

Isolation and characterization of Dehydrin promoter region from sugarcane (*Saccharum officinarum* L.)

Isolasi dan karakterisasi daerah promoter Dehydrin dari tebu (Saccharum officinarum L.)

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Abstrak

Perkembangan teknik biologi molekuler saat ini, memungkinkan untuk merakit tanaman toleran kekeringan melalui teknik rekayasa genetika untuk mempercepat program pemuliaan tanaman. Dehydrin (DHN) berperan penting dalam respons dan adaptasi tanaman terhadap cekaman abiotik (kekeringan, salinitas tinggi, suhu dingin, panas, dll). Pada saat jaringan tanaman mengalami cekaman kekeringan (dehidrasi), protein DHN dapat terakumulasi pada jumlah tinggi di seluruh jaringan baik vegetatif maupun generatif. Penelitian gen DHN pada level molekuler pada tebu belum banyak dilaporkan. Tujuan dari penelitian ini adalah untuk mengisolasi dan mengkarakterisasi promoter DHN dari tebu yang dapat digunakan sebagai salah satu material transformasi dalam perakitan tebu toleran kekeringan. Primer spesifik untuk amplifikasi promoter DHN telah didesain dan area promoter DHN telah berhasil diisolasi dengan metode kloning PCR. Sebanyak dua sekuen promoter putatif DHN yaitu Pr-1DHNSo dan Pr-2DHNSo telah teridentifikasi. Analisis *in silico* telah dilakukan dan beberapa motif *cis-regulatory element* telah teridentifikasi yang berperan dalam adaptasi terhadap cekaman abiotik dan biotik seperti ABRE, MBS, CGTCA-motif, TGACG-motif, GARE-motif, P-box TCA-element dan Box-W1. Promoter Pr-1DHNSo kemudian diklon ke vector ekspresi pBI121 melalui *Overlap Extension PCR (OE-PCR)* untuk dikarakterisasi lebih lanjut. Uji fungsionalitas konstruk promoter pBI-Pr-1DHNSo telah dilakukan melalui transformasi menggunakan *Agrobacterium* ke kalus tebu. Analisis GUS dan PCR menunjukkan bahwa promoter DHN telah tertransformasi dan terekspresi di kalus tebu.

[Kata kunci: cekaman kekeringan, DHN, promoter, *Saccharum* sp.]

Abstract

The development of molecular biology techniques nowadays has enabled to engineer drought tolerant sugarcane by genetic engineering to accelerate the breeding program. Dehydrin (DHN) is known to have an important role in plant response and adaptation to abiotic stresses (drought, high salinity, cold, heat, etc.). While plant tissues are subjected to drought stress (dehydration), DHN protein is accumulated to high content throughout all vegetative or generative tissues. The research aimed to isolate and characterize the DHN promoter from sugarcane that can be used as transformation material in generating drought tolerant sugarcane. Specific primers for DHN promoter amplification were designed and DHN promoter region was successfully isolated by PCR cloning method. Two putative promoter sequences were identified namely Pr-1DHNSo and Pr-2DHNSo. *In silico* analyses were carried out and *cis-regulatory elements* motifs that play a role in adaptation on abiotic stress as well as biotic stress including ABRE, MBS, CGTCA-motif, TGACG-motif, GARE-motif, P-box TCA-element and Box-W1 were identified. The promoter Pr-1DHNSo was then cloned into pBI121 expression vector by *Overlap Extension PCR (OE-PCR)* for further characterization. Functional test of the promoter construct pBI-Pr-1DHNSo was conducted through *Agrobacterium* transformation into sugarcane calli. GUS assay and PCR analysis showed that the DHN promoter was transformed and expressed in the sugarcane calli.

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[Key words: drought stress, DHN, promoter, *Saccharum* sp]

Introduction

Sugarcane tolerant to drought stress is one of the goals of the sugarcane breeding program in Indonesia to achieve national sugar self-sufficiency. Nowadays, with the development of molecular biology techniques, drought tolerant plants are directed through genetic engineering to escalate the breeding program (Beringer, 2000). For instance, overexpression of the P5CS gene in tobacco plants derived in proline accumulation along with alleviation of catalase, ascorbate peroxidase and lipid peroxidation activities increase drought tolerance in tobacco plants (Zarei *et al.*, 2012).

Gene family which encodes a protein and have a role in response to osmotic stress includes genes involves in polyamine and amino acid biosynthesis, sugar and polyols, protein chaperone and transcription regulator. Protein chaperone such as Hsp (*heat shock protein*), dehydrin, and LEA (Late embryogenic abundant) group are known to be induced by drought stress (Hanin *et al.*, 2011). This kind of protein is commonly located at cytoplasm and play a role in protein and membrane structure protection from degradation of proteinase, maintaining water status in the cell, and play a role in folding in the protein synthesis (Ingram & Bartels, 1996). LEA protein gene family firstly identified as inducible gene on seedlings on the maturation and dryness process. On vegetative tissues, LEA protein was induced by osmotic stress and abscisic acid (ABA) (Hand *et al.*, 2011). Unsuitable environment condition may cause oxidative stress and generate a *reactive oxygen species* (ROS) in plants. LEA protein could chelate the ROS component (Liu *et al.*, 2017). Moreover, LEA protein plays a role in tolerant to metal ions such as Cadmium (Cd) and nickel (Cu) (Gao *et al.*, 2012; Reis *et al.*, 2012).

Dehydrin (DHN) is also a member of LEA protein group 2 that has an important role in plant response and adaptation to abiotic stresses (drought, high salinity, cold, heat, etc.) (Graether & Boddington, 2014). When plants subjected to drought stress (dehydration), DHN protein is accumulated up to high concentration on the whole vegetative and generative tissues (Hanin *et al.*, 2011). Some of DHN gene family also played a role in tolerance to biotic stress like DHN-5 that involve in Jasmonic acid (JA) signalling process which can activate PR protein (pathogenesis-related) (Hanin *et al.*, 2011). Therefore, DHN gene is a stress-related gene which very potential and can be used as a marker or gene construct in genetic engineering of sugarcane tolerant to drought.

DHN gene has been studied in many plant species such as *Arabidopsis thaliana*, soybean, wheat, sorghum and grape (Hanin *et al.*, 2011, Savitri *et al.*, 2013; Yang *et al.*, 2012). Overexpressions of DHN gene on transgenic *A. thaliana* and tobacco were proven that it could increase tolerance to osmotic stress and high salinity (Hanin *et al.*, 2011). Previous study explained that there was a rapidly increasing of DHN gene expression on sugarcane culm that subjected to drought stress after 15 days (Iskandar *et al.*, 2011). This was an interesting phenomenon because DHN expression was far higher than other stress-inducible genes such as genes involved in proline biosynthesis. This higher expression of DHN gene was assumed to be triggered by the DHN promoter induced by drought stress.

