Conference Paper

Primer Design for Isolation of Sucrose:Sucrose 1-Fructosyltransferase (1-SST) Gene from Gembili (*Dioscorea esculenta*)

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Abstract

Recent studies have reported the presence of inulin, a prebiotic polysaccharide, in gembili (*Dioscorea esculenta*). Sucrose:sucrose 1-fructosyltransferase (1-SST) is an enzyme that catalyzes the first step of inulin biosynthesis. The identification of this enzyme would be the foundation to improve the yield of inulin in gembili; to modify 1-SST gene for this purpose, its sequence must first be determined. This study aimed to design primers to isolate 1-SST gene from *D. esculenta*. The primers were designed by using the whole-genome sequence of *Dioscorea rotundata* due to the lack of genomic information on *D. esculenta*. Sequences from chromosome 6 and 11 were used as the template of primer design in which four pairs of primers were selected. Amplification products with expected size were gel-purified, then the targets were sequenced and analyzed in-silico. As a result, one of the primer pairs had successfully isolated vacuolar invertase gene, which is closely related to 1-SST gene. On the other hand, the other primer pairs showed either negative or false-positive result. Using the current strategy, 1-SST gene sequence from *D. esculenta* has not been successfully isolated, thus other approaches in primer design should be considered in further studies.

Keywords: 1-SST, Dioscorea esculenta, Gembili, Inulin, Primer design

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Received: 1 February 2020
Accepted: 8 February 2020
Published: 16 February 2020

Publishing services provided by Knowledge E

Selection and Peer-review under the responsibility of the IC-BIOLIS Conference Committee.

1. Introduction

The tuber of a local Indonesian plant called gembili (*Dioscorea esculenta*) has been found to contain inulin up to 14.77% dry weight [1]; this, and the fact that inulin is high in demand, encouraged Indonesian scientists to conduct further studies. Inulin is a type of fructan polysaccharide that is commonly found in dicot plants as their natural storage carbohydrates. Its main feature is its indigestibility in human enzymatic digestion system which makes it a good candidate for a prebiotic agent. Aside from its prebiotic function, inulin can also be used as a carbohydrate-based fat substitute which is used

to increase viscosity, water-holding capacity, mouthfeel, and texture as well as promote gel formation in food products [2]. It could also be used to replace sugar to lower caloric value in chocolates, dairy products, and meat products [3] According to Inkwood Research (2017), the global market for inulin is projected to increase with CAGR of 9.5% from 2018 to 2026. Asia-Pacific is believed to be the fastest-growing region for the market and Indonesia is one of the countries that demand a high amount of inulin to expand its food and non-alcoholic beverages market for the next five years. Indonesia has always imported its inulin supply. Therefore, studies about inulin production in gembili are conducted in Indonesia in the hope of producing inulin on its own.

Due to its many functions, the demand for inulin is high. Usually, inulin is extracted from chicory roots *(Chicorium intybus)* which is easily available in some countries, except Indonesia. Its biosynthesis has also been studied using chicory roots as the model plant. Two main fructosyltransferase enzymes are responsible for the biosynthesis: sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) with sucrose as the precursor [4]. The role of these fructosyltransferase is to transfer fructosyl units from one sucrose molecule to another, resulting in fructose polymer. These enzymes contain glycoside hydrolase 32 protein family along with several other enzymes such as invertase and inulinase [5]. 1-SST enzyme catalyzes the first reaction in which two sucrose molecules are converted into trisaccharide 1-kestose. 1-FFT enzyme then catalyzes the transfer of fructosyl onto the 1-kestose from another 1-kestose, other fructans, or a sucrose molecule depending on the plant species ([6]; [4] Since the information of its biosynthesis is available, the production of inulin in gembili can be manipulated by using genetic modification. However, genetic information of the enzymes in gembili is needed in order to do that.

Furthermore, the gembili plant itself is not well studied; there are no whole genome sequence available for this species which is needed to design a specific primer. Thus far, there are two whole genome sequences of the *Dioscorea* genus; the *Dioscorea rotundata* and *Dioscorea alata*. Though the whole genome sequence of *Dioscorea rotundata* has the highest assembly level, there are still no annotations and gaps still exist. With this in mind, potential candidates of 1-SST in *Dioscorea* is needed to be found *in-silico*. Therefore, this study aims to design primers to isolate 1-SST gene from *Dioscorea esculenta* using *Dioscorea rotundata* genomic sequence as the template.

2. Methods

2.1. Sample preparation

Gembili tubers (*D. esculenta*) was bought from a traditional market in Malang, East Java. They were cultivated until roots, branches, and leaves were produced. Leaves and branches were ground into a fine powder using liquid nitrogen and PVPP. The DNA sample was then extracted using Genomic DNA Mini Kit (Plant) (Geneaid, Taipei, Taiwan). The species of the tubers was confirmed by isolating the DNA barcoding sequence (MaturaseK) which was analyzed in-silico before proceeding to further procedure.

2.2. Primer Design

Since the genomic information of *D. esculenta* is not available, genomic data of *D. rotundata* was chosen as the template of primer design due to the fact that they are closely related. However, the whole genome was not annotated yet thus it was not possible to find 1-SST gene directly. Further procedure was needed to predict the location of 1-SST gene within the whole genome sequence of *D. rotundata*.

Several 1-SST sequences from eight different plant species **(Figure 1)** were aligned using MUSCLE in UGene workbench to find the conserved region among them. The resulting sequence alignment was used as an input for Hidden Markov Model (HMM) to build an HMM3 profile using UGene workbench. The HMM3 profile was then searched against the whole genome information of *Dioscorea rotundata* using *nhmmer* command in terminal (`HMMER," n.d.)[17]. Using Conserved Domains (NCBI), the candidate sequences were analyzed for the presence of conserved protein domains inside. Primers were designed using PrimeQuest Tool (Integrated DNA Technologies). The recommended primers were tested against genomic data of *D. rotundata*.

