ISOLATION OF XET GENE INVOLVED IN WOOD FORMATION IN *SHOREA PARVIFOLIA* DYER *PARVIFOLIA*

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ABSTRACT

Wood formation genes played an important internal factor besides the hormonal aspects in xylogenesis, which control the development of secondary growth in trees. One of the identified enzymes, xyloglucan endotransglycosylase (XET), is discovered to modify intermicrofibrillar xyloglucan chains, a major component of primary cell walls in dicots to allow wall-loosening required for plant cell expansion. In this study, *Shorea parvifolia* Dyer *parvifolia* obtained from Sarawak Forest Seed Bank was chosen due to its economical value and strong adaptability. The total genomic DNA was extracted using a modified CTAB method. A pair of primers, i.e. forward primer (5' – TGGTGACTCAGCTGGAACAG – 3') and reverse primer (5' – AATCATCGGCATTCCATAGG – 3') was constructed based on the known XET mRNA sequences obtained from the databases. Polymerase Chain Reaction (PCR) was performed based on the optimized thermo-cycling profile as follow: 35 cycles of 1 min of denaturing at 94°C, 1 min of annealing at 46°C, and 2 min extension phase at 72°C. A DNA fragment of ~527bp was obtained from the amplification and was cloned using a TA-vector system. Then, the isolated and purified plasmid was sent for sequencing.

Keywords: xylogenesis, xyloglucan endotransglycosylase (XET), xyloglucan, *Shorea parvifolia* Dyer *parvifolia*, CTAB, PCR.

ABSTRAK

Gen yang terlibat dalam pembentukan kayu merupakan satu faktor dalaman yang penting selain daripada aspek hormon dalam proses xylogenesis, yang mengawal perkembangan dalam pertumbuhan sekunder pada pokok. Salah satu enzim yang telah dikenalpasti, xyloglucan endotransglycosylase (XET), ditemui dapat mengubahsuai rantaian intermicrofibrillar pada xyloglucan, satu komponen utama dalam dinding sel primer dikot untuk membenarkan perlonggaran dinding yang diperlukan semasa pemanjangan sel tumbuhan. Dalam kajian ini, Shorea parvifolia Dyer parvifolia yang diperolehi daripada Bank Biji Benih Sarawak telah dipilih disebabkan nilai ekonominya serta kebolehan adaptasi. Genomik DNA telah dipencilkan menggunakan kaedah CTAB yang telah diubahsuai. Sepasang pencetus, iaitu pencetus ke hadapan (5' – TGGTGACTCAGCTGGAACAG – 3') dan pencetus ke belakang (5' – AATCATCGGCATTCCATAGG 3') telah direka berasaskan jujukan mRNA XET daripada pangkalan data. Tindak Balas Berantai Polimerase (PCR) telah dijalankan berdasarkan profil kitaran terma yang telah dioptimakan: 35 kitaran untuk fasa penyahaslian pada 94°C selama 1 minit, 1minit pada 46 C untuk penyepuhan dan 2 minit pada 72°C untuk pemanjangan. Satu serpihan DNA yang bersaiz ~527bp telah diperolehi daripada amplifikasi PCR dan telah diklon menggunakan sistem vektor-TA. Seterusnya, plasmid yang telah dipencilkan dihantar untuk proses penjujukan DNA.

Kata kunci: xylogenesis, xyloglucan endotransglycosylase (XET), xyloglucan, Shorea parvifolia Dyer parvifolia, CTAB, PCR.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

BL	Brassinolide
BR	Brassinosteroids
cDNA	Complementary DNA
CIA	Chloroform-Isoamyl Alcohol
CTAB	Cetyltrimethylammonium Bromide
DBGET	Integrated Database Retrieval System
dH_2O	Distilled water
dNTP	Deoxynucleotide triphosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EBI	European Bioinformatics Institute
EtBr	Ethidium Bromide
EXT	Endoxyloglucan Transferase
GA	Gibberellin
GH	Glycoside Hydrolase
LB	Luria Broth
IPTG	Isopropyl β thiogalactopyranoside
$MgCl_2$	Magnesium Chloride
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
PCI	Phenol:Chloroform:Isoamyl alcohol
PCR	Polymerase Chain Reaction
PVP	Polyvinylpyrrilodone
RE	Restriction Enzyme
RNA	Ribonucleic Acid
RNase	Ribonuclease
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TCH4	Touch genes
TE	Tris-EDTA buffer
T_a	Annealing Temperature
T_m	Melting Temperature
XET	Xyloglucan Endotranglycosylase
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Tree comprises over 90% of the terrestrial biomass of the earth, serve as a primary feedstock for biofuel, fiber, solid wood products, and various natural compounds (Han, 2001). In the formation of environment, forest trees provide soil protection, water retention, CO_2 absorption and carbon storage, recreation and health improvement. Forest tree plays a significant role as the source of renewable raw materials towards the prospective of economic and environment of mankind. In Malaysia, forest tree plays an important role in the economic sector. Forest revenue collected in year 2002 in Sarawak itself was RM895 million. The following year in the Peninsular, was around RM330 million, and the export of major timber products was around RM3.5 billion.

