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Molecular Analysis of Cry1Ab-Cry1Ac Gene Fusion in Transgenic Sugarcane Resistant to Shoot Borer *Scircophaga excerptalis* (Lepidoptera: Pyralidae) and Stem Borer *Chilo sacchariphagus* (Lepidoptera: Pyralidae)

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ABSTRACT

Sugarcane (*Saccharum officinarum*) is a plant with high economic value because it can produce sugar. Shoot borer *Scircophaga excerptalis* (Lepidoptera: Pyralidae) and stem borer *Chilo sacchariphagus* (Lepidoptera: Pyralidae) attacks are one of the issues that limit sugarcane productivity. Establishing transgenic sugarcane is one of the most efficient ways to prevent borer damage. Previously, Cry1Ab and Cry1Ac genes were successfully used to create transgenic sugarcane plants. This study aimed to detect the presence of transgenes and analyze the expression level of the Cry1Ab-Cry1Ac gene fusion in transgenic sugarcane using RT-PCR. The methods used in this study are transgene detection with PCR and gene expression analysis in normalized expression ($2^{-\Delta\Delta Cq}$) with RT-PCR. The Cry1Ab-Cry1Ac gene has been integrated into all lines with varied expression levels. In 311 GV and 333 GV lines, the Cry1Ab-Cry1Ac gene was expressed in the leaf but not in the stem. Shoot and stem borer attack percentage values showed that all lines were lower than the control, with 222 EH as the lowest and 311 GV as the highest. Leaf and stem borer attack levels were compared to gene expression values of Cry1Ab-Cry1Ac. Results may indicate that the 222 EH line was resistant, but the 311 GV and 333 GV lines were not.

1. Introduction

Sugarcane (*Saccharum officinarum*) is a high-value crop because it contains sugar in its stems. Sugarcane productivity in Indonesia is around 5512 kg/ha, which is significantly lower than that in Thailand, where output reached 7610 kg/ha in 2017. This disparity has led to Indonesia becoming one of the world's largest sugar importers, with 4.48 million tonnes (Ditjenbun 2018). Pest and disease attacks, particularly borer infestations, are major contributors to this low sugar productivity, causing significant financial losses. Sugarcane borers are classified into several types; two of the three major species in Indonesia are shoot borer *Scircophaga excerptalis* (Lepidoptera: Pyralidae) and stem borer *Chilo sacchariphagus* (Lepidoptera: Pyralidae), which caused damage around 15.8% of sugarcane fields (Goebel *et al.* 2014).

While conventional breeding strategies can be effective in developing sugarcane pest resistance, they are time-consuming due to the complexity of sugarcane genomes. Bt gene from Bacillus thuringiensis offers a promising solution. When used to develop pest resistance, this gene proves to be both effective and cost-efficient. During sporulation, Bt bacteria make a parasporal crystal that is toxic to the larvae of many insect orders, including Diptera, Lepidoptera, and Coleoptera (Dessoky *et al.* 2020). Cry1Ab and Cry1Ac are genes utilized in the development of borer-resistant sugarcane cultivars (Thorat *et al.* 2017).

The Cry1Ab gene, obtained from the bacterium *Bacillus thuringiensis*, was initially employed in tobacco and sugarcane to provide resistance using

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a CaMV35S promoter. However, the expression was extremely low (Vaeck *et al.* 1987; Weng *et al.* 2011). Following field trials, investigations utilizing transgenic sugarcane with the altered Cry1Ac gene showed that 62% of transgenic plants were pest-resistant, indicating a relatively high resistance value (Gao *et al.* 2016). In the past, hemp, rice, and beans were genetically modified to limit borer infestations by introducing the Cry1Ab-Cry1Ac fusion gene (Qiu *et al.* 2010; Gu *et al.* 2014; Majumder *et al.* 2018). According to the study, this gene can effectively lessen the effects of borer attacks (Majumder *et al.* 2018). Before this study, no research had been done on the association between the rates of the shoot and stem borer attacks and the expression value of sugarcane transgenes containing the Cry1Ab-Cry1Ac fusion gene. The objective of this study is not only to identify the Cry1Ab-Cry1Ac transgenes resulting from the genetic transformation but also to evaluate the rates of shoot and stem borer infestation in leaf and stem, to evaluate the transgene expression levels in genetically modified sugarcane, and to observe and establish a correlation between the transgene expression levels and the rates of shoot and stem borer infestation in leaf and stem samples. This research holds the potential to significantly improve sugarcane productivity and reduce economic losses caused by pest infestations.