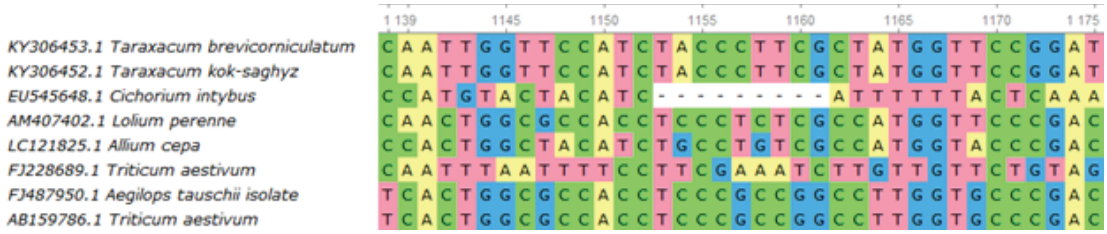


Figure 1: Preview alignment of several 1-SST sequences from different plant species.

2.3. 1-SST gene isolation

Amplification of the isolates were done using PCR with *Taq* Mix Red (PCR Biosystems, London, UK) using Thermocycler (Applied Biosystems, USA). PCR mix composition and

PCR condition for this study are shown in Table 1 and Table 2, respectively. After the isolates were amplified, they were visualized using agarose gel electrophoresis at 100V for 60 minutes using 1.5% (w/v) agarose gel (Lonza, Basel, Switzerland) dissolved in 1x TAE buffer. The resulting gel was visualized using GeneSys transilluminator.

TABLE 1: PCR mix composition for isolation of genes.

Component	Volume (µl)
2x PCRBIO Taq Mix Red	5
Forward Primer	1
Reverse Primer	1
Template DNA	3
Total Volume	10

TABLE 2: PCR conditions for each primer pair.

PCR Stage	*matK*		*Mf-Fr*		*Chr6*		*Chr11*		
	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	
Initial denaturation	95	60	95	60	95	60	95	60	
Denaturation	95	15	95	15	95	15	95	15	40 cycles
Annealing	52	15	52-57	15	50-54	15	50-54	15	
Extension	72	90	72	90	72	90	72	90	

After they were amplified, visualized, and size-confirmed, the PCR products were purified and inserted into pGEM-T Easy Vector (Promega, USA) using DNA ligase. The vector was then transformed into competent *Escherichia coli* and plated onto LB agar. Colony confirmation was done by using blue-white screening and PCR using insert-specific primers. Once confirmed, plasmids were extracted from the colony to be sent for sequencing.

2.4. Insertion and Transformation

Ratio of 5:1 insert:vector was used during the ligation of insert to the pGEM-T Easy vector. The inserted vector was then transformed into competent DH5A *E. coli* by heat shock

method in order to produce cells containing targeted insert. After the transformation, confirmation using colony PCR was done by using insert-specific primers. Colony PCR was done by picking a single colony, submerging the colony into the PCR master mix, and attempting PCR using the optimum condition. The result of PCR was then visualized by agarose gel electrophoresis. The colony that had been submerged into the PCR master mix was directly inoculated on to a fresh LB agar to conserve the confirmed colony.

2.5. 1-SST gene confirmation

The resulting sequences were aligned to form consensus sequences and then analyzed using BLASTn (NCBI) against the NCBI database. Parameters such as query cover, E-value, and identity percentage were taken into consideration in choosing the best hits in order to confirm the genes.

3. Results and Discussion

3.1. Primer choices

The result from HMM was able to find two candidates of 1-SST in *Dioscorea* (Figure 2). By looking at the E-value, the best hits were in chromosome 11 (E-val: 4e-204) and chromosome 6 (E-val: 2.9e-144). Both sequences contain glycoside hydrolases family 32 domain, which is the main protein family of 1-SST and invertase. Thus, both of the sequences were used as a candidate 1-SST gene and used as the sequence template for primer design. Primer pairs with the best specificity and coverage were chosen for 1-SST gene isolation Table 3.

3.2. Isolation of 1-SST gene using primers from chromosome 6

Since the PCR conditions for the primers were not optimized yet, the first trial of 1-SST gene isolation was done by using the primers' determined melting temperatures as the annealing temperature. The average melting temperature for both of the primers (6A and 6B) is 54^{O}C. Aside from using gembili DNA sample, onion DNA sample was also used to check whether or not the primers were working well since 1-SST gene sequence from *A. cepa* (onion) was used in the multiple sequence alignment during primer design.

TABLE 3: List of primer pairs used.

Primers		Primer sequence (5' → 3')	Product size (kb)*
6A	Forward	CACTGATGAGAGGAGGGATTTG	923
	Reverse	CCGGATCGAATCTCATAGCTTAC	
6B	Forward	GGTTGAGTGTTTGGTGTTGTATT	1129
	Reverse	AGGAGATGGATGTTGGGATTG	
11A	Forward	AGATCCGAATGGTGAGTTCTTC	1004
	Reverse	AAGAGAGAGAAGCGTGAATGG	
11B	Forward	CCATTCACGCTTCTCTCTCTTT	968
	Reverse	CCTGCTTCTCCAGGTTTGAATA	

