Menara Perkebunan 2023, 91(1), 1–13
p-ISSN: 0125-9318/ e-ISSN: 1858-3768

http://dx.doi.org/10.22302/iribb.jur.mp.v91i1.512
Accreditation Number: 164/E/KPT/2021

Transformation of DHN1 gene and DHN promoter constructs into sugarcane calli, regeneration of the calli, and acclimatization of the plantlets

Transformasi konstruk gen DHN1 dan promoter DHN ke kalus tebu, regenerasi kalus, dan aklimatisasi planlet

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Received 11 Oct 2022 / Revised 10 Nov 2022/ Accepted 2 Feb 2023

Abstrak

Dehydrin diketahui memiliki peran yang penting pada respons tanaman terhadap cekaman abiotik termasuk kekeringan dan kadar garam tinggi. Penelitian sebelumnya melaporkan bahwa coding sequence (CDS) utuh dari gen DHN1 *telah diisolasi dari tebu varietas PSJT 941 yang memiliki homologi tinggi dengan gen* DHN *pada tebu dan sorgum. CDS lengkap tersebut kemudian diklon di bawah kendali promotor konstitutif CaMV35S dan ditransformasikan ke dalam kalus tebu dengan bantuan* Agrobacterium tumefaciens*. Promotor DHN,* Pr-*1DHNSo, berhasil diisolasi dari tebu varietas PSJT 941 dan konstruk* Pr-1DHNSo *telah dihasilkan pada vektor ekspresi pBI121. Konstruk promotor tersebut juga telah ditransformasikan ke kalus tebu varietas Kidang Kencana menggunakan* Agrobacterium tumefaciens*. Tanaman transgenik yang membawa konstruk gen* DHN *dan yang membawa promotor DHN diregenerasi menggunakan metode standar kultur jaringan tebu. Optimasi metode aklimatisasi menggunakan modifikasi media pasca perakaran dapat menurunkan tingkat kematian dari plantlet transforman. Keberadaan konstruk gen dan promotor secara periodik diuji dengan PCR menggunakan primer spesifik dan menunjukkan bahwa kedua konstruk masih terdeteksi lebih dari satu tahun pasca transformasi.*

[Kata kunci: cekaman kekeringan, dehydrin, daerah promotor, kalus tebu, aklimatisasi]

Abstract

Dehydrin is known to have an important role in plant response and adaptation to abiotic stresses including drought and high salinity. Previous research reported the isolation of the full-length coding sequence (CDS) of DHN1 from sugarcane var. PSJT 941, and it shares a high homology with DHN genes from sorghum and other sugarcane varieties. In this study, the full-length CDS was cloned under the constitutive CaMV35S promoter and transformed into sugarcane calli mediated by *Agrobacterium tumefaciens*. The DHN promoter, Pr-1DHNSo, was also successfully isolated from the sugarcane var. PSJT 941 and cloned into the pBI121 expression vector. The promoter construct was subsequently transformed into sugarcane calli of var. Kidang Kencana. Transgenic sugarcane carrying DHN1 gene and DHN promoter constructs were regenerated according to the standard protocol of sugarcane tissue culture. Optimization of an acclimatization protocol using modified post-rooting media was also conducted and the resulting protocol reduced the total mortality rates of the transformed plantlets. The presence of the gene and promoter constructs was periodically tested by PCR using specific primers. The genotyping results showed that the constructs were present for more than a year after transformation.

[Keywords: drought stress, dehydrin, promoter region, sugarcane calli, acclimatization]

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Menara Perkebunan is DOAJ indexed Journal and accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3125)
How to Cite: Minarsih, H., Fitriyah, F., Aksa, A. A., Turhadi, Sukmadjaya, D., & Sustiprajitno. (2023). Transformation of DHN1 gene and DHN promoter constructs into sugarcane calli, regeneration of the calli, and acclimatization of the plantlets. *Menara Perkebunan, 91*(1), 1–13.
http://dx.doi.org/10.22302/iribb.jur.mp.v91i1.512

Introduction

One of many ways to improve sugarcane production and achieve national sugar self-sufficiency is planting extensification to marginal lands. It may need the use of varieties with high yield and tolerant to drought stress. Therefore, sugarcane breeding was programmed to generate new varieties with especially tolerant to drought conditions. Plants have the ability to develop various drought tolerance strategies, including changes in the life cycle, modulation of growth and development, and regulation of plant functions to overcome growth and stress adaptation (Hu & Xiong, 2014; You & Chan, 2015). To date, numerous plant genes or transcripts that are known to be induced by osmotic stress have been identified and characterized (Ferreira et al., 2017). The function of those genes has been examined by expression analysis and transgenic plants to study their role in tolerance mechanism against stress.

The gene family that encodes for protein and plays a role in response to osmotic stress includes genes from chaperone proteins such as heat shock protein (*Hsp)*, Dehydrin, and Late Embryogenic Abundant (LEA). Those genes have a role in the protection of other proteins from degradation and proteinase effect. Dehydrin (DHN) is a member of LEA proteins group 2 that play a fundamental role in plant response and adaptation to abiotic stresses (drought, high salinity, cold, heat, etc.) on vegetative and generative tissues (Hanin et al., 2011; Ferreira et al., 2017; Yu et al., 2018).

