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# (54) POLYNUCLEOTIDES, PRIMERS, AND METHODS FOR DETECTION OF TRANSGENIC EVENT, GENETIC CONSTRUCT, KIT FOR DETECTION MATERIAL FROM A PLANT SAMPLE, EVENT CTC75064-3, INSECT-RESISTANT SUGARCANE PLANT, AND METHOD FOR PRODUCING AN INSECT-RESISTANT SUGARCANE PLANT, PLANT CELL, PLANT PART OR SEED

(57) The present invention relates to the field of biotechnology. More precisely, a genetic construct and method for producing a transgenic plant event, especially a sugarcane event (*Saccharum* spp.), which is resistant to infestation by the *Diatraea saccharalis* pest, popularly known as ordinary borer, reed borer or just borer. The invention describes the event, the methods for event identification as well as the insertion detection method based on the unique region of intersection between the insert and the host genome and the flanking regions that characterize it.

Figure 1

## Description

Technical Field

**[0001]** The present invention relates to the field of biotechnology. More specifically, it is described a genetic construct and a method for producing a transgenic plant event, especially a sugarcane *(Saccharum* spp.) event, which expresses the CrylAc toxin conferring resistance to infestation by the pest *Diatraea saccharalis,* popularly known as common borer, cane borer or just borer. The invention describes a method for detecting the event and material derived from the event resistant to cane borer infestation, as well as polynucleotides, primers, probes and the flanking regions identifying such an event.

## Background

**[0002]** Sugarcane *(Saccharum* spp.) is a grass belonging to the botanical family Poaceae, originating from Southeast Asia, more precisely from the large central region of New Guinea and Indonesia. It is one of the most important plant species grown in the tropical and subtropical regions, with an area of over 23 million hectares spread over 121 countries (FAO Statistical Yearbook 2012 p. 233).

**[0003]** Sugarcane is a source of raw material for the production of sugar, wine, molasses, rum, cachaça (Brazil's national distillate) and ethanol for fuel. The bagasse that is produced after the sugarcane milling can be used for baling, supplying heat energy, processing at mills, producing electricity (that is typically sold to the consumer's electric grid), or as raw material for the production of sugarcane second-generation ethanol (BR 11 2014 02385-1). Thus, the sugarcane agro-industry has great economic and social importance by generating millions of jobs in the area and fostering foreign exchange through the commercialization of sugar and ethanol and sustainable and optimal use of plant biomass.

**[0004]** More recently, with the advent of global warming and the subsequent desire for alternatives to fossil fuels (biofuels), worldwide interest in sugarcane has increased significantly. The use of sugarcane-based ethanol as a renewable energy source has been considered extremely important for reducing greenhouse gases and dependence on fossil fuels, thus making it a key element in efforts to control global climate change (Savage, 2011).

**[0005]** Due to the economic and social importance of sugarcane, an increasing amount of research has been directed toward defining best agricultural practices for its cultivation and improving the quality of cultivated varieties. Efforts to improve sugarcane agronomic characteristics have focused on increasing sugar production and accumulation, increasing tolerance to biotic and abiotic stresses, resistance and tolerance to pests and pathogens, and developing alternative technologies for the production of sugarcane ethanol from lignocellulosic biomass (PI 0802153-8; PI 0904538-4; PI 1101295-1).

**[0006]** The complexity of the polyploid and aneuploid genome of modem sugarcane varieties, coupled with their relatively restricted genetic base and low fertility, impose great difficulties and numerous limitations on the selection of plants with desirable agronomic characteristics using conventional breeding (Souza et al, 2011; D'Hont & Glaszmann, 2005, Basel, v. 109, no. 1-3, p. 27-33; Cheavegatti-Gianotto et al., 2011). Therefore, high production costs, the necessity of manual labor, and long product-to-market timelines may prevent the sugarcane industry from meeting the growing demands of the global market.

**[0007]** The sugar cane production has been increased over the last 20 years as a result not just of the increase on productivity of plantations, but also as a result of the increase on diversity of cultivated varieties, allowing the expansion of cultivated area to other geographical regions. This geographical expansion demands the development of adapted varieties to different edaphoclimatic conditions, rendering the sustainability of sugarcane market supported by the continuously suppling to the market of new and local adapted germplasm and by the capacity to control diseases and pests.

**[0008]** The conventional breeding methods are important for continuously supplying new varieties, but not sufficient to attend the need of the modern market since part of characteristics are not founded in the genetic background of the varieties, being fundamental the development of efficient methods to introduce exogenous genes. Due to the limitations of conventional breeding methods and the increasing need to rapidly and efficiently incorporate desirable traits, the use of genetic engineering (biotechnology) in sugarcane breeding programs has gained prominence, in particular due to the commercial success of incorporating desirable agronomic traits through genetic engineering into other plant species (soybean, corn, canola, beet and cotton, for example).

**[0009]** Plant genetic engineering involves the transfer of genes-of-interest into plant cells (genetic transformation) in such a way that a fertile and agronomically superior progeny maintain and stably express the gene responsible for the desired trait.

