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LIGHT-HARVESTING CHLOROPHYLL A/B BINDING PROTEIN (LHCB) PROMOTER FOR TARGETING SPECIFIC EXPRESSION IN OIL PALM LEAVES

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ABSTRACT

Oil palm leaves have the potential to be manipulated as a green factory to produce novel metabolites. In order to direct the specific expression of transgenes into the leaf tissue, a leaf-specific promoter is required. In these studies, efforts were carried out to isolate a leaf-specific gene and its regulatory sequence from the oil palm. Rapid amplification of 5'-cDNA Ends (5'-RACE) resulted in isolation of a 962 bp full length sequence of the light-harvesting chlorophyll a/b binding protein (LHCB). The amino acid sequence of this gene exhibited more than 88% homology with the LHCB1 gene isolated from monocots and dicots. Characterization of this transcript using Northern blot analysis revealed LHCB to be abundantly expressed in oil palm green leaf tissues, but not in the non-photosynthetic tissues such as kernel, mesocarp, germinated seedlings and inflorescences. Results from Southern blot analysis confirmed that at least four copies of the LHCB gene are present in the oil palm genome. The promoter region of LHCB was then obtained through genome walking approach. Based on the sequence analysis, it was found that the LHCB promoter lacks a TATA-box. Initiation of transcription can therefore be replaced by an initiator element (Inr) located at positions -1 to -7. Furthermore, a few putative cis*-acting elements responsive to light, wounding, abscisic acid and heat-shock were also found in the distal and proximal regions of the LHCB promoter.*

Keywords: oil palm genetic engineering, leaf-specific promoter, light-harvesting chlorophyll a/b binding protein (*LHCB*).

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INTRODUCTION

Oil palm (*Elaeis guineensis*) is a perennial crop with a long generation time of seven to 10 years. Therefore genetic improvement of oil palm by conventional breeding is rather slow. However, genetic engineering provides an opportunity to fasten the process by being able to introduce specific individual genes into the oil palm genome. Introduction of these transgenes into a specific plant tissue or at certain developmental stages is only possible with the availability of tissue-specific regulatory sequences or promoters.

To ensure focused expression of the transgenes without undue negative impact on normal plant growth and development (Schaart *et al*., 2002), a plant promoter which can target strong tissue-specific expression is needed. A few promoters that exhibit specific expression in fruits, tubers and leaves have been isolated from tomato, potato and *Arabidopsis*, respectively (Lessard *et al*., 2002). In the oil palm, tissue-specific promoters were successfully isolated from the mesocarp and kernel (Siti Nor Akmar *et al*.,

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2003). To our knowledge, no isolation of an oil palm leaf-specific promoter has been reported. Since the leaf is the potential site for accumulation of novel metabolites, efforts have been made to identify such a promoter.

Manipulation of the metabolic pathway for production of biodegradable plastic or polyhydroxybutyrate (PHB) in *Arabidopsis* leaves has been reported by Poirier *et al*. (1992), Nawrath *et al*. (1994) and Mittendorf *et al*. (1998). In transgenic tobacco, leaves were used as the site for accumulation of tumour-specific antibody (Vaquero *et al*., 1999). In a crop improvement programme, Abdullah *et al*. (2000) and Chari *et al*. (2001) reported the production of transgenic oil palm with resistance against bagworm larvae. Cowpea protease trypsin inhibitor protein in the transgenic leaves function as a lethal poison to the bagworm.

Most of the genes isolated from leaves are closely involved in the photosynthetic pathway. One of these genes is light-harvesting chlorophyll a/b binding protein of photosystem II (*LHCB*). Previously known as *Cab*, this gene forms a major protein-pigment complex in the thylakoid membrane of the chloroplast. The protein plays an important role in binding chlorophylls *a* and *b* to form an oligomeric complex involved in capturing and transferring light energy to the photosynthetic reaction center (Becker *et al*., 1992). Jansson and Gustafsson (1990) discovered that approximately 50% of the chlorophyll in plants is bound to the *LHCB* from photosystem II and the ratio of chlorophyll a/b is 1.0 to 1.4.