Iskandar et al. (2011) reported that there was an increase in high-level *DHN* expression on sugarcane stems treated with drought stress for 15 days. *DHN* gene promoter of wheat (*Triticum* sp.) (*PrDHN-5*) (Amar et al., 2013) and pearl millet (*Pennisetum glaucum* L.) (*PgDhn pro*) (Divya et al., 2019) was known as a promoter induced by abiotic stress. Overexpression of *DHN* gene was demonstrated to increase tolerance to osmotic stress and high salinity in tobacco (Hanin et al., 2011), rice (*Oryza sativa*) (Tiwari et al., 2018), *Arabidopsis thaliana* (Cui et al., 2019), pepper (*Capsicum annuum*) (Luo et al., 2019), and wheat (*Triticum aestivum*) (Hao et al., 2020). Previous research on *DHN* gene in sugarcane found that the expression level of *DHN* gene in sugarcane var. PSJT 941 treated with various periods of drought stress increased along with the increasing period of the treatment. The full-length coding sequence (CDS) of *DHN1* gene was isolated with a size of 465 bp. Identification using Blast analysis showed that *DHN1* sequences from sugarcane var. PSJT 941 shared high homology with *DHN* gene on sugarcane and sorghum (Minarsih et al., 2018). The *DHN* promoter, *Pr-1DHNSo* was successfully isolated from sugarcane genomic DNA using the PCR cloning method. It showed that *Pr-1DHNSo* can be considered a constitutive promoter based on functional tests previously conducted (Minarsih et al., 2020). The study of *DHN* promoter also indicated that the *Pr-1DHNSo* promoter from sugarcane was expressed and induced by drought stress treatment in tobacco plants (Iskandar et al., 2020).

Increasing drought tolerance in sugarcane so far was achieved through the overexpression of target genes mostly by biolistic or *Agrobacterium tumefaciens*-mediated genetic transformation (Kumar et al., 2014; Reis et al., 2014; Augustine et al., 2015; Ramiro et al., 2016). However, there are only a few reports of transgenic research that made some successful improvements in drought or water stress tolerance in sugarcane. One of the main challenges in developing drought-tolerant sugarcane is the selection and regeneration of explants before and after the transformation. The media composition is also an important factor to obtain the optimum growth rates on sugarcane tissue culture. The addition of amino acids to the MS media such as glycine and glutamine was reported can improved the growth and healthy of sugarcane plantlet. Hence, the addition of amino acids into the media will be useful for sugarcane micropropagation as well as on regeneration of genetic transformation plants such as on transgenic sugarcane carrying glycine betaine gene (Sugiharto, 2017).

This study aimed to transform sugarcane calli with the DHN1 gene and DHN promoter constructs followed by regeneration of the transformed plantlets. The output of the research will become the basis of the genetic engineering program of sugarcane varieties that are tolerant to drought stress in order to improve sugarcane productivity in marginal lands.

Materials and Methods

Preparation of sugarcane explants with tissue culture

Sugarcane shoots of the Kidang Kencana (KK) variety were grown on solid MS1 (Murashige & Skoog) media and incubated in a dark culture room for 4-6 weeks up to callus formation. After 2–4 subculture cycles, embryogenic calli derived from the shoots were then used as an explant for *Agrobacterium*-mediated genetic transformation of the DHN gene construct and its promoter following previously developed methods (Minarsih et al., 2012).

Transformation of Agrobacterium tumefaciens *LBA4404 and AGL-1 competent cells with pCAMBIA1303-DHN1 and pBI12.1-Pr-1DHNSo constructs*

A total of 5 µl of pCAMBIA-1303 DHN1 and pBI12.1-Pr-1DHNSo recombinant plasmids were added into 200 µl of *Agrobacterium* competent cells, incubated on ice for 15 minutes, then in liquid nitrogen for 5 minutes, and lastly at 37°C for 5 minutes. An amount of 800 µl of liquid YEP media was added into the bacterial suspension and incubated for 3 hours at 28°C. After incubation, the mixture was centrifuged at 6000 rpm for 3 minutes. The supernatant was discarded and 200 µl was left to resuspend the pellet, then spread on selection media containing kanamycin. The plates were incubated for 2 days at 28°C. The transformed colonies were then screened by colony PCR using DHN1-specific primers. PCR with nuclease-free water and plasmid pCAMBIA1303-DHN1 as templates were also included as the negative and positive control, respectively.

Agrobacterium-*mediated genetic transformation of sugarcane with pCAMBIA1303-DHN1 and pBI121-Pr-1DHNSo constructs*

Agrobacterium tumefaciens-mediated genetic transformation of sugarcane was carried out following a method by Sain et al. (1994) using 100 mg L^{-1} acetosyringone. Transformation of sugarcane calli with pCAMBIA1303-DHN1 construct was carried out by immersing the calli of var. Kidang Kencana into a liquid culture of *A. tumefaciens* in MS1 media containing acetosyringone, gently shaken at 60–75 rpm. After being incubated with *Agrobacterium* for 10–15 minutes, the explants or plant cells were dried with sterile tissue paper. Immediately afterward, the explants were co-cultivated on solid MS media without antibiotics in the dark for 2 days. Explants with overgrown *Agrobacterium* were washed with liquid MS media before being cultured on solid selective MS media containing 250 mg L^{-1} cefotaxime. Otherwise, the explants can be directly transferred to the selective MS1 media containing 250 mg L^{-1} of cefotaxime. The cultures were incubated in a dark culture room for 5–7 days. The calli were then subcultured on MS1 selection media containing 50 mg L^{-1} of hygromycin and 250 mg L^{-1} of cefotaxime for 3–4 weeks in a dark room. The calli were subsequently subcultured and incubated in a light room on MS2 selection media (hygromycin 50 mg L^{-1} and cefotaxime 250 mg L^{-1}) for shoot initiation.