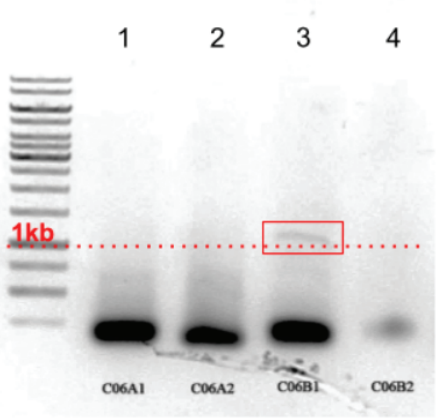
```
# nhmmer :: search a DNA model or alignment against a DNA database
# HMMER 3.1b2 (February 2015); http://hmmer.org/
# Copyright (C) 2015 Howard Hughes Medical Institute.
# Freely distributed under the GNU General Public License (GPLv3).
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
# query file:                      hmm.hmm
# target sequence database:        rotundata_genomic.fna
# number of worker threads:        4
# - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Query:       Multiple_alignment  [M=2013]
Scores for complete hits:
    E-value  score  bias  Sequence          start      end  Description
    ------- ------ -----  --------          -----    -----  -----------
     4e-204  682.7   0.0  BDMI01000011.1   469817   473359  Dioscorea rotundata DNA, contig: Chromosome_11_P1_11_P2_9,
    2.9e-144  484.3   0.0  BDMI01000006.1  5758439  5755711  Dioscorea rotundata DNA, contig: Chromosome_06_P1_6_P2_6,
     3.4e-35  122.8   9.6  BDMI01000010.1   280037   280757  Dioscorea rotundata DNA, contig: Chromosome_10_P1_10_P2_14
     6.7e-34  118.5   0.3  BDMI01000006.1  5759714  5759279  Dioscorea rotundata DNA, contig: Chromosome_06_P1_6_P2_6,
     1.1e-24   88.0   9.1  BDMI01000008.1 10499625 10499364  Dioscorea rotundata DNA, contig: Chromosome_08_P1_8_P2_11,
     8.9e-18   65.1   3.8  BDMI01000001.1  3398892  3398670  Dioscorea rotundata DNA, contig: Chromosome_01_P1_1_P2_18,
     1.9e-17   64.0   5.4  BDMI01000010.1  9546601  9546806  Dioscorea rotundata DNA, contig: Chromosome_10_P1_10_P2_14
```

Figure 2: Preview output of nhmmer with inputs of 1-SST multiple sequence alignment from different species and whole genome sequence of Dioscorea rotundata. Chromosome 11 has the lowest E-value, indicating higher significance.

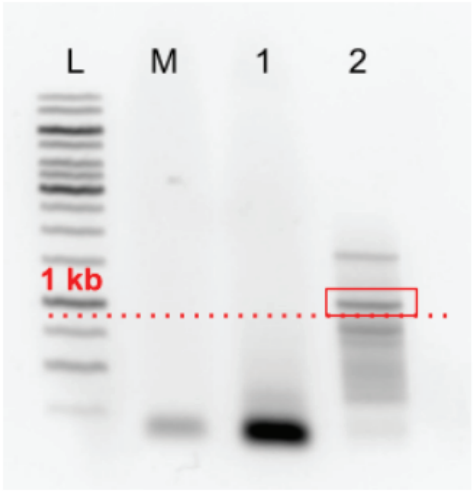
Gene amplification with an annealing temperature of 54^{O}C resulted in a very faint band with the size of approximately 1 kb from Gembili DNA sample using 6B primers (**Figure 3**). However, amplification using 6A primers from Gembili DNA sample did not result in any band with the correct size (**Figure 4**). Meanwhile, amplification of 1-SST gene from onion DNA sample did not result in any band with the correct size when using 6A primers (**Figure 4**). In contrast, amplification using 6B primers from onion DNA

sample did result in multiple bands, with a band of approximately 1 kb showing among them (**Figure 5**).



Lane 1&2: Amplification using 6A primers
Lane 3&4: Amplification using 6B primers

Figure 3: Amplification using 6A and 6B primers with annealing temperature set at 54^{O}C using Gembili DNA sample.

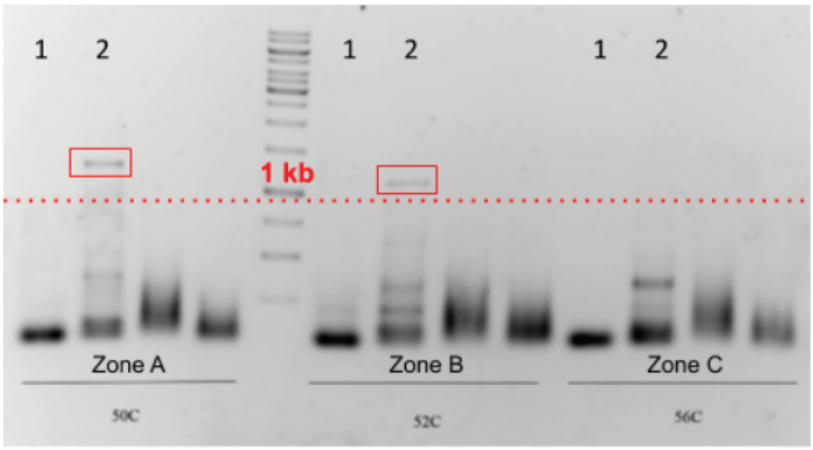


Lane 1: Amplification using 6A primers
Lane 2: Amplification using 6B primers

Figure 4: Amplification using 6A and 6B primers with annealing temperature set at 54^{O}C using Onion DNA sample.

Since the results were insignificant, an optimization of annealing temperatures using gembili DNA sample was done afterward. Temperatures of 50, 52, and 56^{O}C were used during the PCR reaction. As a result, bands with approximately 1 kb size were shown in amplification using 6B primers with annealing temperatures at 50 and 52^{O}C (**Figure 7**). Since the amplification still resulted in multiband, gel purification of the band with 1 kb size was done and the purification product was used as the sample for a further PCR reaction with the same annealing temperatures. Meanwhile, amplification using 6A

primers did not result in bands with the desired size in either one of the reactions with different annealing temperatures.