**[0010]** Despite the potential of sugarcane genetic modification (incorporation of desirable characteristics) by genetic engineering [virus resistance (Guo et al., 2015; Zhu et al., 2011), insects (Kalunke, Kolge, Babu, & Prasad, 2009; Weng et al., 2011), herbicides (Enríquez-Obregon, Vazquez-Padron, Prieto-Samsonov, De la Riva, & Selman-Housein, 1998; van der Vyver, Conradie, Kossmann, & Lloyd, 2013), drought tolerance (Molinari et al., 2007; Reis et al., 2014), salinity

(Kumar, Uzma, Khan, Abbas, & Ali, 2014) and aluminum toxicity (Ribeiro, 2016), increased production and accumulation of sugar (Bewg, Poovaiah, Lan, Ralph, & Coleman, 2016; Mudge et al., 2013)], this approach is limited by intrinsic characteristics of sugarcane. Unlike maize, rice, wheat and other commercial cereals, sugarcane exhibits difficulties in tissue culture propagation, low rates of induction and regeneration of embryogenic calluses, and the impossibility of using the zygotic embryo as a target tissue in genetic transformation [(Anderson & Birch, 2012; Basnayake, Moyle, & Birch, 2011; Molinari et al., 2007)]. Low rates of transformation efficiency and high variability between sugarcane genotypes are frequently observed, and there are still numerous challenges to overcome in order to successfully incorporate desirable agronomic traits into sugarcane using genetic engineering.

**[0011]** Sugarcane is considered a recalcitrant species for genetic transformation, and although several genetic engineering approaches have been evaluated for this species, there are still no standard protocols that guarantee the production of transgenic events (Smith et al. 1992; Rathius & Birch 1992.; Chen et al. 1987; Arencibia 1998; Manickavasagam et al. 2004; Elliott et al. 1998).

**[0012]** In addition to the inherent limitations of the species that prevent the application of existing genetic engineering techniques, the complexity of the sugarcane genome (high ploidy level and aneuploidy), prevents trait introgression into specific cultivars through backcrossing (reconstitution of a specific genotype), as is commonly performed in other crops of commercial interest. Thus, for the "insertion" of the same trait in more than one germplasm, enabling gains in productivity in different geographical regions, it is necessary to carry out a new genetic transformation, incurring the same costs and risks associated with the first event obtained for that trait. Even today, there is very little predictability of success for the insertion of the same characteristic in different varieties of sugar cane, being an extremely genotype-dependent process, which makes the construction of a portfolio of genetically modified products for this species challenging.

**[0013]** The genotypic complexity of the species also significantly impacts the characterization of the events generated in order to ensure the necessary characteristics for their commercialization. The unambiguous identification of transgenic events is fundamental to ensure their traceability and monitoring, which is a regulatory requirement for their commercialization. The high polyploidy of the sugarcane genome and the high number of repeated regions, coupled with the lack of information on their organization and structure, make the characterization of the transgenic events generated even more difficult.

**[0014]** There are several technical challenges to be overcome in the field of sugarcane breeding to increase the predictability of results, even when applying widely known conventional and/or molecular/genetic techniques. Despite all the technical challenges for obtaining more productive varieties of sugarcane, there is no doubt about the urgency of obtaining improved varieties that have characteristics that significantly impact crop productivity and therefore its market.

**[0015]** Historically, agricultural pests are one of the main factors that cause losses in agriculture. In Brazil, the main sugarcane pest is the species *Diatraea saccharalis* (first described by Fabricius in 1794), popularly known as the common borer, cane borer or just borer. It is a member of the Crambidae family and Lepidoptera order. The borer is found practically everywhere the crop is cultivated, an area of approximately 10 million hectares for the 2019/20 crop season (CONAB, 2019).

**[0016]** After mating, the female sugarcane borer lays 200 to 400 eggs distributed on either side of the leaves as well as the leaf sheaths. After hatching, neonate larvae feed on the leaf parenchyma, migrating to the sheath region for shelter. They remain in this region for 7 to 10 days, feeding by scraping the leaf sheath or bark of the young internodes. After an ecdysis, the caterpillars pierce the stalk, penetrating inside. The insect creates tunnels inside the stalk, usually upwards as it feeds. Inside the culm, the caterpillar goes through approximately six ecdysis before becoming a winged adult (DINARDO-MIRANDA, 2014). This is the developmental stage of the insect that causes economic damage to the crop (Figure 1).

**[0017]** The attack of the sugarcane borer also causes serious secondary damage to the quality of the raw material used for sugar and alcohol production, because the drilling of the sugarcane stalk by the borer creates favorable conditions for fungal entry and opportunistic bacteria, especially *Fusarium moniliform* and *Colletotrichum falcatum,* causing red rot (Figure 2). Bacteria associated with the red rot raw material produce undesirable fermentations, resulting in products foreign to industrial alcoholic fermentation. Moreover, these bacteria also produce organic acids and gums (dextrans) from the sugars contained in the wort, which negatively affect the viability of yeast cells, requiring their replacement in fermentation reactors (Prececti and Terán 1983; Prececti et al., 1988; BOTELHO and MACEDO, 2002). Another problem arising from the presence of bacteria in fermentation reactors is the possibility of yeast flocculation occurring. In this case, the contaminating bacteria form mucilage that aggregates the yeast cells, causing them to flocculate. Finally, plants attacked by the borer/red rot complex also have high levels of phenolic compounds (METCALF and LUCKMANN, 1994; PRICE, 1997).

**[0018]** Assuming a 4% borer Infestation Infection Index (typical for Brazilian sugarcane fields), average agricultural losses, and pest control costs, it is estimated that the borer causes economic losses of more than R$5 billion annually to the sugar and ethanol production industries.