The culture of sugarcane calli and shoot regeneration

The media used for sugarcane calli culture was solid MS supplemented with 10% young coconut water, 30 g L^{-1} sucrose with 3.0 mg L^{-1} 2,4 D, and 0.2 mg L^{-1} biotin. Shoots were induced and regenerated on the same media with different hormone contents, that are 2 mg L^{-1} of IAA. Subcultures were done 2-3 times every 4 weeks on the media until the shoots developed into plantlets. To induce roots, plantlets were transferred into liquid media with the same composition.

Transient assay of DHN gene promoter construct in transformed sugarcane calli and shoots

Testing for the presence of the DHN promoter construct in the transformed sugarcane calli and shoots was carried out using PCR with specific primers for DHN, GUS, and hygromycin antibiotic resistance genes. Genomic DNA samples from transgenic plants (about 2 months after transformation) were purified using the Plant Genomic DNA Mini Kit (Geneaid). The DNA samples were then used as templates for PCR. Several controls were included to confirm the results.

The histochemical GUS assay was conducted following the method from Jefferson (1987). The X-Gluc (*5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid*) solution was prepared by mixing 1 mM X-Gluc; 0.1 mM $K_3Fe(CN)_6$; 0.1 mM $K_4Fe(CN)_6$; 50 mM phosphate buffer ($NaH_2PO_4/NaHPO_4$) at pH 7.0. Transformant calli were first sterilized using 500 mg L^{-1} of cefotaxime. Each transformant callus carrying CaMV35S or Pr-1DHNSo promoter was incubated with 4 mL X-gluc solution and incubated at 37°C in the dark for 18-24 hours. The integration of the promoter construct was indicated by the appearance of blue color on the surface of the calli. The explant was then soaked in 90% ethanol followed by observation under the microscope.

Acclimatization of sugarcane transformants and control plants

Before planting, plantlet roots were soaked in 0.1% Benlate solution. Transformant and wild-type plantlets with adequate root lengths were transferred to sterilized soil, sand, and manure/compost (1:1:1) mixed media in plastic containers and placed in a greenhouse. After planting, the soil is watered and treated with Benlate solution. The plants were then placed in a tightly closed plastic cover to reduce transpiration for about 1 week before the covers were removed. After approximately 4 weeks, the plants

were transferred to sterilized media in polybags in nursery and watered daily.

Optimization of post-rooting, hardening, and acclimatization methods

Before the acclimatization process, obtained transgenic sugarcane plantlets were grown in a post-rooting medium modified from Oakes et al. (2016) containing half-strength MS salts supplemented with 10% coconut water, 30 g L^{-1} sucrose, and 2.0 g L^{-1} activated charcoal without a gelling agent. The post-rooting stage involved 19 clusters of transgenic sugarcane plantlets including a non-transformed control from the culture tubes. Eleven transgenic sugarcane plantlets were transferred to half-strength MS medium and the remaining eight clusters were transferred to full-strength MS medium. Plantlets were grown under continuous fluorescence light for 1 week before acclimatization. There was no quantification of the number of shoots in each sugarcane cluster in this experiment, therefore this experiment was for a qualitative purpose only.

An additional post-rooting experiment was performed for contaminated plantlets. Rendered difficulties of advanced rooting systems in sugarcane plantlets include persistent bacterial contamination, which increases plantlets mortality by nearly 100% during acclimatization. This experiment involves 13 clusters of contaminated transgenic sugarcane plantlets and one non-contaminated sugarcane cluster. All plantlets' clusters were grown in half-strength MS medium for 1 week before acclimatization. A flowchart of the post-rooting, hardening, and acclimatization stages is presented in Figure 1.

Plantlets with well-developed root systems were removed from the culture tubes and washed with sterilized distilled water upon transfer to plastic bags containing autoclaved soil for hardening. The plastic bags containing plantlets' clusters were maintained undisturbed under a clear plastic cover to maintain high humidity for one week. The plastic cover was removed, and the hardening process was continued for another week in the culture room and controlled light. Evaluation was conducted at the end of the hardening process. After 30 days the plantlets were separated for each shoot and transferred into a polybag containing a mix of soil: compost (1:1) in a greenhouse (Shafique et al., 2015).

Results and Discussion

Transformation of Agrobacterium tumefaciens *with recombinant plasmids*

a. Transformation of Agrobacterium tumefaciens *LBA4404 competent cells with pCAMBIA1303-DHN1*

Plasmid pCAMBIA1303-DHN1 was first introduced into *A. tumefaciens* prior to sugarcane calli transformation. The presence of plasmid pCAMBIA1303-DHN1 in *A. tumefaciens* was confirmed by colony PCR and electrophoresis in agarose gel. As shown in Figure 2, most of the transformed colonies were confirmed to carry the recombinant plasmid and were ready to be used in calli transformation. Plasmid extracted from one of the transformed colonies (colony 4) was also confirmed to contain the DHN1 gene fragment, which indicates that it is indeed the transformed recombinant plasmid, pCAMBIA1303-DHN1 (Figure 3).