Lane 1: Amplification using 6A primers
Lane 2: Amplification using 6B primers
Zone A: Annealing temperature at 50^{O}C
Zone B: Annealing temperature at 52^{O}C
Zone C: Annealing temperature at 56^{O}C

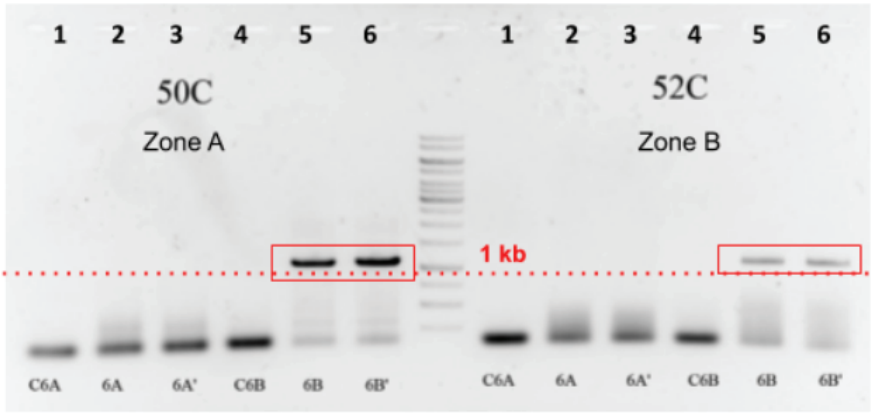
Figure 5: Amplification using different annealing temperatures (50, 52, and 56 ^{O}C) using Gembili DNA sample.

Amplification using purified gel sample with 6B primers and annealing temperature set at 50 and 52^{O}C resulted in distinct bands at 1 kb for both reactions (**Figure 6**). However, since the bands at the reaction with annealing temperatures at 50^{O}C had a higher intensity and more distinctive, it was determined that the optimal annealing temperature for 6B primers is 50^{O}C. Meanwhile, amplification using 6A primers did not result in any bands in both gembili and onion samples. This might be caused by the low specificity of the primer pair towards both samples. It could also be caused by the amplification condition that was not optimum yet.

The gel from amplification with an annealing temperature of 50^{O}C containing the desired band was purified and used as the sample for amplification with 50 µl reaction volume. The amplification product was confirmed before it was cleaned up and inserted into pGEM-T Easy vector.

3.3. Isolation of 1-SST gene using primers from chromosome 11

Two-step annealing temperature conditions were used to isolate DNA fragments on the target amplicon size; this method utilizes the capability of the thermocycler machine by dividing the samples into three separate Zones namely Zone A, Zone B, and Zone C. Each Zone performs a total of 40x cycles PCR with the first 20x cycles under higher



Lane 1: Negative control of amplification using 6A primers
Lane 2&3: Amplification using 6A primers (duplicated)
Lane 4: Negative control of amplification using 6B primers
Lane 5&6: Amplification using 6B primers (duplicated)
Zone A: Annealing temperature at 50^{O}C
Zone B: Annealing temperature at 52^{O}C

Figure 6: Amplification using purified gel sample (6B) at annealing temperature set at 50 and 52^{O}C.

annealing temperature and another 20x cycles under lower annealing temperature (**Table 4**). This method is similar to touchdown PCR where the annealing temperature decreases for every determined cycle (Green & Sambrook, 2018; Korbie & Mattick, 2008); it will reduce off-target priming. This method is particularly useful for primers that might not exactly match from the target such as those designed using other different species as the template (Green & Sambrook, 2018).

TABLE 4: Two-step annealing condition matrix.

	1st 20x cycles	2nd 20x cycles
Zone A	52 ^{O}C	50 ^{O}C
Zone B	56 ^{O}C	54 ^{O}C
Zone C	62 ^{O}C	60 ^{O}C

Higher concentration of agarose (1.5%) were used to further separate the bands; based on the considerations that there were multiple bands appearance below ~1kb. Amplicon size of around ~1004 bp and ~968 bp is expected from 11A and 11B primers respectively. The result indicates (**Figure 7 Image A**) that there were two visible bands close to the target amplicon size. To further separate the bands; the DNA were extracted and amplified under the same conditions; resulting in a more contrast band (**Figure 7**

Image B). Moreover, the bands close to ~1kb (Green rectangle) were extracted and separated until pure.

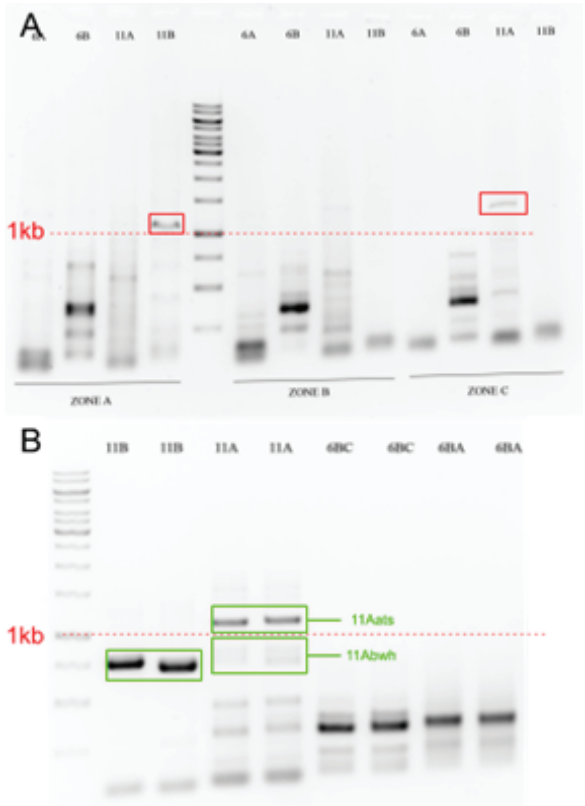


Figure 7: Visualized gel electrophoresis (Agarose 1.5%) of Genomic DNA with two-step annealing conditions. **Image A (1st Gel):** primer 11B produced a band close to 1kb under the condition of Zone A (Red rectangle). Primer 11A produced a relatively larger size of band close to 1kb under the condition of Zone C (Red rectangle). These two bands were then extracted and purified for further downstream process. **Image B (2nd Gel):** amplification of DNA samples extracted from the previous gel with two-step annealing conditions. The bands with desired amplicon size of ~1kb (Green rectangle) were then extracted and purified further.