To date about 53 plant species have been analysed for their dehydrin promoter including *Sorghum bicolor* and *Zea mays* (Zolotarov & Strömvik, 2015). DHN gene promoters of wheat (*Triticum* sp.) have been successfully isolated and the PrDHN-5 was known as a promoter induced by abiotic stress (Ben-Amar *et al.*, 2013). However, no report so far about dehydrin promoter on *Saccharum* sp. The research aimed to isolate DHN promoter from sugarcane in order to establish transformation materials that have tested expressed under drought stress condition as an attempt to generate drought-tolerant sugarcane.

Materials and Methods

Plant materials and drought stress treatment

Seven months old sugarcane var PSJT941 was used as a plant source for promoter isolation. The micro seedlings were originated from the Indonesian Sugar Research Institute (ISRI), Pasuruan, East Java. It has been selected based on the presence or non-presence of internodes, plant height and homologue, and single bud (no-tiller). Canes grew on 2 kg size of polybags with media consists of soil, sand and manure fertilizer (3:1:1). The plant canes were adapted for one week in the greenhouse with normal watering. The plants were then subjected to drought stress by stopped the watering at 0, 4, 8, 12, 16, 20 and 25 days. Whilst plants with regular watering were chosen as control plants. Treatment was repeated three times, with three plants for each replication.

Genomic DNA isolation

Genomic DNA was isolated from sugarcane leaves using Orozco-Castillo *et al.* (1994) method. The quality and quantity of DNA were determined by electrophoretic gel agarose and spectrophotometry on wavelength (λ) 230 nm, ratio A230/280 and ratio A230/260.

Total RNA isolation

The isolation of total RNA was conducted using RNA Total Isolation Kit Geneaid® (*plant*) following the protocol from the company. Total RNA was then purified by adding DNase. Quality and quantity of RNA were determined by electrophoretic gel agarose and spectrophotometry at a wavelength (λ) 260 nm, ratio A260/280 and ratio A260/280.

Bioinformatics analyses

Bioinformatics study was conducted to do the primer designed, specific DHN promoter isolation from sugarcane, and sequencing analyses. Primers were designed using the Primer3 program with the reference gene sequence is DHN gene from *Saccharum* sp R570 clone BAC 051E13 (KF184715.1) and *Sorghum* sp obtained from Genebank NCBI (www.ncbi.nlm.nih.gov). Sequencing analyses were conducted by BLAST analysis with alignment using Geneious, MEGA5 and ClustalW. To isolate the promoter region, the genomic DNA sequence from Genbank database was analysed for its DHN coding sequences. Following that the *cis*-element that activated the gene was marked. To obtain the *cis*-element region, about 2000-5000 bp sequence of *Saccharum* sp particularly 5'UTR region and some parts of the DHN exon were put into a PLACE program. The SnapGene software was used to do the simulation of PCR with the template of *Saccharum* R570 clone BAC 051E13 and the designated *forward* and *reverse* primers.

Promoter isolation by PCR cloning method

Genomic DNA isolated from sugarcane leaves was subsequently amplified by PCR using specific primers designed to amplify the 5'UTR region of DHN gene. The primer pairs used were designed as described above. The PCR amplification was then cloned into pGEM-T Easy vector (Promega) and transformed into *E. coli* following the protocol from the supplier. The construct was then confirmed by sequencing and analysing to reveal the presence of the promoter region.

Construction of DHN promoter into expression vector

The DHN promoter was constructed into the pBI12.1 expression vector by analysing the sites of restriction enzymes on the promoter sequence or at the multiple cloning sites (MCS). The promoter sequence would be inserted to an expression vector by replaced the CAMV-35S constitutive promoter in the vector backbone. Analysis of the restriction sites on DHN promoter and expression vector was done by Snapgene program. Construction of DHN promoter into the expression vector was conducted by Overlap Extension (OE)-PCR method (Bryksin & Matsumura, 2010).

Confirmation of promoter insertion into cloning vector

Confirmation of the promoter as an insert was done using DreamTaq Green PCR Master Mix enzyme (Thermo Scientific™). The total of 20 μ l of PCR reaction consists of 2x *DreamTaq* PCR Master Mix, 0.5 μ M each of Forward and Reverse primers, 50 ng pGEMT-Pr-1DHNSo plasmid and nuclease-free water until the volume of 20 μ l. The PCR reaction were set as: 1 cycle of pre-denaturation 95°C, 1', 18 cycles of denaturation 95°C, 10"; annealing 53°C, 30"; and extension 72°C, 2'30". The reaction was terminated by 1 cycle of final extension 72°C, 7' and preservation 4°C.

Overlap Extension (OE)-PCR primer design

Primers for OE-PCR were designed using SnapGene by creating specific primer that may amplify DHN promoter on pGEMT-Easy cloning vector. Another primer pair was designed on MCS pBI121 plasmid as a region for insertion of the DHN promoter followed by simulation by SnapGene to meet the good primers criteria. Mega primer, the component in the OE-PCR method was generated by PCR reaction with the composition: 2x Q5® High-Fidelity 2X Master Mix, 10 mM Forward and Reverse primer pairs, 50 ng pGEMT-Pr-1DHNSo plasmid and Nuclease free water until the volume of 20 μ l. The PCR reaction was programmed at: 1 cycle pre-denaturation at 98°C, 1'; 18 cycles of denaturation 98°C, 10"; annealing 64°C, 30"; and extension 72°C, 2' 30"; 1 cycle of final extension 72°C, 7' and preservation 4°C. The results were then used as DNA template for 2nd round PCR after being purified using Geneaid™ Gel Extraction Kit following the protocol from the manufacture.

Generating the recombinant plasmid of OE-PCR

The 2nd round PCR was conducted to generate recombinant plasmid with the template of previously amplified using mega primer. The components were consisted of; 2x Q5® High-Fidelity 2X Master Mix, 0.5 μ M Forward and Reverse primer pairs, 50 ng Plasmid pGEMT-Pr-1DHNSo, mega primer 5 μ l, and Nucleus free water up to the volume of 20 μ L. The PCR program was consisted of pre-denaturation, 98°C 3', denaturation 98°C, 10", annealing 62°C, 1", extension 72°C, 8'30", final extension 61°C and final extension 72°C, 15', and preservation 4°C. Following the PCR reaction, digestion with *DpnI* enzyme was done to cut the methyl compound which only occurs on the wild type parental pBI121 plasmid.