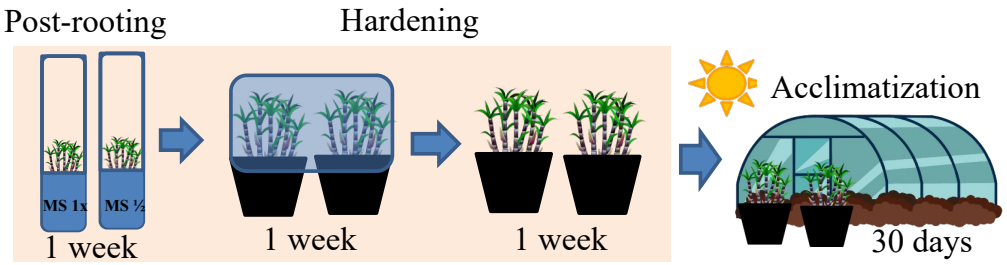

Figure 1. Post-rooting, hardening, and acclimatization stages of micropropagation of transgenic sugarcane plantlets
Gambar 1. Tahapan pasca perakaran, penguatan, dan aklimatisasi mikropropagasi pada planlet tebu transgenik

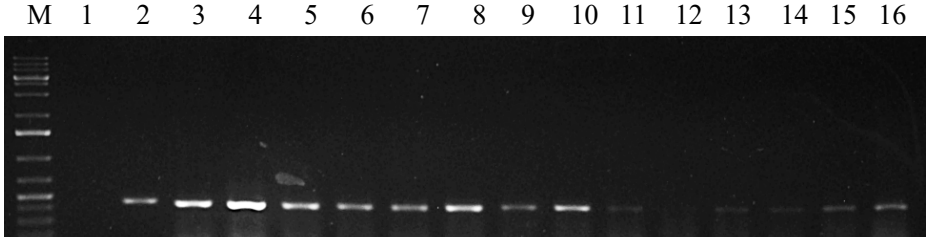

Figure 2. Colony PCR results of transformed Agrobacterium LBA4404 with DHN1 primers (lane 3–16). PCR with water (lane 1) and plasmid pCAMBIA1303-DHN1 (lane 2) as templates acted as control

Gambar 2. Hasil PCR koloni dari transforman Agrobacterium LBA4404 dengan primer DHN1 (lajur 3–16). PCR dengan air (lajur 1) dan plasmid pCAMBIA1303-DHN1 (lajur 2) sebagai templat dilakukan sebagai pembanding

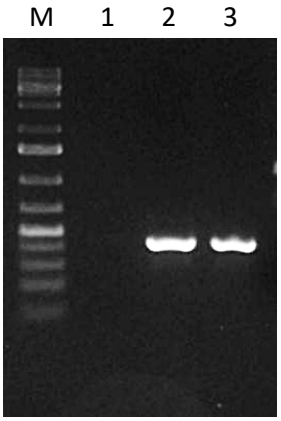

Figure 3. PCR results of pCAMBIA1303-DHN1 extracted from the transformed Agrobacterium LBA4404 (colony number 4) with DHN1 primers. PCR with water (lane 1) and plasmid pCAMBIA1303-DHN1 (lane 2) as templates act as controls

Gambar 3. Hasil PCR dari pCAMBIA1303-DHN1 diekstraksi dari Agrobacterium transforman LBA4404 (koloni nomor 4) dengan primer DHN1. PCR dengan air (lajur 1) dan plasmid pCAMBIA1303-DHN1 (lajur 2) sebagai templat dilakukan sebagai pembanding

b. Transformation of Agrobacterium tumefaciens *AGL-1 competent cells with pBI121-Pr-1DHNSo promoter construct*

The constructed plasmid (confirmed by sequencing) was used to transform *A. tumefaciens* strain AGL-1. Plasmids from transformed colonies were isolated using the alkaline lysis method and confirmed by PCR. This aims to determine whether the isolated bacteria carry the correct plasmids or not. PCR results using DHN-specific primers performed to confirm the inserted promoter fragment showed that the plasmid in *Agrobacterium tumefaciens* was the correct plasmid. This plasmid carries the Pr-1DHNSo construct which is shown as a 2000-bp band in the electrophoresis gel (data not shown). This confirmed that the transformed *Agrobacterium* was ready to be used in sugarcane calli transformation.

Regeneration of sugarcane calli transformed with pCAMBIA1303-DHN1 gene construct

Figure 4 shows the growth comparison of wild-type (4B) and transformed (4C) calli on selective media containing 10 ppm hygromycin. Some of the wild-type calli on selective media turned dark brown. Browning indicates the death of the calli cells due to the addition of hygromycin. In transformed calli, the integration of T-DNA carrying the hygromycin resistance gene helps the calli to survive on selective media, as indicated by the normal color and growth. The transformed calli then regenerated further to form plantlets as shown in Figure 4D. During the regeneration phase, plantlets were subcultured on MS2 media without antibiotics (data not shown).

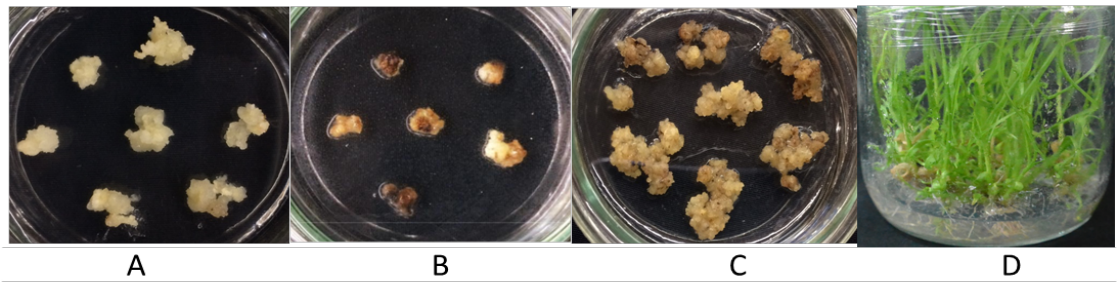

Figure 4. Kidang Kencana (KK) sugarcane calli: A) wild type (non-transformed) in non-selective MS media, B) wild type (non-transformed) in selective MS media (10 ppm hygromycin, 250 ppm cefotaxime), C) transformed calli in selective MS1 media (10 ppm hygromycin, 250 ppm cefotaxime). D) Transformed plantlets in MS 2 media without antibiotics, 4 months post-transformation

Gambar 4. Kalus tebu Kidang Kencana (KK): A) wild type (tidak ditransformasi) pada media MS non-selektif, B) wild type (tidak ditransformasi) pada media MS selektif (10 ppm hygromycin, 250 ppm cefotaxime), C) kalus tebu hasil transformasi dengan pCAMBIA1303-DHN1 pada media MS1 selektif (10 ppm higromisin, 250 ppm cefotaxime). D) planlet tebu hasil transformasi dalam media MS2 tanpa antibiotik, 4 bulan pasca transformasi.