**[0019]** The sugarcane borer is difficult to control with chemical insecticides due to the feeding behavior of the larva in the stalk, which prevents the insecticide from effectively contacting the insect. As an alternative to chemical insecticides, insecticidal proteins, mainly identified from the bacterium *Bacillus thuringiensis* (Bt), have been used to control agricultural pests, including *Diatraea* sp. Among the insecticidal proteins derived from Bt strains, Cry crystalline proteins stand out for their specific toxicity to larvae of common lepidopteran, dipteran and coleopteran species. These proteins, produced as protoxins (65-149 KDa), are solubilized and activated in the intestines of susceptible insects by proteolysis and bind to the intestinal cell membrane, inducing osmotic lysis of the epithelium, which causes the insect to die.

**[0020]** Cry proteins are classified into several groups according to sequence homology, among them, the protein group classified as Cry1 presents high specificity against lepidopteran insects, making it an excellent candidate for introduction into sugarcane germplasm to produce sugarcane borer resistant varieties. The heterologous expression of Cry1 proteins in sugarcane varieties, although challenging, has great potential for sugarcane borer control, reducing economic losses to the sugarcane industry, as well as the release of chemical insecticides in the environment.

**[0021]** Therefore, there remains a need to develop strategies to mitigate the damage caused to sugarcane crops by pest infestation, especially by infestation by the sugarcane borer pest. By offering to sugarcane growers varieties with both high yield and borer resistance traits to different edaphoclimatic environments, agricultural biotechnology makes an important contribution to the sugarcane industry and to the sugarcane growers.

**[0022]** The event CTC75064-3 disclosed in the present invention was generated to provide to the sugarcane growers a new variety, resistant to sugarcane borer pest and with the genetic background of RB 867515 (Plant Variety Protection/PVP - protocol number - 21806.000439/2000-45; PVP Certificate № 271). In the 2018/19 season, RB 867515 sugarcane variety was grown on approximately 1,730 thousand hectares in the Brazilian Center-South region. The cultivation of this variety in the Northeast region was approximately 57 thousand hectares. Considering the sugarcane cultivated area (CONAB, 2019), this variety has a market share in Brazil of approximately 21%.

<u>Embodiments</u>

**[0023]** In a first embodiment the invention provides polynucleotides that unambiguously identify the event CTC75064-3.

**[0024]** The invention also identifies primers pairs and probes able to identify polynucleotides that characterize the event CTC75064-3.

**[0025]** In a third embodiment the invention provides methods for detecting plant material derived from the CTC75064-3 event.

**[0026]** Other embodiment of the invention defines a kit for detecting the presence of event CTC75064-3 in a sample of plant material.

**[0027]** The fifth embodiment of the present invention is a genetic construct capable of imparting to a sugarcane *(Saccharum* spp.) plant, resistance to insect infestation, particularly by *Diatraea saccharalis* pest.

**[0028]** Also, the invention provides a genetically modified sugarcane, a plant part, plant cell, plant tissue, or seed comprising the genetic construct of interest located at a site defined in the genome of the transformed sugarcane plant, characterized by specific flanking sequences.

**[0029]** The seventh embodiment of the present invention is to provide a commodity product.

**[0030]** The eighth embodiment of the present invention is a method of producing an insect resistant plant.

**[0031]** Finally, the ninth and tenth embodiments of the invention are to provide a method of making and cultivating an insect resistant sugarcane plant and/or plant cell, plant part, or seed.

## Summary of the Invention

**[0032]** The first embodiment is achieved by providing polynucleotides comprising at least 14 to 26 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 18, SEQ ID NO: 19, and SEQ ID NO: 22.

**[0033]** The second embodiment of the invention is achieved by providing primers pairs wherein the forward primer consists of SEQ ID NO: 6 and the reverse primer consists of SEQ ID NO: 7 and/or the forward primer consists of SEQ ID NO: 8 and the reverse primer is SEQ ID NO: 9.

**[0034]** The third embodiment is achieved by a method of detecting plant material derived from event CTC75064-3 comprising the steps of:

a) obtaining a sample for analysis;
b) extracting DNA from the sample;
c) providing primer pairs comprising at least a forward and a reverse primer;
d) amplifying a region between the primer pair; and detecting the presence of a product from amplification.

**[0035]** Also to achieve the third embodiment the primer pairs in step c) are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO:

29, wherein at least one pair of primers comprises contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31.

**[0036]** Still to achieve the third embodiment, the present invention describes a method of detecting plant material derived from event CTC75064-3 comprising the steps of:

a) obtaining a plant material sample for analysis;
b) extracting DNA or RNA from the sample;
c) providing a probe designed to bind to the complement of a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33;
d) hybridizing said probe with the sample; and
e) detecting the actual hybridization of the probe.

**[0037]** The fourth embodiment of the invention is evidenced by a kit for detecting the presence of event CTC75064-3 in a plant sample, the kit comprising a means to detect the presence of a polynucleotide comprising, at least, 14 contiguous nucleotides of SEQ ID NO: 18 and/or of SEQ ID NO: 19 and/or a pesticidal crystal protein (Cry).

**[0038]** The fifth embodiment is achieved through providing a genetic construct comprising SEQ ID NO: 1 or 2.

**[0039]** The sixth embodiment is achieved through a genetically modified sugarcane (Saccharum spp.) plant, a plant part, plant cell, plant tissue, or seed comprising SEQ ID NO: 18 or SEQ ID NO: 19.