Cloning Recombinant Plasmid Containig DHN Promoter to *E. coli* and its verification

Cloning was conducted by heat shock method and confirmed by PCR analysis. Plasmid isolation

was performed using the alkaline lysis method (Sambrook *et al.*, 1989). The pellet was dissolved in 50 µl TE Buffer at pH 8.0. PCR analysis was conducted using *DreamTaq* Green PCR Master Mix (2x) (Thermo Scientific™). The promoter construct was subsequently sequenced and the results were confirmed by BLASTN and examined for its motifs on the promoter region using ProScan Software.

Transformation of pBI212- Pr-1DHNSo into Agrobacterium LBA4404 competent cells

The plasmid pBI 212- Pr-1DHNSo was transformed into 200 µl of competent cells of *Agrobacterium* followed by incubation on ice for 15 min and liquid Nitrogen for 5 min and then in incubator 37°C for 5 min. About 800 µL YEP liquid medium was added and shake for 3 hours at 28°C. The mixture was then centrifuged at 6000 rpm for 3 min. The supernatant was then removed and 200 µl of it was kept and spread onto selection medium containing Kanamycin antibiotic. Transformant shoots were then incubated for 2 days at 28°C. Colonies grown on the selection medium were isolated for colony PCR using DHN1 primer pair.

Transformation of Pr-1DHNSo promoter construct into sugarcane calli using Agrobacterium

Sugarcane var Kidang Kencana was used as plant material for producing calli as an explant for genetic transformation. Spindle leaf roll was cut into pieces and cultured on a solid MS1 medium supplemented with 10% coconut water, 30 g/L sucrose and 3,0 mg/L 2,4 D, incubated in the darkroom for 4-6 weeks until callus performed. The embryogenic calli were then subcultured 2-4 times before used as explants.

Genetic transformation to sugarcane cells via *Agrobacterium* was conducted by the method of Sain *et al.* (1994) with the addition of 100 mg/L acetosyringone. After the inoculation with *Agrobacterium* for 10-15 minutes, the explant was dried off using sterile tissue paper. Explants were then co-cultivated on a solid MS medium without antibiotic in the dark for two days. If the *Agrobacterium* was significantly grown, explants were washed using liquid MS medium and subsequently cultured on a solid MS medium containing cefotaxime 250 mg/L. On the opposite, the explants were cultured directly to selectable solid medium with cefotaxime 250 ppm and incubated in the dark for 5-7 days. Calli were then subcultured into a selectable MS1 medium with the addition of hygromycin 50 ppm and cefotaxime 250 ppm for 3-4 weeks in the dark. After that, calli were subcultured into selection media MS2 added with hygromycin 50 ppm and cefotaxime 250 ppm for shoots initiation under light condition.

Transient expression analysis of GUS on transformant sugarcane calli

Histochemical GUS assay was conducted following the method of Jefferson (1987). The transformed calli were previously sterilized with cefotaxime 500 ppm and incubated with 4 mL X-gluc solution in 50 mL Falcon tube, at 37°C in the dark during 24 hours. Positive results were shown by the appearance of blue colour on callus tissue. Callus with positive results was then soaked in 90% ethanol until the green chlorophyll disappeared. The observation was conducted under the binocular microscope. Another test to the transformant sugarcane was done by PCR using specific primers of resistance to Kanamycin antibiotic, NPTII. Genomic DNA was isolated from the transformed calli using Genomic DNA Mini Kit (Plant) from Geneaid following the protocol provided by the manufacture, and used as templates for PCR analysis.

Results & Discussion

Isolation of DHN promoter region from sugarcane

Bioinformatics study was conducted based on the NCBI database. The *dehydrin* sequence of *Saccharum* was determined for its coding sequence (CDS) following the identification of the *cis*-element which activated the gene. About 2000 basepairs of *Saccharum* sequence particularly the 5'UTR and partly the exon region of the dehydrin gene were input into the PLACE software to find the *cis*-element. Annotation was done using Geneious software. Primers were then designed that amplified the area which contains *cis*-element related to drought stress such as Abre-related and TATABOX. Two primer pairs were selected which can be used to amplify the promoter region of 5'UTR DHN gene.

Primers sequence that was used for PCR analysis of genomic DNA firstly designed by aligned the sequence of *Saccharum* R570 clone BAC 051E13 (Acc no: KF184715.1) with sequences of *expression sequence tags* (ESTs). The EST sequences were obtained from the BLAST results of *Saccharum* R570 clone BAC 051E13 using NCBI with the keywords *Saccharum* organism (taxid: 4546). The alignment did not show the presence of a conserved region at 5'UTR site due to the EST sequences were not long enough and the 5'UTR was not covered by. Therefore, only *Saccharum* R570 clone BAC 051E13 sequence that was used as a template and designed by Primer3 program. Selected primers are shown in Table 1.

Primers were designed based on the good characteristic parameters according to Sasmito *et al.* (2014). The expected length of the amplicon was 1992 bp covering the 5'UTR of DHN gene. Optimization of PCR reaction was previously

Table 1. Primer designed to amplify 5'UTR region of DHN gene
 Tabel 1. Primer yang dirancang untuk mengamplifikasi daerah 5'UTR gen DHN

Primer/ Primer	Primer sequence/ Sekuen primer	Note/ Keterangan
Fprom (forward primer)	AGCTTGTCCTTGCCACCATA	%GC: 50.0.; Tm: 58.4°C
Rprom (reverse primer)	CAACTGGATTGCCGTACT	%GC: 50.0.; Tm: 53.9°C

conducted using gradient PCR with temperatures of 59°C, 60°C and 61°C to obtain the best annealing temperature.

The result from PCR amplification showed that there were two bands of genomic DNA with the size of ~2000 bp and ~1700 bp. The band of ~2000 bp was appropriated with the expected amplicon size using primers Fprom and Rprom which was 1992 bp on the 5'UTR of DHN gene on sugarcane. The two DNA bands obtained might indicate that there were two different promoters with different length. This could be due to the presence of more than single-gene member in DHN family in sugarcane.

The PCR products were then purified and cloned into pGEM-T Easy plasmid and the confirmation is shown in Figure 1. It is shown that two inserts with the size of ~2000 bp (Pr-1DHNSo) and ~1700 bp (Pr-2DHNSo) were obtained after digestion with *EcoRI* restriction enzyme. Therefore, it confirmed that the 5'UTR of DHN gene was successfully cloned into the plasmid vector.