2. Materials and Methods

2.1. Plant Materials

Three transgenic and one non-transgenic sugarcane samples of variation Bulu Lawang (BL) were collected from the greenhouse of BB Biogen's limited test facility (RK FUT) in Bogor. The genetic transformation experiments previously conducted by Sukmadjaja *et al.* (2021) and the plasmid binary pCambia5300 containing the *RuBisCO* (rbcS) gene promoter controls the Cry1Ab-Cry1Ac gene fusion in its multi-cloning site were applied to developing transgenic sugarcane lines. The three transgenic sugarcane lines used in this study were 311 GV, 333 GV, and 222 EH. The Agrobacterium strains used for plant transformation were EHA105 and GV3101. As a result, samples were identified with the initials EH and GV. For this study, samples were taken from five-month-old sugarcane. A sample of about 2-3 cm long was taken from a young leaf found at the curled section of the leaf. Before the treatment, the leaf and stem samples were placed in a sealed plastic container and frozen at -20°C.

2.2. Identification of Cry1Ab/Ac Gene in Transgenic Lines

DNA was isolated from sugarcane leaf and stem samples using the CTAB method that was modified from Doyle and Doyle (1987) (Vaze *et al.* (2010). DNA obtained was used as a template to identify the Cry1Ab-Cry1Ac gene using the PCR. The pCambia5300 plasmid containing the Cry1Ab-Cry1Ac gene was used as a positive control for PCR using Cry1Ab-Cry1Ac 139 forward (TCGGTATTGTTCTCTATCTC) and reverse (GCAATAGCTCGGTGTCCATT) primers. The PCR running program starts with a pre-denaturation procedure at 94°C for 3 minutes. The following stages were repeated 35 times, beginning with denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The final stage is the post-extension procedure at 72°C for 5 minutes, followed by the temperature reduction to 15°C.

The isolated DNA is quantitatively analyzed to ensure its purity from contamination. A NanoDrop™ 2000c Spectrophotometer (Thermo Scientific) was used for quantitative testing at 230, 260, and 280 nm wavelengths. The amplified DNA was observed by electrophoresis with 1% agarose and 1x TAE buffer for 45 minutes at 50 volts. Agar was then stained with ethidium bromide solution, and the electrophoresis results were visualized with Gel-Doc™.

2.3. RNA Extraction and cDNA Synthesis of Sugarcane Leaves and Stems

RNA was isolated using the Total RNA Mini Kit for Plant (Geneaid 2017). The leaf and stem samples weighed around 50 mg each. The total RNA extracted is subsequently converted into cDNA using the iScript™ cDNA Synthesis Kit. The sample was then loaded into a T100 Thermal Cycler (Bio-Rad) with the PCR running software set to the iScript™ cDNA Synthesis Kit protocol.

2.4. Cry1Ab-Cry1Ac Gene Expression Using RT-PCR

The expression of the Cry1Ab-Cry1Ac gene in each sugarcane leaf and stem sample was measured using a modified RT-PCR method (Ganguly *et al.* 2014). GAPDH was used as a reference gene for gene expression comparisons. Master mix solution was made from a mixture of 5 µL of Evagreen, 0.2 µL of

each primer, one µL of cDNA, and enough NFW to a total volume of 10 µL, then put it into an 8-strip PCR tube and then closed with a stripped cap. The RT-PCR program was used to run the samples, which included pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 62°C for 30 seconds, dissociation at 65°C for 5 seconds, and a final temperature of 95°C for 5 seconds. These stages were repeated 45 times from denaturation to annealing.