Result on Figure 8shows that both 11A and 11B primers produced a clear band on around 1kb. Although 11A sample had undergone multiple purification using PCR and gel electrophoresis, it has unsuccessfully produced a single band. Plausibly, this phenomenon can be caused by the primers attaching to the middle part of the target sequence, instead of only attaching to the termini. This mispriming will cause the amplification of smaller sequence within the target itself; and thus, producing multiple bands even after multiple purification.

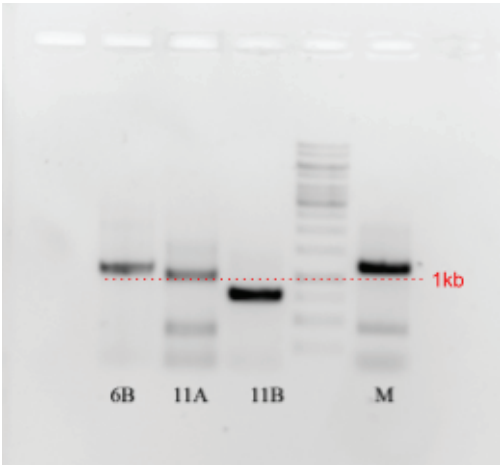


Figure 8: Confirmation of DNA sample prior insertion into pGEM-T Easy Vector.

3.4. Insertion and transformation of 1-SST gene

Prior to insertion into the plasmid, the samples were confirmed using gel electrophoresis (Agarose 1%). Result suggests that some of the samples were not pure (**Figure 9**); however, due to time constraints, further purification could not be carried.

Unfortunately, this study was only able to obtain one plasmid sample for sequencing, pla11A. The rest of the samples, 11B and 6B, were sent in the form of PCR product since the bacteria did not grow in the LB Broth medium. Furthermore, the pcr11A PCR reaction sample were sequenced with 11A self-designed primers, while the pla11A plasmid were sequenced with M13-pUC universal primer provided by the sequencing vendor.

Prior to inoculation into LB Broth + Ampicillin, the bacteria were grown in LB Agar + Ampicillin with a noticeable slow growth. Normally, a blue-white screening with *E. coli* takes around 16-24 hours after inoculation (New England BioLabs, n.d.)[17]. However, in this case, some of the bacteria took more than one day to grow; which might be caused by non-optimal insert-vector ligation conditions [7]. With this in mind, the antibiotic in the agar have been degraded and exacerbated by enzymes released by resistant bacteria ([8];[9]) allowing the growth of satellite colony that does not contain the plasmid. Therefore, the bacteria that was inoculated into the LB Broth + Ampicillin might be those from the satellite colony since they do not possess the antibiotic resistant gene.

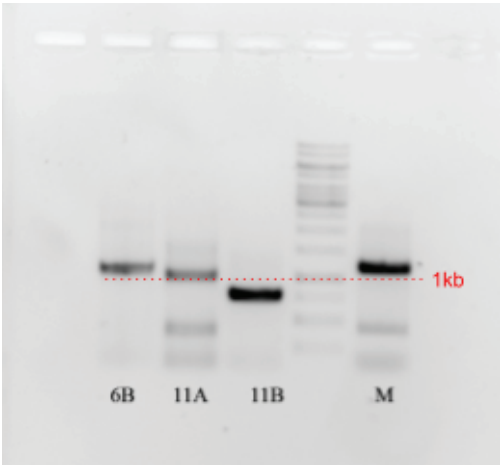


Figure 9: Confirmation of DNA sample prior insertion into pGEM-T Easy Vector.

3.5. Sequencing results and analysis

The consensus of 6B sequence with the size of 1033 bp (**Appendix 4**) was generated from the forward and reverse sequences of the respective primers using UGENE workbench. The sequence was analyzed using BLAST (NCBI) and the result is shown below (**Figure 10**).

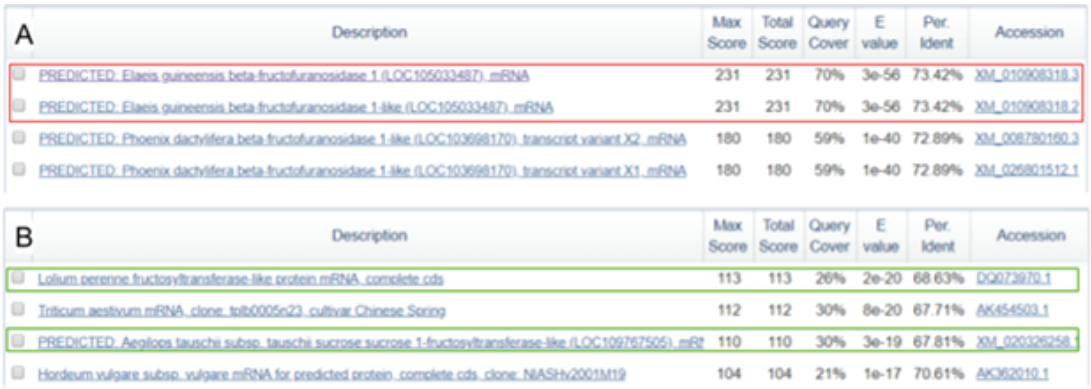
Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Steroidobacter denitrificans strain DSM 18526, complete genome	324	324	83%	6e-84	69.10%	CP011971.1
Caulobacter mirabilis strain FWC 38 chromosome, complete genome	143	411	34%	1e-29	74.34%	CP024201.1
Phenylobacterium zucineum HLK1, complete genome	115	419	47%	6e-21	72.89%	CP000747.1
Pseudoxanthomonas suwonensis strain J1, complete genome	113	189	29%	2e-20	72.05%	CP011144.1
Lysobacter gummosus strain 3.2.11, complete genome	104	162	34%	1e-17	71.24%	CP011131.1

