino acid residues and 45.8 kDa, respectively. The results thus confirm the earlier indication of subcellular localization of the MEP pathway enzymes in plastids of higher plants (Carretero-Paulet *et al*., 2002),

4. Phylogenetic tree analysis of HbDXR and other DXRs

Phylogenetic tree were generated by MEGA3 program based on degree of similarity between 22 cDNA sequences of plants DXRs. Sequence analysis was performed using CLUSTAL W and the branches were validated by bootstrap analysis from 500 replications. The resulting phylogenetic tree demonstrated that the plants DXR enzymes are highly conserved in evolution. These results concur with sequence comparison showed a high degree of similarity among DXR protein member from the plant kingdom, more than 75% sequence identity, and at least 87 % conservation. Sequence analysis has shown that all of the genes in the MEP pathway are highly conserved (Lange *et al*., 2000). This is also true for the *dxr* gene. There appeared to be three major lineages of these plants genes, in evolution order; gymnosperms, monocots, and eudicots. The eudicot genes formed two major clades, rosids and asterids. The *Hevea* gene belonged to rosids clads and was most closely related to the one from the same Euphorbiaceae family, *Croton stellatopilosus*.

Plastids are thought to have arisen as a result of an endosymbiotic event in which a cyanobacteria invaded a primitive eukaryotic host. It has been accepted that the genes encoding the MEP pathway enzymes in plants have been acquired during the endosymbiotic event with an ancestral cyanobacteria (Sprenger *et al*., 1997). According to phylogenic tree from an alignment of DXR amino acid sequence from 14 plant species, 6 cyanobacteria species, and 3 proteobacteria in Figure 18, the plant enzymes share the great similarity with the homologues from ***cyanobacteria***, providing a reasonably straightforward argument that

this nuclear encoded enzyme was acquired through gene transfer to the nucleus in the process of the endosymbiotic origin of plastids.

5. Overproduction of the recombinant HbDXR protein in *E. coli* cells

The full-length *Hbdxr* sequences were applied for the recombination reaction with Gateway destination vectors, pDEST15 or pDEST17 to construct plasmids for the expression of HbDXR fused with glutathione S-transferase (GST)-tag (pDEST15) or Histidine-tag (pDEST17). Since an overexpression of exogenous DXR in *E. coli* was supposed to affect the viability of the host cells causing perturbation of the endogenous IDP biosynthetic pathway. The resulting expression plasmids were, therefore, introduced not only into BL21(DE3) but also BL21-AI, which can strictly regulate the arabinose-inducible expression of recombinant proteins. Accordingly, we succeeded in the overexpression of HbDXR both in BL21-AI and BL21(DE3) (Figure 19). By induction with IPTG and arabinose for BL21(DE3) and BL21-AI, respectively, the *E. coli* cells harboring pDEST17-HbDXR produced recombinant proteins at 54 kDa, 51 kDa HbDXR with 2.7 kDa His-tagged, but non-induced *E. coli* cells harboring pDEST17-HbDXR did not show detectable expression proteins. However, most of the recombinant proteins were included in insoluble pellet fractions as inclusion body. In order to obtain soluble proteins, the insoluble fractions were subjected to affinity purification in denaturing condition, followed by renaturation by step-wise dialysis. Although the purified HbDXR were obtained, the recombinant protein did not show distinct DXR activity. These result suggested that the predicted transit peptide at the N-terminal region may affect the protein folding or solubility of recombinant HbDXR in *E. coli* as the case of the recombinant DXR protein from *A. thaliana* (Rohdich *et al*., 2006). The recombinant expression vector, containing the complete DXR protein sequence of *Arabidopsis* (residues 1-477) could be expressed at a low level in *E.coli* host cells, but the protein was insoluble, and *in vitro* renaturation experiments were unsuccessful. Rohdich and coworkers then investigated the expression of the *Arabidopsis* DXR protein in a pseudomature form. They constructed several expression constructs directing the expression of recombinant proteins starting with amino acid residues 57–95 and only the clone

specifying the protein extending from amino acid residues 57 to 477 afforded enzymatically active protein. Further investigate the expression of HbDXR in a psudomature form may required for our recombinant HbDXR activity.