Sequence characterization of putative DHN promoter regions

Sequencing results of the first insert namely Pr-1DHNSo can only be read about 1211 bp of 2109 bp, with 66 bp of it was the sequence of DHN gene. Another sample with the insert size ±1700 bp namely Pr-2DHNSo was sequenced of 1761 bp from the 5'UTR region with 69 bp hanging of, was the sequence of DHN gene. The part of DHN gene was not included in the *cis*-element analysis. The sequence was identified with BLASTn and showed 97% and 96% homology for Pr-1DHNSo and Pr-2DHNSo respectively with the *Saccharum* hybrid cultivar R570 clone BAC 051E13 sequence from GenBank. Therefore, it can be concluded that the 5'UTR sequence of DHN gene on sugarcane was determined. It was then completed by *cis*-acting elements analysis and the result is shown in Table 2.

Sequencing analysis using Proscan software (<http://www-bimas.cit.nih.gov/molbio/proscan/>) found a putative *transcription start site* (TSS) at base 98 (-98) from the start codon. Besides, it is showed in Table 2 that the promoter common motif was also found such as TATA-box at -128 base from DHN

start codon or at base 30 (-30) from TSS. Another *cis*-element that was determined on Pr-1DHNSo primarily related to the abiotic stress response. ABRE is a *cis*-element that has an important function in the adaptation of vegetative tissue to drought and salinity stress. ABRE also act in the process of seeds maturation and dormancy as well as DRE (Shinozaki et al., 2003 in Kumar et al., 2009). Whilst CGTCA-motif and TGACG-motif are *cis*-elements that have a response to methyl jasmonate compound (MeJA). It is a so-called primer intracellular transducer that usually accumulates when the plants wounded and subjected to drought stress (Sasaki et al., 2001). Our results also reveal those *cis-regulatory elements* which respond to light to induce DHN expression. CREs motifs were included TC-rich repeat, LAMP-element, MNF1, SP-1, Box-1, G-box, TCCC-box, I-box, and GAG-motif. GARE-motif and P-box are *cis*-element which responsive to gibberellin located at the 5'UTR of DHN gene in sugarcane. Gibberellin is a plant hormone that has a role in a response to abiotic stress. Decreasing of gibberellin level and signaling process may cause plant growth subjected to several stresses such as drought, cold, or high salinity (Colebrook et al., 2014).

The MBS motif has a role as a location of the attachment of myeloblastosis (MYB) that involve in drought response. Drought and high salinity stress could induce the accumulation of ABA that activated MYB. Following that the MYB will attach to the receptor (MYBR) which is a *cis-regulatory element* MBS at the promoter region resulted in the expression of DHN gene (Singh & Laxmi, 2015).

Despite the abiotic stress response, this research found some *cis*-regulatory elements that have a role in biotic stress such as TCA-element which is a place where protein induced by Salicylic acid (SA) attached. Moreover, SA is a signalling molecule in plants that have a function in the defence mechanism against pathogen and some abiotic stresses (Hayat et al., 2010). Another putative DHN promoter Pr-2DHNSo showed almost all the CREs that has been found in Pr-1DHNSo listed above. Some motifs such as HSE, TC-rich repeat, CATT-motif, W-box, and Box-W1 were not found in *cis-regulatory elements* Pr-1DHNSo (data not shown).

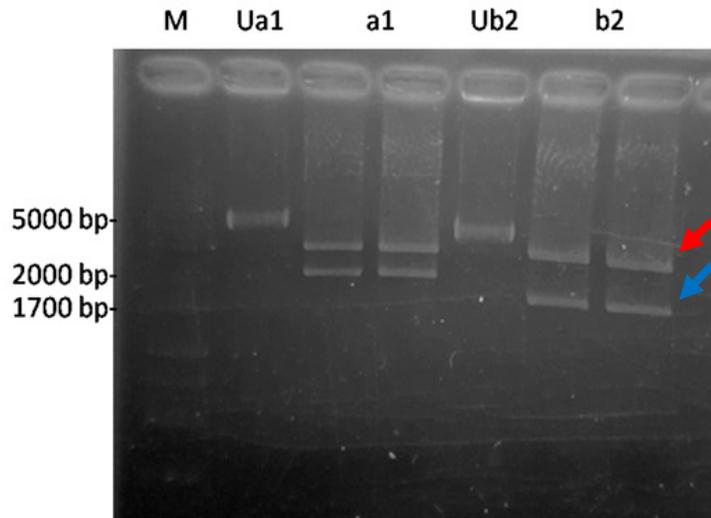


Figure 1. Confirmation of plasmid isolation carrying DHN promoter inserts (Red arrow indicates PGem-T Easy; blue arrow indicates the insert. M: ladder 1 kb; Ua1: plasmid with insert ± 2000 bp undigested; a1: digested plasmid with *EcoRI* containing insert with the size of ± 2000 bp; Ub2: plasmid with the insert ± 1700 bp undigested; b2: plasmid digested with *EcoRI* and containing insert with the size of ± 1700 bp

Gambar 1. Konfirmasi hasil isolasi plasmid yang membawa insert promoter DHN (panah merah menunjukkan PGEM-T Easy; panah biru menunjukkan bagian insert. M: marka 1 kb ladder; Ua1: plasmid dengan insert ± 2000 bp tanpa dipotong; a1: plasmid yang dipotong dengan *EcoRI* yang membawa insert dengan ukuran ± 2000 bp; Ub2: plasmid dengan insert ± 1700 bp tanpa dipotong; b2: plasmid yang dipotong dengan *EcoRI* dan membawa insert dengan ukuran ± 1700 bp

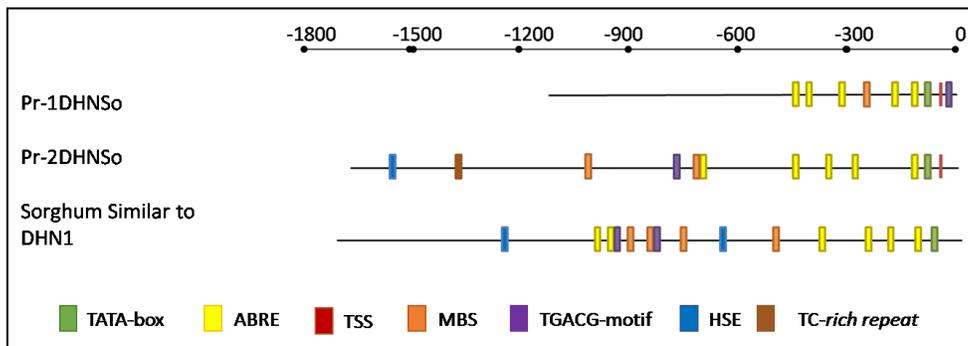


Figure 2. Comparison between CREs motifs acts in abiotic stress at Pr-1DHNSo, Pr-2DHNSo and *Sorghum bicolor* DHN1 promoter

Gambar 2. Perbandingan antara motif CREs yang berperan dalam cekaman abiotik pada Pr-1DHNSo, Pr-2DHNSo dan promoter DHN1 *Sorghum bicolor*

Pr-1DHNSo and Pr-2DHNSo were then subsequently aligned with DHN1 gene promoter from *Sorghum bicolor* which is the closest related species with *Saccharum* spp. Figure 2 shows the result of a comparison between several CREs motifs related to abiotic stress. It can be observed that there are differences between CREs among the three DHN promoters. Hence, we have found two putative DHN promoters that have not been discovered previously.