Figure 10: BLAST result of 6B consensus sequence.

The best hit was shown on a complete genome sequence of *Steroidobacter denitrificans* strain DSM 18526 with an E value of 6e-84 and query cover of 83%. The percent identity showed around 69%. Even though the percent identity is quite low, the query cover of the sequence against the whole genome sequence of the bacteria is the highest among all hits that mostly showed <50% query cover. This indicates that the alignment of the query sequence with the genome of *Steroidobacter denitrificans* was more specific than the others. Therefore, the isolated sequence belongs to *Steroidobacter denitrificans*. This is contradictory with the target of the primer which is 1-SST. Since amplification using 6B primer showed a false positive result, it could be concluded that both of the primers designed from chromosome 6 failed to isolate 1-SST gene.

The consensus of pla11A (**Appendix 1**) and pcr11A (**Appendix 2**) were generated from the forward and reverse sequences of the respective primers using UGENE workbench. This consensus sequence was then used as an input for BLAST to determine the gene

with closest similarity to the sequence. The BLAST result of pla11A suggests that the isolated sequence is 73.42% identical to beta-fructofuranosidase 1 (**Figure 11 Image A**). Further, the result from pcr11A reveals that the isolated sequence is 68.63% and 67.81% identical to fructosyltransferase-like protein and 1-SST respectively (**Figure 11 Image B**).



A

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Elaeis guineensis beta-fructofuranosidase 1 (LOC105033487), mRNA	231	231	70%	3e-56	73.42%	XM_010908318.3
PREDICTED: Elaeis guineensis beta-fructofuranosidase 1-like (LOC105033487), mRNA	231	231	70%	3e-56	73.42%	XM_010908318.2
PREDICTED: Phoenix dactylifera beta-fructofuranosidase 1-like (LOC103698170), transcript variant X2, mRNA	180	180	59%	1e-40	72.89%	XM_008780160.3
PREDICTED: Phoenix dactylifera beta-fructofuranosidase 1-like (LOC103698170), transcript variant X1, mRNA	180	180	59%	1e-40	72.89%	XM_026801512.1

B

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Lolium perenne fructosyltransferase-like protein mRNA, complete cds	113	113	26%	2e-20	68.63%	DQ073970.1
Triticum aestivum mRNA, clone: tolb0005n23, cultivar Chinese Spring	112	112	30%	8e-20	67.71%	AK454503.1
PREDICTED: Aegilops tauschii subsp. tauschii sucrose sucrose 1-fructosyltransferase-like (LOC109767505), mR	110	110	30%	3e-19	67.81%	XM_020326258.1
Hordeum vulgare subsp. vulgare mRNA for predicted protein, complete cds, clone: NIASHv2001M19	104	104	21%	1e-17	70.61%	AK362010.1

Figure 11: BLAST result of self-designed 11A primer. Image A: pla11A plasmid with insert isolated using 11A primers sequenced using M13-pUC primers. Highest similarity to beta-fructofuranosidase 1 (Red rectangle). Image B: pcr11A DNA sample isolated and sequenced using 11A primers. Highest similarity to fructosyltransferase-like protein and 1-SST (Green rectangle).

Phylogenetic tree was constructed from the sequence of pla11A, Invertases, 1-SSTs of several different plant species as well as the top two results from the BLAST using PhyML Maximum Likelihood (**Figure 12.**). The result suggests that pla11A are closely related to Vacuolar Invertase and 1-SST of *Triticum aestivum* as they belong to the same clade. Furthermore, it can be inferred that the pla11A is unlikely to be a cell wall Invertase as they are located in a distant clade.

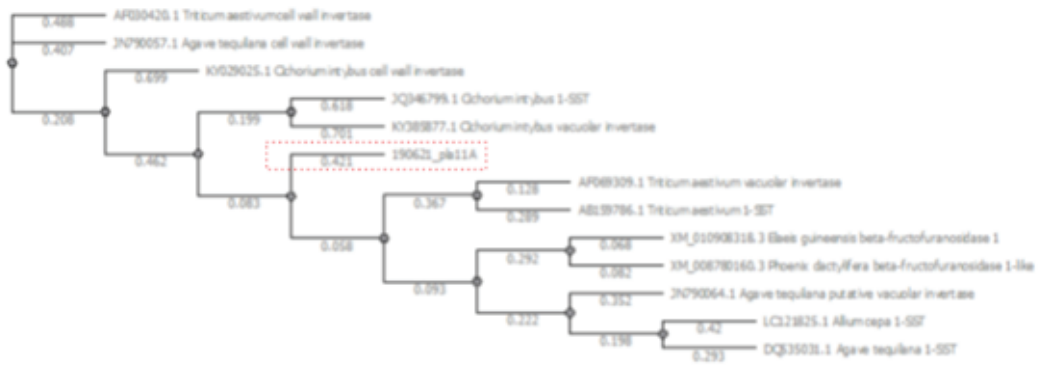


Figure 12: Phylogenetic tree of Invertases and 1-SST in different plant species. Dotted red square: pla11A.