6. Expression analysis of HbDXR mRNA

The levels of RT-PCR products in the inflorescences and young stem were higher than that of the latex tapped from the tree. The RT-PCR product was, however, barely detected in the mature leaves (Figure 21). These results indicate that HbDXR function especially in young actively dividing tissues, which is consistent with the report that the MEP pathway is active in young organs as found in the case of *Arabidopsis dxr* expression (Carretero-Paulet *et al*., 2002). The MEP pathway might having roles in growth and tissues development, the development of these tissues of the rubber tree require some special plastid isoprenoids, which are synthesized by the MEP pathway. The HbDXR was also transcriptionally expressed in laticifer of *H. brasiliensis*, where rubber is accumulated, and that may also function to supply IDP in latex for rubber biosynthesis, in addition to other minor isoprenoids synthesized in FW particles, although other enzymes of the MEP pathway in *Heve*a latex remain to be elucidated.

Because carotenoids and phytol side chain of chlorophyll are derived from MEP pathway, the *dxr* expression should be distinguished in photosynthetic tissues. It is notable that HbDXR show low mRNA expression in mature leaves, which contains photosynthetic plastids, compared with that of the latex. This result suggested that this HbDXR homologue might more specific expressed for predicted plastids, FW particles, in latex and might play some roles in rubber biosynthesis.

7. Subcellular localization of HbDXR

In order to demonstrate that DXR enzyme is localized to the plastid, we fused HbDXR at the C-terminal with reporter green fluorescent protein (GFP). The resulting HbDXR-GFP fusion constructs were transformed into *Arabidopsis* cells and observed under

confocal laser scanning microscope. As expected, fluorescence of HbDXR-GFP was detected in chloroplasts of *Arabidopsis* cultured cells, and this was confirmed by an overlapping of the autofluorescence of chloroplasts. This result consistent with the result that HbDXR was predicted by the ChloroP algorithm to have a putative plastid transit peptide in the 80 amino acid region at the N-terminus, indicate the function of HbDXR in the plastid. In higher plant, the MEP pathway is known to localize in plastid, such as chloroplast. On the other hands, latex has been known to contain plastid-like organelles termed Frey-Wyssling particle, which contains carotenoids. Localization of HbDXR in chloroplast of *Arabidopsis* cells indicate that HbDXR has distinct transit peptide and that the transit peptide may function in translocation of HbDXPR to Frey-Wyssling particle in latex.

8. Measurement of Pigments in Arabidopsis cultured cells expressing of HbDXR

In order to investigate effect of HbDXR on the flux of MEP pathway in plant cells, HbDXR was expressed constitutively under the control of cauliflower mosaic virus 35S promoter in *Arabidopsis* T87 cells. The expression levels of HbDXR in independent transgenic T87 lines were analyzed by semi-quantitative RT-PCR All of transgenic lines tested showed distinct expression of HbDXR. Total amounts of chlorophylls calculated from absorbance values of pigments were slightly varied among the transgenic cell lines, were decreased in first transgenic cell line, increased in second line, and unchanged in third line, compared with wild type line. Moreover, we could not find significant difference in total carotenoids among the transgenic lines. In conclusion, significant correlation between the expression level of *HbDXR* and the accumulations of plastidial pigments could not be observed in this study. These results have been suggested to be implicated in post-transcriptional and post-translational regulations of plastidial MEP pathway proteins. Post-transcriptional and post-translational regulations of the enzymes in MEP pathway have been reported in several reports (Guevara-García *et al*., 2005; Sauret-Güeto *et al*., 2006; Xia *et al*., 2008) but the mechanism by which plastid-encoded proteins directly or indirectly affect the accumulation of certain plastid-targeted proteins remains unknown.

