Screening of environmental stress tolerant superior sugarcane (Saccharum officinarum L.)

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Abstract. Sugarcane (Saccharum officinarum L.) is among the important commodities of the commercial sector since it accounts for 65% of the world’s sugar production. Indonesia is the world’s eighth largest country in terms of productivity, planting area and yield of sugarcane. Environmental stresses due to climate change are among factors that may cause negative impacts on the production of sugarcane. The purpose of the present study was to determine sugarcane cultivars tolerant to environmental stress based on a multiplex analysis of SCDR 1 and P5CS, as well as sugarcane defense mechanisms against environmental stress conditions. The methods used in this study were quantitative test with spectrophotometry, qualitative test with electrophoresis, DNA amplification of the target SCDR 1 and P5CS by the PCR method. The presence of these two genes in the tested sugarcane cultivars indicates a tolerant trait of environmental stress. The study found that 24 tested sugarcane cultivars are potentially tolerant to environmental stress due to the presence of those two genes.

1. Introduction
Sugarcane (Saccharum officinarum L.) is among the most important commodities of the commercial sector and accounts for 65% of sugar production worldwide [1]. Indonesia is the world’s eighth largest country in terms of productivity, planting area and yield of sugarcane [2]. In 2016, the area of sugarcane plantations in Indonesia was 444,220 ha with a total productivity of 2,222,971 tons and predicted to increase in 2017 of up to 453,456 ha for the planting area and 2,465,450 tons for its total productivity [3]. The level of sugar demand in Indonesia was 4.6 million tons and would continue to rise yearly [4], but this was not offset by domestic sugar production due to declining conditions of sugarcane planting in Indonesia.

Sugarcane is a unique plant capable of accumulating high sugar levels and is a source of commercial biomass for second-generation bioelectricity and bioethanol [5]. Modern sugarcane cultivars are highly productive in the tropics. Environmental stress conditions can affect sugarcane productivity; for example, drought stress can reduce productivity due to a morphophysiological effect
that causes water deficiency in sugarcane as well as affecting the inhibition of photosynthetic activity and growth. These drawbacks led to the development of new sugarcane cultivars with better water consumption efficiency [6]. In addition, the salinity stress is also an environmental stress that does not only affects the growth rate, but also affects the accumulation of sucrose in sugarcane stems [7]. In some sugarcane cultivars, stress conditions affect the shoot growth [8]. Oxidative stress by ROS can be fatal to plants since it can attack cellular metabolic processes, such as the cellular respiration process associated with energy production for cells [9].

SCDR 1 is among the examples of a gene capable of responding to multiple stresses, such as salinity, drought, and oxidative stresses. SCDR 1 is strongly expressed in tolerant sugarcane varieties, whereas it is not expressed in susceptible varieties. An overexpression of SCDR 1 will reduce the harmful effects of environmental stress conditions [9]. In drought stress conditions, sugarcane plants expressing P5CS will be resistant to the stress conditions [10]. In the present study, tolerant sugarcane cultivars were screened by means of SCDR 1 and P5CS molecular markers to detect cultivars tolerant to drought, salinity and oxidative stress conditions. PCR (polymerase chain reaction) method was used for the screening process to amplify specific fragments in nucleic acids by regulating their temperature to run the reaction [11]. In the present study, the specific fragments in question were SCDR 1 and P5CS fragments found in the sugarcane genome.

Identification of sugarcane genotypes tolerant to environmental stress conditions at the early stages of growth is important for plant breeding programs as a means to increase processed sugarcane productivity. The purpose of the present study was to perform early screening and detection of superior sugarcane cultivars resistant to environmental stress conditions in order to increase sugar production in Indonesia. Therefore, the mechanism of sugarcane defense responses to environmental stress conditions can be determine based on the multiplex analysis of SCDR 1 and P5CS.

