Isolation and Characterization of Partial cDNA of Sucrose Synthase Putative Gene in Palmyra Palm (*Borassus flabellifer*)

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ABSTRACT

Intensification of biofuel resources is urgently needed considering decreased availability of world's fossil fuel. Palmyra palm (*Borassus flabellifer*) is highly potential to be developed as bioethanol source regarding the high sucrose content in its *nira*. It was observed that nira produced in dry season is sweeter than that in rainy season, which presumed to be influenced by a difference in expression level of sucrose-related genes during the two seasons. Study of Sucrose Synthase (SUS) gene of palmyra are therefore required prior to study of the gene expression. Palmyra SUS gene sequence is currently unavailable in GenBank, thereby pair of primers was designed from highly conserved region of SUS proteins among monocots. A 1866 bp partial cDNA fragment of SUS putative gene has been succesfully isolated from RNA of the young leaves of *B. flabellifer*. BLASTn and BLASTp aligments showed that either *BfSUS* cDNA or BfSUS polypeptide has high similarity with SUS cDNA and proteins from diverse plant species with the highest similarity shown by *Tulipa gesneriana*. The phylogenetic tree showed that SUS protein sequences of monocot species were distinctively grouped and splitted from those of dicot species. The BfSUS was clustered in monocot group, although not specifically grouped with particular monocot species. Nevertheless, *B. flabellifer* showed nearest genetic distance with *Tulipa gesneriana* and *Oncidium cv.'Goldiana'*. Characterization of BfSUS polypeptide using Geneious 4.6.2 indicated the presence of sucrose synthase (SUS) and glycosyl transferase (GT) domains, four putative UDP-glucose binding pockets within the GT domain, and a calcium-dependent Ser/Thr protein kinase binding site within the SUS domain. These domains and motifs are highly conserved in SUS proteins across plant species, confirming that the cDNA fragment obtained in this study is very likely cDNA encodes sucrose synthase in *B. flabellifer*.

Keywords: *Borassus flabellifer*, *nira*, sucrose, sucrose synthase, SUS domain, GT domain, Palmyra palm.

INTRODUCTION

Palmyra (*Borassus flabellifer*) is a *palmae* species with high potency to be developed as a source of bioethanol. The plant is typically grown in dry areas in strictly seasonal tropical or subtropical climate. The palmyra palm is known to be very adaptive in dry areas with only 500-900 mm annual rainfall (Flach and Rumawas, 1996). The main product of palmyra is the sweet liquid produced from its inflorescence, called *nira,* or locally known as *tuak* (Fox, 1977). The nira of palmyra palm contains 17-20% dry matters comprises of sucrose, amino acids, proteins, vitamins, and other essential minerals. Sucrose ($C_6H_{12}O_6$) that constitutes 13-18% per liter *nira* is the principal material in the production of bioethanol through the process of fermentation. It was observed in East Nusa Tenggara, particularly in Timor and Rote islands, that *nira* tapping is more

preferable in dry season rather than in rainy season. Beside the safety reason, it is probably because palmyra nira in dry season is sweeter than in rainy season. It becomes interesting to study whether this phenomena is solely caused by high level of water contained in the nira, by a higher rate of photosynthesis occurred in dry season, or it has something related with sucrose genes expression level. A preliminary study on sucrose related gene expressed in palmyra palm is therefore needed to answer the question.

Sucrose is the most important plant disaccharide; it is the principle form by which photosynthetic product is transported throughout plant tissues from the source photosynthetic tissue to the sink non-photosynthetic tissues (Bush, 1999). This disaccharide consists of one molecule of glucose and one molecule of fructose that is bounded by a glycosidic bond (Lodish *et al*., 1999). Many enzymes involved in metabolism of sucrose. The closest related enzymes are sucrose-synthase (SUS: EC 2.4.1.13), sucrose phosphate synthase (SPS: EC 2.4.1.14), sucrose-6-phosphate-phosphatase (SPPase: EC 3.1.3.24), and invertase (EC 3.2.1.26). The reversible and irreversible reactions of sucrose hydrolysis are catalyzed, respectively, by SUS and invertase. The biosynthesis of sucrose is catalyzed by the sequential action of SPS and SPPase (Winter and Huber, 2000). Sucrose can be synthesized from hexose monophosphates by SUS or SPS. In the case of SUS, the *in vivo* sucrose concentrations are always much higher than fructose or UDP-glucose, resulting in reaction that is essentially always towards the direction of sucrose cleavage. Invertase, on the other hand, only catalyzes the cleavage of sucrose into glucose and fructose.

All enzymes catalyze transfer of sugar moieties from activated donor molecules to specific acceptor molecules by forming glycosidic bonds are classified in the glucosyltranferase (GT) family of enzyme. Sucrose synthase (EC 2.4.1.13) is a key enzyme involved in sucrose metabolism, included in the GT4 family regards to its role in transferring the glucose from UDP-glucose to fructose molecule to form sucrose or, in reverse, hydrolyze the sucrose into glucose and fructose. SUS enzyme plays significant role in food storage of many plants, either in the form of starch or sugar (Baud *et al*., 2004). SUS was shown to control the mobilization of sucrose into various pathways that important for the metabolic, structural, and storage functions of the plant cell. Phloem-loading, a process by which sucrose is transported from photosynthetic to non-photosynthetic tissues, is facilitated by SUS. Inhibition of SUS-encoding genes had shown significant reduction in sucrose import capacity of floem tissues, thereby causing less sucrose content in tomato fruit (D'Aoust *et al*., 1999). Conversion of sucrose to UDP-glucose that is catalyzed by SUS provides substrate for cell wall biosynthesis and starch synthesis in plant storage organs (Sun *et al*., 1992; Zrenner *et al*., 1995; Dejardin *et al*., 1997; Hendrik, 1990). SUS activity also associated with development of nodules in legume plants and in regulation of apical meristem function (Craig *et al*., 1999). Studies on SUS-encoding genes in many plant species revealed that *in vitro* environmental conditions, such as light, temperature, and water availability have a significant influence on the expression of SUS-encoding genes. Palmyra (*B. flabellifer*) which has higher sugar content in dry season than in wet season, probably reflects a likewise relation.

SUS genes have been isolated from various starch and sugar-storing plants, such as citrus (*CitSUSA* and *CitSUS2*) (Komatsu *et al*., 2002), sugarcane (Kumar *et al*., 2007), rice (Wang *et al*., 1992), maize (*Sus1* and *Sh1* gene) (McCarty *et al*., 1986), wheat (Marana *et al*., 1988), cotton (Ruan *et al*., 2003), tomato (Sun *et al*., 1992), and potato (Zrenner *et al*., 1995).The enzyme is found in all plant tissues but is highly expressed particularly in sink tissues (Baud *et al*., 2004). According to those studies, the full length SUS gene has in average 4970 bp length, while its cDNA length is only about half of it, ranging from 2400 to 2500 bp. This research was aimed to study the characteristic of SUS gene in *B. flabellifer*. A comprehensive knowledge of the SUS gene characteristics is an initial requirement to study its expression level, and further to conduct manipulation or control of those genes, which enables efforts to enhance the productivity of *B. flabellifer* as a potential source of sugar and bioethanol.

RESULTS AND DISCUSSION

RNA Isolation from *Borassus flabellifer*

Total RNA of *B. flabellifer* was isolated from the young leaves of 35-40 years old-plants. Young leaves was chosen as it was reported that SUS genes are commonly expressed in newly developed tissues that require supply of either sucrose or sucrose derived-compounds (Kumar *et al*., 2007). The results of five methods tested for RNA isolation from *B. flabellifer* is summarized in Table 1. The modified method of Apt *et al*. (1995) resulted in good quality of RNA although the purity and quantity were rather low. The RNA isolation was best accomplished by using $TRI_{ZOL}^{®}$ Reagent (Invitrogen). The reagent, a mono-phasic solution composed of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). This method successfully generated two distinct bands of 18s and 28s ribosomal RNA that were clearly occurred in 1% gel electrophoresis (Figure 1), which indicated a good RNA quality. The RNA yield was relatively high, 1600 µg/ml, with low protein contamination as shown by the ratio 1.62. We noted that the success in obtaining high RNA yield from *Borassus* leaves is greatly determined by the finest powder that could be recovered from the leaves tissue.

