C synthetic gene of $\text{CryIAb-CryIAc}$ fusion to generate resistant sugarcane to shoot or stem borer

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Abstract. The sugar yield lost caused by borer is about 10% or equal to 1000 kg of sugar/ha. There is no resistance trait to stem or shoot borer available in sugarcane germplasm. Genetic engineering by expressing of the $\text{CryIAb-CryIAc}$ gene fusion is efforts to develop resistant variety to borer. A synthetic gene which consists of $\text{Rubisco}$ gene promoter, chloroplast specific transit peptide (CTP) and the $\text{CryIAb-CryIAc}$ was designed and assembled for a total size of 4019bp. It was inserted into pU3775CE plasmid cloning, and then into pCAMBIA5300_Ubi-tNOS plasmid vector at HindIII and KpnI sites, producing of pCAMBIA5300_RbcS::CryIAb-CryIAc. This plasmid was transforming into $\text{Agrobacterium tumefaciens}$ and then transformed into Bulu Lawang (BL) sugarcane calli. Research aims were to identify lines produced from plant transformation molecularly and to evaluate their resistances against shoot borer. Methods applied were a DNA isolation and PCR using KAPA 2G ready mix and CryIAc-316F and CryIAc-316R primers, and plant bioassay with larva instar 1. Research progresses were 30 lines had been identified and proved containing of the $\text{cryIAc}$ gene. Preliminary results of bioassay showed that there was variation among 10 BL Cry lines on shoots (from healthy to wilt and dry) and also on shoot borer conditions.

Key words: $\text{CryIAb-CryIAc}$ gene fusion, Bulu Lawang. Resistance to shoot bore.

1. Introduction
There is a big gap between sugar consumption and sugar production in Indonesia from 2004 to 2016. A lackness of good quality variety was identified as one of the main problems cause this gap and a high yileding sugarcane ($\text{Saccharum officinarum}$ L.) variety was suggested to use [6]. Bulu lawang (BL) is a high yielding sugarcane variety and widely planted by farmer in Indonesia, as it has about 94.3 tons of yield per ha and 7.51% of sugar content, but it does not resistant to stem or shoot borers [5]. Insect attack is a major issue in sugarcane cultivation in all countries producing sugar from sugarcane, resulting in yield losses and sucrose content reductions. Among insects, shoot or top and stem or stalk borer cause serious yield losses in sugarcane worldwide. In 2016, it was reported sugarcane production reached about 318 721 tones and this level will increase if insect borers can be controlled and good quality of stumps are available [1].

$\text{Scirphophaga excerptalis}$ (Walker) is one of the most important lepidopteran pests attacking shoot part of sugarcane plants, whereas $\text{Chilo sacchariphagus}$ attacking of stem part of sugarcane plants. These may cause more than 10% losses in sugarcane yield worldwide, including in Indonesia [4]. Research in East Java indicated that about 14.5% of damage due to by these borers [4]. $\text{S. excerptalis}$, is Lepidopteran of Crambidae causes damage to sugarcane especially during early phase of the growth.
(1.5-2 months), commonly known as top or shoot borer, whereas *C. sacchariphagus* is lepidopteran of Pyralidae causes damage to crop at the later stage, known as stalks or stem borer. Larvas of the first one bore into the shoot and feed there. They cut off the growing point which position in the central whorl of leaves, and produces a symptom of wilt and dry of young leaves. The central dead shoot is also called "dead-heart" in which plants never grow further, but sprouts and side shoots are produced from the dormant buds. When it attacks after formation of canes, the borer attack does not produce "dead-hearts", and only affected to a few internodes. Nevertheless, there is a significantly reduction in cane yield and sugar contents [3] [4]. The second one attacks the stalk and clearly affected the quality of sugar due to increasing in fiber and reducing of water uptake [4].

Common approaches to solve insect-attack problems are the use of insecticides, cropping systems and sugarcane plant resistant variety. Selective insecticide such as carbofuran is recommended to use [8], but it is not effective enough to control the insect after the larva reach the plant base, bore into the shoot and feed there. Types of cropping system were also investigated to find out which one is effective to reduce the insect-attack level. Results showed that the wide double rows cropping system was recommended than single row or narrow double rows cropping system [6]. However, the last approach is the most desirable. Unfortunately, an insect-resistant trait is not available in any collection of sugarcane’s germplasms all over the world. Due to that reason, plant genetic engineering is the only possible approach in order to generate sugarcane resistant variety to borer. Mostly efforts to generate resistant crops to borer were to insert *cry* genes that was originally from *Bacillus thuringiensis* into crops genome.