Interestingly, although pcr11A shows that the isolate sequence is indeed 1-SST (**Figure 11, Image B**) the result from pcr11A cannot be used as a conclusion as the consensus sequence is low in quality. On the other hand, beta-fructofuranosidase is also known as invertase [11];[4]. This enzyme has the capability to synthesize fructooligosaccharide which is a short-chain inulin. Some studies consider short-chain fructans as

fructooligosaccharide (FOS) and long-chain fructans as inulin [11]. A small amount of 1-kestose (simplest inulin) can be produced by vacuolar invertase at high sucrose [12]; [13]). Looking at the phylogenetic tree (**Figure 12**) of pla11A, several 1-SSTs and Invertases; it is obvious that the isolated sequence is not a cell wall invertase as it is closely related to 1-SST and vacuolar invertase of *Triticum aestivum*. Therefore, it is not a surprise that the isolated sequence fragment was identified as beta-fructofuranosidase.

Moreover, based on sequence analysis it was postulated that 1-SST evolved from vacuolar invertase [13]; [14]. Also, a study had successfully transformed wheat (*Triticum aestivum*) vacuolar invertase into 1-SST [10]; the transformation involves the disruption of highly conserved WMNDPNG motif that is present in vacuolar invertase but not in 1-SST. With this in mind, it is possible to identify the 1-SST in *Dioscorea esculenta* by observing the presence of the motif. However, since this is a partial sequence obtained from genomic DNA, further study is needed to confirm the identity of the complete 11A sequence.

The consensus sequence of Sample 11B (**Appendix 3**) was obtained through the same method. The BLAST result of 11B consensus sequence (**Figure 13**) indicates that sequence isolated is similar to protein kinase with highest similarity of 82.91%. Protein Kinase is a housekeeping gene and plays an important role in plant defense response towards pathogen [15]. With the fact that the template whole genome sequence from *Disocorea rotundata* has gaps and no annotations, it is difficult to design a very specific primer. Consequently, it is not a surprise that result shows a gene fragment from plant-related gene.

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
PREDICTED: Setaria italica calcium-dependent protein kinase 13 (LOC101771161), mRNA	496	496	71%	1e-135	82.85%	XM_004976547.4
PREDICTED: Phoenix dactylifera calcium-dependent protein kinase 26 (LOC103717884), transcript variant X2, r	490	490	78%	4e-134	80.64%	XM_008806438.3
PREDICTED: Phoenix dactylifera calcium-dependent protein kinase 26 (LOC103717884), transcript variant X1, r	490	490	78%	4e-134	80.64%	XM_008806447.3
PREDICTED: Musa acuminata subsp. malaccensis calcium-dependent protein kinase 26-like (LOC103977087),	487	487	70%	5e-133	82.91%	XM_009392508.2

Figure 13: BLAST result of 11B self-designed primer with high similarity to protein kinase.

4. Conclusion

Gene amplification using 6B, 11A, and 11B primer pairs resulted in amplicons with expected size. However, sequence analysis of 6B and 11B amplicons resulted in false positive results. In-silico analysis of 11A sequence showed that the isolated sequence has a close identity with the target gene, 1-SST. New approach of primer design is needed to successfully isolate 1-SST gene from *D. esculenta*.

Appendix

Appendix 1: Consensus sequence of pla11A plasmid with histogram

> 190621_pla11A
TGTAATACGATTTACTAAAGGGCGAATTGGGCCCGAAGTTGCATTCTCCCGGCCGCCATGGCGGCCG
CGGGAATTTGATTAGATCCGAATGGAGAGTTCTTCTTCTCTTCGTTGAAGGCTTATTGATTGATTTTT
TTTTTTTTTTTTGCTTCCTTGTTTGGATGGTAATGGTGGTGGGATTAATGGCAGGGCCGGTGTTCTAC
AACGGATGGTATCATTTGTTCTACCAGTACAACCCTGATTCGGCGGTGTGGGGGAACATCACTTGGG
GCCACGCCGTGTCGCGCGATCTCATCCGGTGGCGGCATCTCCCTCTCGCCATGGTTCGTGATCAATG
GTACGATTCCGATGGCGTGTGGACCGGCTCCGCCACTGTCCTCCCAGACGGCAGCCTCATTATGCTC
TACACCGGGTCCACCAACTCGTCCGTACAGGTCCAGAACCTCGCCATCCCCGCCGATCCCTCAGATC
CGCTCCTTCTCGAGTGGACAAAGTCTGATTCGAACCCCGTCCTCCTCCCGCCGCCAGGAGTGGGGCT
AAAGGATTTCCGGGATCCGACCACGGCGTGGTACGACGAGGACGCGGAGGCGTGGCGGTTCGTGAT
CGGGACGAAAGAGGACAAGGGCCACTCGGGGATCGCGCTGGTGTACCAGACGAAGGACTTCTTGA
ACTACGAGCTGCTGCCGGGGAGGCTCCACGCGGTGGCCAGGACGGGGATGTGGGAATGCGTCGACT
TCTACCCTGTAGCGACGACGGCAGGGGTTGGTGGGCTGGATACGTCAGTGATGGGGGAGAAGGTGA
AGCATGTCCTGAAGGCGAGCATGGACTACGACGAGGCACGACTACTACGCGATCGGAACGTACAAG
AGCGATAAGGTGAAGTGGGTACCTGATGATCCGGATGAAGACGTATGGTATAGGCCTGAGGTACGA
CTGAGGCAAATT