Reverse Transcription- PCR

A total cDNA from the young leaves of *B. flabellifer* were obtained by cDNA synthesis. They then used as template for the next PCR reaction. The annealing of SUS-spesific primers to the target cDNA were best achieved in temperature 52ºC. As well, 2 mM of Mg^{2+} and 1.5 µg/50 µl of template cDNA were found to be the most suitable concentration for the PCR mix. Total 30 cycles of amplification using the gene specific primers, SSFw and SSRv, has successfully produced a single fragment of 1866 bp that was detected in 1% agarose gel (Figure 2). According to the expected product size, this 1866 bp fragment was predicted as the target fragment of the SUS gene.

Cloning of SUS Putative Gene and Transformation of *E. coli*

Prior to sequencing reaction, the SUS putative fragment is purified from the agarose gel and then cloned into a plasmid vector, pGEM®-T easy (PROMEGA), which then delivered to *E. coli* strain DH5α. *E. coli* cells that had been transformed were able to grow in the ampicilin-containing medium. Transformed cells containing SUS gene fragment were selected by blue-white screening method. Transformation of *E. coli* strain DH5α with pGEM®-T containing SUS fragment resulted in blue and white colonies growth on the ampicillin contained-LB media.

Plasmid Isolation and Confirmation of Gene Insertion

Plasmid isolation from the *E. coli* white colony and subsequent restriction cut using particular restriction enzymes are purposed to confirm the presence of the SUS gene fragment within the plasmid of *E. coli*. When cut with *EcoRI*, the plasmid produced two unexpected fragments, ± 1300 bp and ± 500 bp, instead of 1866 bp (Figure 3a). It was presumed that the SUS fragment of *B. flabellifer* has an *EcoRI* restriction site within its sequence, thereby produced shorter fragment than expected. It was found later that the sequence of SUS gene of *B. flabellifer* does have the recognition site for *EcoRI*. The plasmid was then cut with another endonuclease enzyme, *NotI*. As expected, cutting with *NotI* generated a single 1866 bp fragment (Figure 3b). This result indicated that the SUS gene fragment had been successfully inserted into the pGEM®T *easy* plasmid.

PCR screening with SP6 and T7 primers was further conducted to prove the presence of SUS fragment within the multiple cloning sequence (MCS) region in the plasmid. Those two primers

flank the MCS region in the pGEM®-T easy plasmid. As expected, a ±1995 bp fragment was occurred (Figure 3c). This length corresponds to the length of the SUS fragment, ±1866 bp, plus 129 bp lengths of the SP6 and T7 MCS region.

Sequencing and Characterization of SUS Putative Gene and Polypeptide

Two steps of sequencing were required since SUS putative fragment of *B. flabellifer*, further stated as *BfSUS,* is relatively long for a single reading by the sequencer system. The SP6 and T7 primers were used in the former sequencing, followed by specific internal primers to read the gap sequence within the fragment. The later primers were designed from the read of the former sequence. Using Geneious 4.6.2 program, an overlapped reading occurred from those two steps of sequencing, thus finally assembly the whole sequence of the *BfSUS* putative gene fragment. The assembled *BfSUS* fragment was trimmed from pGEM®T vector region by using VecScreen program (www.ncbi.nlm.nih.gov), followed by determination of SSFw and SSRv primers annealing region. The final construction of *BfSUS* partial gene, which total length is 1866 bp, was successfully obtained (Figure 4).

The sequence of *BfSUS* putative gene was first analyzed by BLASTn program (Altschul *et al*., 1997) to figure out the similarity level of *BfSUS* putative gene with other *SUS* gene sequences recorded in the GenBank. The percentage of identical sites and query coverage indicated that *BfSUS* putative gene shares quite high similarity, 70.8% – 80.6%, with *SUS* genes from various monocots. Thus, confirming that the isolated 1866 bp *BfSUS* fragment is most likely a sucrose synthase gene. *BfSUS* sequence shows the highest similarity with *SUS* genes of *Tulipa gesneriana* (80.6%), followed by *X. mokara* (78.8%), *Potamogeton distinctus* (78.7%), *Oncidium sp. cv Goldiana* (78.6%), and *Bambusa oldhamii* (78.5%).

Translation of *BfSUS* cDNA sequence resulted in BfSUS polypeptide sequence consists of 622 amino acids (Figure 5). The *BLASTp* program showed that *BfSUS* polypeptide sequence is highly similar with SUS protein sequences of many plant species, ranged from 78.3 % to 87.5%, confirming that the BfSUS gene expresses SUS protein. The highest similarity, 87.5%, are showed by *Tulipa gesneriana*, a *Liliaceae* plant widely known as *tulip*, and Oncidium cv 'Goldiana', a genus that contains about 330 species of orchids from the *Orchidaceae* family. *Oryza sativa* (86.8%), *Bambusa oldhamii* (86.5%), *Zea mays* (86.2%), and other monocots also showed high similarity.

In order to elucidate relationship between BfSUS putative protein and SUS proteins from other species, and between monocot and dicot species, a phylogenetic tree is generated using Neighbor-Joining method (Figure 6). The following things can be interpreted from the phylogenetic tree. First, the similarity of SUS polypeptide sequence in certain taxonomic group tends to be higher than another group. The similarity of SUS among monocot species, for instance, is distinctively higher than those of dicot species. This also showed by species coming from the same genus, for instance, between *Solanum tuberosum* and *Solanum lycopersicum*. Second, *B. flabellifer* is shown to be clustered with the monocot group, although not specifically grouped with particular monocot species. It is however shown that *Borassus flabellifer* has the nearest genetic distance with *Tulipa gesneriana*. Although these two species live in distinctively different climate regions, presumably they both evolve similar mechanism to adapt to osmotic stress in their surroundings, probably by accumulating sucrose. In water stress conditions, plants are able to keep their osmotic gradient lower than their surrounding by accumulating more solutes, including sucrose, in their tissues, thereby preventing loss of water.

Multiple sequence alignment of SUS polypeptides indicated two main domains that typically occurred in all plant species. Those are a *sucrose synthase* domain located upstream toward the N-terminal and a *glucosyltransferase* domain that is located downstream towards the C-terminal

of the protein. These domains differs SUS from other glycosyltransferase enzymes. Using *pFam* database, it was shown that those two domains are occurred in the *BfSUS* polypeptide albeit in partial length. The moiety of sucrose synthase and glucosyltransferase domain is found toward the N and C-terminal region, respectively, of the *BfSUS* polypeptide. The overlapped region between these domains is spanned from Asn283 to Ser559. Despite found to be highly conserved among plant species, the structural and functional sites and motifs within sucrose synthase domain are still unrevealed yet. Instead, four putative functional motifs have been reported for the second domain, the glycosyl transferase.

It is known that structure of a protein determine its function. Therefore, function of a protein could be deduced by comparing protein sequences and structures with homolog proteins of known function. Similar motifs between two proteins generally will have same function, especially when they are homolog (Horton *et al*., 2006). Functional motifs within the *BfSUS* polypeptide were elucidated by finding functional conserved motifs within other enzymes of GT family which had been previously annotated. Buschiazzo *et al*. (2004) had successfully isolated and crystallized another GT family enzyme from *Agrobacterium tumefaciens*, the Glycogen Synthase (GS). GS catalyses the synthesis of the α-1,4-glucose backbone in the reaction of glycogen biosynthesis. This enzyme possesses the GT domain, a domain also found in SUS enzyme, with some annotated motifs within it. According to homolog motifs in GS (Buschiazzo *et al*., 2004), four functional motifs predicted as urasile diphosphate (UDP)-binding pockets were detected in the BfSUS polypeptide.