LHCB forms a multigene family in higher plant genomes (Jansson, 1994; Yuzura *et al*., 1998). The gene is generally divided into Types I, II and III based on differences at the amino acid level and specific location of the intron in the genomic sequences (Demmin *et al*., 1989). Synthesis of this nuclear encoded gene occurs in the cytosol, followed by post-transcriptional processing in the chloroplast. It was noticed that the presence of a transit peptide eases the transport of *LHCB* into the chloroplast organelle (Jansson, 1994).

The *LHCB* gene and its promoter have been widely studied in various plants like *Arabidopsis* (Mitra *et al*., 1989), petunia (Gidoni *et al*., 1989), rice (Luan and Bogorad, 1992), maize (Becker *et al*., 1992), wheat (Villegas-Sepulveda *et al*., 1994), *Lemna gibba* (Kehoe *et al*., 1994), pea (Jofre-Garfias *et al*., 1997) and sugar beet (Stahl *et al*., 2004). Moguel *et al*. (2000) reported on the application of a *LHCB* promoter from alfalfa for directing the expression of the β-zein gene from maize into alfalfa and also tobacco. On the other hand, this promoter has also been used as a model system for studying the interaction between the phytochrome system and circadian clock in plant development (Yao and Carl, 2001).

Expression of the *LHCB* transcript in the angiosperm is regulated by light. Major induction of *LHCB* mRNA was observed when the green tissues of maize were exposed to light (Becker *et al*., 1992; Knight *et al*., 1992). In the study by Bansal *et al*. (1992), it was concluded that the transient expression of *LHCB* promoter was specific to the mesophyll cells rather than the bundle sheath cells. In this article, isolation and characterization of the first *LHCB* gene from oil palm was reported. Tissue-specific expression of this gene will enable the use of its promoter for targeting a controlled expression of transgenes in the oil palm genome through genetic engineering.

MATERIALS AND METHODS

Plant Materials

Oil palm (*Elaeis guineensis*, var *tenera*) tissues, such as different developmental stages of leaflets, germinated seedlings, inflorescences, mesocarp and kernel, were obtained from MPOB-UKM Research Station, Bangi, Malaysia. The samples were stored at –80°C prior to RNA extraction.

Rapid Amplification of 5′-cDNA Ends (5′-RACE)

A full length sequence of the *LHCB* gene was isolated from cDNA pool via 5′-RACE using SMARTRACE cDNA Amplification Kit and Advantage 2 PCR Kit (CLONTECH). The expected fragment was purified and cloned into pCR® II-TOPO vector from a TOPO-TA Cloning® Kit (Invitrogen™). Automated sequencing was performed using ABI PRISM 377. Analysis of the nucleotide sequences was carried out with DNAsis Max version 1.0.

Northern Blot Analysis

Total RNA was extracted from various oil palm tissues according to the method of Rochester *et al*. (1986). A complete nucleotide sequence of the *LHCB* gene was used as a probe in Northern analysis. The PCR-derived fragment was purified using the QIAquick Gel Extraction Kit (QIAgen), then labelled with ^{32}P-dCTP using the Megaprime DNA Labeling Kit (Amersham Pharmacia Biotech).

Northern blot analysis was carried out according to McMaster and Carmicheal (1977) and, Kroczek and Siebert (1990). In this study, 5 μg total RNA were heat denatured at 55°C for 15 min in 18 μl GFP mixture [78% (v/v) deionized formamide, 16% deionized glyoxal and 10 mM sodium phosphate buffer]. The RNA was then cooled on ice immediately prior to electrophoresis on 1.2% agarose gel with 1x TAE buffer (40 mM Tris-acetate and 1

mM EDTA). The RNA was transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech) via a vacuum blotter using 20x SSC as transfer buffer. Pre-hybridization of the membrane was performed at 65 °C for 4 hr in buffer containing 5x SSC (1x SSC is 0.15 M NaCl, 15 mM trisodium citrate), 5x Denhardt's (1x Denhardt's is 0.02% each Ficoll 400, bovine serum albumin and polyvinylpyrrolidone), 0.5% SDS and 100 μg ml^{-1} denatured herring sperm DNA. This was followed by overnight hybridization of the membrane with ^{32}P-labelled probe at 65°C. Washing of the membrane was performed with 4x SSC/0.1% SDS at 65°C for 15 min, followed by 2x SSC/0.1% SDS at 65°C for 15 min. Exposure to X-ray film was carried out at -80°C for 48 hr.