**[0040]** The seventh embodiment is achieved by providing a commodity product produced from the genetic modified sugarcane from the present invention.

**[0041]** The eighth embodiment is achieved by a method of producing an insect resistant plant comprising SEQ ID NO: 20 and SEQ ID NO: 21

**[0042]** The ninth embodiment of the present invention provides a method of making an insect resistant sugarcane plant comprising introducing a genetic modification to a sugarcane

**[0043]** (*Saccharum* spp.) plant comprising SEQ ID NO: 5 or SEQ ID NO: 22 to produce a genetically modified sugarcane (*Saccharum spp*.) plant of event CTC75064-3.

**[0044]** Finally, the tenth embodiment of the invention describes a method of cultivating a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3, comprising growing a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3 comprising SEQ ID NO: 5 or SEQ ID NO: 22 under conditions comprising insect infestation.

**Brief Description of the Figures**

**[0045]**

Figure 1 exemplifies the damage to sugarcane stalks caused by *D. saccharalis* (sugarcane borer).

Figure 2 exemplifies the red rot-borer complex due to *D. saccharalis* (cane borer) attack.

Figure 3 represents the map of the T-DNA introduced in the event of the present invention.

Figure 4 represents the plasmid used as a base for constructing the plasmid used in the present invention.

Figure 5 represents the resulting plasmid used to obtain the event of interest.

Figure 6 is the graph of qPCR amplification via Taqman® (relative fluorescence x cycle) for the event of interest.

Figure 7 is the qPCR melting curve via SYBR GREEN ™ (relative fluorescence x temperature) for the event of interest. The arrows indicate the specific amplification peak of the event of interest and the baseline indications of no amplification for the negative events and controls.

Figure 8 is the result of comparing means of Cry1Ac protein expression in leaves (Fresh tissue) of the event of the invention during the sugarcane cultivation cycle. Combined analysis for Barrinha, Piracicaba, Valparaiso (SP), Quirinopolis (GO), Mandaguaçu (PR) and Juazeiro (BA). Bars followed by the same letter do not differ by T test at the 5% probability level. Bars means SE.

Figure 9 is the result of comparing means of CrylAc protein expression in leaves (Dry tissue) of the event of the invention during the sugarcane cultivation cycle. Combined analysis for Barrinha, Piracicaba, Valparaiso (SP), Quirinopolis (GO), Mandaguaçu (PR) and Juazeiro (BA). Bars followed by the same letter do not differ by T test at the 5% probability level. Bars means SE.

Figure 10 is the expression means of CrylAc protein in fresh (A) and dry base (B) of the stalk, leaf and root tissues of CTC75064-3 event. Bars followed by the same letter do not differ by T test at the 5% probability level. The bars represent the mean ± standard error.

Figure 11 is the result of comparing means of NptII protein expression in leaves (Fresh tissue) of the event of the invention during the sugarcane cultivation cycle. Combined analysis for Barrinha, Piracicaba, Valparaiso (SP), Quirinopolis (GO), Mandaguaçu (PR) and Juazeiro (BA) locations. Bars followed by the same letter do not differ by T test at the 5% probability level.

Figure 12 is the result of comparing means of NptII protein expression in leaves (Dry tissue) of the event of the invention during the sugarcane cultivation cycle. Combined analysis for Barrinha, Piracicaba, Valparaiso (SP), Quirinópolis (GO), Mandaguaçu (PR) and Juazeiro (BA) locations. Bars followed by the same letter do not differ by T test at the 5% probability level.

Figure 13 is the expression means of NptII protein in fresh (A) and dry base (B) of the stalk, leaf and root tissues of CTC75064-3 event. Bars followed by the same letter do not differ by T test at the 5% probability level. The bars represent the mean ± standard error.

Figure 14 is the result of the Western blot methodology for identifying the CrylAc protein. M: molecular weight marker (kDa). R1-R4: biological repeats of the event of the invention; WT (1): negative control-total protein extracted from parental cultivar. CP: 1ng of purified CrylAc protein; WT+CP: 1ng of purified CrylAc protein diluted in total proteins of WT; ø: empty lane.

Figure 15 is the result of the Western blot methodology for identification of *NptII.* M protein: molecular weight marker (kDa). R1 and R2: biological repeats of the event of the invention. CP: 5ng *NptII* protein; WT+CP: 5ng *NptII* protein diluted in total protein extracted from parental cultivar. WT: total protein extracted from parental cultivar.

Figure 16 represents: **A)** Infestation index (I.I.%), **B)** Relative length of damage from event CTC75064-3 and from controls (plants in the first harvest). The letters represent the result of the analysis of variance by the T test and the bars refer to the standard error.

Figure 17 represents Borer Control Effectiveness of event CTC75064-3 from the 4 locations (1.1% = infestation index). The bar refers to the standard error of the analysis.

Figure 18 shows in A: larvae mortality in the Leaf disc assay with plant material from the insect (sugarcane borer) resistant CTC75064-3 event in comparison to the parental non-genetically modified CTC75-TC (RB867515); B: Borer Control Effectiveness of event CTC75064-3; C: exemplary results of larval size and development after seven days of feeding in the Leaf disc assay. On left are exemplary larvae that were fed with plant material from the insect resistant CTC75064-3 event, and on right (B) are exemplary larvae that were fed with plant material from the parental non-genetically modified CTC75-TC (RB867515).