2.5. Leaves and Stem Borer Test

Three number (lines) of transgenic sugarcane and one non-transgenic control plant (BL) were tested for their resistance to stem borer in the screen house. Five plants per number were planted and grown. Each plant produced three to five shoots, and we chose a somewhat similar size of three shoots for the experiment. The attack data for the shoot borer was gathered using the bioassay method (Dessoky *et al.* 2020). Infestation of stem borer on these genetic materials (transgenic and non-transgenic sugarcane) was conducted following the method applied by Sutejo (2008). Ten of the first instar larvae of the stem borer were applied onto the corner of the leaf sheath, and observation was carried out every week within three weeks to measure the level of borer attack. The first instar larvae actively bore into the leaf's epidermal tissue while still inside the leaf, causing crushing wounds to appear when the leaves opened. From a distance, the wound seems like white dots (Sutejo 2008). The non-transgenic plants were destroyed by the third week of observation because the larvae were at their most active developmental stage. Shoot borer observations were carried out by observing the number of plants that show symptoms of leaf cracking and dieback attacked by shoot borers. The percentage of sugarcane stem borer attacks was assessed using the formula referred from Sujak *et al.* (2021):

$$P = \frac{\sum \text{infested plant}}{\sum \text{Total plant}} \times 100\%$$

2.6. Data Analysis

The Cq value obtained after the RT-PCR process is then utilized to calculate normalized expression using the Livak method (Livak and Schmittgen 2001). The percentage value of sugarcane stem borer assaults was descriptively examined using the formula provided. The percentage of attacks on transgenic sugarcane plants was then compared to other transgenic samples.

3. Results

3.1. Confirmation of Cry1Ab-Cry1Ac Gene in Sugarcane Leaves and Stems

Before employing the PCR method to confirm the Cry1Ab-Cry1Ac gene, the isolation results were quantitatively analyzed to verify the amount of DNA purity from impurities. Table 1 shows each sample concentration and absorbance ratio using a nanodrop. The seven sugarcane DNA samples and the pCambia5300 plasmid containing Cry1Ab-Cry1Ac gene fusion as a positive control were then qualitatively evaluated with the Cry1Ab-Cry1Ac F and R primers (producing 139 base pairs in length). The DNA bands of sugarcane samples were visually observed using the electrophoresis technique, and the molecular size was compared to the 100-base pair ladder (Vivantis) (Figure 1). According to Figure 1, both the positive and negative controls are appropriate, and all samples display bands.

3.2. Preparation of Sugarcane cDNA for RT-PCR

The Total RNA Mini Kit for Plant (GeneAid) was used to isolate RNA from sugarcane leaf and stem samples, following the recommended protocol for the kit. The concentration and purity of sugarcane stem RNA were quantitatively assessed using a nanodrop spectrophotometer at wavelengths 260 and 280 (Table 2). Messenger RNA (mRNA) constitutes 1–5% of total RNA and serves as the template for cDNA synthesis. The ribosome subsequently used an mRNA template to create an amino acid sequence. The nucleotide

Table 1. DNA concentration and purity of transgenic sugarcane leaves and stems

Sample name	Concentration (ng/µL)		$A_{260}/_{A280}$		$A_{260}/_{A230}$	
	leaf	stem	leaf	stem	leaf	stem
311 GV	2734.90	186.40	1.85	1.57	1.97	1.90
333 GV	1099.80	1135.70	1.92	1.85	0.86	2.74
222 EH	6373.10	2471.30	1.80	1.85	1.94	2.01