The first putative UDP binding pocket found in BfSUS polypeptide is Gly169, a residue located in a glycine rich motif, 166-DTGGQ-170 (Figure 7) (Huber and Huber, 1996; Buschiazzo *et al*., 2004). The Glycine (G) residue is predicted to directly contact with phosphate group of UDP molecule that binds to SUS protein. Buschiazzo *et al*. (2004) discovered that, in the close conformation of the GS protein, the glycine residues in the KXGGL motif come into contact with the phosphate groups of UDP. This role had been reported also in the GS of *Escherichia coli* (Furukawa *et al*., 1993). They reported that only the glycine residues but not the basic side chain appear to be essential for GS enzymatic activity. The DTGGQ motif was found to be conserved in sucrose phosphate synthase (SPS), another member of GT family, which functioned as fructose-6-P binding site (Huber and Huber, 1996).

The three subsequent UDP-putative binding pockets in *BfSUS* polypeptide are 444-MAR-446 residues, N520, and T546 (Figure 8 and 9). It was observed that the MAR motif within the BfSUS polypeptide is located at the C-terminal end of a β-strand (Figure 8), a pattern that is also occurred in ISR motif of GS polypeptide, albeit in different number of β-strand. This probably reflects their similar role. It was observed that the guanidinium group of R299, in GS, interacts with the phosphate group of ADP via a hydrogen bonding (Buschiazzo *et al*., 2004). The MAR residues of BfSUS polypeptide thereby suggested as binding site for phosphate group of UDP via a hydrogen interaction. The Asn520 (N520) motif of BfSUS polypeptide is predicted to bind with the adenine ring of the UDP molecule. According to GS, the carbonyl group of protein backbone in Asn520 may be interacted with atom N6 of the adenine ring of UDP molecule via a weak hydrogen interaction. The last putative UDP binding pocket, the T546 was suggested to bind ribose sugar of UDP molecule. The side chain of T546 may interact via hydrogen bond with O_2 atom of the ribose sugar.

The last conserved region of SUS protein that is also found in *BfSUS* polypeptide is the Ser170 putative phosphorylation site. This essential site was found within a typical serine residue containing- motif, 'RHLSS', which lay between Arg167 and Ser171. In the partial BfSUS polypeptide this motif is located between Arg30 and Ser34 (30-RHLSS-34). This motif was firstly detected in *Zea mays SUS* protein, spanned from Arg159 to Ser163 (Hardin *et al*., 2003). This motif was also found in *Oryza sativa*, *Saccharum officinarum* (R159 to S163), *Bambusa*

oldhamii, *Triticum aestivum* (R167 to S171), and *Tulipa gesneriana* (R161 to S165). Hardin *et al* (2003) reported that SUS protein of *Zea mays* is phosphorylated by calcium-dependent protein kinases (CDPKs) at the Ser170 residue, within the RHLSS motif, in addition to Ser15 (Asano *et al.*, 2002). Ser15 was reported as a major phosphorylation site that affects cleavage activity and membrane association, whereas Ser170 is a minor phosphorylation site that may trigger enzyme degradation via the ubiquitin/26S proteasome (Qiu *et al.*, 2007). Since phoshorylation of Ser170 is important for enzyme degradation but not directly involved in the catalytic activity of SUS, this site is suggested as one of the allosteric sites of the enzyme.

Those all results confirm that the isolated 1866 bp of *BfSUS* gene fragment is most likely the sucrose synthase- encoding gene in *B. flabellifer* that encodes SUS protein. Further expression study however is required to prove that the *BfSUS* gene actually expresses sucrose synthase protein in *B. flabellifer*. The sequence of partial *BfSUS* has been submitted to the GenBank with accession number **GQ265926**.

MATERIALS AND METHODS

Plant Material

Young leaves were picked from the apical shoot of 35-40 years age-plant of Palmyra palm (*Borassus flabellifer*) grown in Lasiana shore area in Kupang, East Nusa Tenggara. Leaves samples were cleaned, wrapped, and immediately stored in cold condition before being taken to Bandung. The whole processes of sampling were done with RNase-free standard work procedure. Leaves samples were then frozen in liquid nitrogen and were stored in -80^{0}C refrigerator before RNA isolation. All the laboratory works took place in the Laboratory of Genetics and Molecular Biology, in SITH-ITB, Bandung.

RNA Isolation

Total RNA of *B. flabellifer* was extracted from the fresh young leaves that were previously stored in -80ºC. All chemicals were diluted in RNAse-free water and prepared using RNAse-free equipments. All equipments were formerly treated with dietylpyrocarbonate (DEPC) before use. Grinding equipment, such as mortar, pestel, and scissors were chilled before use, either by storing in -80ºC or by soaking in liquid nitrogen.

Five protocols have been tested to optimize RNA isolation method from young leaves of *B. flabellifer*. Those are method of Seki *et al.* (2002), method of Khemvong and Suvachittanont (2005), modified-method of Apt *et al.* (1995), TRIzol-modified (I) method, and TRIzol modified (II) method (Invitrogen). Quality of RNA is determined by absorbance ratio in 260 nm and 280 nm UV light in the range of 1.80 – 2.00 which indicated low contamination of protein. Appearance of two typical ribosomal RNA bands, 18s and 28s, in electrophoresis gel also indicated good quality of RNA. The quantity of RNA was determined by RNA yield (µg) per total solution volume (ml).

Primer Design

Sequence of *B. flabellifer* SUS gene is currently unavailable in the GenBank, thereby pair of primer was designed from highly conserved region of SUS proteins among monocot plant species. Sucrose synthase protein sequences of *Oryza sativa*, *Zea mays*, *Saccharum officinarum*, and other monocots, were accessed from NCBI GenBank database (www.ncbi.nlm.nih.gov). Multiple sequence alignment of SUS proteins was conducted using Clustal X software. The most conserved regions in SUS proteins among those species were chosen for primer sequence

determination. Primer sequences were deduced from those regions by choosing a particular group of species whose cDNA sequence is highly conserved. This was accomplished by BioEdit and CodeHop programs. Nucleotide number 412 to 432 of *SUS* gene of *Zea mays* was chosen as forward primer; while reverse primer was taken from nucleotide number 2277 to 2252. Primer physical characteristics were measured by *sigma-genosys* primer calculator. Specificity of both primer pairs were tested by nucleotide BLAST program (Altschul *et al*., 1997). The chosen primer sequences showed eligible characteristics, and more importantly, exhibited high specificity for SUS gene (Table 2).

Reverse Transcription-PCR

Isolation of sucrose synthase (SUS) gene from *B. flabellifer* was accomplished by *two step* reverse-transcription polymerase chain reaction (RT-PCR) method. In the first step, total cDNA is synthesized from the total RNA by using *Superscript II cDNA synthesis kit* (Invitrogen). In the second step, PCR amplification using SUS gene-specific primers was carried out to specifically amplify fragment of SUS gene from the previously isolated cDNA.

Total cDNA synthesis

Total cDNA synthesis of *B. flabellifer* was conducted using *Superscript II cDNA synthesis kit* (Invitrogen). Composition of each component in cDNA synthesis reaction is described in Table 3. All solutions are diluted in DEPC-treated deionized water. All steps were carried out according to RNAse-free standard work procedure to avoid or minimize degradation of the RNA.