Southern Blot Analysis

Genomic DNA was extracted from oil palm spear leaves following the method of Doyle and Doyle (1990). A total of 20 μg genomic DNA was digested with *Hind*III and *Xba*I. The digested products were size fractionated on 1.0% agarose gel at 100V for 5 hr in 1x TAE, pH 7.9. This was followed by immobilization of the DNA onto nylon membrane via vacuum blotting of the gel at 60 psi for 1 hr using 0.4 N NaOH as transfer buffer. At the end of the process, the membrane was rinsed with 2x SSC prior to uv-crosslinking. Hybridization and washing of the blot were performed as stated above for Northern blot analysis.

Promoter Isolation

A leaf-specific promoter was isolated from oil palm genomic DNA using Universal GenomeWalker™ Kit and Advantage Genomic PCR Kit (CLONTECH). Two antisense gene-specific primers, designated LS14 and LS12 were designed based on the 5'-terminal of the coding region and within 5'-UTR of the oil palm *LHCB* gene, respectively. Four GenomeWalker libraries were obtained through digestion of 2.5 μg leaf genomic DNA with *Dra*I, *Eco*RV, *Pvu*II and *Stu*I prior to ligation with the GenomeWalker adaptor. Aliquots of 12 μl of these libraries were used in the primary PCR reaction with the antisense gene-specific primer (LS14) and adaptor primer (AP1) provided with the kit. This was followed by secondary PCR of the 50x diluted primary library using the antisense nested gene-specific primer (LS12) and adaptor primer (AP2) supplied in the kit. The PCR was carried out using GeneAmp® PCR System 9700 (Applied Biosystems) under the conditions recommended by the GenomeWalker Kit. The expected band was gel purified and cloned into pCR®II-TOPO vector (Invitrogen™) prior to sequencing.

RESULTS AND DISCUSSION

Oil Palm *LS01* Sequence Coding for Full Length *LHCB1*

The 5'-RACE approach resulted in amplification of a 1.0 kb distinct band from the 5'-RACE-Ready cDNA pool (*Figure 1*). This clone was designated *LS01*. Analyses of the nucleotide and deduced amino acid sequences revealed that *LS01* represents the full length oil palm *LHCB* gene. Adenine at the 5' end region of the *LS01* is predicted to be the putative transcription start site. This clone contains a 5'-untranslated region (5'-UTR) of 78 bp, an open reading frame (ORF) of 795 bp and a 3'-untranslated region (3'-UTR) of 89 bp. A schematic diagram of this clone is shown in *Figure 2*. The ORF of this clone encoded for 265 amino acids. The first 33 amino acids represent the transit peptide and the remaining 232 amino acids make up the mature protein. As a nuclear encoded gene, a transit peptide is required for transport of the *LHCB* gene from the cytoplasm into the chloroplast (Mullet, 1993).

As shown in *Table 1*, LS01 exhibited more than 88% homology at the amino acid level with *LHCBI* of photosystem II identified in other monocots and dicots. The amino acid alignment between LS01 and other plants revealed that the region coding for mature protein is highly conserved compared to the region of transit peptide (data not shown). The oil palm *LS01* gene also has a comparable structure and number of amino acids with *LHCB1* isolated from *Glycine max (*Stockinger and Walling, 1994), *Gossypium hirsutum* (Anderson *et al*., 1993) and *Solanum tuberosum* (Fernandez *et al*., 1995). It was reported by Buetow *et al*. (1988) that *LHCB1* ORF usually comprises 31 to 37 amino acids for the transit peptide and 231 to 235 amino acids for the mature protein. Based on the BlastX identity search and amino acid characteristics, it is predicted that the oil palm *LS01* belongs to the *LHCB1* gene family.