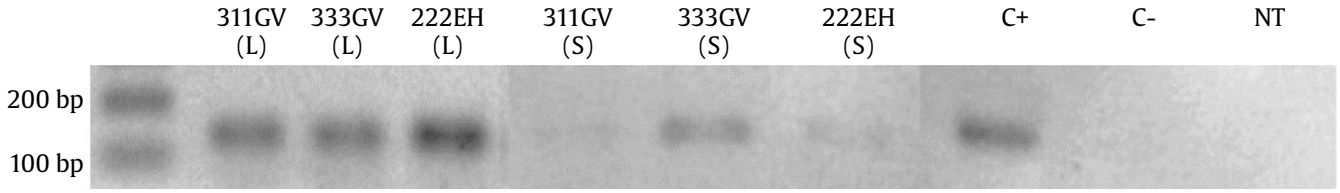

Figure 1. Electropherogram of transgenic sugarcane leaves, stems, positive control (plasmid pCambia5300 with Cry1Ab-Cry1Ac gene 139 bp), and negative controls (template without sample and non-transgenic sugarcane) DNA with 100 base pairs ladder (Vivantis); (L): leaf, (S): stem

Table 2. RNA and cDNA concentration and purity of transgenic sugarcane leaves and stems

Sample name	RNA conc. (ng/µL)		$A_{260}/_{A280}$		cDNA conc. (ng/µL)		$A_{260}/_{A280}$		$A_{260}/_{A230}$	
	leaf	stem	leaf	stem	leaf	stem	leaf	stem	leaf	stem
311 GV	163	47.00	1.90	2.16	1275	512.50	1.82	1.80	2.20	2.05
333 GV	117	63.40	1.97	2.07	1099	623.50	1.82	1.84	2.22	2.19
222 EH	99.30	67.70	2.01	2.10	1035	1014.00	1.83	1.83	2.23	2.23

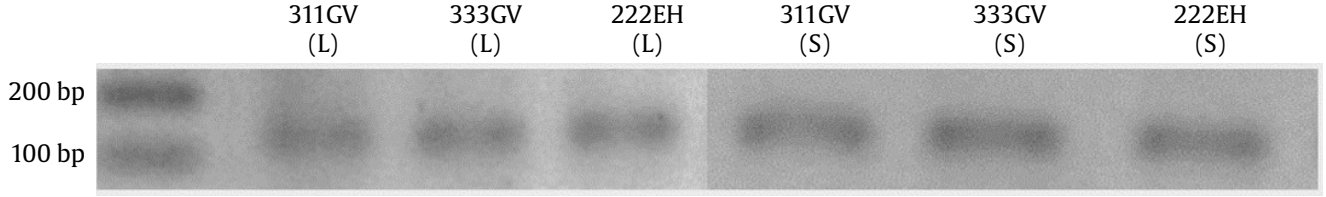

Figure 2. PCR analysis of transgenic sugarcane leaves and stems cDNA with Cry1Ab-Cry1Ac gene 139 base pairs with 100 base pairs ladder; (L): leaf, (S): stem

sequence of mRNA is complementary to that of cDNA. Sugarcane shoot and stem complementary DNA (cDNA) were synthesized from RNA samples using the iScript kit, and the results were quantitatively and qualitatively assessed (Table 2). Figure 2 depicts the cDNA electropherogram of sugarcane leaves and stems obtained by the PCR procedure as part of the cDNA qualitative test. According to Figure 2, all samples display bands in the targeted base pairs.

3.3. Analysis of Cry1Ab/Ac Gene Expression by RT-PCR

Cry1Ab-Cry1Ac's gene expression levels were determined in three (repetitions) transgenic sugarcane leaf and stem samples using the CFX96 Real-Time System, and the results were analyzed using Bio-Rad CFX Maestro software. Table 3 displays the Cq values for the reference gene (GAPDH) and the target gene (Cry1Ab-Cry1Ac). Gene expression levels will be shown in $2^{-\Delta\Delta Cq}$ units (normalized expression) (Livak and Schmittgen 2001). Figure 3 displays the expression values of transgenic sugarcane samples and non-transgenic control. Based on Figure 3, 311 GV and 333 GV had normalized expression values higher than NT, while 222 EH was lower than NT. The only stem sample with a normalized expression value higher than NT is 222 EH.