A master mix solution was prepared by mixing buffer RT with $MgCl_2$, Dithiothreitol (DTT), and RNase out ^{TM} recombinant RNase. Separately, RNA sample was mixed with oligo dT primer and dNTPs prior to five minutes incubation in 65ºC and then one minute in ice. The master mix was then added to the RNA sample mixture and then diluted with DEPC-treated water to 20 µl final volume. This final mixture was incubated for two minutes in 42^0C for primer annealing. Superscript^{TM} II- Reverse Transcriptase was subsequently added to the mixture, gently homogenized, and then incubated for 50 minutes in 42ºC for complete reaction of cDNA synthesis. The reaction was terminated by 15 minutes incubation in 70ºC. To eliminate any remaining RNA strands in the cDNA solution, RNaseH were added to the solution and then incubated in 37^0C for 20 minutes. Total cDNA of *B. flabellifer* young leaves was obtained at the end of this step.

PCR Amplification for Isolation of *SUS* gene

A standard PCR amplification technique was conducted following the cDNA synthesis to specifically isolate the SUS gene. Optimization of PCR profile, especially annealing temperature, was carried out to find the best condition that favor specific amplification of the SUS gene fragment. A range of annealing temperature, from 45ºC to 55ºC, was tested. Concentration of cDNA template, ranging from 0.5 to 2 µg /50 µl, and Mg^{2+}, ranging from 1 to 2.5 mM, were tested as well to obtain best fragment of the target gene. The PCR reaction was carried out using a *Long PCR Enzyme Mix* product (FERMENTAS). Composition of the PCR mixture is described in Table 4.

Gene Cloning and Transformation of *E. coli*

The product of RT-PCR was observed by electrophoresis using 1% agarose gel. Detected SUS fragment was purified from the agarose gel and then cloned to the pGEM-T Easy® vector by employing T4 ligase enzyme. To amplify the SUS fragment, the recombinant plasmids were subsequently delivered into bacterial cell of *Escherichia coli* strain DH5α using heat-shock

treatment. The transformed cells of *E. coli* that contain recombinant plasmid were selected through screening of blue-white colony. The transformed cells were cultured for 16 hours in Luria-Bertany (LB) media containing 0.1 ng/µl ampicillin. The plasmids DNA of the transformed cells were then extracted by alkaline lysis method (Sambrook *et al*., 1989). Insertion of SUS fragment into plasmid was confirmed by cutting the plasmid with two endonuclease restriction enzymes, *EcoRI* and *NotI*.

Sequencing and Characterization of BfSUS Gene and Polypeptide

SUS putative fragment that have been cloned to pGEM-T easy plasmid were further analyzed by dideoxy chain-termination method to read the nucleotide sequence of the isolated fragment. Minimum 150 ng/µl of plasmid DNA is required to a single sequencing reaction. Since the 1866 bp gene fragment is relatively long for a single accurate reading by the sequencer system, two steps of sequencing are needed to accomplish the total fragment reading. The first sequencing employed T7 and SP6 universal primers, which followed by second sequencing using gene internal–primers. Gene-internal primers were designed from the first sequencing result. Sequencing result from those four primer directions were finally assembled using Geneious 4.6.2 program. All sequencing process was conducted by Macrogen Inc. in South Korea.

Characterization of *BfSUS* putative gene comprises nucleotide and polypeptide sequence analysis. The nucleotide sequence of the putative *BfSUS* was aligned with SUS cDNA sequences deposited in GenBank (www.ncbi.nlm.nih.gov) using BLASTn program in order to find their similarity. The *BfSUS* cDNA sequence was then translated into BfSUS polypeptide using Geneious 4.6.2, followed by searching in the GenBank using BLASTp program. The highly conserved regions, including essential domains and motifs, were searched within BfSUS putative polypeptide, either by employing Geneious 4.6.2 program or by comparing the polypeptide with other related proteins that have been previously annotated. Other proteins from the same family are preferable since it is expected to share similar properties with the SUS. To reveal genetic relationship between *Borassus flabellifer* and other plant species based on their SUS polypeptide sequence, a neighbor- joining (NJ) phylogenetic tree was generated using Geneious 4.6.2 tree builder.

CONCLUSION

A partial cDNA fragment of sucrose synthase (SUS) putative gene has been successfully isolated from the young leaves of *B. flabellifer*, henceforth named as *BfSUS*. The sequence has been submitted to the Genbank with accession number **GQ265926**. The sequence of *BfSUS* cDNA, and the corresponding BfSUS polypeptide, is highly similar with *SUS* cDNA and SUS protein from various plant species recorded in GenBank, with highest similarity shown by *Tulipa gesneriana* and *Oncidium sp*. The *BfSUS* putative polypeptide performs all typical characteristics of SUS protein, including most conserved domains and motifs. Presence of sucrose synthase (SUS) and glycosyl transferase (GT) domains, four putative UDP-glucose binding pockets within the GT domain, and a calcium-dependent Ser/Thr protein kinase binding site (Ser170) within the SUS domain, confirms that the *BfSUS* cDNA fragment obtained in this study is most likely encodes sucrose synthase in *B. flabellifer*. This also assuring that *BfSUS* gene is expressed in the young leaves of *B. flabellifer*.

Table 1. Summary of five tested methods for RNA isolation from young leaves of *B. flabellifer*. TRIzol- modified II found to be the best method for RNA isolation from the young leaves of *B. flabellifer*

M e t h o d	Ratio of UV absorbance (A_{260}/A_{280})	RNA quality in gel electrophoresis	RNA yield (µg/ml) *)	Total time (hour)
TRIzol-modified I (Invitrogen)	1.23 – 1.24	Average	114.75	5
Seki *et al.*, 2002	1.44 – 1.47	Poor	93.50	8
Khemvong &Suvachittanont (2005)	1.67 – 2.00	Poor	44.625	12
Apt *et al.* (1995)-modified	1.45 – 1.47	Good	148.75	18
TRIzol-modified II (Invitrogen)	**1.61 – 1.62**	**Good**	**1600**	**4**

**) RNA yield = (Absorbance in 260 nm) x (42.5 µg/ml) x (dilution factor)*

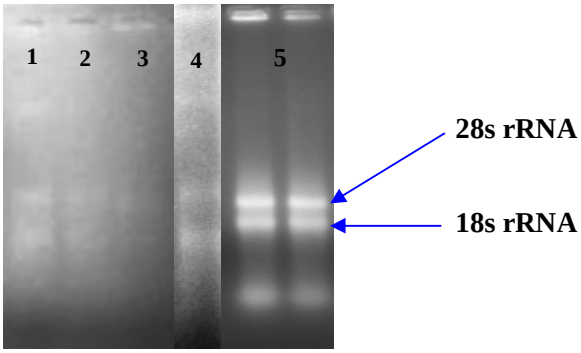


Figure 1. Comparison of RNA quality obtained by the five tested methods. Two bands of ribosomal RNA, the 18s and 28s rRNA, that were occurred in electrophoresis gel indicated that total RNA of *B. flabellifer* has been successfully obtained. TRIzol-modified II method is the best method for RNA isolation from the young leaves of *B. flabellifer*.

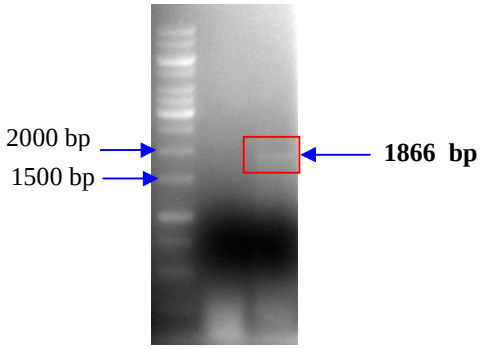


Figure 2. A single cDNA fragment of 1866 bp, generated by reverse transcription-PCR, predicted as the target fragment of the SUS gene of *B. flabellifer*.