Forest trees improvement promises turnover in producing tree with rapid growth rate, good stem and branching form, high quality wood, resistant to pest and diseases and tolerant to environment stresses. Nowadays, the modern improvement programs aims in conserving the genetic variation of the species under consideration for future needs (protection of natural resources), and improvement of economic important characteristics of the trees to fit better to human needs (Jochen and Andreas, 1990).

Wood formation is a secondary cell wall biosynthesis, which offers great significance especially for the pulp and paper industry, and for the ecology of the

world's forests. Understanding of the wood formation pathway (xylogenesis) will enable the domestication of forest trees, and the improvement of wood qualities.

In this region, the most sought after commercial valuable tropical tree are Dipterocarp species. At the present, *Shorea* are economically the most important timber species in tropical Asia. *Shorea* or meranti is a genus of Dipterocarpaceae, a family which consist of over 500 species. It is widely distributed in IndoChina, Thailand, Sri Lanka, India, South China, Malaysia and Maluku. They grow well in low lands to upper hill at the altitude of 700m and on a variety of usually well-drained clay soils. Plantation trials have shown that it grows better in the foothills than on ridge tops.

Shorea parvifolia Dyer *parvifolia* or also known as meranti sarang-punai, is an important source of quality wood, resin, food, material for producing health care and other commercial products. This species has suffered a massive population reduction mainly because of the rates of exploitation of its timber. Despite the importance of wood, there are too little emphasis on exploring and enhancing the forest industry through biotechnological intervention because trees are more difficult to work with than other plants.

Despite the all-round efforts to improve forest tree biological properties, there is still a lack of understanding in the cellular and molecular regulation of tree growth. Most of the studies agreed that several factors make the studies of trees, especially at the molecular level difficult (Kriebel, 1988; Ahuja, 1988; Han, 2001; Chaffey, 2002). For example, lack of tree model system for study, forest tree species are usually large in genome, large in size, and long generation time for tree growth are the main

obstacles which hinder the progress of the trees molecular fundamental study. As mention, tree like pine, has extremely large genome, about 200 to 400 times larger than the genome of *Arabidopsis thaliana* (Somerville and Somerville, 2000).

Currently, not many studies have been carried out on the regulatory mechanism and genomic approach in woody species. The published findings are focused more to non-woody plants such as maize, rice, tomato, pea, Arabidopsis, etc. Functional genomic studies on wood forming tissue are mainly from temperate timber species such as pine (Allona *et al.*, 1998; Whetten *et al.*, 2001), poplar (Sterky *et al.*, 1998; Hertzberg *et al.*, 2001) and black locust (Yang *et al.*, 2003). Therefore, the molecular information of wood formation on tropical tree species remains poorly understood.

The objective of this study is to isolate the XET gene involved in wood formation in *S. parvifolia* Dyer *parvifolia* via PCR method.

CHAPTER II

LITERATURE REVIEW

2.1 Selection of Species Studied

2.1.1 Family Dipterocarpaceae

Under the order of Guttiferales, family Dipterocarpaceae comprises of about fifteen genera, which is divided into three subfamilies. Geographical distribution of Dipterocarpoideae, Monotoideae and Pakaraimoidene are found in tropical Asia, tropical Africa and America. The Asiatic subfamily Dipterocarpoideae is further divided into four tribes, Dipterocarpeae, Dryobalanopseae, Shoreae and Vaticeae (Ashton, 1982).

There are two genus under the Dipterocarpeae tribe, which are the *Dipterocarpus* and *Anisoptera*. *Dryobalanops* is the only genus under Dryobalanopseae. Shoreae tribe consists of four genus which are the *Hopea*, *Neobalanocarpus*, *Shorea* and *Parashorea*. Vaticeae tribe consists of genus *Cotylelobium* and *Vatica* (Ashton, 1982).