Figure 19 demonstrates examples of cassettes for generation of event CTC75064-3 through gene editing approach. Cassette A comprises a sugarcane codon optimized Cas9 driven by pZmUbi promoter and T-35s terminator; Cassette B comprises crRNA for Cas9 driven by wheat U3 promoter and Cassette C comprises the HR template comprising CTC75064-3 T-DNA region (SEQ ID NO 2) with homologous arms for site directed integration.

Figure 20 represents an example of a gene editing construction comprising Cas 9 and crRNA Cassettes.

Figure 21 shows an example of a gene editing construction comprising the HR template comprising CTC75064-3 T-DNA region (SEQ ID NO 2) with homologous arms for site directed integration.

Figure 22 represents a gene editing construction comprising the all the cassettes for generation of event CTC75064-3: a codon optimized Cas9 driven by pZmUbi promoter and T-35s terminator; a crRNA for Cas9 driven by wheat U3 promoter and the HR template comprising CTC75064-3 T-DNA region (SEQ ID NO 2) with homologous arms for site directed integration.

Figure 23 A) is a schematic representation of Southern blot strategy using *Hind*III and *EcoRV* restriction enzymes for identifying the T-DNA inserted in the event CTC75064-3. Gray arrows below the T-DNA scheme indicate the predicted sizes of the T-DNA fragments from CTC75064-3 event generated. B) is a schematic representation of Southern blot strategy for detecting vector (backbone) fragments using the restriction enzyme *Sph*I.

Figure 24 represents the Southern blot using the restriction enzymes *Hind*III (left) and *EcoRV* (right). Results for the cry probe that recognizes the *crylAc* gene. M: molecular weight marker; WT: negative control; 1C: positive control 1 copy. T0: plant from the 1st harvest. T1: plant from the 2nd harvest; T2: plant from the 3rd harvest; T3: plant from the 4th harvest.

Figure 25 represents Southern blot using the restriction enzymes *EcoRV* (left) and *Hind*III (right). Results for the 35s probe that recognizes 35s promoter. M: molecular weight marker; WT: negative control; 1C: positive control 1 copy. T0: plant from the 1st harvest. T1: plant from the 2nd harvest; T2: plant from the 3rd harvest; T3: plant from the 4th harvest.

Figure 26 represents the Southern blot using the restriction enzymes *Hind*III (left) and *EcoRV* (right). Results for the *nptII* probe that recognizes the *nptII*gene. M: molecular weight marker; WT: negative control; 1C: positive control 1 copy; T0: plant from the 1st harvest. T1: plant from the 2nd harvest; T2: plant from the 3rd harvest; T3: plant from the 4th harvest

Figure 27 A) represents the Southern blot using the restriction enzyme *Sph*I and the backbone probes BB1 and BB3. M: molecular weight marker; WT: negative control; 1C: positive control 1 copy with pCTC146; 1C*: positive control 1 copy with pCTC302. BB1: positive control for the probe backbone 1; BB2: positive control for the backbone 2 probe; BB3: positive control for the backbone probe 3. The red arrows indicate two repetitions of the CTC75064-3 event. Lanes 1: other events. B) represents the Southern blot using the *Sph*I restriction enzyme and the BB2 backbone probe. M: molecular weight marker; WT: negative control; 1C: positive control 1 copy with pCTC146; 1C*: positive control 1 copy with pCTC302. BB1: positive control for the probe backbone 1; BB2: positive control for the backbone 2 probe; BB3: positive control for the backbone probe 3. The red arrows indicate two repetitions of the CTC75064-3 event. Lines 1 are other events.

Figure 28 represents the confirmation of the integrity of the flanking regions of the T-DNA inserted in the event CTC75064-3. (A) Confirmation of integrity of the left border (5'). (B) confirmation of integrity of the right border (3'). Event: DNA from event CTC75064-3; WT: DNA CT 7515 parental (RB 867515 variety); C-: negative control.

**Detailed Description of the Invention**

**[0046]** First, the term "event" refers to the transgenic plant produced through genetic transformation that stably expresses the desired trait conferred by the introduced transgene. More particularly, in the present case, the term "event" is considered to be the transgenic plant, preferably a sugarcane transgenic plant *(Saccharum* spp.) which, after genetic modification, expresses the characteristic of pest resistance, particularly resistance to the pest *Diatraea saccharalis* (sugarcane borer). In a preferred embodiment, the transgenic sugarcane produced through the genetic transformation is designated 'CTC75064-3' and may alternately be referred to as "CTC75064-3 event".

**[0047]** Also, for reference purposes, unless expressly mentioned otherwise, "LB region" means the left border (edge) of T-DNA transfer (5'), and "RB region" means the right border (edge) of T-DNA transfer (3').

**[0048]** Additionally, all biological sequences describe herein, except otherwise explicitly stated, encompass sequences having at least 80%, preferably 85%, 90%, 95%, 98%, 99% or 100% of identity with the described sequences.

**[0049]** Finally, "plant material" means any and all plant tissue or derivatives thereof, such as, but not limited to, seeds, stems, stalks, leaves, straws, bark, roots, cells, molecules of plant origin, among others. In addition, "plant material" may include any product of a plant or derivative thereof, for example, but not limited to, sap, sugar, ethanol, among others.

