Screening and characterization of molecular markers for sugarcane stem borer (Saccharum officinarum L.) resistance genes

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Abstract. Sugarcane (Saccharum officinarum L.) constitutes a main crop for production of sugar that serves as the main calorie source for public consumption. It is a particular challenge for the Government and related parties to ensure its availability. A decline in sugarcane production on agricultural land occurs due to disturbances by plant pests, especially stem borers. Stem borers can reduce sugar production by 52 to 73%. The purposes of the present study were to screen and detect genes resistant to stem borer attacks in 22 sugarcane cultivars and to characterize SacBBI4 as among the resistance gene markers in sugarcane. The present study produced 3 primer designs. The primer P-SacBBI4-A successfully amplified SacBBI4 gene at 729 bp, the primer P-SacBBI4-B at 624 bp, while the primer P-SacBBI4-C successfully amplified the target gene at 671 bp and same value were also obtained when analyzing the sequences on the Genstudio. As shown by the phylogenetic tree, two large clusters were formed. The first cluster was the WIP1 coding cluster, while the second one was the BBI coding cluster. The phylogenetic tree and genetic distance showed that the primer P-SacBBI4-C was the primer design closest to the target gene SacBBI4 on the NCBI data.

Keywords: PCR, primer design, stem borer, Saccharum officinarum L.

1. Introduction
Sugarcane (Saccharum officinarum L.) constitutes a main crop for production of sugar that serves as the main calorie source for public consumption [1]. The government’s policy of deciding sugar as one of the nine essential culinary ingredients has consequences for the government, in particular, and every walk of life, including academics, professionals and industries to secure the availability of sugar in sufficient quantities and quality, at affordable prices and with an easy access to the public, which is
a challenge amidst the declining condition of sugarcane industry in Indonesia [2]. Sugarcane cultivars cultivated in Indonesia include VMC (Victoria Miling Company) 76-16, TLH (Tolongohula) 2, PSDK (Pasuruan Desa Kemlagi) 923, PS (Pasuruan) 862, PSBM (Pasuruan Bunga Mayang) 901, KK (Kidang Kencana), BZ 132, BL (Bulu-Lawang), PSCO 92, Kentung, PS 864, PS 865, PS 881, PS 891, PS 921, PS 951, PS 80.910, PS 41, PS 58, PS 384, PS 851, and PS 80.1649 [3].

During its cultivation, sugarcane production encounters some obstacles and disturbances, especially those originating from pests that may cause diseases for sugarcane plants [4]. More than 100 species of pests can attack sugarcane, mainly insect species [5]. Stem borers are pests that frequently attack sugarcane, resulting in a decrease in sugar production by 52 to 73% [6]. In several areas, stem borers are the main pests of sugarcane and research currently focused on dealing with these pests. Stem borers have attacked several countries in general, such as Indonesia, America, Australia, Brazil, Africa etc [7]. Several species grouped as stem borers are Chilo sacchariphagus, Chilo infuscatellus and Diatraea saccharalis [8]. The life cycle of the borers consists of four stages: egg, larva, pupa, and adult. The egg stage lasts until the egg becomes a pupa which grows in the body of the plant, while at the adult stage the borer lives freely [9]. Basically, plants naturally have innate defense systems, one of which being the Bowman-Birk inhibition pathway [10, 11]. The Bowman-Birk peptidase inhibition was first isolated and characterized in soybean seeds and later found in the Poaceae group, such as in sugarcane (SacBBI, Saccharum officinarum Bowman-Birk Inhibitor) [12]. This protein is related to regulation of endogenous seed peptidase, storage of sulfur amino acids, and plant defenses against pathogens and insects [12].

A control technique that has not been developed optimally but has good prospects with regard to overcome stem borers in sugarcane while increasing production is to develop plant cultivation starting from creating resistant and good quality plant varieties [13]. Plant breeding efforts are inseparable from the development of molecular markers where the use of the markers is adjusted to the objectives of the study so purposes of the present study were to screen and characterize molecular markers of resistance to stem borers while simultaneously characterizing the resistance gene (SacBBI4) in 22 sugarcane cultivars cultivated in Indonesia.