2. Materials and Methods

The purpose of the method of early detection of sugarcane cultivars was to determine sugarcane cultivars adaptive to environmental stress conditions using molecular markers. The advantage of this method is quick and easy determination of sugarcane cultivars of superior quality that can be planted in various locations. This method of detection consists of several stages, including the isolation of genomic DNAs in order to obtain the genomic DNAs from the leaf samples of 24 sugarcane cultivars to be tested. The genomic DNAs obtained were then subjected to a qualitative test to determine the genome size of each isolated sample. The quantitative test was carried out to determine the concentration and purity of isolated genomic DNAs; when the results of qualitative and quantitative tests were compatible, the next stage could be performed.

2.1. Genomic DNA Isolation

Samples of sugarcane leaves from 24 sugarcane cultivars were used in the study, namely PS 41, PS 58, PS 384, PS 851, PS 864, PS 865, PS 881, PS 882, PS 891, PS 921, PS 951, PS 80.910, PS 80.1649, PS CO 902, PS JT 941, Kentung, BZ 132, KK, TLH 2, PSDK 923, BL, PS 862, PSBM 901, and VMC 76-16, obtained from the results of cultivation of seedlings by PT. Madu Baru in Bantul, Special Region of Yogyakarta, and the results of cultivation of seedlings at the Indonesian Sweetener and Fiber Crops Research Institute (Balittas) Malang, East Java. Genomic DNAs were isolated using the Nucleon Phytopure reagent kit. The first step was to chop leaf samples and weigh them of 0.3 grams using an analytical scale. Subsequently, the weighed leaf samples were put into a mortar and added with liquid nitrogen to facilitate grinding. The leaf samples were ground to fineness and to have powder-like texture. The fine samples were then put into a labeled 1.5-mL microtube and gradually added with Phytopure reagent along with other reagents in accordance with the prescribed protocol. Upon completion of isolation of genomic DNAs from 12 samples of sugarcane leaves, those samples were then stored in a freezer at -20°C.
2.2. Quantitative Test of Genomic DNAs
The genomic DNAs isolated at the previous stage were subjected to a DNA quantitative test to determine its concentration and purity spectrophotometrically. The absorbance value at λ 260 nm showed the DNA concentration in 50μg/mL. Subsequently, the DNA concentration was calculated using the absorbance formula of λ 260 x 50 x dilution μg/mL. In addition, the results of DNA purity ratio would also be displayed on the monitor of the device. The purity of DNA could be measured by the absorbance of λ 260/λ 280, with a good DNA purity ratio ranging from 1.8 to 2.0 [12].

2.3. Qualitative Test of Genomic DNAs
The isolated genomic DNAs were also subjected to a DNA qualitative test to demonstrate that the isolated samples were DNAs and to determine the size of the sugarcane genomic DNAs in base pair (bp). The sugarcane genomic DNAs were qualitatively tested by means of the electrophoresis technique using agarose gel.

2.4. DNA Amplification by the PCR Multiplex Method
The next stage was DNA amplification using specific target gene primers. Amplification was performed using a PCR machine (Veriti Thermal Cycler Applied Biosystems). The PCR multiplex premixes were made according to the amount of samples to be amplified. The premixes contained sterile ddH₂O, diluted specific primers (Table 1) and Bioline PCR kit. Subsequently, all PCR premix reagents were put into a special 1.5 mL microtube without previously adding DNA templates (isolated genomic DNA) (Table 2).

| No. | Primer        | Sequence (5’-3’)       |
|-----|---------------|------------------------|
| 1   | M.SCDR 1-Forward | AGAAGAAGGTGGGTGGGTTG   |
|     | M.SCDR 1-Reverse | CAGGCTTAGACTTGGGCTTG   |
| 2   | P5CS- Forward  | ACAGATGATAAAGTAGCAGAGAC|
|     | P5CS- Reverse  | AGACCTTCAACACCCACAG    |

| Mixture       | Volume | Concentration |
|---------------|--------|---------------|
| Sample DNAs   | 2 μL   | 20 ng/μL      |
| PCR Kit       | 12.5 μL| -             |
| Forward Primer M.SCDR 1 | 1 μL | 25 μM         |
| Reverse Primer M.SCDR 1 | 1 μL | 25 μM         |
| Forward Primer P5CS  | 1 μL  | 25 μM         |
| Reverse Primer P5CS  | 1 μL  | 25 μM         |
| Sterile ddH₂O  | 6.5 μL | -             |