**[0050]** Recombinant DNA technology has enabled the isolation of genes and their stable insertion into a host genome. This technique, also called genetic transformation, can be defined as the controlled introduction of nucleic acids ("DNA" or DNA) into a recipient genome, excluding introduction by fertilization. It is a controlled process where a defined DNA fragment is introduced into the host (or recipient) genome and must be integrated into it. The stable insertion of these molecules into a host genome gives rise to an individual with the genome equal or substantially equal to the recipient (host) of the recombinant molecule, but with a new and particular feature. "Substantially equal" means a genome with more than 80%, preferably 85%, 90%, 95%, 98%, 99% or 100% of identity in relation to the recipient.

**[0051]** There are several plant genetic transformation techniques grouped into two main categories: indirect and direct gene transfer. Indirect transfer is when exogenous DNA is inserted into the genome by the action of a biological vector, while direct transfer is based on physical-biochemical processes.

**[0052]** Different tissues and/or cells could be used according to the genetic transformation technique and according to the species or genotypes to be transformed. Generally, these tissues or cells include, without limitation, embryogenic callus, callus, protoplasts, embryos, somatic embryos, meristematic tissues, and any other part, tissue or cell of plant with regenerative capacity.

**[0053]** Indirect transformation is based on the bacterium-mediated system of the genus *Agrobacterium* and has been the most widely used method for obtaining transgenic plants. Advantages to this method include the ability to transfer relatively long DNA segments without rearrangement while maintaining low copy number integration of the transgenes, thus ensuring greater genotypic stability for the generated events. Several *Agrobacterium* species and strains, plasmids and protocols have been developed and adapted for genetic transformation of several plant species. The advantages of these methods include higher probabilities to single copy events, stable integration, and genetic heritage of the introduced genetic traits, as well as, consistent genic expression through generations and lower rates of gene silencing.

**[0054]** *Agrobacterium tumefaciens* and *A. rhizogenes* are gram negative soil phytopathogenic bacteria belonging to the Rhizobiaceae family that cause diseases in dicotyledons, known as crown and hairy root galls, respectively. In this plant-pathogen interaction there is a process of natural gene transfer between the agrobacterium and the plant cell wherein fragments of bacterial DNA are transferred into the plant cell (T-DNA), integrating with the nuclear genome. In its natural form, the bacterium transfers T-DNA ("transferred DNA"), which is part of the bacterial plasmid called Ti ("tumor-inducing") and integrates into the genome of infected plant cells. The T-DNA fragment that is transferred to the plant cell is comprised of genes involved in the constitutive biosynthesis of phytohormones (auxins and cytokinins), which alter the normal developmental program of infected tissue and cause tumor formation. In addition, it also contains oncogenes for the synthesis of sugars and amino acids called opines, which serve as carbon and nitrogen sources for bacteria (Oger et al. 1997). Repeated ends of 25 base pairs (bp) at the right and left borders delimit the T-DNA and are essential for its transfer. Phenolic compounds released by injured plant tissues activate specific regions (vir regions), initiating the process of transfer of T-DNA to the plant cell. *Agrobacterium* also has chromosomal (chv) genes that promote binding between bacterial and host cells, allowing the formation of the pore passage of the T-DNA-containing complex (Sheng & Citovsky. 1996).

**[0055]** Since the segment to be transferred is defined by its borders, any sequence flanked by the borders can be transferred to a plant by means of agrobacteria, making it possible to manipulate these sequences in order to transfer coding sequences of interest. The replacement or deletion of the coding regions of wild-type T-DNA (oncogenes) allows for the generation of non-oncogenic (disarmed) *Agrobacterium* strains, which can carry the sequences of interest. The modified T-DNA is able to transfer the sequences of interest to plants because the virulence genes (vir region) remain intact.

**[0056]** Additionally, the *Agrobacterium* indirect transformation system allows for the transfer of artificial plasmid constructs to plants as long as the constructs contain such T-DNA borders, which enables the flexibility to use molecular tools and materials developed for other bacterial strains.

**[0057]** These artificial plasmid constructs have promoters from different origins, as for example, plant promoters, viral promoters, bacterial and or chimeric promoters, besides genes that confer antibiotic resistance, herbicide resistance or tolerance, or enzymatic activity (phosphomannose isomerase (PMI)/mannose (Man)), so these markers can be used for the selection of transformed cells or plants.

**[0058]** These constructions also can contain auxiliaries genes which interfere with relevant morphogenesis signaling pathways, enhancing the efficiency of the genetic transformation process and regeneration of vegetal tissues. Included, without limitations, LEAFY COTYLEDON1 (Lotan et al., 1998), Lecl (Lowe et al., 2002), LEAFY COTYLEDON2 (Stone et al., 2001), WUSCHEL (WUS; Zuo et al., 2002), e BABY BOOM (BBM; Boutilier et al., 2002), among others.