Table 3. Cry1Ab-Cry1Ac gene expression in leaf and stem

Sample name	Cry1Ab-Cry1Ac (Cq)		GAPDH (Cq)	
	leaf	stem	leaf	stem
311 GV	36.78±1.78	36.26±0.33	44.25	41.86
333 GV	34.78±1.61	32.92±0.16	44.03	37.06
222 EH	38.51±2.42	29.55±0.77	40.47	35.91
NT	37.33±0.93	30.96±2.92	44.01	36.05

*GV: plants transformed with *A. tumefaciens* strain GV3101; EH: plants transformed with *A. tumefaciens* strain EHA105; NT: non-transgenic plants

3.4. Percentage of Leaf and Stem Borer Attacks

The percentage level of shoot borer attacks on several transgenic sugarcane samples previously revealed that the three samples used in this study had the lowest attack rates (Table 4). These findings form

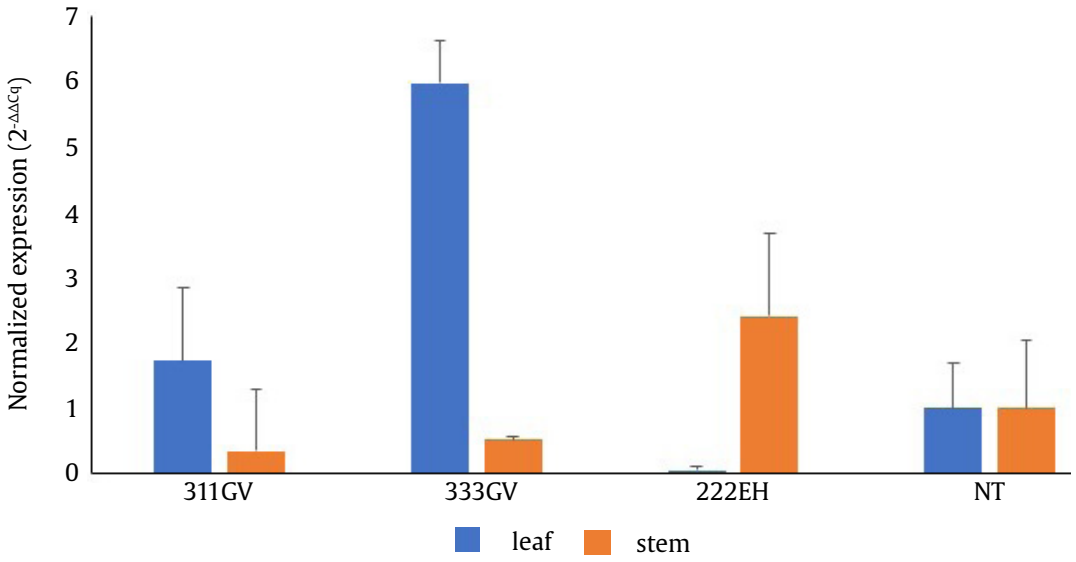

Figure 3. Expression of Cry1Ab-Cry1Ac gene in three transgenic sugarcane leaves and stems in normalized expression ($2^{-\Delta\Delta Cq}$) to reference gene GAPDH with three repetitions. Bar = standard error. *GV: plants transformed with *A. tumefaciens* strain GV3101; EH: plants transformed with *A. tumefaciens* strain EHA105; NT: non-transgenic plants

Table 4. Percentage of transgenic and non-transgenic sugarcanes attacked by shoot borer

Sample name	Number of plants (replications)	Damaged shoots (%)
311 GV	5	20.00
333 GV	7	28.57
222 EH	9	22.22
NT	6	100.00

*GV: plants transformed with *A. tumefaciens* strain GV3101; EH: plants transformed with *A. tumefaciens* strain EHA105; NT: non-transgenic plants

the foundation for further detailed measurements of stem borer attacks. In their early stages, striped stem borer attacks are distinguished by irregular elongated transparent patches on the leaves. The caterpillars that attacked entered through the sugarcane stem's midribs. Figure 4 shows the percentage of stem borer attacks transgenic and control sugarcane samples over the three-weeks period. The data show that 222 EH had the lowest percentage of attacks, while sugarcane 311 GV had the highest rate.