2. Methods

2.1. Materials

Twenty-two sugarcane genomes were used in the present study, including 4 cultivars from the sugar company PT. Madu Baru Bantul, Yogyakarta (BZ 132, TLH 2, PSDK 923, VMC 76-16) and 18 other cultivars from the Indonesian Sweetener and Fiber Crops Research Institute, Malang, East Java (PS 41, PS 58, PS 384, PS 851, PS 862, PS 864, PS 865, PS 881, PS 891, PS 921, PS 951, PS 80.910, PS 80.1649, PSCO 902, PSBM 901, BL, Kentung and KK. The samples used were the 5th sugarcane leaves from the top of sugarcane plants with good stature (not too old or young). The primers used in the present study were those gene primers resulting from primer designs using the Primer3 application.

2.2. Primer designs

The primers were designed using the online application Primary3 versions 4.1 and 4.0 referring to SacBBI4 from the GenBank number: CA272687.1 of NCBI database based on the study of Medeiros et al. (2016). Upon obtaining the primer design in accordance with good primer criteria, the primers were subsequently ordered online at PT. Genetika Science.
2.3. DNA amplification and sequencing

DNA amplification was successfully carried out using the PCR (polymerase chain reaction) method. The composition of PCR buffer consisted of KAPA Extra HotStrart of 12.5 µL, specific primers of 2 µL (25 µM), including forward and reverse primers, 2 µL (20 ng/µL) of DNA template and distilled water of 8.5 µL. Furthermore, the amplification process took place in the PCR machine with the following settings: pre-denaturation at 94°C for 1 minute, denaturation at 94°C for 45 seconds, annealing at 54.5°C for 1 minute, elongation and post-elongation at 72°C for 1 minute and 15 seconds with repetition of 40 cycles.

2.4. Data analysis

The PCR-amplified SacBB14 was analyzed by regressing DNA bands from gel electrophoresis visualization using the Microsoft Excel application (quantitative). Furthermore, the sequencing results were analyzed using the Genstudio application, the alignment process was performed using the Mesquito application, and the genetic distance calculation and phylogenetic tree reconstruction were performed using the Mega application.

3. Results and discussion

3.1. Primer design

The primers in the present study were designed using the application Primary3 versions 4.1 and 4.0. The Primer3 version 4.1 selected 3 primers, while the version 4.0 selected 6 primers. The 9 primer were selected on the basis of good primer criteria indicators. Furthermore, 3 primer designs with good primer criteria were tested again for their ability in the amplification process of SacBB14.

There are several criteria for a good primer, including primer length, primer melting temperature (Tm), primer annealing temperature (Ta), primer melting temperature difference (ΔTm), GC contained, GC clamp, secondary structure, self-complementary (SC) and pair-complementary (PC), repeats & runs, specificity and product length [14]. The primer designs selected by the Primer3 versions 4.0 or 4.1 (tables 1 and 2) were in the range of 18 to 22 bases. In accordance with the theory, [15] used the 18–22 base limit.

| No. | Primer | Start | Len | Tm | GC% | Any th | 3’ th | Pro Size | Pair any th compl | Pair 3’ th compl |
|-----|--------|-------|-----|----|-----|--------|-------|----------|------------------|-----------------|
| 1.  | Left Primer | 6     | 20  | 58.25 | 55.00 | 0.00 | 0.00 | 522 | 0.00 | 5.93 |
|     | Right Primer | 527   | 20  | 59.31 | 50.00 | 0.00 | 0.00 | |
| 2.  | Left Primer | 3     | 20  | 57.51 | 50.00 | 0.00 | 0.00 | 518 | 0.00 | 0.00 |
|     | Right Primer | 520   | 20  | 58.30 | 50.00 | 13.15 | 0.00 | |
| 3.  | Left Primer | 7     | 22  | 57.21 | 45.45 | 0.00 | 0.00 | 524 | 6.61 | 0.00 |
|     | Right Primer | 530   | 20  | 57.76 | 50.00 | 0.00 | 0.00 | |