According to Ng (1991), 9 genus and 155 species were found in Malay Peninsula. The family is characterized by winged fruits in which the wings are developed from persistent sepals, fleshly bilobed unequal cotyledons, simple stipulate leaves, and dimorphic shoot system.

2.1.2 Genus *Shorea*

Shorea or meranti consists of about 194 species, 163 of which occur in Malaysia and is widely distributed from Sri Lanka through Indo-China towards Malaysia. The greatest diversity occurs in Borneo, followed by Sumatra, Peninsular Malaysia, the Philippines and the Moluccas (PROSEA, 1994). Meranti species also can be characterized according to its colour and intensity: light red, dark red, white or yellow colour.

Shorea refers to small to large deciduous or evergreen trees, and it is the largest and economically most important genus in the family of Dipterocarpaceae. The genus has been divided into 10 sections based on the appendages to the connectives, the form of the anthers and the number of pollen sacs (Ashton, 1982). The flower bud is almost sessile and petals usually partially joined at base or free. The number of stamens is mostly fifteen or more. Fruiting calyx imbricates in the bud, developing into wings that are usually two or three lobes larger than the others.

2.1.3 Species *parvifolia* Dyer *parvifolia*

In general, *S. parvifolia* Dyer is further divided into subspecies *parvifolia* and *velutinata*. This two subspecies can be distinguished based on the leaf morphology, where *parvifolia* is smooth and small - leaved, meanwhile the indumentum of *velutinata* is covered with minute and rough bundles of red-brown, brown or grey-brown hairs (Newman *et al.*, 1996).

In Malaysia, *S. parvifolia* Dyer *parvifolia* is locally known as meranti sarang punai or light-red meranti, meranti samak (Sarawak) and seraya punai (Sabah) (Figure 2.1). Other name such as abang gunung (East Kalimantan), kontoi burung or Tengkawang (West Kalimantan), saya-luang (Thailand) also exists. It is found throughout the peninsular except Perlis, Nothern Kedah and Langkawi. The tree can grow up to 65m tall, and over 190cm in dbh with short and sharp buttresses up to 4m high (FDRM, 1999). The thickness of the sapwood is 5-8cm. The sapwood is pale but heartwood is dark red (Figure 2.1b). The stem usually is straight cylindrical trunks and buttresses up to 4m high. Frequent brittle-heart and black holes, with the presence of white resin streaks are found on the log. The tree barks are smooth and usually thick. It is grayish brown outside and reddish, pink or orange inside. The wood texture is rather coarse but even, and there is an absent of growth ring.

(a) (b)

Figure 2.1 *Shorea parvifolia* Dyer *parvifolia.* (a) Mature Tree, and (b) Sapwood (Retrieved from http://www.edinburgh.ceh.ac.uk/tropical/Shorea.htm)

The leaves are ovate-elliptic or oblong lanceolate and the size is 5 – 13 cm × 2.5 – 5cm (Figure 2.2a) with the present of 10-13 pairs of secondary veins not prominent beneath surface. Domatia pubescent or scale-like is frequently one or two pairs at base. Flower buds are ovoid; petals are falcate oblong and white tinged pink at base or pinkish-red in colour. Stamens are glabrous and curved downwards, exceeding the anthers. The anthers are sub-globose with short appendages, stylopodium ovoid to conical. Flowering begins in January-November. The fruit are a nut borne on short stalks with ovate to oblong shape and is shorter than 1cm in length (Figure 2.2b). It contains three outer wings and two inner wings embrace the lower portion of the nut. The fruit calyx lobes are up to 9cm × 1.5cm. The fruiting starts in January-December.

(a)

(b)

Figure 2.2 *Shorea parvifolia* Dyer *parvifolia.* (a) Leaf is ovate or oblong in shape, and (b) Fruit is nut born shape.
(Adapted from Forestry Department of Peninsular Malaysia, 1999)

Shorea parvifolia Dyer *parvifolia* grows well in humid climate, where annual rainfall not exceeding 1600 mm and with a dry season of less than 6 months. It grows well in tropical forest and on a variety of soils but does not tolerate waterlogged sites, especially peat soils. It grows better in well-drained clay soils up to 800m altitude.