4. Discussion

Bt (*Bacillus thuringiensis*) produces cry protein during sporulation, which might dissolve in the larvae's digestive tract (Chattopadhyay and Banerjee 2018). Cry toxin will be activated by proteolytic enzymes in the insect's digestive fluid, which has a pH of 8-10. Some peritrophic membranes then bind to specific receptors on the intestinal epithelial cell membrane, forming holes. Toxin-penetrated cells enlarge until they lyse and detach from the basal membrane of the intestinal epithelium. The alkaline fluid from the intestinal cells then combines in the insect's body cavity, causing the pH of the hemolymph to rise. This technique causes paralysis, which kills the insect (Abbas 2018). The next generation of Bt transgenic plants is designed to prevent pest adaptability by combining two or more Bt toxins with diverse modes of action and expression levels (Carriere *et al.* 2015). The Cry1Ab-Cry1Ac fusion gene previously introduced into *Corchorus* sp. resulted in considerable protein production in the leaves and stems (Majumder *et al.* 2018). In rice, the expression of the Cry1Ab-Cry1Ac gene increases in the following order: leaves, stems, and roots (Wang *et al.* 2016). Feed safety tests on monkeys proved the safety of the Cry1Ab-Cry1Ac protein (Mao *et al.* 2016).

Figure 1 depicts Cry1Ab-Cry1Ac fusion gene confirmation in three lines of transgenic sugarcane leaf and stem samples successfully integrated with a borer-resistant gene. The PCR analysis revealed band visibility in three samples and plasmid as a positive control in the expected base pair range of 100-200 bp ladder bands. This band shows a positive outcome when comparing the positive control plasmid to two negative controls (non-transgenic and no template). Three samples confirmed to contain the