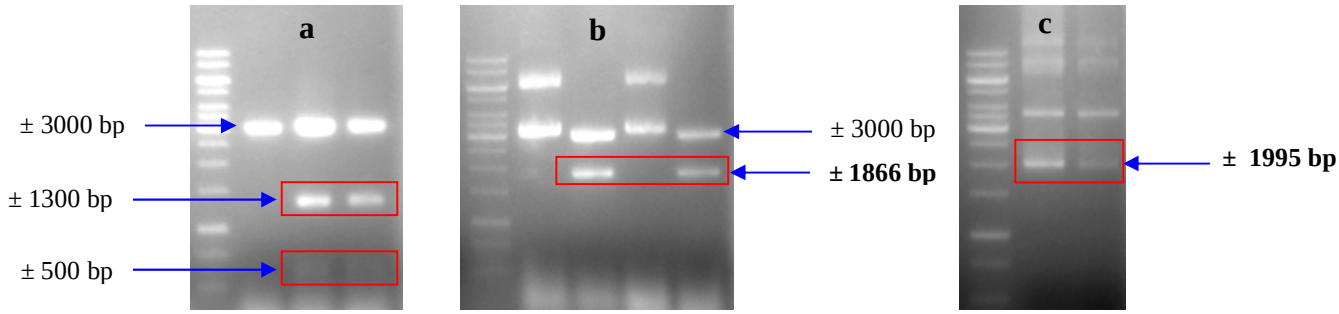


Figure 3. (a) The recombinant plasmid cut with *EcoRI* generated two unexpected ± 1300 and ± 500 bp fragments indicating gene internal cut by this enzyme; **(b)** plasmid cut with *NotI* generated a single expected fragment of length ±1866 bp, indicating that SUS gene of *B. flabellifer* had been successfully inserted in the MCS region of the plasmid; **(c)** PCR screening using SP6 and T7 primers resulted in ±1995 bp fragment confirmed the presence of SUS fragment within the MCS region of the pGEM®-T easy plasmid.

Table 2. Pair of forward and reverse primers used to isolate sucrose synthase gene from total RNA of *Borassus flabellifer* young leaves.

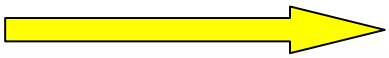
Primer Code	Primer Sequence	Start	Primer Length	Product Size (bp)	GC (%)	Tm (ºC)	Sec. Structure	BLAST Output (Σ species)
SUSFw	CTTGAGCTGGACTTTGAGCCA	412	21		52.38	62.92	Weak	10
SUSRv	CTTCCAGGTGTACTTCTCCTCGATAC	2277	26	1866	50.00	62.72	Weak	8

Table 3. Chemical composition in cDNA synthesis

No.	Component	Final concentration
1	Total RNA of *B. flabellifer*	2 µg/20 µl
2	dNTPs	0.5 Mm
3	Oligo $(dT)_{12-18}$ primer	0.025 µg/µl
4	Buffer RT	1x
5	$MgCl_2$	5 mM
6	Dithiothreitol (DTT)	0.01 M
7	RNase out™ Recombinant Rnase	1 u/20 µl
8	Superscript™ II- Reverse Transcriptase	1u/20 µl
9	DEPC-treated deionized water	until 20 µl
10	*E. coli* RNaseH	1 µl/20 µl

Table 4. Composition of PCR mixture for SUS cDNA isolation

No.	Component	Final concentration
1	10x long PCR buffer	1x
2	2 mM dNTP mix	0.2 mM
3	25 mM $MgCl_2$	2 mM
4	Primer (forward and reverse)	0.2 µM
5	Total cDNA	0.03 µg/µl
6	Long PCR enzyme mix	1.25 unit/ 50 µl
7	Nuclease free water	to 25 µl



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1     CTT GAG CTG GAC TTT GAG CCA TTC AAT GCT TCC TTT CCT CGG CCT   45
1     Leu Glu Leu Asp Phe Glu Pro Phe Asn Ala Ser Phe Pro Arg Pro   15

46    TCA CTG TCG AAA TCC ATT GGT AAT GGA GTG CAG TTC CTC AAT CGC   90
16    Ser Leu Ser Lys Ser Ile Gly Asn Gly Val Gln Phe Leu Asn Arg   30

91    CAC CTC TCT TCA AAA CTG TTT CAT GAC AAA GAA AGC ATG TAC CCA   135
31    His Leu Ser Ser Lys Leu Phe His Asp Lys Glu Ser Met Tyr Pro   45

136   CTG CTT AAT TTT CTT CGG GCA CAC AAA TAT AAG GGA ATG ACA ATG   180
46    Leu Leu Asn Phe Leu Arg Ala His Lys Tyr Lys Gly Met Thr Met   60

181   ATG TTA AAT GAT AGA ATA CAA AGC CTG AGC GCT CTC CAA GCT GCA   225
61    Met Leu Asn Asp Arg Ile Gln Ser Leu Ser Ala Leu Gln Ala Ala   75

226   TTA AGA AAG GCA GAG GAA TAT CTG TTG AGT ATC CCA GCA GAC ACT   270
76    Leu Arg Lys Ala Glu Glu Tyr Leu Leu Ser Ile Pro Ala Asp Thr   90

271   CCT TAT TCG GAG TTT AAT CAC AGG TTT CAA GAG CTT GGT TTA GAG   315
91    Pro Tyr Ser Glu Phe Asn His Arg Phe Gln Glu Leu Gly Leu Glu   105

316   AAG GGT TGG GGT GAT ACA GCT CAG CGT GTT GGT GAG ACT ATT CAT   360
106   Lys Gly Trp Gly Asp Thr Ala Gln Arg Val Gly Glu Thr Ile His   120

361   CTA CTC CGT GAT CTT CTT GAG GCA CCT GAT CCT TGC ACC CTA GAG   405
121   Leu Leu Arg Asp Leu Leu Glu Ala Pro Asp Pro Cys Thr Leu Glu   135

406   AAA TTT CTT GGG ACA ATT CCT ATG GTC TTT AAT GTT GTC ATT TTA   450
136   Lys Phe Leu Gly Thr Ile Pro Met Val Phe Asn Val Val Ile Leu   150

451   TCT CCA CAC GGT TAC TTT GCC CAA GCT AAT GTT TTG GGA TAC CCT   495
151   Ser Pro His Gly Tyr Phe Ala Gln Ala Asn Val Leu Gly Tyr Pro   165

496   GAC ACT GGA GGC CAG ATT GTC TAT ATT TTG GAT CAA GTT CGT GCA   540
166   Asp Thr Gly Gly Gln Ile Val Tyr Ile Leu Asp Gln Val Arg Ala   180

541   CTA GAG AGT GAG ATG CTT CTT AGA ATG AAG CAG CAA GGT CTC AAT   585
181   Leu Glu Ser Glu Met Leu Leu Arg Met Lys Gln Gln Gly Leu Asn   195

586   ATC ACT CCT CGA ATT CTT ATT GTA ACT AGA TTA CTA CCT GAT GCA   630
196   Ile Thr Pro Arg Ile Leu Ile Val Thr Arg Leu Leu Pro Asp Ala   210

631   ATT GGG ACC ACT TGC GGT CAG CGG CTT GAG AAA GTC CTA GGC ACA   675
211   Ile Gly Thr Thr Cys Gly Gln Arg Leu Glu Lys Val Leu Gly Thr   225

676   AAG CAC ACG CAC ATT CTG CGG GTT CCA TTT AGA AAT GAA AAG GGA   720
226   Lys His Thr His Ile Leu Arg Val Pro Phe Arg Asn Glu Lys Gly   240

721   ATC CTT CGC AAA TGG ATC TCA CGC TCC GAT GTA TGG CCT TAC CTT   765
241   Ile Leu Arg Lys Trp Ile Ser Arg Ser Asp Val Trp Pro Tyr Leu   255