Appendix 2: Consensus sequence of pcr11A sample with histogram

>190621_pcr11A
TTTTTTTTTTCTTTTTTTTTTGAAGGGATTGAAAGGGGATTTAAGGATGATGTCAGTTCCTTTTTCGTA
CAAAGGATGTATTATTTATTCCAGCAGTACAACCTGATTTGTCGGTTTTGGAGAATATCATTGGGGG
CACGCCGTGTCGCGCGATTTCCATCCGGTGGGGGCATCTCCCTCTCGCCATGGTTTGTGATTAATGGT
AAAAATCCAAAGGGGTGTGGAACGGGTCCGCCACTATCCTCCCCAAAGGGAGCCCTTATTATTTTTT
ACACGGGATCCACCAACTCTTCTTTAGAGGTCAAAAACCTCGCCATCCCCGCCTATCCCTTAAAAAA
ACTCCTTTTCAAGTGGAAAAATTTTTATTAAAACCCCGTCCTCCTTCCGCCGCCAGGAGTGGGGCCA
AAGAATTTCCGGTATCCGACCACGGCGTGGTATAAAAAAAAAGCGAAAGCGTGGGGTTTTTTTTATC
GGAAAAAAAAAAAAAAAAGGGCCATTCGGGGATCGCTTTTGTGTACCAAAAAAAAAAATTTTTTAAT
TAAAAACATTTTCCGGGGAGGGTCCACGCGGTGGTTTATAAGGAGAATGTGAAATTGCGTTAATTTC
TATCCCTTTAGAGACAACGATTGGGGGACTTGGTGGAGTGAATTTTGATACTTCAGGGTAATGAAGA
ATATTAAGCAGTCCACAGCC

Appendix 3: Consensus sequence of 11B sample with histogram

>190621_11B
TGATGGGCAACACTTGCCGCGGCTCTTTGACGAGCAGCAAGTTTTTCCGCAGCTTCGCCGAGCCCAC
CGAGCGCTTGTCCTCCTCCTCCAGCCGCACCCATCCTCACAGCTCCTTCTCCGACGCCGACGAACCCT
CCAAACCCGCCCCCAAGCCCGCCACCATGCGCCGCGGTGCTGACATGCACTCCAGCTGCATCCTCGG
CCACAAGACCGCCAACATCCGCGATCTCTACACCCTCGGCCGCAAACTCGGCCAGGGCCAGTTCGG
CACCACCTATCTCTGCACCGAGATTGCCACCGGCGTCGAGTACGCCTGCAAGTCAATCTCCAAGCGC
AAACTCATCTCCAAGGACGACGTCGACGATGTGCGCCGTGAGATCCAGATCATGCACCATTTGTCCG
GGCATCGCAACGTGGTGACCATTAAGGGCGCTTACGAGGATCCGCTCTATGTGCACATCGTGATGG
AGCTCTGCGCTGGCGGGGAGTTGTTTGATCGGATTATTCAGAGGGGGCATTACAGCGAACGAAAGG
CGGCCGAGCTCACGAGGATCATTGTTGGAGTCGTCGAGGCCTGCCACTCTCTCGGTGTCATGCATCG
GGATTTCAAGCCGGAGAAACTTCTTATTGGTGAACAAGGACGGTTGTTCCTCTCTCAAGGCCATCGA
TTTCG

Appendix 4: Consensus sequence of 6B sample with histogram

>190621_6B
ATTGGCACGCATGAGGAACGAGGCACCGATCGGTTGCTCGAACGTCGCGGACAGGCTGGAGGACCA
TTTCGGCGCGAACGACAGCGTGTTGTTCTTGCGCTCGGTGCGGAAGAACTGTCCCGCCGTGCCGAAG
TCTTCGATGGTCGTATCGGCGTAGGTCACGCCGCCTTGCAGGCTCAGCTGTTCGAGCGGGGTGTACC
ACACGAAGTCGAGGTCCACGCCCTTTCGACACCACCTGCGGCAGCGAAGTCACGATGAACTGCAGG
CCGGTGAAGGTGTTGAGCTGGAAGTCCGTGTAGTCCTGATAGAACAGCGCGCCGTTCACCAACAGG
CTGTTGCCGGCCCACTGCGTCTTCATGCCGAGCTCGTACGAATCAACCGTTTCCTTGTTGAAGTGCGT
GTCGGGATCGACTGTGGCCCCTTGCAGAACCACGCCATTCGGCAGCGTCAGATTGCTGGCCAGGAA
GAACTGACCATTGCGCTCGCGATAGAGATTGAAGCCGCCACCTTTGTAGCCCTTCGCGTACGACAGG
TACGACATCACGTCCGGGGTGAAGCGATACGCAACTTTGGCAGTGCCGCTCCATTCCTGTTCGTCTC
GCGATTGCGAGGTATCGACGTTGTTGAAGATCGGATCGGCGTACGTGACGCTGCAGCCAATGCCGA
ACAGCGTCTGGAGCGCGGCCTGTTGGGCCGGCGTTCCCGTCAGCAGGGCGGTTCTCAGCGCGGCGC
TGGAGCGCAGGTAGGTGCAACCCCGGCCGCCGTGCTCGTTTCGATAGTGCGAGTCCAGGTCCTTCGA
TTCATCCGTATAGCGCAGGCCCAGGGTGAGCTCCAGCGCCTCGGTGAAGCGAATGCTGTTGTTGGTG
AACAGTGCCCAGCTCTTGGATTCCTGCTCGTACACGTCACGTGTCGCGCCGTTAGCGGGGTAGGCGT
CGGCAGGCAATGAGGCGATCGGGCTGGGCGTGGCTGATGTCCTCACCGCGGTGAAATATGGCCGCA
CCTGCGTGCCCTGAATCAACTGGTCATGCGAGTC

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