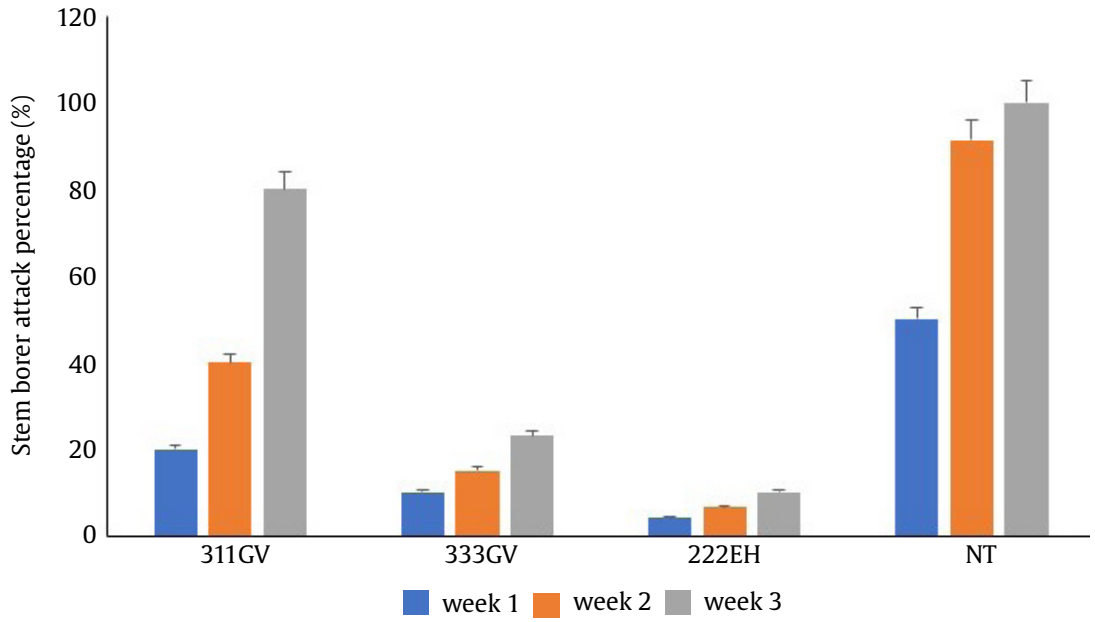

Figure 4. Percentage of transgenic and non-transgenic sugarcanes attacked by stem borer in five plants of each number. Bar = standard error. *GV: plants transformed with *A. tumefaciens* strain GV3101; EH: plants transformed with *A. tumefaciens* strain EHA105; NT: non-transgenic plants

Cry1Ab-Cry1Ac gene were then used in the RT-PCR method to evaluate the expression level. The DNA is considered excellent and free of impurities or contaminants when the A_{260}/A_{280} ratio is between 1.8 and 2 (Novita 2013; Abdelhai *et al.* 2019). A260/A230 absorbance ratios between 2.0 and 2.2 are generally free of contaminants; however, values outside this range suggest the presence of contaminants such as protein, EDTA, polysaccharides, phenols, and others (Usman *et al.* 2014). The three samples examined met the acceptable value of A_{260}/A_{280}, except the 311 GV stem sample, which was categorized as low. This data suggests that sample 311 GV contains components like protein or phenol. The ratio of A_{260}/A_{230} in three samples suggests that sample 333 GV contains impurities in the leaf and stem samples. Considering samples of the same purity, samples with a lower DNA concentration will have low A_{260}/A_{230} due to absorption by salt at an absorbance of 230 nm (Lucena-Aguilar *et al.* 2016).

Figure 2 demonstrates that all sugarcane RNA samples tested were free of contaminants and can proceed to the next stage because their absorbance ratio values were close to 2.0, the recommended range (Ahlberg *et al.* 2021). All RNA samples are subsequently used in the cDNA synthesis process. The absorbance ratio of 260/280 cDNA is near 1.8, indicating that it is devoid of contaminants (Hassan *et al.* 2015). The A_{260}/A_{230} cDNA was likewise free of contaminants because all samples fell within the acceptable range (2.0-2.2) (Usman *et al.* 2014). The qualitative test results show that the bands generated in all samples are the expected size, around 139 base pairs. This is evidenced by the three samples' band positions, which are below the 200 bp ladder band and above the 100 bp ladder band. Quantitative and qualitative tests indicated that all synthesized transgenic sugarcane cDNA samples were of good quality, making them eligible for use in the RT-PCR method to determine the expression level of the Cry1Ab/Ac gene.

The quantification cycle (Cq) denotes the number of cycles required to produce a given fluorescence response in each sample. The Cq value is used as data to calculate gene expression levels based on RT-PCR results. A low Cq value indicates a significant amount of amplification product in the sample, which increases the amount of fluorescence (Pabinger *et al.* 2014). The Livak approach was used to calculate the expression level of the Cry1Ab-Cry1Ac gene based on the Cq value of the gene of interest and the Cq value of the reference gene, GAPDH. GAPDH is a suggested reference gene for gene expression investigations due to its excellent stability across tissues and genotypes (Crystian *et al.* 2018). Gene expression levels will be quantified

in $2^{-\Delta\Delta Cq}$ units, representing normalized expression (Livak and Schmittgen 2001).