766   GAA ACT TAT GCA GAG GAT GTT GCA AAT GAA CTG GCT GGA GAA CTG   810
256   Glu Thr Tyr Ala Glu Asp Val Ala Asn Glu Leu Ala Gly Glu Leu   270

811   CAG GCA ACC CCG GAT CTC GTT ATT GGA AAC TAC AGT GAT GGA AAC   855
271   Gln Ala Thr Pro Asp Leu Val Ile Gly Asn Tyr Ser Asp Gly Asn   285

856   CTA GTA GCA TCT TTG TTG GCA CAT AAA CCA GGG GTT ACT CAG TGT   900
286   Leu Val Ala Ser Leu Leu Ala His Lys Pro Gly Val Thr Gln Cys   300

901   ACT ATT GCC CAT GCC TTG GAG AAA ACA AAG TAT CCA AAT TCA GAT   945
301   Thr Ile Ala His Ala Leu Glu Lys Thr Lys Tyr Pro Asn Ser Asp   315
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946 ATA TAC TGG AAA AAG TTT GAG AAC CAG TAC CAT TTT TCT TCT CAG 990
316 Ile Tyr Trp Lys Lys Phe Glu Asn Gln Tyr His Phe Ser Ser Gln 330

991 TTT ACT GCT GAT TTA ATT GCT ATG AAT CAT GCT GAT TTC ATT ATC 1035
331 Phe Thr Ala Asp Leu Ile Ala Met Asn His Ala Asp Phe Ile Ile 345

1036 ACC AGT ACT TTC CAA GAG ATT GCC GGA AGC AAG GAC ACC GTT GGG 1080
346 Thr Ser Thr Phe Gln Glu Ile Ala Gly Ser Lys Asp Thr Val Gly 360

1081 CAG TAC GAG TCT CAC ATT GCA TTT ACC CTC CCT GGG CTC TAC CGA 1125
361 Gln Tyr Glu Ser His Ile Ala Phe Thr Leu Pro Gly Leu Tyr Arg 375

1126 GTG GTT CAT GGA ATT GAT GTA TTT GAC CCA AAG TTC AAC ATT GTT 1170
376 Val Val His Gly Ile Asp Val Phe Asp Pro Lys Phe Asn Ile Val 390

1171 TCC CCT GGA GCT GAT ATG TCT ATT TAC TTC CCT TAC ACG GAA GAG 1215
391 Ser Pro Gly Ala Asp Met Ser Ile Tyr Phe Pro Tyr Thr Glu Glu 405

1216 AGT AAG AGA TTG ACT TCT CTT CAC CCT GAA ATT GAG GAG CTC CCC 1260
406 Ser Lys Arg Leu Thr Ser Leu His Pro Glu Ile Glu Glu Leu Pro 420

1261 TTC AGT TCT GTG GAG AAC TCT GAA CAC AAG TTT GTA TTG AAG GAC 1305
421 Phe Ser Ser Val Glu Asn Ser Glu His Lys Phe Val Leu Lys Asp 435

1306 CGG AAT AAG CCT ATT ATC TTC TCA ATG GCA AGA TTG GAC CGT GTG 1350
436 Arg Asn Lys Pro Ile Ile Phe Ser Met Ala Arg Leu Asp Arg Val 450

1351 AAG AAC ATG ACA GGT CTA GTT GAG CTA TAT GGA AGG AAT GCC CGT 1395
451 Lys Asn Met Thr Gly Leu Val Glu Leu Tyr Gly Arg Asn Ala Arg 465

1396 CTG AGG GAA CTG GTC AAC CTT GTC GTT GTG GCT GGA GAC CAT GGG 1440
466 Leu Arg Glu Leu Val Asn Leu Val Val Val Ala Gly Asp His Gly 480

1441 AAG GAA TCG AAG GAT CTT GAG GAA CAA GAA GAG CTA AAG AAG ATG 1485
481 Lys Glu Ser Lys Asp Leu Glu Glu Gln Glu Glu Leu Lys Lys Met 495

1486 TAC AGG CTC ATT GAT CAG TAC AAA TTG AAT GGC CAG ATC CGC TGG 1530
496 Tyr Arg Leu Ile Asp Gln Tyr Lys Leu Asn Gly Gln Ile Arg Trp 510

1531 ATC TCT GCC CAG ATG AAC AGG GTT CGC AAT GGT GAG CTA TAC CGC 1575
511 Ile Ser Ala Gln Met Asn Arg Val Arg Asn Gly Glu Leu Tyr Arg 525

1576 TAC ATT GCC GAT ACC GGA GGA GCC TTT GTT CAG CCG GCA TTT TAT 1620
526 Tyr Ile Ala Asp Thr Gly Gly Ala Phe Val Gln Pro Ala Phe Tyr 540

1621 GAA GCA TTT GGG CTC ACA GTT ATC GAA GCC ATG ACG TGT GGC CTG 1665
541 Glu Ala Phe Gly Leu Thr Val Ile Glu Ala Met Thr Cys Gly Leu 555

1666 CCC ACA TTT GCA ACA GCC AAC GGA GGG CCA GCT GAG ATC ATA GTT 1710
556 Pro Thr Phe Ala Thr Ala Asn Gly Gly Pro Ala Glu Ile Ile Val 570

1711 CAT GGC GTT TCC GGC TTC CAC ATT GAT CCT TAC CAG GGT GAC AAA 1755
571 His Gly Val Ser Gly Phe His Ile Asp Pro Tyr Gln Gly Asp Lys 585

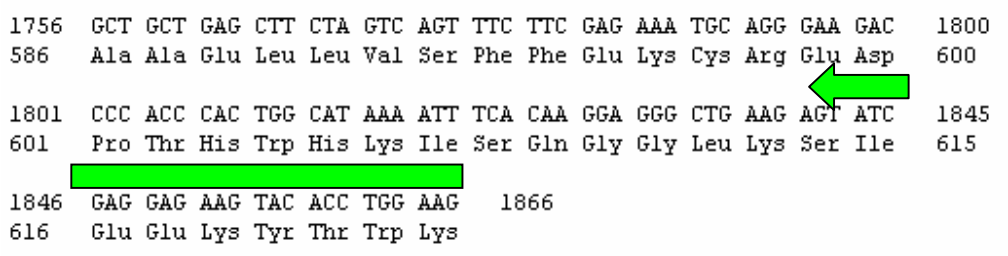

Figure 4. Sequence of partial cDNA of *Borassus flabellifer SUS* putative gene. The yellow arrow indicates forward primer, SSFw, and the green arrow indicates reverse primer, SSRv. The red arrow indicates internal primer p1, and the blue arrow indicates internal primer p2. The red box indicates restriction site of *EcoRI* endonuclease restriction enzyme. The brown box indicates putative phosphorylation site (30-RHLSS-34). The blue boxes indicate four putative UDP binding pockets (166-DTGGQ-170; 444-MAR-446; N520; and T546)

Figure 5. The BfSUS putative polypeptide sequence with total length 622 amino acids was generated by translating the sequence of *BfSUS* cDNA, using Geneious 4.6.2.