Testing of transformed calli and shoots were carried out in two ways which include a screening for reporter gene expression (e.g., GUS gene) and selection of transformant that were resistant to selection agents (hygromycin) (Jouanin et al., 1993). Expression of the GUS gene encoding the enzyme β-glucuronidase was indicated by the formation of the blue color (Figure 5) in the transformed explants, thus indicating that transformation of the DHN gene construct has been successfully carried out into sugarcane. The enzyme activity can be visualized by the addition of X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) which is decomposed to intermediate compounds through oxidative dimerization reactions that produce the blue dichloro-dibromoindigo (ClBr-indigo). The GUS gene will be expressed only in plant cells and not in *Agrobacterium* due to the presence of an intron in the N-terminal region of the gusA gene sequence (Jouanin et al., 1993). Divya et al. (2019) reported that tissue-specific expression of the three pearl millet promoters (*PgApx pro*, *PgDhn* pro, and *PgHsc70 pro*) using *GUS* assay also showed in vegetative organs of transgenic tobacco.

Transformed calli resistance to hygromycin is conferred by the integration of a gene encoding hygromycin phosphotransferase (*HPT*) that is included in the pCAMBIA1303-DHN1 T-DNA construct. Thus, the integration of the T-DNA can also be confirmed by PCR using *HPT*-specific primers. PCR amplification using *HPT*-specific primers resulted in a 200-bp DNA fragment, the expected fragment size that indicates the presence of the HPT gene in transgenic plants. The band did not appear on wild-type sugarcane (Figure 6). This result, in addition to GUS assay results and transformed calli resistance in the selective media, confirmed the integration of DHN1 gene expression construct in the transformed sugarcane calli.

Transformation of pBI121-Pr-1DHNSo in sugarcane calli and its regeneration

Similar to the confirmation of the DHN1 gene expression construct in transformed calli, the confirmation of the Pr-1DHNSo construct was conducted by PCR using GUS and NPTII-specific primers. Instead of *HPT*, the T-DNA carrying Pr-1DHNSo construct consists of neomycin phosphotransferase II (NPTII) encoding gene that confers plant resistance to neomycin and kanamycin. Figure 7 showed the comparison of wild-type and transgenic calli transformed with pBI121-Pr-1DHNSo in selective media containing kanamycin. While wild-type calli turned dark brown, transgenic calli survived in the selective media and regenerate further to become sugarcane shoots and plantlets.

PCR amplification of genomic DNA from transformed sugarcane calli and leaves using NPTII-specific primers resulted in the expected 700-bp fragment (Figure 9), indicating the successful integration of the Pr-1DHNSo construct. This was also confirmed by PCR using GUS-specific primers that resulted in a 300-bp DNA fragment (Figure 8).

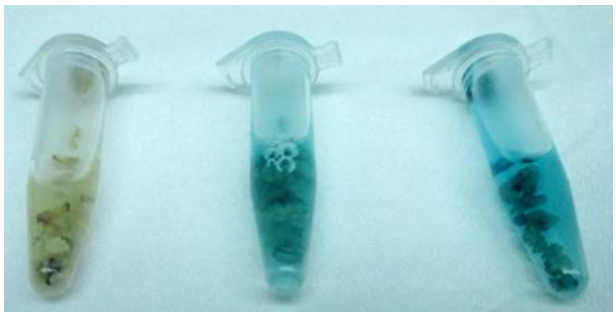

Figure 5. GUS test results on sugarcane var. KK calli carrying T-DNA from pCAMBIA1303-DHN1 construct after 2 days in co-cultivation media: (A) wild-type and (B & C) transformed sugarcane calli

Gambar 5. Hasil uji GUS pada tebu varietas KK yang membawa konstruk T-DNA dari pCAMBIA1303-DHN1 setelah 2 hari di media ko-kultivasi: (A) control (non transforman), (B&C) kalus tebu transforman

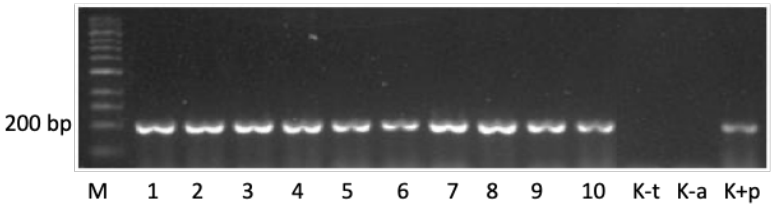

Figure 6. PCR amplification results of genomic DNA from transgenic sugarcane calli (lane 1-10), wild-type calli (K-t), non-template control (K-a), and plasmid pCAMBIA1303-DHN1 (K+p) with HPT gene primers