**[0059]** In a first aspect of the present invention, foreign or exogenous DNA to be introduced into the plant is cloned into a binary plasmid between the left and right border consensus sequences (T-DNA). The binary plasmid is transferred to an *Agrobacterium* cell, which is subsequently used to infect plant tissue. The T-DNA region of the vector comprising the exogenous DNA is inserted into the plant genome. The marker gene expression cassette and the characteristic gene expression cassette may be present in the same region of T-DNA, in different regions of T-DNA on the same plasmid, or in different regions of T-DNA on different plasmids. In one embodiment of the present invention, the cassettes are present in the same region as the T-DNA. One of skill in the art is familiar with the methods of indirect transformation by *Agrobacterium.*

**[0060]** Alternatively, direct DNA transfer can be used to directly introduce DNA into a plant cell. One method of direct DNA transfer is to bombard plant cells with a vector comprising DNA for insertion using a particle gun (particle-mediated biolistic transformation). Other methods for transformation of plant cells include protoplast transformation (optionally in the presence of polyethylene glycols); ultrasound treatment of plant tissues, cells, or protoplasts in a medium comprising the polynucleotide or the vector; microinjection of the polynucleotide or vector into plant material; microinjection, vacuum infiltration, sonication, use of silicon carbide, chemical transformation with PEG, electroporation of plant cells and the like. Between the disadvantages of direct transformation are challenges related to regeneration of plant tissue and the low transgene expression.

**[0061]** In addition, genetic transformation could be performed by site direct insertion through homologous recombination mediated by nucleases (genome editing). In recent years, genome editing technology based on use of engineered or chimeric nucleases has enabling the generation of genetically modified organisms in a more precise and specific way. The introduction of exogenous or foreign genes occur by homologous recombination through introduction of a Homologous recombination template (HR) having the exogeneous DNA linked to a DNA fragment homologous to the genome of the receptor organism. Between the tools available are the chimeric enzymatic system CRISPR (clustered, regularly interspaced, short palindromic repeats) - Cas, the Zinc finger (ZFN)nucleases and TAL effector nucleases (TALENs). Crispr-Cas systems are enzymatic systems comprising two main components: an endonuclease (Cas) and a guide-RNA (single-guide RNA - sgRNA; a guide to the specific cleavage site of Cas endonuclease). The guide RNA may also comprise of two components: a Crispr RNA (crRNA) - a sequence of 17-20 mer complementary to specific DNA genomic sequences and, optionally, of a tracrRNA. The specific cleavage performed by endonuclease and guide by the sgRNA would be repair by homologous recombination, specifically inserting the exogenous DNA flanked by the homologous sequences to the cleavage site. The introduction of this enzymatic system to the cell could occur by several manners, using plasmids, through direct or indirect transformation, or using carriers like proteins and other chemical agents. The expression of the system components would occur in a transient or stable manner, using the cellular machinery of the receptor organism or being realized in a exogeneous way, in vitro, delivering to the target cell or tissue all the components ready to use (endonucleases + sgRNA, in vitro transcribed and combined before cell delivery). The description presented herein is not exhaustive and should not limit the use of different variations, systems and methods of genome editing on scope of the present invention, known in the State of the Art and even the ones not yet discovered.

**[0062]** Following transformation, transgenic plants are regenerated from the transformed plant tissue and the progeny that have exogenous DNA can be selected using an appropriate marker such as kanamycin or ammonium glufosinate resistance. One skilled in the art is familiar with the composition of suitable regeneration media.

**[0063]** Alternatively, other selection methods could be applied, without the insertion of any gene marker in the host genome (receptor organism) as described before.

**[0064]** In a preferred embodiment, genetic transformation is mediated through a bacterium of the genus *Agrobacterium.*

**[0065]** In an even more preferred embodiment, genetic transformation is mediated by *Agrobacterium tumefaciens.*

**[0066]** CTC75064-3 event exhibits a new genotype comprising two expression cassettes. The first expression cassette comprises a promoter suitable for plant expression operably linked to a gene encoding a Cry1Ac insecticide toxin useful in controlling lepidopteran insect pests and a suitable polyadenylation signal. The second expression cassette comprises a promoter suitable for plant expression operably linked to a gene encoding a protein used as a selective marker in obtaining the event of the present invention.

**[0067]** Promoters suitable for plant expression may be isolated from plants or from other organisms. Several promoters have been isolated or developed including constitutive promoters, "on and off" promoters, and promoters that are responsive to tissue-specific abiotic stresses, among others. Many of these promoters have intronic sequences described as relevant for proper gene expression. In a preferred aspect of the invention, promoters are constitutive promoters and may be selected from the non-limiting group consisting of CaMV 35s, CoYMV (Commelina yellow mottle virus), FMV 35s, Ubiquitin, Actin Rice Promoter (Act-1), Act -2, nopaline synthase promoter (NOS), octopine synthase promoter (OCS), corn alcohol dehydrogenase promoter (Adh-1), PvUbi1, among others.

**[0068]** Additional elements such as introns, enhancer sequences and transporters may also be incorporated into the expression cassette for the purpose of enhancing gene expression levels, for example, transcriptional or translation enhancers such as CaMV 35s enhancers, FMV 35s, Nos, supP, non-translated leader sequence from wheat major Chlorophyll a/b-Binding Polypeptide (L-Cab), kosak sequences 5' upstream from the translational start site, among others.

**[0069]** In one embodiment of the invention, the promoter is Cauliflower Mosaic Virus 35s (CaMV 35s). In an even more preferred embodiment, the promoter CaMV 35s is a doubled enhanced CaMV 35s promoter (2xCaMV35s).

**[0070]** In one embodiment of the invention Kosak sequence 5' upstream from the translational start site and the *Oryza sativa* Actin 1 intron (OsACT1) are contemplated in the CaMV 35s promoter region. Additionally, L-Cab leader sequence is also contemplated in the CaMV 35s promoter region.