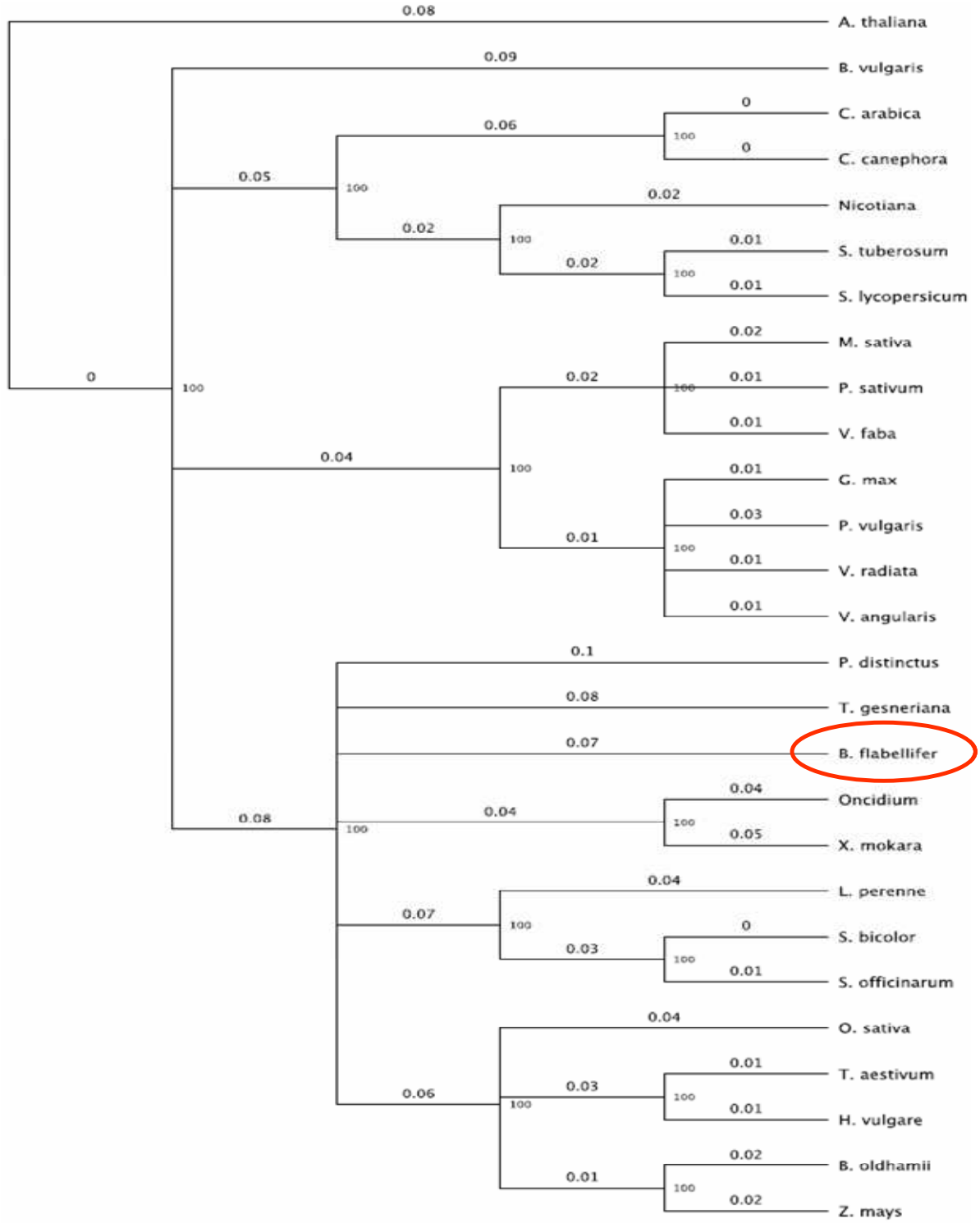

Figure 6. A phylogenetic tree based on sucrose synthase polypeptide sequence of various monocot and dicot species. Similarity of SUS polypeptide among monocot species is distinctively higher than those of dicot species. *Borassus flabellifer* is clustered with the monocot group, although not specifically grouped with particular monocot species. *B. flabellifer* showed the nearest genetic distance with *Tulipa gesneriana*.

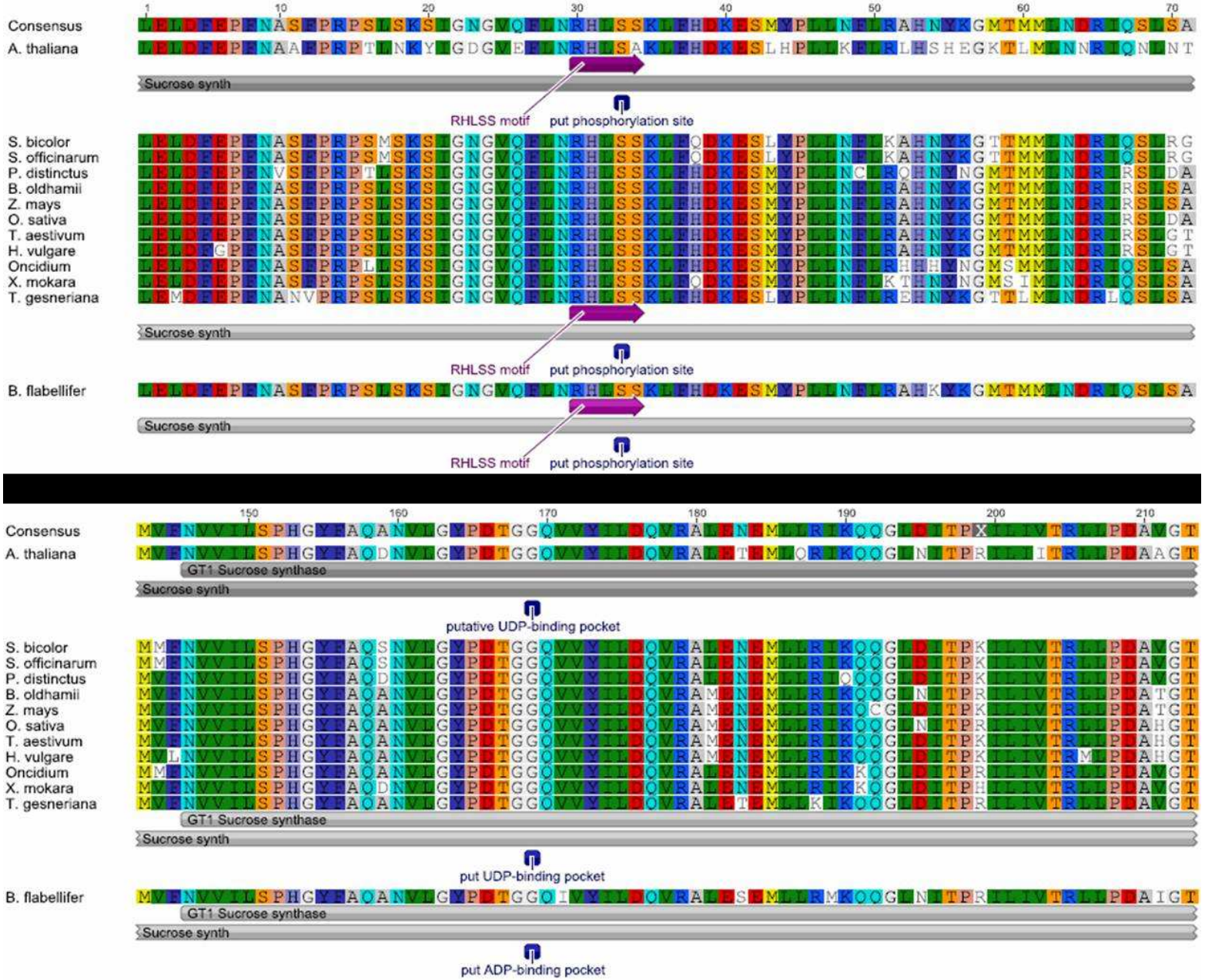

Figure 7. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3)Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