Gambar 6. Hasil amplifikasi PCR DNA genom dari kalus tebu transgenik (lajur 1-10), kalus wild-type (K-t), kontrol non-templat (K-a), dan plasmid pCAMBIA1303-DHN1 (K+p) dengan primer spesifik gen HPT

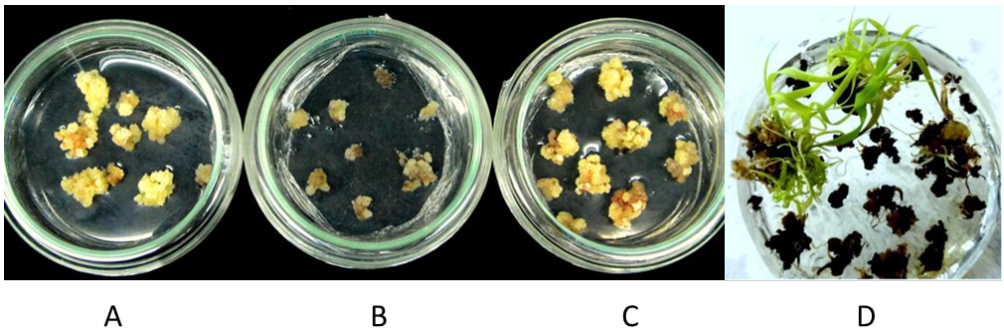

Figure 7. Kidang Kencana (KK) sugarcane calli: A) wild type (non-transformed) in non-selective MS1 media, B) wild type (non- transformed) in selective MS1 media (50 ppm kanamycin, 250 ppm cefotaxime), C) transformed calli in selective MS1 media. D) Transformed shoots in MS2 media without antibiotics, 3 months post-transformation

Gambar 7. Kalus tebu Kidang Kencana A) wild type (tidak ditransformasi) pada media MS1 non selektif, B) wild type (tidak ditransformasi) pada media MS1 selektif (50 ppm kanamycin, 250 ppm cefotaxime), C) kalus transforman pada media MS1 selektif. D) Tunas tebu hasil transforman dalam media MS2 tanpa antibiotik, 3 bulan pasca transformasi

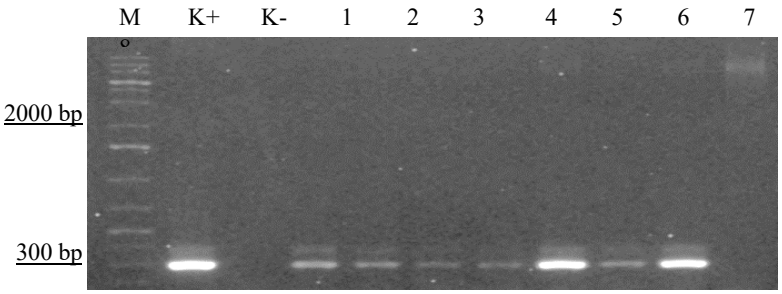

Figure 8. PCR amplification results of (lane 1-7) genomic DNA from transformed sugarcane leaves, (K+) plasmid pBI12.1-Pr-1DHNSo as a positive control, (K-) non-template control, and (lane 8) wild-type leaves with GUS marker gene primers

Gambar 8. Hasil amplifikasi PCR dengan templat (lajur 1-7) DNA genom daun tebu hasil transformasi, (K+) plasmid pBI12.1-Pr-1DHNSo sebagai kontrol positif, (K-) kontrol non-templat, dan (lajur 8) daun tebu wild-type dengan primer gen penanda GUS

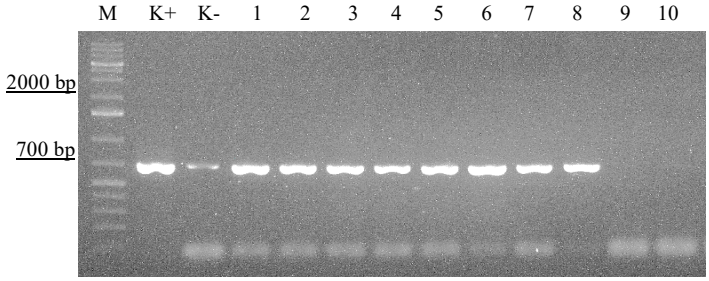

Figure 9. PCR amplification results of (lane 1-9) genomic DNA from sugarcane leaves transformed with pBI12.1-Pr-1DHNSo, (K+) plasmid pBI12.1-Pr-1DHNSo as a positive control, (K-) non-template control, and (lane 10) wild-type leaves with NPTII marker gene primers

Gambar 9. Hasil amplifikasi PCR dengan templat (lajur 1-9) DNA genom daun tebu hasil transformasi dengan pBI12.1-Pr-1DHNSo, (K+) plasmid pBI12.1-Pr-1DHNSo sebagai kontrol positif, (K-) kontrol non-templat, dan (lajur 10) daun tebu wild-type dengan primer gen penanda NPTII

The use of constitutive promoters such as CaMV35S and ubiquitin is the most common approach in sugarcane transformation that resulted in a high expression level of transgenes (Porto et al., 2014; Dutt et al., 2014). In prior research, we have successfully isolated the DHN promoter region that was drought-inducible. The Pr-1DHNSo promoter sequence was inserted into the pBI121 expression vector by replacing the CaMV35S promoter region (Minarsih et al., 2020). Transformation of the Pr-1DHNSo promoter construct into calli was carried out following the method by Sain et al. (1994) as previously done in the transformation of the DHN gene construct with the CaMV35S promoter.