Then, when all the premixes have been distributed into each PCR microtube, the DNA templates were then added to each microtube. The total volume of each PCR reaction was 25 μL for each microtube (Table 2). The mixture was then rehomogenized using a vortex. Subsequently, the mixture containing microtube was put into the PCR machine with preset time, temperature and number of replications for each cycle during the process of DNA amplification.

During amplification of samples using the PCR machine, the pre-denaturation cycle was carried out at 94°C for 1 minute, denaturation at 94°C for 45 seconds, annealing at 62°C for 1 minute, elongation at 72°C for 75 seconds and post-elongation at 72°C for 75 seconds. The denaturation, annealing and elongation cycles were repeated 40 times. The multiplex method was used for M.SCDR 1 and P5CS
specific primers. SCDR 1 gene was amplified using M.SCDR 1 specific primers, when P5CS gene was amplified using P5CS specific primers.

2.5. Qualitative Analysis of PCR Products
The previously obtained PCR amplification products were subsequently analyzed qualitatively by the electrophoresis technique. The agarose gel that contained samples and had been run was then observed with Gel Doc and Optilab connected to a computer. The gel was placed on the UV light to visualize DNA bands. The single band appearing during agarose gel visualization showed the presence of PCR-amplified specific target genes.

2.6. Analysis of Results
Results of the qualitative test were used to determine the size of sugarcane genomic samples by comparing them on the ladder. Genomic DNAs will appear right below the well because of its large size from 760 Mbp to 926 Mbp for its total basic genome size [13]. Data obtained from genomic DNA isolation were tested quantitatively. When the measured purity of the DNAs is less than 1.8, the DNA samples are contaminated with protein; when the measured purity is more than 2.0, the samples are contaminated with RNA molecules.

The next stage after qualitative and quantitative test was DNA amplification using the PCR method. PCR is a method for multiplication of DNA fragments in a short time in vitro [14]. PCR technique is capable of multiplying a specific fragment in the nucleic acid (DNA or RNA) by adjusting its temperature to run the reaction [11]. The DNA amplification products with specific target gene primers were then analyzed using electrophoresis method or qualitatively to determine the presence of those genes. The presence of target genes in the samples can be detected through the length of DNA bands produced by the amplification process. SCDR 1 (Sugarcane drought related 1) target gene has an amplicon size of 315 bp, while the P5CS (Delta -1-pyrroline -5-carboxylate synthetase) has an amplicon size of 167 bp [10]. When the results of agarose gel visualization indicate DNA bands similar to the size of the target gene amplicons, a conclusion can be drawn that the tested samples contain the specific target genes desired, namely SCDR 1 and P5CS.

3. Results and Discussion
As shown by the results of qualitative tests of genomic DNAs of 24 sugarcane cultivars, the 24 sugarcane cultivars had a fairly large genome size greater than 1 Kbp (Figure 1). The obtained genome size did not reach the Mbp size since the genomic DNAs were cut into smaller sizes during the isolation treatment, which was also supported by the appearance of smears in the electrophoresis visualization results. Electrophoresis visualization indicated that all samples showed DNA bands, meaning that genomic DNA isolation from sugarcane leaf samples had been successfully performed.