Characterization of Oil Palm *LS01* Gene

Northern blot analysis was carried out to determine the expression patterns of the *LS01* gene across various oil palm tissues. It was observed in *Figure 3a* that the PCR produced probe hybridized strongly to a single transcript of approximately 1.0 kb. Specific expression of the *LS01* gene was detected at different stages of oil palm leaves. High expression of *LS01* transcript was detected in the young and mature green leaves as compared to yellowish spear leaves. In the non-photosynthetic tissues, such as kernel, mesocarp, germinated seedlings and inflorescences, expression of *LS01* transcript was not detected. This result is in agreement with Northern analysis performed on cotton. Expression of cotton

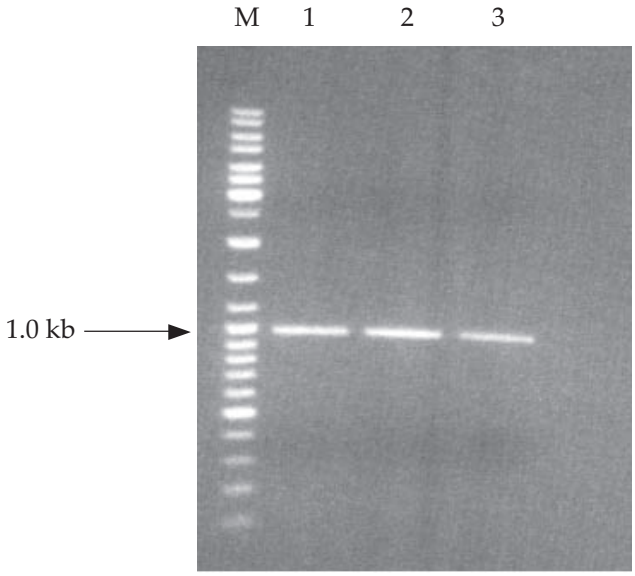

Figure 1. Amplication of oil palm Lhcb gene from 5'-RACE-Ready cDNA pool using a gene-specific primer. Lane M is the DNA Ladder Mix Marker. Lanes 1, 2 and 3 are the 1.0 kb amplified products of SMARTRACE.

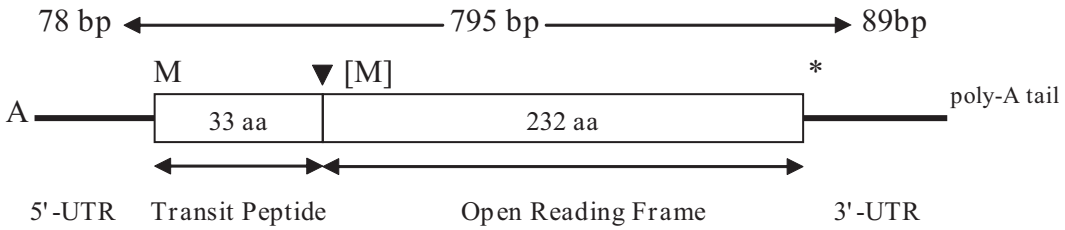

Figure 2. Schematic diagram of the oil palm full length LHCB *gene (designated LS01). The total length of the LS01 sequence is 962 bp. M (ATG) is the start codon for LS01 precursor protein. An arrowhead shows the putative cleavage site between the transit peptide and LS01 mature protein. [M] (ATG) indicates the first predicted amino acid of the LS01 mature protein. The stop codon (TGA) is denoted by an asterisk (*). Nucleotide A in the 5'-UTR represents the putative transcription start site for the LS01 gene.*

TABLE 1. BLASTX 2.2.17 (24 June 2007) ANALYSIS OF OIL PALM *LHCB* GENE AGAINST THE GENBANK DATABASE

Organism	Accession No.	Score (Bits)	E-value	Identity (%)
Musa balbisiana	ABF70112	521	2e-146	95
Vitis vinifera	CAN67709	511	3e-143	94
Solanum tuberosum	AAA80589	509	1e-142	92
Nicotiana sylvestris	BAA25389	508	2e-142	93
Solanum lycopersicum	P07370	508	3e-142	92
Gossypium hirsutum	AAA18529	506	6e-142	93
Glycine max	AAA50172	499	1e-139	92
Zea mays	P06671	481	4e-134	88
Oryza sativa	BAF06004	484	4e-135	89

LHCB transcript was observed in leaves, stem of germinated seedlings and also pericarp tissues, but not in roots, seeds and ovules (Anderson *et al*., 1993).