Sugarcane DHN promoter construction into expression plasmid vector

The isolated DHN promoter region was constructed into a pBI121 expression vector. At the beginning, restriction site analysis was done at the promoter sequence as well as the expression vector multiple cloning sites (MCS). This was done in order to determine the sites of enzyme restriction that would be used to insert the promoter sequence into

Table 2. Putative Cis-element identified on Pr-1DHNSo DHN gene
Tabel 2. Cis-element putatif teridentifikasi dari Pr-1DHNSo pada gen DHN

Function/ Fungsi	Putative Cis-acting elements Elemen Cis-acting putatif	Position ^a Letak	Reference Referensi
Response to ABA and drought / Respons ABA dan kekeringan	ABRE	+233, +307, +430, +441, & -177	PlantCARE, Softberry
	DRE1/ABRE1	-303	Softberry
Common cis-acting elements found on promoter/enhancer regions / Elemen cis-acting ditemukan di promoter/daerah enhancer	CAATBox	+779 +351, +202, +795, +380, +813, +350, +779, -120, -345, -805, & -545,	PlantCARE
Response on MeJA / Elemen respons terhadap MeJA	CGTCA-motif	-66	PlantCARE
	TGACG-motif	+70	PlantCARE
Responsive element to gibberellic acids / Elemen responsif terhadap asam giberelin	GARE-motif	+602	PlantCARE
	P-box	+147 & -684	PlantCARE
Responsive element on light / Elemen responsif terhadap cahaya	GATA-motif	+562 & -144	PlantCARE
	LAMP-element	+820	PlantCARE
	MNF1	+276 & -360	PlantCARE
	Sp-1	+175, +220, -246, & -216	PlantCARE
	Box-1	+912	PlantCARE
	G-box	+182, -436, -425, -301 & -228	PlantCARE, Softberry
	GAG-motif	+542	PlantCARE
	TCCC-motif	-34	PlantCARE
	I-box	-553 & -726	PlantCARE
Attachment site of myeloblastosis (MYB) as a response to drought stress / Situs penempelan myeloblastosis (MYB) sebagai respons terhadap cekaman kekeringan	MBS	+359	PlantCARE
Core element of promoter commonly located at 30 bp upstream from transcription start site / Elemen inti promoter yang terletak 30 pb upstream dari situs inisiasi transkripsi	TATA-box	+128	PlantCARE
Responsive element on salicylic acid/ Elemen responsif terhadap asam salisilat	TCA-element	-449	PlantCARE

^aRelative position to ATG; +: positive strand; -: negative strand

the expression vector. The Pr-1DHNSo was inserted into pBI121 by replaced the CAMV35S promoter in the vector backbone.

Functional characterization of DHN promoter was undertaken by observing the GUS gene expression which located adjacent to the inserted DHN promoter. However, the cut and paste method was unsuccessful due to the undetermined specific restriction sites on the sugarcane DHN promoter. Therefore, the construction was then continued using the Overlap Extension PCR (OEP) method. DHN

promoter with the size 2109 bp was cloned in pGEMT-Easy vector and *E. coli* confirmed by PCR analysis using primers listed in Table 3 with annealing illustrations in Figure 4. Specific bands at 2109 bp reveal that the pGEMT-Easy plasmid has been inserted with Pr-1DHNSo (Figure 3).

The insertion of DHN promoter in exchanged with CAMV35S constitutive promoter in the pBI121 was conducted by Overlap Extension PCR Cloning followed the method of Bryksin & Matsumura (2010) (Figure 5). The method was initiated by

designed specific primers according to Bryksin & Matsumura (2010) which one of them has a longer bp than the common primer length and has two sides of primer annealing. The first annealing site is on the promoter fragment which runs in PCR I following with the 2nd annealing on the vector region which runs in PCR II. The designed primers were listed in Table 4.

OEP method is one of the DNA fragment construction method into plasmid vector without the use of restriction enzyme ligation. Therefore, when we do the point mutation or additional sites on a particular area on the directed plasmid vector, the

specific restriction sites on the attachment or mutation by PCR was unnecessary (Suhandono *et al.*, 2014).

The first step performed on OEP was done to obtain mega primer with an annealing temperature of 64°C obtained from a gradient PCR analysis. Table 4 shows that reverse primer has a quite high GC content and long bp that has a consequence in high annealing temperature as well. Figure 6A shows a light smear of primer dimer due to a high percentage of GC content (58%) (Table 4). Reducing of primer concentration at the range 0.1-0.2 µM was also needed to obtain the right amplification.

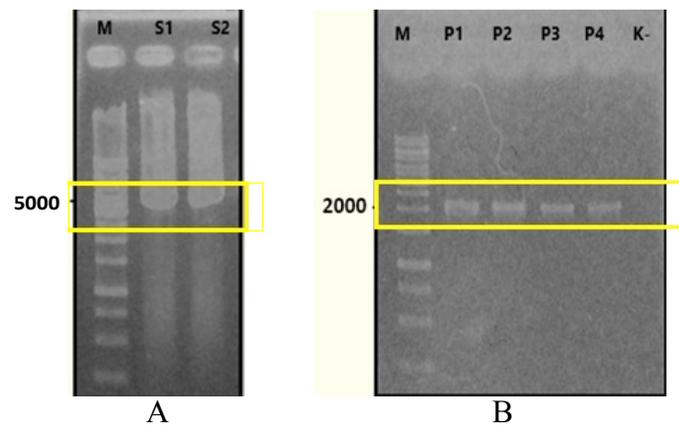


Figure 3. Plasmid isolation of pGEM-T vector inserted with Pr-1DHNSo; A. *pGEMT-Pr-1DHNSo*, M: DNA Ladder 1Kb; S1&S2: plasmid sample; B) PCR confirmation of pGEMT-Pr-1DHNSo. M: DNA Ladder 1Kb; P1-P2: plasmid sample S1; P3-P4: plasmid sample S2; K-: negative control