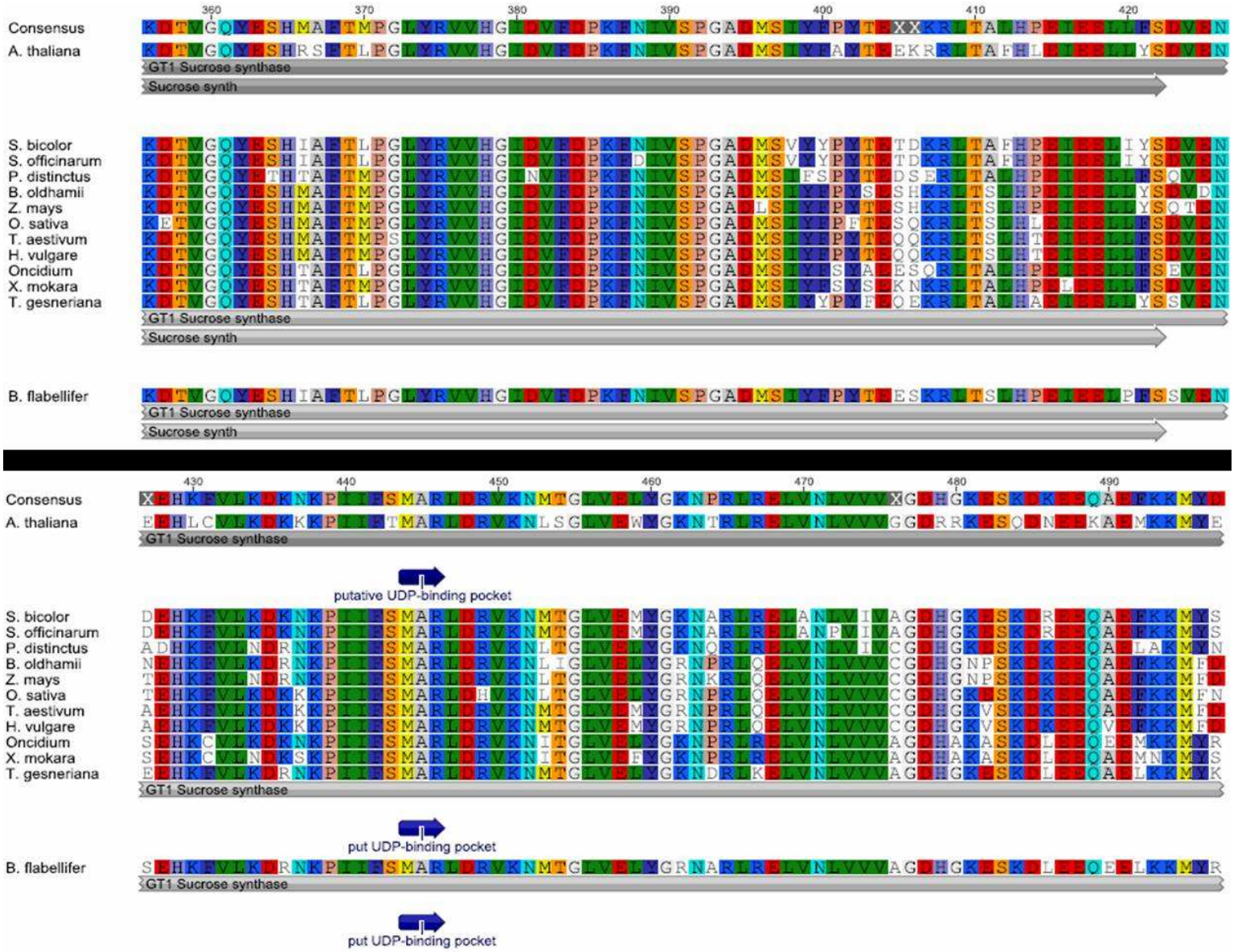

Figure 8. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3)Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

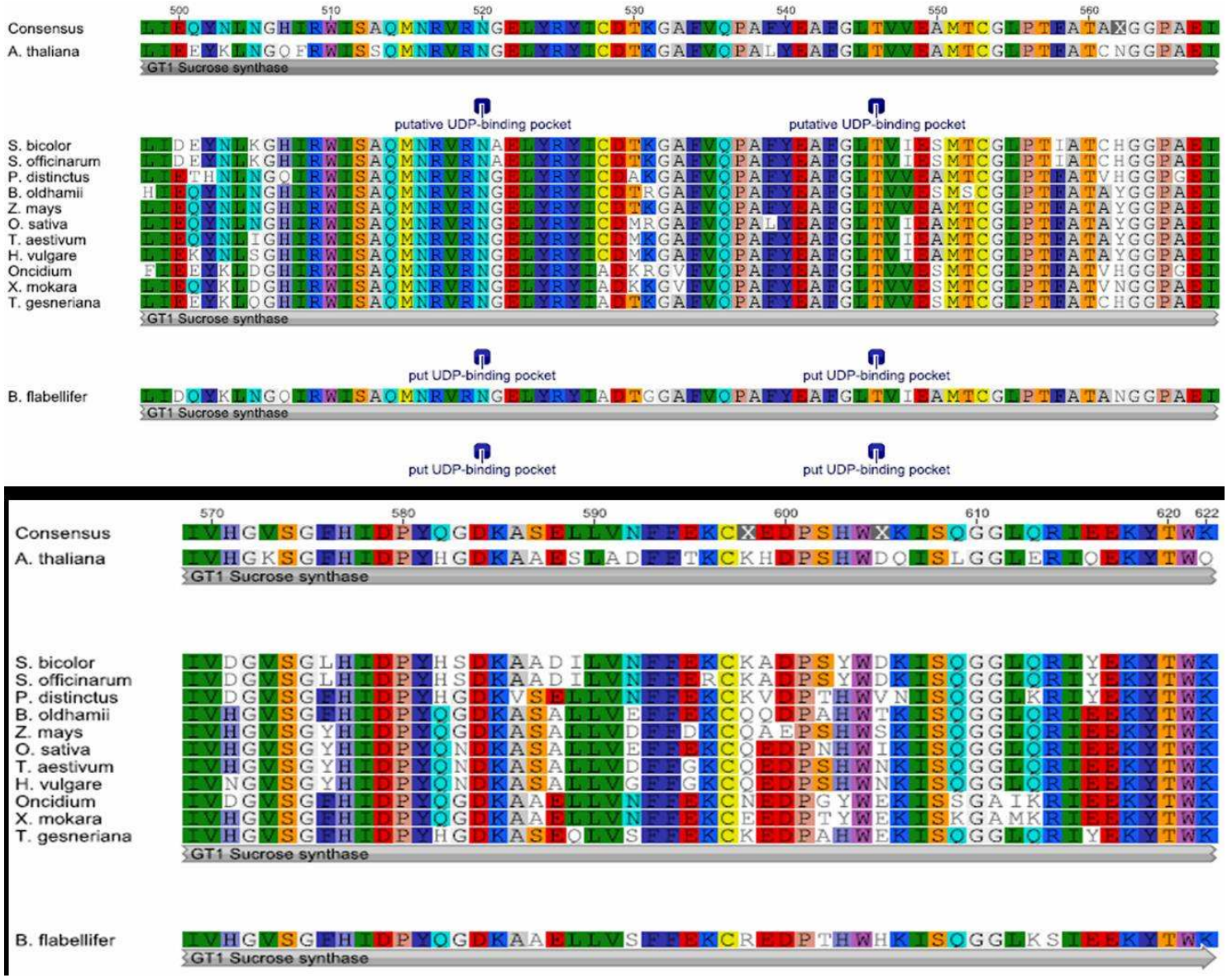

Figure 9. Seven regions in BfSUS polypeptide that highly conserved among monocot species: (1) Sucrose synthase domain spanning from N-terminal region to Ser422; (2) A putative calcium-dependent Ser/Thr protein kinase binding site in the 30-RHLSS-34 motif within the SUS domain. This site may have role in triggering enzyme degradation via the ubiquitin/26S proteasome; (3)Glycosyltransferase (GT) domain spanning from Asn146 to the C-terminal region. Four putative UDP-binding pockets are detected within this domain. They are: (4) Glycine residues in 166-DTGGQ-170 motif, (5) 444MAR446, (6) N520, and (7) T546.

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