Genomic Southern analysis was performed to determine the gene copy number of the oil palm *LS01*. The same PCR produced probe in Northern analysis was used to hybridize the Southern blot containing genomic DNA digested with *Hind*III (Lane H) and *Xba*I (Lane X). It was observed that the *LS01* hybridized to four different fragments in both lanes *(Figure 4*). This demonstrates that there are at least four members of the *LHCB* gene in the oil palm genome. Literature review showed this number to be rather small in comparison to the gene copy number reported in cotton and soyabean genomes. The cotton genome contains seven to 10 copies of

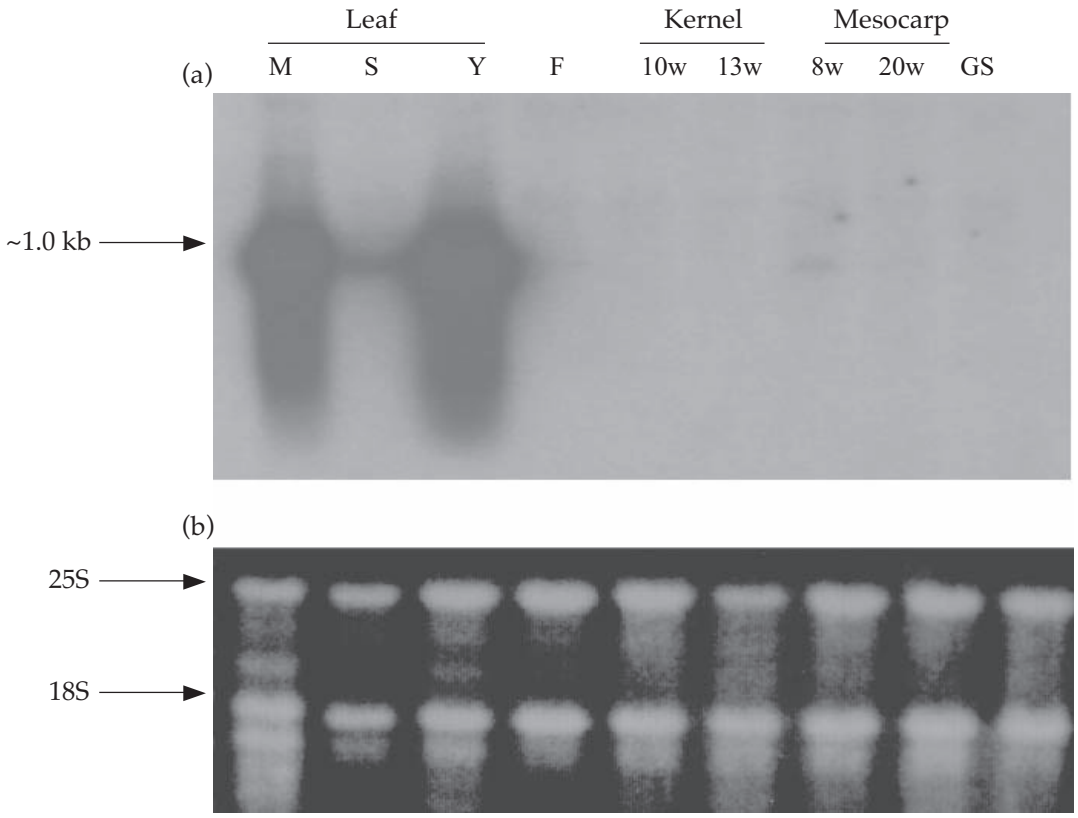

Figure 3. Northern blot analysis using the oil palm full length LHCB *cDNA as probe. (a) Total RNA (5 μg/lane) was size fractionated on 1.2% agarose gel and transferred to nylon membrane prior to hybridization with ^{32}P-labelled probe. M, S, Y, F and GS represent the total RNA isolated from mature leaves, spear leaves, young leaves, inflorescences and germinated seedlings. Total RNA from kernel (10 weeks after anthesis -10w and 13 weeks after anthesis -13w) and mesocarp (eight weeks after anthesis -8w and 20 weeks after anthesis -20w) at two different stages were also used. (b) An ethidium bromide stained gel was included to show the equal loading of total RNA from the various oil palm tissues.*

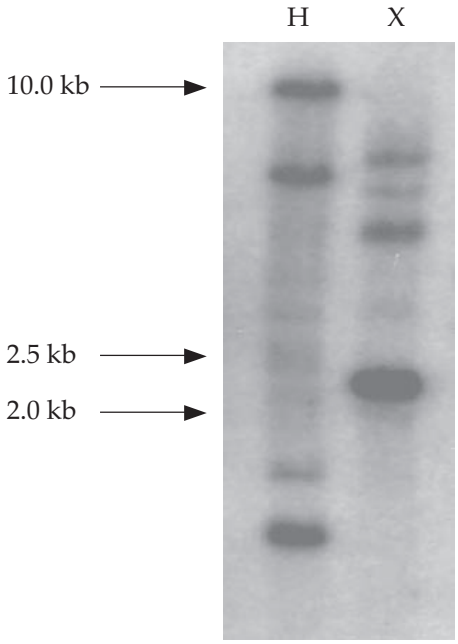

Figure 4. Southern blot analysis for determination of oil palm LHCB *gene copy number. A total of 20 μg genomic DNA from oil palm leaves was digested with* Hind*III (Lane H) and* Xba*I (Lane X) prior to size fractionation on 1.0% agarose gel. The digested DNA was transferred to nylon membrane and hybridized with ^{32}P-labelled probe of full length* LHCB *cDNA.*