Table 1. Primer designs produced by the Primary3 version 4.1 application.
Table 2. Primer designs produced by the Primary3 version 4.0 application.

| No. | Primer | Start | Len | Tm  | GC% | Any th | 3’ th | Pro Size | Pair any th compl | Pair 3’ th compl |
|-----|--------|-------|-----|-----|-----|--------|-------|----------|------------------|-----------------|
| 1.  | Left Primer | 1     | 22  | 58.89 | 50.00 | 4.00  | 0.00  | 570      | 5.00             | 0.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 0.00  |          |                  |                 |
| 2.  | Left Primer | 4     | 21  | 59.24 | 57.14 | 2.00  | 0.00  | 567      | 5.00             | 1.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 2.00  |          |                  |                 |
| 3.  | Left Primer | 20    | 21  | 58.73 | 47.62 | 4.00  | 2.00  | 551      | 4.00             | 1.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 2.00  |          |                  |                 |
| 4.  | Left Primer | 6     | 21  | 58.68 | 52.38 | 2.00  | 0.00  | 565      | 5.00             | 0.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 2.00  |          |                  |                 |
| 5.  | Left Primer | 21    | 20  | 57.67 | 50.00 | 4.00  | 2.00  | 550      | 4.00             | 1.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 2.00  |          |                  |                 |
| 6.  | Left Primer | 21    | 21  | 59.61 | 47.62 | 4.00  | 3.00  | 550      | 4.00             | 1.00            |
|     | Right Primer | 570   | 22  | 57.85 | 40.91 | 3.00  | 2.00  |          |                  |                 |

The next criterion is the primer melting temperature (Tm). According to the primer design guide of the Hopkins Laboratory, the Tm value of the two primer pairs cannot be more than 1 to 2ºC. Tables 1 and 2 show that the ΔTm of the Primer3 output is in the range of Tm values as recommended by the Hopkins Laboratory where the 9 primer designs selected have values ranging from 0.18 to 1.76ºC. With regard to the primer annealing temperature (Ta), the general rule followed by most primer design programs is to use percent G and C bases of 40% to 60% [16]. Tables 1 and 2 show that the 9 primer designs selected have GC contents in range of 40.91 to 55%, which are consistent with those recommended by [16]. The criteria of primer specificity for the primer designs selected in the present study have been fulfilled it since the gene source or reference for primer designs was the specific gene, SacBBI4. Thus, the primer designs selected as offered by the Primer3 would be adjusted to the referred target gene. The subsequent criterion is the distance between the 5’ ends of both primers, known as amplicon or product length. In general, the product length used is <2000 bases. This distance is considered adequate for the amplification process on the template [14]. With regard to the last criterion, namely product size, the primers selected in the present study were also appropriate since they were <2000 bases, or more precisely ranging from 518 to 570 bp. The product size produced was also adjusted to the SacBBI4 length of 596 bp.

Table 3 shows the selected final primers from sorting the output of the Primer3 versions 4.1 and 4.0 based on the criteria of a good primer design as described above. Determination of the three
primer designs selected for subsequent use as molecular markers in the PCR amplification of \textit{SacBBI4} was also confirmed by the laboratory optimization results. The primer designs produced in the present study are new ones that have never been studied before; thus, the designation for primer designs 1, 2 and 3 is P-SacBBI4-A, P-SacBBI4-B and P-SacBBI4-C, respectively.

| No | Primer       | Start | Len | Tm   | GC% | Any th | 3’ th | Pro Size | Pair any th | Pair 3’ th |
|----|--------------|-------|-----|------|-----|--------|-------|----------|-------------|-------------|
| 1. | Left Primer  | 1     | 22  | 58.89| 50.00| 4.00   | 0.00  | 570      | 5.00        | 0.00        |
|    | Right Primer | 570   | 22  | 57.85| 40.91| 3.00   | 0.00  | 517      | 0.00        | 0.00        |
| 2. | Left Primer  | 4     | 21  | 59.24| 57.14| 2.00   | 0.00  | 520      | 13.15       | 0.00        |
|    | Right Primer | 520   | 20  | 58.30| 50.00| 13.15  | 0.00  | 522      | 5.00        | 0.00        |
| 3. | Left Primer  | 6     | 21  | 58.68| 52.38| 2.00   | 0.00  | 527      | 5.00        | 0.00        |
|    | Right Primer | 527   | 22  | 59.31| 50.00| 0.00   | 0.00  |          |             |             |