Gambar 3. Hasil isolasi plasmid vector pGEM-T yang diinsersi dengan Pr-1DHNSo; A: pGEMT-Pr-1DHNSo; M: marka DNA 1Kb ladder; S1&S2: sampel plasmid; B) konfirmasi PCR dari pGEMT-Pr-1DHNSo. M: marka DNA 1Kb ladder; P1-P2: sampel plasmid S1; P3-P4: sampel plasmid S2; K-: kontrol negatif

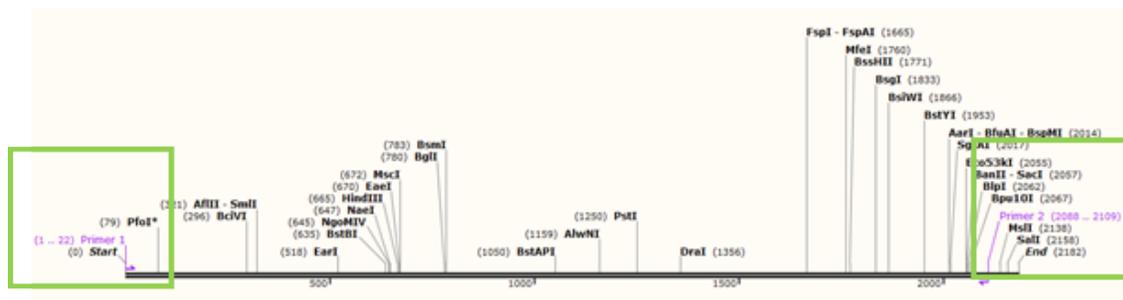


Figure 4. Restriction map of Pr-1DHNSo construct where specific primer annealed.
Gambar 4. Peta restriksi konstruk Pr-1DHNSo dimana primer spesifik menempel.

Table 3. PCR primer for DHN promoter confirmation in plasmid vector
Tabel 3. Primer PCR untuk konfirmasi promotor DHN di dalam vector plasmid

Primer/ Primer	Primer sequence 5' -> 3' / Sekuen primer 5' -> 3'	Note/ Keterangan
DHNF (Forward)	AGCTTGTCCTTGCCACCATAAA	%GC: 45% TM: 58 °C
DHNR (Reverse)	GTCGTGCGGCTTGGTGGTTTCT	%GC: 59% TM: 63 °C

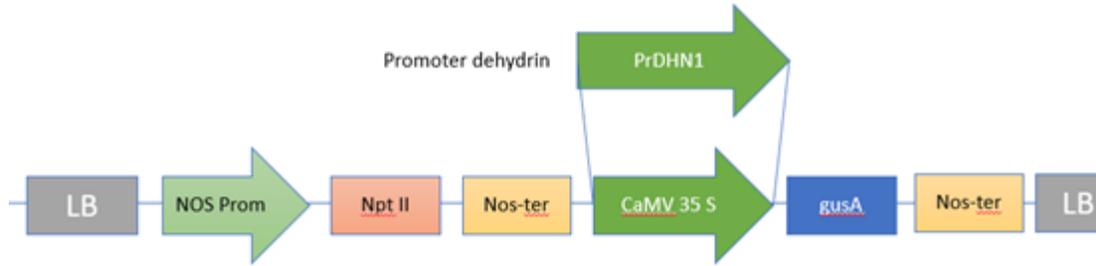


Figure 5. Construction design of Pr-1DHNSo in pBI121 vector using OE-PCR method
 Gambar 5. Desain konstruksi dari Pr-1DHNSo pada vector pBI121 menggunakan metode OE-PCR

Table 4. Primers for the insertion of Pr-1DHNSo fragment into pBI121 vector using OE-PCR method
 Tabel 4. Primer untuk menyisipkan fragmen Pr-1DHNSo ke dalam vector pBI121 menggunakan metode OE-PCR

No.	Primer/ Primer	Primer sequence 5' -> 3' Sekuen primer 5' -> 3'	Note Keterangan
1	OEPC- DHNF	<u>CATGATTACGCCAAGCTTGCATGCCTGCAGAGCTTGTCC</u> <u>TTGCCACCATAAA</u>	%GC: 50 TM: 67
2	OEPC_DH NR	<u>AGGACGTAAACATAAGGGACTGACCACCCGGGTCGTGCG</u> <u>GCTTGGTGGTTTCT</u>	%GC: 58 TM: 69

Note: Blue color indicates specific primer of DHN promoter fragment while the underlined sequences are OE-PCR specific primers

Ket: Warna biru menunjukkan fragmen primer spesifik promoter dehydrin, sedangkan urutan yang digaris bawah merupakan primer spesifik OE-PCR

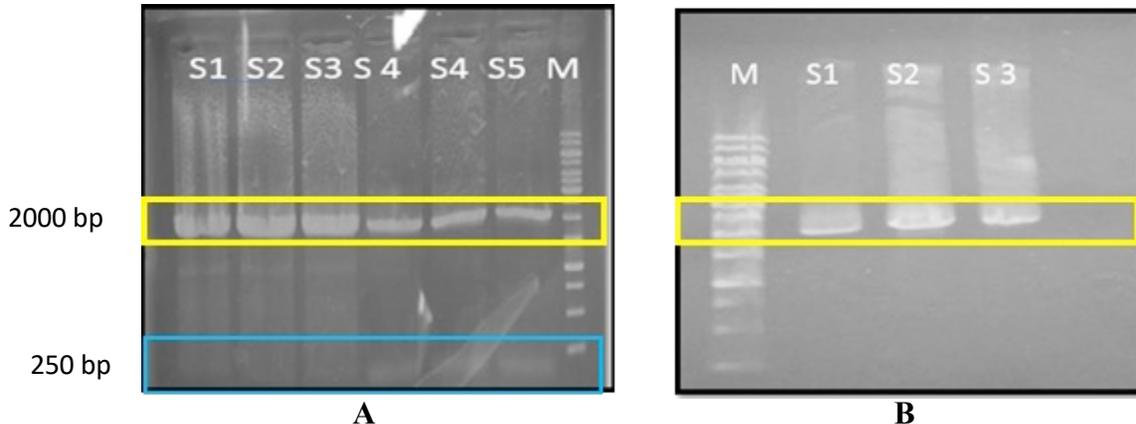


Figure 6. PCR analysis of megaprimer generation of OE-PCR A) PCR I result; B) agarose gel purification
 Gambar 6. Analisis PCR generasi megaprimer dari OE-PCR A) Hasil PCR I; B) Hasil pemurnian gel agarose

Figure 6B demonstrates that the purification of the mega primer from agarose gel was successfully done. The second round of PCR was running to finalised the pBI121-Pr-1DHNSo construct using NEB Q5 polymerase with more rapid extension up to 10 seconds per 1000 bp. The addition of *DpnI* enzyme restriction into the PCR product was done to degrade the pBI121 plasmid. It worked with methylated sites of pBI121 parental plasmid which act as an imprint on the OEP process. The recombinant plasmid pBI121-Pr-1DHNSo that has

been digested with *DpnI* was then cloned into *E.coli* DH5 α (Data not shown).