Basically, the physical and mechanical properties can vary greatly depending on origin and growth conditions. The density of the wood is very variable and ranges between 290 kg/m^3 and 835 kg/m^3 at 15% moisture content (PROSEA, 1994). The physical properties consist of monin hardness 2.4 g/cm^3, coef of volumetric shrinkage 0.48%, total tangential shrinkage 7.1%, total radial shrinkage 3.6%, fibre saturation point 29% and it is moderately stable to stable. For the mechanical properties, crushing strength is 42 MPa, static bending strength is 86 MPa and modulus of elasticity is 13620 MPa (CIRAD Forestry Department, 2003).

Meranti which has different intensity of red colour indicates its weight and usage. Due to its advantage of not having siliceous content, it is good for production of various wood works and products, e.g. plywood, veneer, hardboard and particleboard. This light red meranti is suitable for floorings, fittings, paneling, ceiling, shelving, interior partitions, joinery, low-grade decking and boat planking, concrete shuttering, musical instruments (organ pipes), coffin, boxes, toys, turnery and matches.

2.2 Secondary Growth

Secondary growth is a process occurs most extensively in dicotyledons, and in some monocotyledons. This process occurs in the later development of woody plants to adjust to the demands of water transport required by the leaf biomass and the mechanical strength which is necessary to support the crown and to withstand wind forces (Zimmermann and Brown, 1971). Secondary growth begins after cell expansion involves the activity of the vascular cambium which produces secondary xylem and phloem cells, and the cork cambium which produces secondary dermal tissue, called periderm that comprised of cork, cork parenchyma and cork cambium.

Vascular cambium originates from the procambium in the vascular bundles and the interfascicular parenchyma cells between vascular bundles. It is a lateral cambium composed of two types of initials: fusiform initials that produce tracheary elements and xylary fibers in the longitudinal system of wood, and ray initials that produce ray parenchyma cells in the transverse system of wood (Esau, 1977; Mauseth, 1988). Fusiform initials are tapered, prism-shaped cells that are vertically elongated and form the axial growth of the stem. They produce secondary xylem and secondary phloem between the rays. Ray initials are small, elongate cells oriented laterally out to the axis of the stem (towards the epidermis). Their derivatives form the rays of the wood and function to transport water and dissolve solutes radially.

The cells are meristematic active and undergo mitosis to produce layers of cell on either side of the cambium. Derivative cells that mature toward inner side of cambium become secondary xylem, whereas cells that mature toward outer side of cambium become secondary phloem.

Along the secondary growth path, dermal system also undergoes expansion. A layer of the cortical cells becomes meristematic, differentiates soon after the vascular cambium forms, forming a cork cambium (phellogen). The cork cambium produces derivatives that mature to the inside, the phelloderm (secondary cortex), and derivatives that mature to the outside, cork (phellem), a densely packed dead cells which are thickened and waterproofed with suberin. Lenticels, loosely packed cell eruptions that enable gas exchange are raised.

As the epidermis layer is being stretched, and sloughed off, they are replaced by cork. The same goes to the primary phloem. The cork tissue interlaces with secondary phloem tissue to form bark. Old tissues are eventually replaced with more bark. This continuous alternating process can often be distinguished by the layers of phloem fibers, which give rise to interesting patterns in bark. These bark pattern of a tree is also a species characteristic.

2.2.1 Xylogenesis

Xylogenesis, a term describing the development of secondary xylem or wood formation involves the initiation of the vascular derivatives into xylem cells through the process of cell division, cell expansion, secondary wall formation, lignification and finally, programmed cell death (Hetzberg *et al.*, 2001). Cell division in pine produces cambial cells known as fusiform initials and ray initials that give rise to phloem mother cells or xylem mother cells that differentiate into tracheids and ray parenchyma cells, respectively (Whetten *et al.*, 2001) (Figure 2.3). Xylem is composed of conducting tracheary elements such as vessels elements in angiosperm and tracheids in gymnosperms, and nonconducting elements such as xylary parenchyma cells and xylary fibers (Ye, 2002).