Toxins Cry is a parasporal crystal consisting one or several α-endotoxins, produced by bacteria gram-positive *Bacillus thuringiensis*. It was isolated for the first time when Shigetane Ishiwatan investigated the cause of Sotto disease (the suddenly death) attacked silk worm (*Bombyx mori*) in 1901 [7]. Bt was started used as pesticide in 1920, when French produced Bt spore for commercial scale named Sporine, and it was mainly applied for moth of flour. In 1956, three German researchers (Hannay, Frizt-James and Angus) found that insecticidal Bt is a parasporal crystal [7]. Bravo et al. (2013) reported about Cry-3D domain family which relates to how the toxin works in the insect midgut. The first domain is responsible for insertion into the gut membrane and to develop pore and the second one is responsible for binding to receptor in the epithelium of midgut. The third domain has not yet defined, but it facilitates the oligomeric structure and induces the toxin insertion into the midgut [2]. This cry-3D works specifically against target-insect and only kills small number of species, so it is safe for human and animals’ vertebrate and also to plants. Therefore, when it is expressed in plants, it contributes to control pest efficiently and reduces the use of chemical pesticides.

To solve the stem or shoot insect borer problem in Indonesia, we applied such a similar approach mentioned above. In 2017, Koerniati and Trijatmiko developed a vector expression containing of a synthetic gene fusion of *cry* for controlling of borer [7]. In 2018, a vector containing of the synthetic gene has successfully been transformed into calli of Bulu lawang sugarcane variety, mediated by *Agrobacterium tumefaciens* [11]. This research aims were to identify sugarcane lines produced from plant genetic transformation molecularly and to evaluate sugarcane lines for their resistance performances against shoot borer. It is a short report about that research progress.

### 2. Materials and Methods

#### 2.1 Molecular identification
Materials used in this experiment were 30 lines of Bulu lawang sugarcane generated from plant genetic transformation mediated by *Agrobacterium* (coded by BL), KAPA 2G Ready mix for Taq polymerase enzyme, and larva of shoot borer (*Diatraea saccharalis* F.). DNA Molecular identification of lines was carried out by direct Polymerase Chain Reaction (PCR) using KAPA 2G RM Taq Polymerase enzyme and *CryIAc316-Forward* (5’GCCCAACAACTGTGAC CTG3’) and *CryIAc316-Reverse* (5’GGAGAGCTCCTG TTC3’) primers. PCR reactions were run using PCR machine (Biorad). Program used was {95°C, 3 minutes, (95°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute)
repeated 29 cycles, 72°C for 5 minutes}. PCR result was checked by gel electrophoresis (1% agarose gel and 1 x TAE buffer) and then were visualized using chemidoc (Biorad).

2.2 Bioassay
Plants were generated vegetative and maintained in the glass house of the BB BIOGEN containment facility (FUT). Resistance trait of Bulu lawang sugarcane Cry lines against shoot borer was investigated through efficacy of five larva (1st instar) of shoot borer (S. excerptalis Walker) onto 10 putative transgenic lines and wild type of Bulu Lawang sugarcane. Indications that caterpillars feed in the stem and cut off the growing point (the most inner part of the leave whorl), causing the youngest leaf to wilting and drying were observed among lines tested after three weeks of efficacy.

3. Results and Discussions

3.1. Molecular identification
Transformation conducted on Bulu Lawang sugarcane variety produced about 50 plantlets, but only 30 were survive from acclimatization and produced plants that were analyzed for their molecular characteristics. PCR was carried out to analyze whether transfer DNA (T-DNA) were inserted in these sugarcane genomes or were not. Primers (cryIAc-316F and cryIAc-316R) used were designed to amplify a DNA fragment of the CryIAc gene and will produce about 316bp of DNA. PCR results indicated that 30 lines of sugarcane Cry produced fragments of DNA with the size of about 300bp (based on 100bp ladder from NEB). These mean that all lines had T-DNA. Electrophoresis some of those 30 lines were shown in Figure 1.

![Electropherogram of PCR results of Sugarcane BL-Cry lines transformed by Agrobacterium strain GV3101. L: Ladder 100bp (NEB), 1-10: BL-Cry lines (2.1.2 GV, 2.1.3 GV, 2.1.4 GV, 2.1.5 GV, 2.2.2 GV, 2.2.3 GV, 2.2.4 GV, 3.1.1 GV, 3.1.2 GV, and 3.1.3 GV)](image)

3.2. Bioassay test
To identify whether these putative transgenic sugarcane lines have crystal (cry) endotoxin that able to kill or to stop the growing of shoot borer larva, a bioassay test was set up. Larva of S. excerptalis (Walker) was produced from hatch of eggs laid by adult moths that were collected from the sugarcane plantation at Lampung. Five of the first instar of larva were applied to the shoot of sugarcane plant. This treatment was applied to three plants (as replications).
Caterpillars (larva) naturally will feed in the stem and cut off the growing point (central whorl of the leaf), causing the shoot to wilt and dry (called die heart). This symptom will appear within one week after efficacy of larva. The trace of larva feeding the leaf tissue can be noticed in the leaf as a brown scare, for example shown in Figure 3 (magnifying).

Figure 3. Performance of sugarcane lines after 3 weeks of efficacy test with larva of shoot borer. A. Line 2.1.1 GV has a healthy shoot, B. Line 2.2.1 EH has a wilt and dry shoot.