Based on the quantitative test of genomic DNAs, the 24 sugarcane cultivars under study had a quite varied range of sugarcane leaf-isolated DNA concentration of 76 to 1875ng/µL, with a purity range of 1.4 to 2.0. The data of isolated genomic DNA concentration (Figure 2) showed that the PSDK 923 cultivar had the lowest concentration of 76ng/µL, whereas the Kidang Kencana (KK) cultivar had the highest concentration of 1875ng/µL. Low concentrations could be due to the accumulation of phenolic compounds, too old leaf samples, or damaged DNAs due to extraction treatment. The data of DNA purity (Figure 3) showed that the PS 41 cultivar had the lowest purity of 1.448, while the PSDK 923 cultivar had the highest purity of 2.071. The relatively low DNA purity was caused by protein contamination.
Figure 1. Result of electrophoresis visualization of genomic DNAs of 24 sugarcane cultivars. Note: M: 1 Kbp Marker, 1: PS41, 2: PS58, 3: PS384, 4: PS851, 5: PS862, 6: PS864, 7: PS865, 8: PS881, 9: PS882, 10: PS891, 11: PS921, 12: PS951, 13: PS80910, 14: PS801649, 15: PSCO982, 16: PSBM901, 17: PSJT941, 18: BL, 19: Kentung, 20: KK, 21: BZ 132, 22: TLH 2, 23: PSDK 923, and 24: VMC 76-16.

Figure 2. Genomic DNA concentration of 24 sugarcane cultivars.
According to Sambrook and Russell [12], PS 58; PS 384; PS 851; PS 864; PS 865; PS 881; PS 882; PS 891; PS 921; PS 951; PS 80.910; PS 80.1649; PS CO 902; PS JT 941; Kentung; KK; PSDK 923; BL; PS 862; and PSBM 901 cultivars have the good level of purity. Based on quantitative test they have purity levels in range 1.8-2.0. It can be assumed that the twenty sample are free from contamination, but the four sample (PS 41, BZ 132, TLH 2, and VMC 76-16) was contaminated with protein and polysaccharide because they have low purity (<1.8) (Figure 3).

**Figure 3.** Genomic DNA purity of 24 sugarcane cultivars.

**Figure 4.** Results of electrophoresis visualization of PCR products with the multiplex analysis of SCDR 1 and P5CS target genes in 12 sugarcane cultivars. Note: M: 100 bp Marker, 1: PS41, 2: PS58, 3: PS384, 4: PS851, 5: PS862, 6: PS864, 7: PS865, 8: PS881, 9: PS882, 10: PS891, 11: PS921 and 12: PS951. Remarks: The appearance of a band of 315 bp in size indicates the presence of SCDR 1, while the appearance of a band of 167 bp in size in the tested sugarcane cultivars shows the presence of P5CS.
Figure 5. Result of electrophoresis visualization of PCR products with the multiplex analysis of SCDR 1 and P5CS target genes in 12 sugarcane cultivars. Note: M: 100 bp Marker, 13: PS80910, 14: PS801649, 15: PSCO982, 16: PSBM901, 17: PSJT941, 18: BL, 19: Kentung, 20: KK, 21: BZ 132, 22: TLH 2, 23: PSDK 923, and 24: VMC 76-16. Remarks: The appearance of a band of 315 bp in size indicates the presence of SCDR 1, while the appearance of a band of 167 bp in size in the tested sugarcane cultivars shows the presence of P5CS.