The Cry1Ab-Cry1Ac gene is expressed in 311 and 333 GV leaf samples and 222 EH stem samples (Figure 3). This is demonstrated by comparing the normalized expression value of NT. Normalized expression values in 311 GV and 333 GV are 1.72 and 6 times higher than non-transgenic control, respectively. However, only the 222 EH stem sample has a normalized expression value greater than NT, 2 times higher than the control. In this study, the *Rubisco* (RbcS) promoter was used to control the Cry1Ab-Cry1Ac gene. Previous research using the RbcS promoter to control the Cry1Ac gene in chickpeas demonstrated that leaf tissue produced more toxins than other tissues (Chakraborty *et al.* 2016). Studies comparing the RbcS promoter to other promoters (such as CaMV35S and Actin1) in rice and jute demonstrated that the Cry1Ab-Cry1Ac gene is expressed more in the leaves than in other plant tissues, like stems and roots (Jiang *et al.* 2016; Wang *et al.* 2016; Majumder *et al.* 2018). The Cry1Ab-Cry1Ac gene in the *Jatropha curcas* (jute) plant had expression values (normalized expression) ranging from 1 to 2. These findings show that all samples used exhibited expression levels higher than those found in non-transgenic plants (Gu *et al.* 2014). Variations in expression values are induced by randomly integrating transgenes into host genes, which may result in varied gene expressions in each plant line (Negawo *et al.* 2016).

Scirpophaga excerptalis, which causes shoot borer, significantly impacts sugar productivity because it destroys crops at all stages of growth (Geetha *et al.* 2018). Young shoot borer larvae penetrate via the central core of an unopened leaf spindle and prepare to build a pupal chamber in the apical area when they mature. Consequently, young branches die, and prominent axillary buds to form on growing plants. The symptoms of shoot borer infestation on the leaves include a line pattern and a bunchy top. The stem borer pest (*Chilo sacchariphagus*) is a major pest in most sugarcane-growing locations worldwide (Sanghera and Kumar 2018). This pest also drastically lowers food crop yields, including rice, sorghum, and corn (Vargas *et al.* 2015). Stem borer pests attack sugarcane plants at rates ranging from 23 to 36% (Rahman *et al.* 2013). The degree of attack on sugarcane shoots and roots by borer pests is relatively modest compared to the amount of attack in the stem. The life cycle of *C. saccariphagus* lasts 58-87 days. It begins with the egg phase, which develops into a 6-7-day larva. The imago is the final stage, lasting seven to ten days. The larval stage of pests poses the most significant harm to sugarcane due to its high levels of activity (Muliasari and Trilaksono 2020).

The implications of our research findings for genetically modified sugarcane resistance to pests are significant. The damage caused by shoot borers to sugarcane was quantified using the bioassay method. Three transgenic sugarcane lines demonstrated shoot borer damage values in the 20-28% range. At the same time, the non-transgenic plant was destroyed. These are the lowest percentage values reported for numerous samples tested in prior bioassays. Figure 4 further illustrates that all transgenic sugarcane samples had lower attack rates than controls up to the third week, suggesting that the Cry1Ab-Cry1Ac gene inserted into these three transgenic sugarcane lines may provide some resistance to leaf and stem borer pests. However, the expression values of 311 GV lines were not strong enough to be classified as resistant lines to stem borers. This is evidenced by a higher increase in the percentage of plants destroyed each week compared to other samples, although the increase was lower than that of the non-transgenic control (BL). Sample 222 EH has been certified as resistant to leaf and stem borer pests. This sample also had a higher expression value than the NT control. Sugarcane stem borer attack rates are categorized as moderate (<5), medium (5-10), and heavy (>10) (Sujak *et al.* 2021). A stem borer attack percentage of more than 10 indicates substantial damage; hence, 311 GV and 333 GV with attack percentages greater than 10 are termed non-resistant.

These research findings demonstrate the successful incorporation of the Cry1Ab-Cry1Ac gene fusion into the genome of three transgenic sugarcane lines, a significant step towards developing pest-resistant sugarcane. Results showed that one line (222 EH) has a higher Cry1Ab-Cry1Ac gene expression than the other two lines (311 GV and 333 GV), thus producing the resistance trait to both leaf and stem borer. However, further research is crucial. DNA sequencing analysis to determine the transgene (T-DNA) site in the genome and detect Cry1Ab-Cry1Ac protein in this particular line. It is a necessary step for releasing a transgenic new

variety, underscoring the importance of our future work.

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