The result of plasmid isolation shown in Figure 7 reveals a faint band on the size at above 10 Kb indicated that the plasmid has been isolated. The inserted fragment was then confirmed by PCR using primers listed in Table 4 and is illustrated in Figure 8. The electrophoregram in Figure 7B shows a band on \pm 2000 bp indicating the presence of DHN promoter constructs on pBI121. The construct was sequenced afterwards using specific primers stated in Table 4.

Verification of the promoter by sequencing analysis was done by aligning the DNA sequence of Pr-1DHNSo with the known promoter sequence using BLASTn program. The result showed that the alignment has a 99% identity of the 84% Query cover.

The plasmid construct that has been confirmed by sequencing was subsequently transformed into *A. tumefaciens* cells strain LBA 4404. Plasmid isolation was conducted from the transformant bacteria colonies using alkaline lysis method followed by PCR confirmation analysis. This aimed to know whether the isolated bacteria carrying the correct plasmid construct. PCR was also carried out to confirm the insert of promoter fragment. The insert confirmation was also done using DHN specific primer as listed in Table 3. Figure 9 shows that the plasmid in *Agrobacterium tumefaciens* was the ones which carry the Pr-1DHNSo construct determined by the gel agarose band with the size of ± 2000 bp. The *Agrobacterium* carrying the construct was then ready for transformation into the sugarcane calli.

Transformation and expression analysis of PrDHN construct to sugarcane callus

The pBI121-Pr-1DHNSo construct was transformed into the calli using the method of Sain *et al.* (1994). Calli of sugarcane var Kidang Kencana

were incubated in the liquid culture of *A. tumefaciens* strain LBA 4404 in the MS1 media supplemented with acetosyringone for 10-15 min with agitation at 60-75 rpm. Explants were then dried off with sterile tissue paper followed by co-cultivation on MS1 solid media with the addition of acetosyringone 100 ppm and incubated in the dark for two days. Cultures were then transferred into solid MS media containing cefotaxime 250 ppm for 7 days. Transformants calli were then subcultured to the selection media (MS1 with the addition of kanamycin 100 ppm and cefotaxime 250 ppm) during four weeks incubation in the dark (Figure 10).

The transient analysis results are shown in Figure 11 where the GUS assay reacted in blue colour on the transformed sugarcane and not on the control or non-transformed plant. Figure 12 also shows that PCR analyses using NPTII primers resulting in an amplification band of 700 bp indicated that the DHN promoter construct was integrated into sugarcane calli cells. The NPTII gene encodes the neomycin phosphotransferase which confers the resistance ability towards the kanamycin that allow the transformed calli to grow on the selectable media (Ghanem, 2011). Hence, results from the genetic transformation support that the functional test of putative DHN promoter was successfully done in sugarcane.

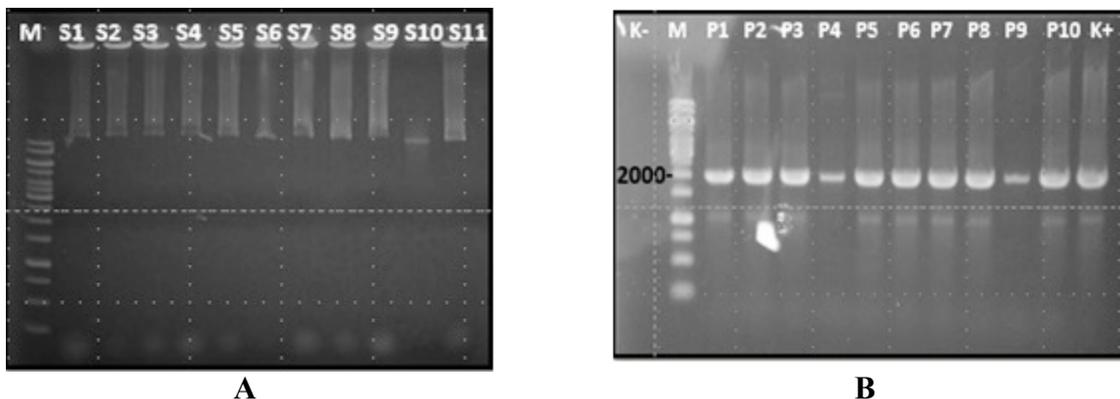


Figure 7. Plasmid isolation and PCR confirmation after construction in pBI121; A) OE-PCR result; B) Plasmid confirmation of pBI121-Pr-1DHNSo.

Gambar 7. Isolasi plasmid dan konfirmasi dengan PCR setelah konstruksi di pBI121; A) hasil OE-PCR; B) konfirmasi konstruk plasmid pBI121-Pr-1DHNSo

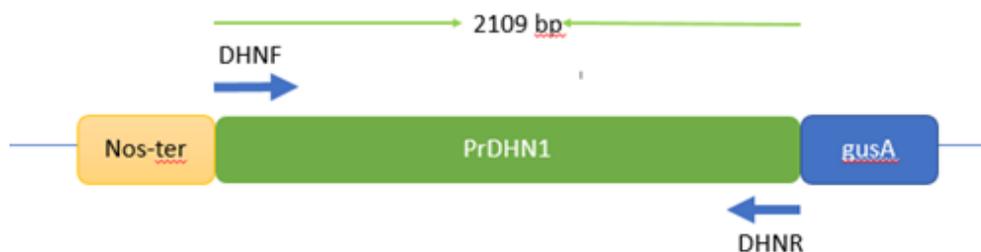


Figure 8. Illustration of PCR to confirm that the Pr-1DHNSo fragment has been inserted to pBI121
 Gambar 8. Ilustrasi PCR untuk mengkonfirmasi bahwa fragmen Pr-1DHNSo telah terinsersi pada pBI121