Figure 4 shows that PS 41, PS 58, PS 384, PS 851, PS 862, PS 864, PS 865, PS 881, PS 921 and PS 951 cultivars are detected to have SCDR 1 marked by the appearance of a DNA band of 315 bp in size in the electrophoresis results. Additionally, they are also detected as having P5CS marked by the appearance of a DNA band of 167 bp in size in the electrophoresis results. The similar results were obtained from the other twelve cultivars. Figure 5 shows that PS 80910, PS 801649, PSCO 982, PSBM 901, PSJT 941, BL (BuluLawang), Kentung, KK (KidangKencana), BZ 132, TLH 2, PSDK 923, and VMC 76-16 cultivars are also detected as having SCDR 1 and P5CS. Based on these results, those 24 sugarcane cultivars can be categorized as superior cultivars that potentially tolerant to drought, salinity and oxidative stress conditions due to the presence of SCDR 1 and P5CS. According to Prabawanti [15], PS 864, PS 865, PSJT 941, PS 881 and Kentung cultivars are known as sugarcane cultivars resistant to drought stress conditions. Based on that research, PS 864, PS 865, PSJT 941, PS 881 and Kentung cultivars already proven that all of them are potential sugarcane who have resistant traits to environmental stress like drought stress.

The morphologic and physiologic responses of sugarcane vary by genotype, duration (fast or gradual) and intensity (heavy or light) of the stresses and also by the type of the affected tissues. Drought conditions also substantially affect sugarcane productivity and sugar produced [5]. Plants also respond and adapt to conditions of fluid deficits at cellular and molecular levels, for example by the accumulation of osmolytes, proteins specifically involved in stress tolerance, hormones (ABA) and certain genes [16]. Salinity conditions not only affect the growth rate, but also affect the sucrose contents in sugarcane stems [7]. Several sugarcane cultivars also show its effect on shoot growth [8]. ROS (reactive oxygen species) can be fatal to plants since it attacks cellular metabolic processes, such as the cellular respiration associated with energy production for cells [9].

Electropherogram shows the visualization results of SCDR 1 and P5CS in multiplexes, leading to an interpretation that the two primers are able to amplify the two regions that match the primers and the genes in the genomes of each sugarcane cultivar under study. Based on the multiplex analysis, the 24 sugarcane cultivars under study had SCDR 1 and P5CS in their genomes, which were shown by the
appearance of bands with lengths that matched the size of the target genes, and had equally parallel bands between cultivars (Figure 4 and 5).

SCDR 1 is strongly expressed in tolerant sugarcane varieties, whereas this gene is not expressed in susceptible varieties. This gene is capable of responding to multiple stresses, such as salinity, drought, and oxidative stresses. Photosynthetic parameters, such as transpiration rate, net photosynthesis, stomatal activity, and CO₂ concentration, are not significantly affected by drought and salinity stress conditions due to the activity of this gene. The percentage of carbon fixation is higher than respiration rate in drought stress conditions since plants will optimize photosynthetic activity, rather than cellular respiration. An overexpression of SCDR 1 will reduce the harmful effects of environmental stress conditions by reducing the initially high cellular respiration rate [9].

P5CS is a gene that plays a role in proline amino acid biosynthesis. Proline amino acid is one of the main components that make up enzymes and proteins that play a role in the response to drought stress conditions. The activity of this gene proves to be negatively correlated to sucrose biosynthesis; therefore, in drought stress conditions sugarcane expressing this gene would tend to increase its proline biosynthetic activity and decrease sucrose productivity [17]. In drought stress conditions, P5CS-expressing sugarcane would resistant to the stress conditions so that it can still grow optimally; however, the side effect is that sugarcane would reduce sucrose productivity in the stem by decreasing the sucrose accumulation in the stem. This is because the sucrose produced would be used to support the metabolic, growth and development processes during the stress conditions, leading to decreased levels of accumulated sucrose in the stem [10].

Based on the foregoing, the 24 sugarcane cultivars under study are found to have SCDR 1 and P5CS in their genomes, thus having potentially multiple defenses in response to drought stress conditions and tolerant to salinity and oxidative stress conditions. The disadvantage is that when these 24 sugarcane cultivars are exposed to drought stress conditions, the accumulation of sucrose in the stem will decrease, resulting in low sucrose productivity in stressful conditions.