Prior research also showed that the activity of the sugarcane DHN promoter that controls the expression of the GUS promoter gene was greatly increased in drought-stressed transgenic tobacco plants, relative to well-watered tobacco plants (Iskandar et al., 2020). Utilization of the inducible DHN promoter can be very helpful in designing drought-resistant sugarcane plants.

Optimization of acclimatization process of sugarcanes transformed with pCAMBIA1303-DHN1 and pBI121-Pr-1DHNSo

The presence of roots and shoot tips showed the successful acclimatization of tissue culture-produced transgenic sugarcane plantlets. Transgenic sugarcane plantlets of the var. Kidang Kencana that had been transformed with the pCAMBIA-DHN or Pr-1DHNSo promoter constructs with sufficient root sizes, about 3-4 months in regeneration media, were ready for acclimatization in the greenhouse. Based on the plantlet morphology, it was shown that the growth of transgenic sugarcane plants post-acclimatization was relatively as good as the control plants (Figure 10 and Figure 11).

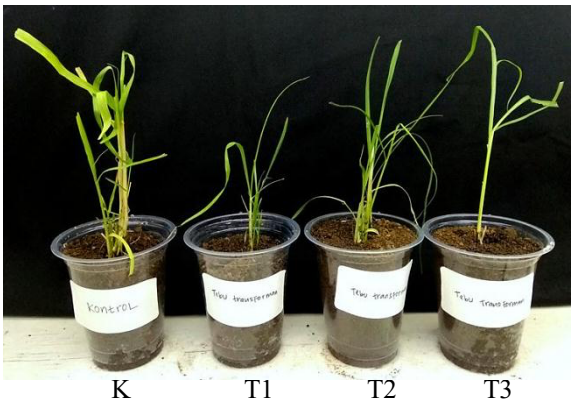

Figure 10. Transformed sugarcane with pCAMBIA1303-DHN1 construct after 3-4 weeks of standard acclimatization. K: non-transformant sugarcane, T1-T3: transformed sugarcanes

Gambar 10. Tebu transforman yang membawa konstruk pCAMBIA1303-DHN1 3-4 minggu setelah aklimatisasi standar. K: tebu non-transforman, T1-T3: tebu transforman

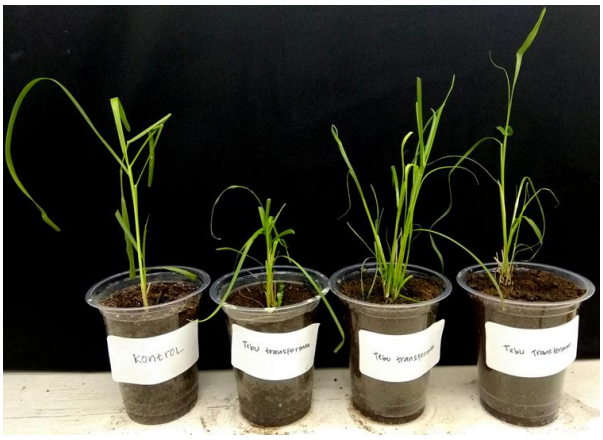

Figure 11. Transformed sugarcanes with DHN promoter pBI construct after 3-4 weeks of standard acclimatization. K: non-transformant sugarcane, T1-T3: transformant sugarcane

Gambar 11. Tebu transforman dengan konstruk promoter pBI121-Pr1DHNSo, 3-4 minggu setelah aklimatisasi standar. K: tebu non-transforman, T1-T3: tebu transforman

Table 1. Response of plantlets of all treatments applied
T*abel 1. Respons planlet terhadap seluruh perlakuan yang diberikan*

Experiment *Percobaan*	Treatment *Perlakuan*	Survived *Hidup*	Dead *Mati*	Note *Catatan*
Post-rooting	Full-strength MS medium	5 clusters	3 clusters	Many plants died within the same cluster and were still considered survived by at least one shoot alive
	Half-strength MS medium	11 clusters	0 clusters	No dead shoot within all clusters with higher plant vigor
	Control	1 cluster	-	Non-transgenic plantlets in each treatment
Contaminated plantlets	Half-strength MS medium	7 clusters	6 clusters	Of 7 plantlets, the growth strives after old leaves dry and new leaves grow
	Control	1 cluster	-	Non-contaminated in ½MS

Survival improvement of micro-propagated transgenic sugarcane

Transgenic sugarcane plantlets var. Kidang Kencana transformed with pCAMBIA-DHN construct or DHN Pr-1DHNSo promoter construct is susceptible to the acclimatization process. Some studies mentioned that reducing MS media strength may reduce osmosis levels and improve the acclimatization process in some plants i.e., *Typhonium flagelliforme* (Rezali et al., 2017), date palm cv. Aziza Bouzid (Elmaataoui et al*.,* 2020), and apple (*Malus domestica* Borkh) 'Golden Delicious', 'Maksat', and 'Voskhod' (Kabylbekova et al*.,* 2020). Nine out of a total of 25 tubes or clusters (Table 1) of both transgenic and control (wild type) acclimatization-ready sugarcane plantlets died after the acclimatization process. In the post-rooting treatment using 1x MS media, 3 out of 5 plant clusters died. Meanwhile, the use of ½ MS media was shown to reduce the post-acclimatization mortality rate. None of the 11 plant clusters grown in ½ MS media experienced death. This shows that post-rooting treatment with ½ MS media may be applied to obtain transgenic plants with better viability post-acclimatization. Figure 12 also shows the plantlet responses to 1x MS and ½ MS media during the hardening phase in which plants grown in ½ MS media were shown to be healthier. While many sugarcanes without pre-acclimatization experienced death or leaf drying.