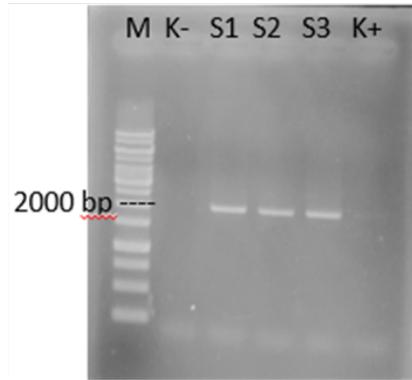


Figure 9. Confirmation PCR result of the insert in *Agrobacterium tumefaciens*
Gambar 9. Hasil konfirmasi PCR dari insert pada *Agrobacterium tumefaciens*

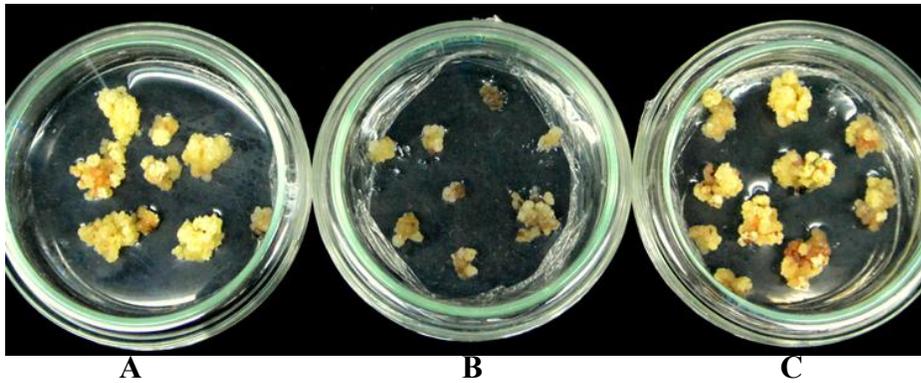


Figure 10. Sugarcane calli of Kidang Kencana variety after one month transformation with pBI-PR-1DHNSo construct, (A) Control on MS1 media without antibiotic, (B) Control on MS1 media with antibiotic Kanamycin 50 ppm and Cefotaxime 250 ppm, (C) Transformant calli on MS1 media with antibiotic

Gambar 10. Kalus tebu varietas kidang Kencana satu bulan setelah transformasi dengan konstruk pBI-PR-1DHNSo, (A) Kontrol pada media MS1 tanpa antibiotik, (B) Kontrol pada media MS1 dengan antibiotik Kanamisin 50 ppm dan Sefotaksim 250 ppm, (C) Kalus transforman pada media MS1 dengan antibiotik

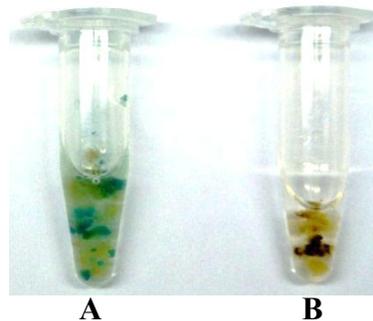


Figure 11. Result of GUS assay on transformant sugarcane Kidang Kencana variety carrying pBI- Pr-1DHNSo construct; (A) Transformant calli, (B) Non transformant (control) calli

Gambar 11. Hasil uji GUS pada transforman tebu varietas Kidang Kencana yang membawa konstruk pBI-Pr-1DHNSo; (A) Kalus transforman, (B) Kalus non-transforman (kontrol)

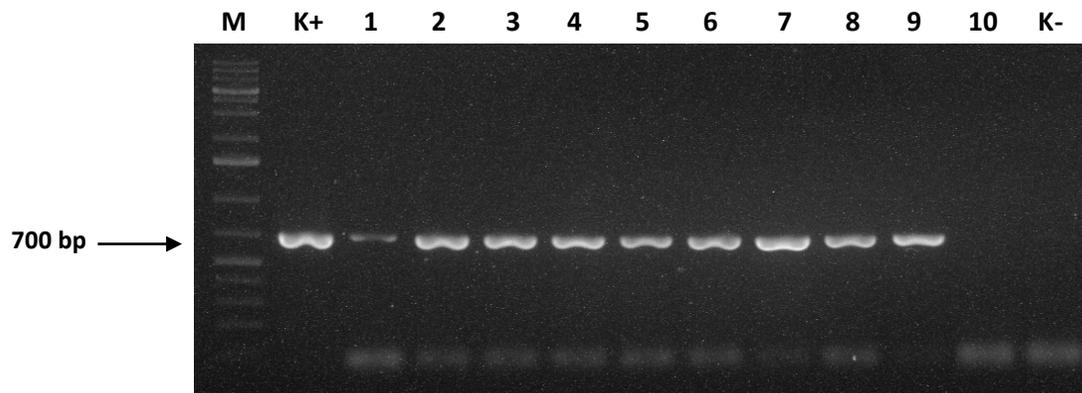


Figure 12. Result of PCR analyses of Kidang Kencana calli transformed with PBI-Pr-1DHNSo using NPTII primer, (M) Marker 1 Kb plus, (K+): positive control (pBI-Pr-1DHNSo plasmid, (K-) Negative control (H₂O), (1-9) transgenic sugarcane calli, (10) Non-transgenic sugarcane calli

Gambar 12. Hasil analisis PCR kalus Kidang Kencana yang ditransformasi dengan pBI-Pr-1DHNSo menggunakan primer NPTII; (M) marka DNA 1 Kb plus, (K+): kontrol positif (plasmid PBI-Pr-1DHNSo), (K-): kontrol negatif (H₂O), (1-9): kalus tebu transgenik, (10): kalus tebu non-transgenik

Conclusion

The two sequences of DHN promoter, Pr-1DHNSo and Pr-2DHNSo, were isolated from sugarcane genomic DNA using PCR cloning method. *In silico* characterization of these two putative promoters found that there were *cis*-regulatory elements motifs that play a role in adaptations to abiotic stress such as ABRE, MBS, CGTCA-motif, TGACG-motif, GARE-motif, and P-box as well as to biotic stress such as TCA-element and Box-W1. The construct of Pr-1DHNSo promoter in pBI121 expression vector was transformed into *Agrobacterium* for functional test in sugarcane calli. Transient assay depicted that the DHN promoter construct was successfully transformed and expressed in sugarcane calli. Based on our results we hypothesize that Pr-1DHNSo can be considered as a constitutive promoter. The DNA sequence identified here is a novel plant promoter that can be a potential candidate for genetic engineering of sugarcane or other plants.

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