3.2. \textit{PCR} amplification of \textit{SacBBI4}

The electrophoregram of \textit{PCR}-amplified \textit{SacBBI4} using the primer P-SacBBI4-A on the 22 sugarcane cultivars under study shows that all samples indicate DNA bands in the visualization results ‘figure 1’. The primer P-SacBBI4-A was able to amplify the sequence of \textit{SacBBI4} of 729 bp, while the primer P-SacBBI4-B was able to amplify the sequence of \textit{SacBBI4} of 624 bp and the primer P-SacBBI4-C was capable of amplifying the sequence of \textit{SacBBI4} of 671 bp.

Additionally, visualization of the \textit{PCR}-amplified \textit{SacBBI4} using the primer P-SacBBI4-A also shows a DNA band of 1154 bp. Furthermore, the electrophoregram of the primer P-SacBBI4-C shows DNA bands of 533 bp and 1190 bp. The present study found three bands of a size beyond the target gene, namely 1154 bp, 533 bp and 1190 bp. The appearance of those DNA bands is thought to occur because the gene sequences amplified by the primer bind to similar sequences but in other regions of the sugarcane genomes.

Figure 2 shows that the cultivars PS 41, PS 58, PS 384, PS 851, PS 862, PS 864, PS 865, PS 881, PS 882, PS 891, PS 921, PS 951, PS 80910, PS 801649, PSBO 982, PSBM 901, PSJT 941, BL (Bulu Lawang), Kentung, KK (Kidang Kencana), BZ 132, TLH 2, PSDK 923, and VMC 76-16 are detected as having \textit{GAPDH} as marked by the appearance of a DNA band of 402 bp on electrophoresis results based on regression calculations and confirmed in journal references via the accession number at NCBI. In order to verify that the DNA band appearing on the visualization results was the target gene of the sugarcane genome used, an internal control was required. The present study used \textit{GAPDH} as the internal control, which was also amplified.

3.3 Analysis of \textit{SacBBI4} sequence

The next process was the sequencing of \textit{SacBBI4} of the cultivar Kidang Kencana as a representative of cultivars based on the optimization results, which showed DNA bands in the gel electrophoresis with good visualization results. The sequence of \textit{SacBBI4} was first analyzed using the Genstudio application. This application provides information on the nucleotide sequence length of each sample. The sequencing results of the amplified \textit{SacBBI4} samples using the primers P-SacBBI4-A, P-
SacBBI4-B and P-SacBBI4-C produced nucleotide sequences of 729 bp, 624 bp, and 671 bp, respectively. The length of nucleotide sequences was obtained by regression calculations and the values obtained were in accordance with the visualized gel electrophoresis of amplification products.

![Figure 1](image.png)

**Figure 1.** Electrophoregram of PCR products with the target gene *SacBBI4* in 22 sugarcane cultivars. M: 100-bp Marker, 1: PS 41, 2: PS 58, 3: PS 384, 4: PS 851, 5: PS 862, 6: PS 864, 7: PS 865, 8: P 881, 9: PS 891, 10: PS 921.11: PS 951, 12: PS 80.910, 13: PS 80.1649, 14: PSCO 902, 15: PSBM 901, 16: BL, 17: Kentung, 18: Kidang Kencana, 19: BZ 132, 20: TLH 2, 21: PSDK 923, 22: VMC 76-16 using (A) P-SacBBI4-A primer, (B) P-SacBBI4-B primer, (C) P-SacBBI4-C primer.