Transgene stability of DHN1 gene and its promoter in transgenic sugarcane plants

Testing the presence of transgene constructs in post-acclimatization sugarcane explants was carried out using PCR with specific primers. Amplification results using hygromycin resistance (HPT) gene primers are indicated by the presence of a band with a size of 200 bp, as expected in transgenic plants. Figure 13 shows that most of the sugarcane plants transformed with the DHN gene construct in the year 2017 and DHN promoter constructs in 2018 still showed amplification bands after PCR analysis using HPT primers in 2019. In control (wild-type) plant samples, no such amplification band was observed (Figure13).

This study shows the opportunity to develop drought-tolerant sugarcane through *Agrobacterium*-mediated calli transformation. However, further optimization and improvement are still needed to increase the transformation success rate and reduce the frequency of somaclonal variation that often accompanies the application of plant tissue culture. Two approaches that can be applied are the use of different explant types in transformation and the addition of amino acids in the growth media of transformed explants. The use of *in vitro*-propagated axillary shoots has been shown to increase the transformation success while suppressing the genetic abnormalities of the transformed plants (Sugiharto & Safitri, 2011). The use of amino acids in growth media can also help plant development during the regeneration and selection process of transformed explants (Sugiharto, 2017).

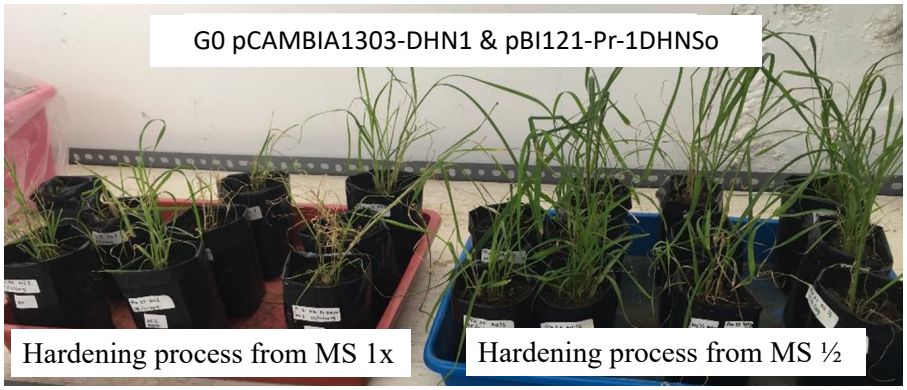

Figure 12. Response of sugarcane plantlets grown in different strength MS medium after the hardening stage

Gambar 12. Respons dari planlet tebu pada konsentrasi media MS yang berbeda setelah tahap penguatan

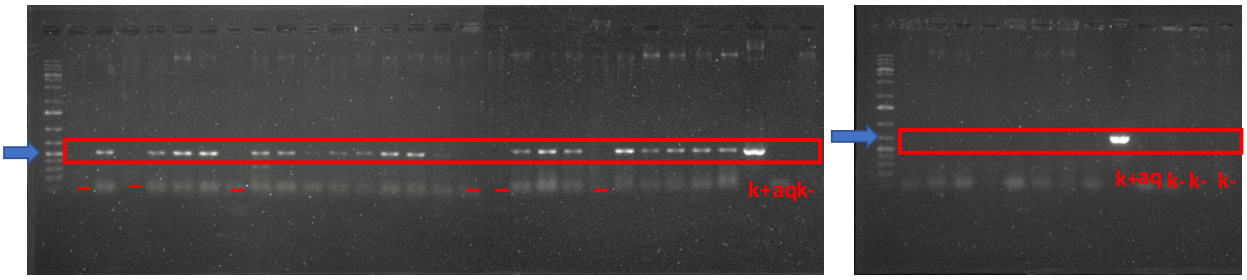

Figure 13. Transgene confirmation of transgenic sugarcane plantlets. PCR amplification results of genomic DNA from sugarcane plants transformed with (left) pCAMBIA1303-DHN1 with HPT primers; (right) pBI121-Pr-1DHNSo with NPTII primers; (k+) plasmid positive control; (aq) water negative control; (k-) non-transgenic sugarcane; Ladder 1kb Plus

Gambar 13. Konfirmasi transgen dari planlet tebu transgenik: (kiri) pCAMBIA1303-DHN1 dengan primer HPT; (kanan) pBI121-Pr-1DHNSo dengan primer NPTII; (k+) kontrol positif plasmid; (aq) kontrol negatif air; (k-) tebu non-transgenik; marka Ladder 1kb Plus

Dehydrin is one of the proteins that play a role in dehydration tolerance in plants (Hanin et al., 2011). The protein plays an important role in maintaining the structural stability of proteins and other macromolecules when plants are dehydrated either caused by drought or high salt levels. Overexpression of the DHN-encoding gene in transgenic plants is expected to provide a buffer system for plants when facing drought stress without sacrificing sugarcane productivity.

Conclusion

Transformed sugarcane plantlets carrying the DHN gene construct under the control of the constitutive promoter CaMV35S were obtained and successfully acclimatized in the greenhouse. Sugarcane calli transformed with DHN gene promoter Pr-1DHNSo cloned into the expression vector pBI121 have also been regenerated and acclimatized. Post-rooting treatment using ½ MS media was shown to reduce the mortality rate of acclimatized plantlets. Most of the transgenic plants carrying the DHN gene construct or the DHN promoter constructs grown in the greenhouse were confirmed to carry the integrated transgenes more than one year after transformation.

Acknowledgments

The present study was supported by a grant from the 2018 KP4S of the Indonesian Agency for Agricultural Research and Development, Indonesian Ministry of Agriculture.

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