LHCB1 members (Anderson *et al*., 1993) and the soyabean genome has at least nine members (Stockinger and Walling, 1994).

Isolation of Oil Palm *LHCB* TATA-less Promoter

Primary PCR of the GenomeWalker libraries with a gene-specific primer (LS14) and adaptor primer (AP1) resulted in the amplification of genomic fragments of different sizes from the *Dra*I, *Eco*RV and *Pvu*II digested libraries. The largest fragment of 1.2 kb was amplified from the *Dra*I digested library. This library was then selected as template for secondary PCR using nested primer pairs (LS12 and AP2). This resulted in the amplification of a 1.0 kb PCR product. Since LS12 is located 228 bp upstream of LS14, a smaller amplified genomic fragment was to be expected. This clone was designated pGWLS01.

Based on the schematic diagram of pGWLS01 in *Figure 5*, the promoter region obtained for the oil palm *LHCB* gene is around 932 bp. At the expected distance of 32±7 bp upstream of the transcription start site, no TATA-box consensus sequence was observed (Joshi, 1987). However, at positions –1 to –7, an initiator element (Inr) which is pyrimidine rich, PyTCANTPyPy, was identified (Nakamura *et al*., 2002). This finding revealed that the oil palm *LHCB* promoter is TATA-less and the initiation of basal transcription can be directed by the Inr motif. The same trend was also reported in the majority of nuclear encoded photosynthesis genes. It was strongly agreed that TATA-independent transcription mechanisms are crucial for the regulated expression of photosynthesis nuclear genes (Nakamura *et al*., 2002).