Beside that, observation on the larva within the sugarcane tissue, specifically in the whorl of leaves was carried out. All plants of lines tested which showed the symptom of die heart were dissected to search for larva. Results showed that there was variation in the size or weight of larva in the whorl of leave of lines. Line 2.2.2EH had a symptom of dead heart. The shoot leaf became wilt and dry. When its whorl was dissected up to the inside part, a healthy larva of instar 5 was found. The larva ate the middle part of the leave whorl, made a tunnel and killed the growing point of the plant (Figure 4, Left). While line 3.3.5 GV which had similar symptom in its shoot leaf, a smaller larva of instar 3 was found in the whorl of leaves (Figure 4, Right).
Figure 4. Performances of the shoot leaf of sugarcane lines after 3 weeks of efficacy with the 1st instar larva of shoot borer. Left: plant with symptoms of wilt and dry shoot and instar 5 of larva, Right: plant with symptom of wilt and dry shoot and instar 3 of larva.

It was observed that the larva were at various stage of developments, sizes and healthiness among sugarcane transgenic lines. Larva found were from instar 3 up to instar 5. Larva of instar 3 was found in the line 3.3.5 GV, instar 4 was found in the line 4.2.1 EH (Figure 5A), and larva of instar 5 was found in the line 2.2.2 EH. Beside those healthy and alive larvas, it was also observed unhealthy (looked burnt or dark brown) and die instar 5 of larva in the line 3.3.3 GV (Figure 5B). Larva found in the line 4.2.1 EH was about instar 4 (Figure 5A), while larva found in the non-transgenic sugarcane plant (wild type) had entered a pupa stage of development (Figure 5C).

Figure 5. Performances of larvas within the whorl of leaves of transgenic and non transgenic sugarcane plants. A: line 4.2.1 EH, instar 4 and life, B: line 3.3.3 GV, instar 5 and die, and C: wild type (non-transgenic) BL plant.

Figure 6. Weight and approximate size of shoot borer larva observed in the preliminary bioassay test of sugarcane transgenic lines.
Beside the stage of development, the weighed the larva are also varied. It ranged from the lightest of 0.0074g up to the heaviest of 0.193g (Figure 6). Based on these preliminary data, there was differences in response among larvas when they were grown (efficacy) within transgenic sugarcane BL lines or within wild type of sugarcane BL variety (control). These might indicate that there was a different concentration of endotoxin produced by each line and these must be investigated further.

It was previously reported about efforts to improve sugarcane borer resistance in FN15 cultivar with cryIAc gene [3]. ELISA carried out to analyze Cry1Ac protein levels in seven transgenic lines showed that it was varied from 0.85μg/g\(^{-1}\) Fresh Weight (FW) to 70.92μg/g\(^{-1}\) FW in leaves and 0.04μg/g\(^{-1}\) FW to 7.22μg/g\(^{-1}\) FW in stes [3]. Observation on FN15 transgenic sugarcane lines showed that 10 to 20% of borers survived but these appeared in weak and were small [3]. In contrast, larva found in non-transgenic sugarcane was larger and with higher survivorship.

Earlier works on sugarcane with cryIAc had been reported [12]. They reported about efforts to increase expression level by synthesizing of a truncated insecticidal gene modified-cryIAc by increasing its GC content from 37.4 to 54.8%. Transgenic sugarcane expresses the m-cryIAc has five fold higher of protein than that of harbouring of the partially synthesis cryIAc (GC%=47.5%). Results showed that all transgenic lines with medium copy numbers significantly improved sugarcane borer resistance, which lowered susceptibility to damage by insects compared to non-transgenic sugarcane [12]. These indicated that transgenic sugarcane harbouring CryIAc more resistance to sugarcane borer than the wild type. Transgenic sugarcane lines with high levels of insect resistance showed similar agronomic and industrial traits as untransformed control plants.

4. Conclusions

The synthetic gene which consists of fusion Cry lab and CryIAc, promoter Rubisco and chloroplast transite protein (CTP) (about 4019bp in size) were proved inserted in the genome of these 30 lines of Bulu Lawang sugarcane, by PCR analysis. Expresion of Cry lab and CryIAc gene fusion in the chloroplast is expected to affect the healthyness of the shoot borer (Scirpophaga excerptalis Walker.) larva, and this expectation was shown by this preliminary bioassay test results on 10 lines of sugarcane Cry tested. Various stage of development and healthiness of larvas were observed among 10 BL sugarcane transgenic lines from healthy and big size (instar 5, 0.143g) of larva to small (instar 3, 0.0074g) one. Unhealthy (looks like burnt and die) and big size (instar 5, 0.193g) larva found in transgenic line, in contrast a healthy larva which entered a pupa stage found in non-transgenic sugarcane plant (wild type). These may indicate the healthiness of borer was reduced by BL transgenic lines compared to by BL wild type. Analysis on protein level using ELISA should be carried out to support that evidence.

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