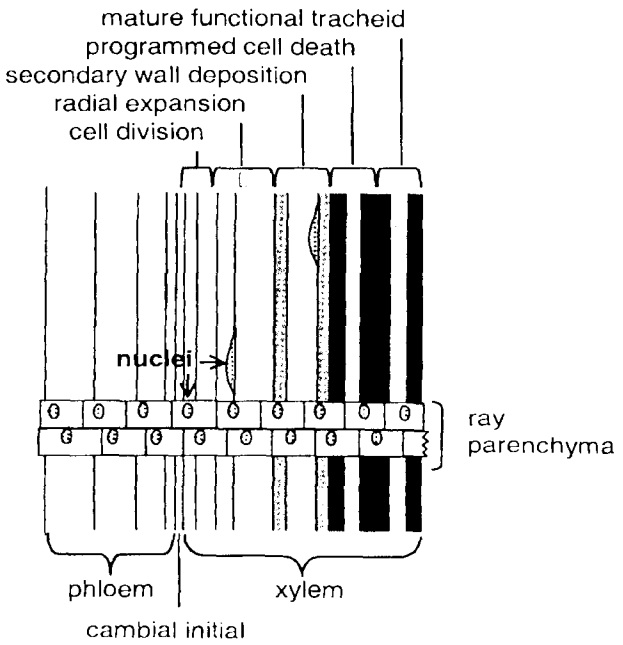

Figure 2.3 A schematic diagram of differentiating pine secondary xylem. The cambium is a meristem comprised of a single cell layer. This layer contains both elongated fusiform initials that gives rise to phloem precursors to the outside and xylem precursors to the inside, as well as ray initials that give rise to parenchyma cells that form continuous rays across the xylem and phloem.
(Adapted from Whetten *et al.*, 2001)

Tracheary element differentiation has been divided into two processes: the "early" process involves the origination and development of procambial initials and the "late" process, involves secondary wall thickenings and programmed cell death (Fukuda, 1996). Secondary wall thickening with annular, helical, reticulate, scalariform, and pitted patterns (Mauseth, 1988) provides mechanical strength to the vessels for withstanding the negative pressure generated through transpiration. There has been a significant progress in the characterization of genes involved in the synthesis of secondary wall, including synthesis of cellulose and lignin (Ye, 2002).

Programmed cell death is the execution of cell death initiated by disruption of the vacuole membrane, resulting in release of hydrolytic enzymes including cysteine proteases (Beers and Freeman, 1997, Obara *et al.*, 2001; Ye and Varner, 1996,; Zhao *et al.*, 2000), serine proteases (Beers and Freeman, 1997; Groover and Jones, 1999), and nucleases (Aoyagi *et al.*, 1998; Thelen and Northcode, 1989; Ye and Droste, 1996) into the cytosol (Groover and Jones, 1999; Kuriyama, 1999; Obara *et al.*, 2001). Little is known about the possible signals that trigger the biosynthesis of hydrolytic enzymes and the final disruption of vacuoles, except for a possible involvement of calcium influx and an extracellular serine protease in the initiation of cell death of tracheary elements (Groover and Jones, 1999).

The hormonal aspects of xylogenesis by Roni Aloni (1991), discussed the role of growth regulators in controlling vessel and fiber differentiation in hardwood trees. Based on recent studies using functional genomic approach (Ko *et al.*, 2002; Milioni *et al.*, 2001; Allona *et al.*, 1998, Sterky *et al.*, 1998, and Hertzberg *et al.*, 2001), significant progress has been made in the study of the genes and signaling

mechanisms responsible for secondary wall formation, lignin and cellulose biosynthesis (Arioli *et al.*, 1998) and xylem development (Fukuda, 1997). These genes and proteins need to be further studied in order to understand wood formation, and the potential targets for the directed modification of wood properties (Whetten *et al.*, 2001).

2.3 Xyloglucan

Xyloglucan is a soluble hemicellulosic polysaccharide of dicots that associates especially with cellulose microfibrils in the primary cell walls of plants or accumulates as a storage polysaccharide in some seeds (Edwards *et al.*, 1985). This polymer consists of a β(1-4)-linked glucose residue that can bind specifically to cellulose microfibrils by hydrogen bonding and extends the *in vivo* xyloglucan-cellulose network (Figure 2.4). Some xylose residues are further substituted by the disaccharide galactosyl and fucosyl-galactosyl groups (Vissenberg *et al.*, 2000). Primary walls of monocotyledonous plants contain small amounts of xyloglucan, with a lower xylose : glucose ratio than in dicot primary walls. Some xyloglucan structures found in certain seed endosperm cell walls are lack of fucose.

Xyloglucan binds non-covalently to cellulose, coating and cross-linking adjacent cellulose microfibrils (McCann *et al.*, 1992). The extensive interlink xyloglucan-cellulose network serve as the major tension-bearing structure in the primary wall. Such xyloglucan crosslinks might restrain cell expansion and allow the generation of turgor pressure. Hydrolytic enzymes that break crosslinks in the cell