At the distal region of the pGWLS01 promoter, a few interesting *cis*-acting elements were identified. Light-responsive elements, such as GATA, CCAAT, and G-box, were commonly found in the light-responsive promoter (Arguello-Astorga and Herrera-Estrella, 1998). Two separated regions that contain GATA and CCAAT motifs at positions –68 and –88 relative to the transcription start site were postulated to be phytochrome-responsive. These motifs were also identified in the *LHCB* promoter of *Lemna gibba* and other plants (Kehoe *et al*., 1994). In the upstream region of the promoter, a putative wound-responsive element (WUN), CAAATTCCAAA, nearly identical to the WUN of the pathogenesis-related gene, AAATTTCCT, in potato was identified at position –424 (Matton *et al*., 1993), whereas at positions –657 and –836, an abscisic acid-responsive element (Knight *et al*., 1992) and heat-shock responsive element (Pastuglia *et al*., 1997) were identified, respectively. The presence of these elements indicated that the expression of *LS01* gene could be regulated by environmental cues such as light, mechanical wounding, abscisic acid and heat.

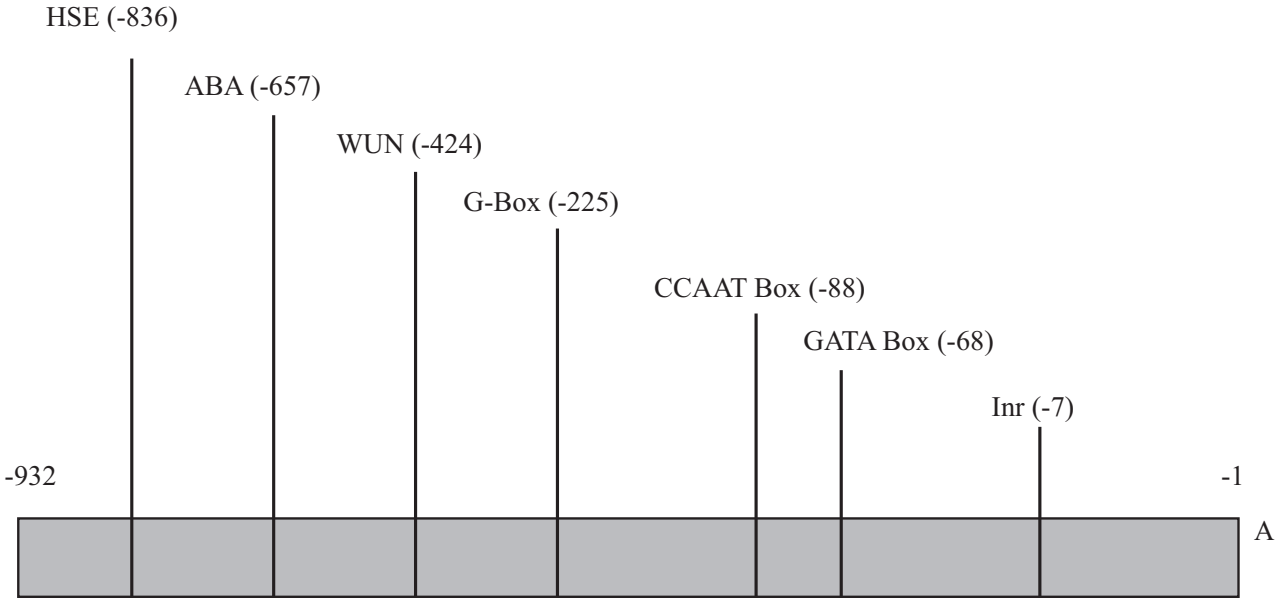

Figure 5. Schematic diagram of the oil palm LHCB promoter (designated pGWLS01). Several putative cis*-acting elements were identified in the distal and proximal regions of the promoter. These consist of an initiator element (Inr), GATA box, CCAAT box, G-box, wound responsive element (WUN), abscisic acid responsive element (ABA) and heat-shock responsive element (HSE). Nucleotide A represents the putative transcription start site.*

CONCLUSION

A full length oil palm LHCB*1* gene was successfully obtained via the 5′-RACE approach. Northern blot analysis confirmed the specific expression of this transcript in oil palm leaf tissues. The promoter of the LHCB gene was found to be TATA-less. Therefore, initiation of basal transcription in this promoter can be replaced by an initiator element, Inr, a common feature in photoregulated promoters.

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