The next process was the alignment of all nucleotide sequences compared, including 3 nucleotide sequences of *SacBBI4* using the primers P-SacBBI4-A, P-SacBBI4-B and P-SacBBI4-C, hereinafter referred to as SeqSacBBI 1, 2, and 3. The other ten sequences were the BLAST-produced analog genes, hereinafter referred to as EU725407, EU725429.1, AF396273.1, EU725407.1, AY525558.1, AY525553.1, AY549635.1, AY525662.1, XM021457487.1 and XM004967999.2, according to the accession number of each nucleotide sequence. One nucleotide sequence derived from a reference journal which was the source during the primer design and then was known as CA272687.1
(according to the accession number). Therefore, the total number of nucleotide sequences to be compared was 14 sequences as shown in figure 3.

**Figure 2.** Electrophoregram of PCR products using the target gene *GAPDH* in 22 sugarcane cultivars. M: 100-bp marker, 1: PS 41, 2: PS 58, 3: PS 384, 4: PS 851, 5: PS 862, 6: PS 864, 7: PS 865, 8: PS 881, 9: PS 891, 10: PS 921.11: PS 951, 12: PS 80.910, 13: PS 80.1649, 14: PSCO 902, 15: PSBM 901, 16: BL, 17: Kentung, 18: Kidang Kencana, 19: BZ 132, 20: TLH 2, 21: PSDK 923, 22: VMC 76-16.

**Figure 3.** Neighbor-joining tree of *SacBBI4* of the sugarcane cultivar Kidang Kencana with analog genes from the NCBI data mining and reference genes from journals in the forms of cladogram (A) and real tree (B). The numbers on each node indicate the bootstrap values from 1000 replications (x10\(^1\)).

The phylogenetic tree as shown by figure 3 is in general divided into two large clusters. The first cluster consists of EU725407, EU725429.1, AF396273.1, EU725432.1, AY525558.1, AY525553.1,
AY549635.1, and AY525662.1, while the second cluster consists of SEQSacBBI1, SEQSacBBI2, SEQSacBBI3, CA272687.1, XM021457487.1, and XM004967999.2. The division shows that the clustering is based on the genes of each sequence in which the first cluster consists of WIP1 gene sequences, while the second cluster is the BBI cluster. The first cluster of the phylogenetic tree consists of a group of genera Zea comprising the species *Zea mays* subsp. parviqlumis isolate sn9a (EU725432.1), isolate snf6c (EU725429.1), isolate a12L (EU725407); *Zea perennis* (AY549635.1); *Zea mays* clone wipZmays6b (AF396273.1); *Zea diploperennis* strain Ames21884 (AY525558.1), strain 10003 b (AY525553.1) and strain 10003 (AY525552.1). The second cluster consists of 3 samples of sequencing results in the present study, including SEQSacBBI1, SEQSacBBI2, SEQSacBBI3 derived from sugarcane (*Saccharum officinarum* L.): *Saccharum hybrid* (CA272687.1); *Sorghum bicolor* (XM021457487.1) and *Setaria italica* (XM004967999.2).

WIP1 or serine wounding-induced proteinase inhibitor and BBI or Bowman-Birk Inhibitor are 2 gene families of the gene group of proteinase inhibitors (PIs). The proteinase inhibitor group is genes that play a role in plants’ response to a biotic stress of insect attacks. WIP1 and BBI have the same role. This explains the appearances of both WIP1 and BBI during the BLAST on the online NCBI application, which is because both have the same function [17].

4. Conclusion

Three primer (P-SacBBI4-A, P-SacBBI4-B and P-SacBBI4-C) were produced and successfully amplified *SacBBI4*, which was confirmed by the qualitative testing of gel electrophoresis with Kidang Kencana (KK) sample. The primer P-SacBBI4-A successfully amplified *SacBBI4* at 729 bp, the primer P-SacBBI4-B at 624 bp, and the primer P-SacBBI4-C succeeded in amplifying the target gene at 671 bp. The same values were also acquired when analyzing the sequence of *SacBBI4* on the Genstudio application. The phylogenetic tree shows two large clusters formed. The first is the WIP1 coding cluster, while the second is the BBI coding cluster. WIP1 and BBI have the same role in plant defenses against insect attacks. Based on the phylogenetic tree and the genetic distance produced, the primer P-SacBBI4-C is the primer design closest to the target gene *SacBBI4* on the NCBI data.

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