4. Conclusion
The present study shows that PS 41, PS 58, PS 384, PS 851, PS 864, PS 865, PS 881, PS 882, PS 891, PS 921, PS 951, PS 80.910, PS 80.1649, PS CO 902, PS JT 941, Kentung, BZ 132, KK, TLH 2, PSDK 923, BL, PS 862, PSBM 901, and VMC 76-16 sugarcane (S. officinarum) cultivars are potentially tolerant to environmental stresses, particularly drought, salinity and oxidative stresses, due to the presence of SCDR 1 and P5CS. With the presence of P5CS, the twenty-four cultivars are likely to reduce their sucrose productivity under drought stress conditions. Further research is recommended to identify other sugarcane cultivars in Indonesia that are potentially tolerant to other environmental stress. Further research is needed to determine the function of these two genes in response to other environmental stress conditions.

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References
[1] Carson D L and Botha F C 2002 Genes expressed in sugarcane maturing internodal tissue
Plant Cell Rep. 20 1075-81.

[2] Zhao D and Li Y R 2015 Climate change and sugarcane production: potential impact and mitigation strategies Int. J. Agron. 2015 1-10.

[3] Bambang 2016 Statistik Perkebunan Indonesia Komoditas Tebu (Jakarta: Directorate of the General of Plantation) p 4.

[4] Voboril D 2010 Indonesia Sugar Annual Report 2010 (Jakarta: GAIN (Global Agriculture Information Network)) p ID1014.

[5] Ferreira T H S, Tsunada M S, Bassi D, Araujo P, Mattiello L, Guidelli G V, Righetto G L, Goncalves V R, Lakshmanan P and Menossi M 2017 Sugarcane water stress tolerance mechanisms and its implications on developing biotechnology solutions Front. Plant Sci. 8 1077.

[6] Ghannoum O 2009 C4 photosynthesis and water stress Ann. Bot. 103 635-44.

[7] Rozeff N 1995 Sugarcane and salinity a review paper Sugar Cane 5 8-19.

[8] Akhtar S, Wahid A, Akram M and Rasul E 2001 Some growth, photosynthetic and anatomical attributes of sugarcane phenotypes under NaCl salinity Int. J. Agric. Biol. 3 439-43.

[9] Begcy K, Mariano E D, Gentile A, Lembke C G, Zingaretti S M, Souza G M and Menossi M 2012 A novel stress-induced sugarcane gene confers tolerance to drought, salt, oxidative stress in transgenic tobacco plants PloS ONE 7 1-14.

[10] Matin R, Ebrahimi M A and Niazi A 2014 Quantitative expression analysis of P5CS and BADH genes in cultivated wheat plants under salt and ABA treatments Iran. J. Genet. Plant Breed. 3 43-8

[11] Joshi M and Deshpande J D 2010 Polymerase chain reaction: methods, principles and application Int. J. Biomed. Res. 1 81-97.

[12] Sambrook J and Russel D W 1989 Molecular Cloning: A Laboratory Manual 2nd ed (New York: Cold Spring Harbor Laboratory Press) pp 165-6.

[13] Menossi M, Silva-Filho M C, Vincentz M, Van-Sluys M A, and Souza G M 2008 Sugarcane functional genomics: gene discovery for agronomic trait development Int. J. Plant Genom. 2008 1-11.

[14] Bintang M 2010 Biokimia: teknik penelitian (Jakarta: Erlangga) pp 236-48.

[15] Prabawanti Y W 2012 Biosistematika keanekaragaman tanaman tebu (Saccharum officinarum) melalui pendekatan morfologi (Jawa Timur: Universitas Airlangga).

[16] Shinozaki K and Yamaguchi-Shinozaki K 2007 Gene networks involved in drought stress response and tolerance J. Exp. Bot. 58 221-7.

[17] Iskandar H M, Casu R E, Fletcher A T, Schmidt S, Xu J, Maclean D J, Manners J M and Bonnett G D 2011 Identification of drought-response genes and a study of their expression during sucrose accumulation and water deficit in sugarcane culms Plant Biol. 11 1-14.