Despite the impressive progress in the elucidation of the MEP pathway in bacteria and plants, much work is still ahead to analyze the contribution of the different enzymes to the control of the flux of intermediates through the pathway that will eventually determine the supply of IDP and DMAPP for the synthesis of plastid isoprenoid end products. The first studies have been carried out with DXS and DXR (Mandel *et al*., 1996; Bouvier *et al*., 1998; Lange *et al*., 1998; Lange and Croteau, 1999; Schwender *et al*., 1999; Araki *et al*., 2000; Chahed *et al*., 2000; Estévez *et al*., 2000, 2001; Lois *et al*., 2000; Veau *et al*., 2000; Walter *et al*., 2000; Mahmoud and Croteau, 2001; Rodríguez-Concepción *et al*., 2001; Carretero-Paulet *et al*., 2002). To date, DXS is the only enzyme of the MEP pathway that has been shown to have a limiting role for isoprenoid biosynthesis in all the systems analyzed, including Arabidopsis (Estévez *et al*., 2001), tomato (Lois *et al*., 2000), and bacteria (Harker and Bramley, 1999; Miller *et al*., 1999; 2000; Kuzuyama *et al*., 2000b; Matthews and Wurtzel, 2000). The role of DXR is less clear. Overexpression studies suggest that DXR activity is not limiting for isoprenoid biosynthesis in bacteria (Miller *et al*., 2000). The dramatic accumulation of carotenoids that takes place during tomato fruit ripening does not require increased levels of *dxr* transcripts and encoded protein either (Rodríguez-Concepción *et al*., 2001). By contrast, overexpression of DXR in peppermint led to increase isoprenoid synthesis (Mahmoud and Croteau, 2001), and a positive correlation was found between enhanced isoprenoid biosynthesis and accumulation of transcripts encoding both DXS and DXR in monocot roots (Walter *et al*., 2000) and periwinkle cell cultures (Veau *et al*., 2000). The distribution of *dxr* and *dxs* transcripts in the *Arabidopsis* plant is similar, with highest levels in light-grown seedlings and inflorescences (Carretero-Paulet *et al*., 2002). However, *dxs* expression precedes that of *dxr* in some organs, such as developing inflorescences, suggesting that DXR instead of DXS might be limiting for the onset of plastid isoprenoid biosynthesis in this case (Carretero-Paulet *et al*., 2002). Together, the results support a general regulatory role for DXS in controlling the metabolic flux through the MEP pathway, whereas DXR activity may be limiting or not depending on the species, organ, and/or developmental stage. It is likely that other enzymes of the MEP pathway may also contribute to regulate the supply of intermediates for plastid isoprenoid biosynthesis, but this remains to be established.

CHAPTER 5

Conclusion

The membrane-free preparation obtained from FW particles was shown to be active in converting [^{14}C] glucose into radio labeled allylic diphosphate products and more efficiently inhibited by fosmidomycin, the MEP pathway inhibitor, than mevilonin, the inhibitor used to block the MVA pathway. This result confirmed the presence of the MEP pathway in the FW particles and may play role in supplying both IDP monomer as well as initiating allylic diphosphate primer, FDP (Tanaka et al., 1996), for the *in vivo* rubber formation. The *Hevea* latex thread-like reticulum comprising FW particles had previously been proposed as a site for rubber biosynthesis (Southorn 1961), similarly to the reported finding on the role of endoplasmic reticulum in the ontogeny of rubber formation in guayule, *Parthenium argentatum* (Backhaus *et al*., 1983). Accordingly, the *Hevea* rubber latex gene (*Hbdxr*) encoding for the first committed step enzyme in the MEP pathway was successfully cloned from rubber latex cDNA. This gene contains a putative ORF encoding a protein consisting of 471 amino acid residues with a predicted molecular mass of 51 kDa. The study on the localization of HbDXR in the chloroplast of *Arabidopsis* cells indicated that HbDXR has a distinct transit peptide function in translocation of the HbDXR to a plastid. Latex FW particles have been assumed to be modified plastids due to the presence of plastoquinone, plastochromanol and isoprenoids (Ohya and Koyama, 2001; Phatthiya *et al*., 2007). Hence, the *Hbdxr* may be expressed and localized in the latex FW plastid-like particles. Moreover, the levels of *Hbdxr* mRNA transcripts in actively young latex containing tissues, inflorescences and seedling stems, were shown to be higher than that of the latex tapped from mature latex vessels localized in the bark of the rubber tree. This result is opposite to the barely detectable transcript shown in the leaves rich in light gathering plastids. Thus, this *Hbdxr* homologue may be specific to the putative FW plastids specialized in the biosynthesis of rubber. It remains to be seen whether the IDP and FDP required for the biosynthesis of rubber polymer, in the proposed thread-like reticulum, is also mainly contributed by the FW particles.

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List of Publication and Proceeding

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