**[0071]** In a preferred embodiment, the cry1Ac gene expression is regulated by the combination of elements selected from the group consisting of doubled enhanced promoter CaMV 35s, non-translated leader sequence from wheat major Chlorophyll a/b-Binding Polypeptide (L-Cab), OsACT1 intron and Kosak sequence 5' upstream from the translational start site. In a preferred embodiment, *cry1Ac* gene expression is regulated by a promoter region comprising doubled enhanced promoter CaMV 35s, wheat leader sequence L-Cab, OsACT1 intron and Kosak sequence 5' upstream from the translational start site.

**[0072]** In another embodiment the promoter is the maize Ubiquitin (pUBI) gene promoter. In an even more preferred embodiment, the maize Ubiquitin promoter contains an intron in the 5' sequence of the leader RNA.

**[0073]** The promoter region (UBI-1) of the present invention has 1992 base pairs which are subdivided into: promoter fragment (899 bases), first exon of the polyubiquitin-1 gene (83 bases) and first intron (1,010 bases).

**[0074]** In one embodiment, the *nptII* gene expression is regulated by maize Ubiquitin (pUBI) gene promoter. In a preferred embodiment, *nptII* gene expression is regulated by maize Ubiquitin promoter contains an intron in the 5' sequence of the leader RNA. In a specific embodiment, *nptII* gene expression is regulated by a maize Ubiquitin (pUBI) gene promoter region comprising 1992 base pairs which are subdivided into: promoter fragment (899 bases), first exon of the polyubiquitin-1 gene (83 bases) and first intron (1,010 bases).

**[0075]** Terminator sequences are also contemplated on the expression cassettes. Examples of suitable and functional plant polyadenylation signals include those from the *Agrobacterium tumefaciens* nopaline synthase gene (nos), proteinase inhibitor II gene rbcS (pea ribulose-1,5-bisphosphate carboxylase small subunit), Lhcb1 (tobacco chlorophyll a/b-binding proteins), CaMV 35s, octopine synthase, alpha-tubulin gene, among others.

**[0076]** In one embodiment of the present invention, the polyadenylation signal is that derived from the CaMV 35s gene (T35s). In another embodiment of the present invention, the polyadenylation signal is that derived from the *Agrobacterium tumefaciens* nopaline synthase (T Nos) gene.

**[0077]** Preferably, the polyadenylation signal for cry1Ac cassette is CaMV 35s terminator (T-35s) and polyadenylation signal for *nptII* gene is *Agrobacterium tumefaciens* nopaline synthase terminator - NOS.

**[0078]** The cry1Ac gene encodes a 615 amino acid toxin with an estimated molecular weight of 68 KDa, originating from *Bacillus thuringiensis* serovar kustaki (strain HD73), which confers resistance to *Diatraea saccharalis* (cane borer). The present invention contemplates gene modifications for expression of the active tryptic nucleus of native CrylAc protein only. Thus, in a preferred embodiment of the present invention, the polynucleotide encoding the CrylAc protein is truncated, encoding the 52 KDa tryptic insecticide nucleus. In a more preferred embodiment, the CrylAc protein is SEQ ID NO 34. The present invention also contemplates sequences having at least 80%, preferably 85%, 90%, 95%, 98%, 99% or 100% of identity with SEQ ID NO: 34. The tryptic nucleus is responsible for the insecticidal activity of the protein, binding to specific proteins of the insect's gut leading to disruption of the functional and anatomical integrity of this organ. Ingestion of the CrylAc protein by the target insect causes altered nutrient absorption, which leads to rapid toxicity and subsequent death of the insect.

**[0079]** According to the invention, the polynucleotide encoding the CrylAc protein may have optimized (or otherwise altered) codons to improve expression in plant material. Such codon optimization may be used to alter the predicted secondary structure of the RNA transcription product produced in any transformed cell or to destroy the cryptic RNA instability elements present in the unchanged transcription product, thereby enhancing the stability and/or availability of the transcription product in the transformed cell.

**[0080]** Preferably, the cry1Ac gene present at the event of the invention corresponds to a truncated synthetic DNA sequence optimized with preferred sugarcane codons. In an even more preferred aspect of the present invention, the cry1Ac gene has the sequence SEQ ID NO: 20. The invention also contemplates sequences having at least 80%, preferably 85%, 90%, 95%, 98%, 99% or 100% of identity with SEQ ID NO: 20.

**[0081]** Several marker genes for plant event selection have already been characterized, including some that confer tolerance to antibiotics and others that confer resistance to herbicides. Examples of marker genes that may be selected for use in the present invention include those that confer resistance or tolerance to hygromycin, kanamycin, gentamicin, geneticin, glyphosate, ammonium glufosinate or resistance to toxins such as eutypine. Other forms of selection are also available such as hormone-based selection systems, visual selection through expression of fluorescent proteins, mannose isomerase, xylose isomerase, among others. In one embodiment of the present invention, the event selection marker gene is one which confers tolerance to kanamycin and geneticin.

**[0082]** In a preferred embodiment of the invention, the marker gene used in the second expression cassette is the *nptII* gene, which encodes the 265 amino acid neomycin phosphotransferase II (*nptII*) enzyme with an estimated molecular weight of 29.2KDa. In an even more preferred aspect of the present invention, the *nptII* gene has the sequence SEQ ID NO 21. Neomycin phosphotransferase II confers resistance to antibiotics of the aminoglycoside class, such as kanamycin and geneticin. The *nptII* gene used as a selection marker in obtaining transformed events is derived from the Escherichia coli Tn5 transposon as described by BECK et al. (1982). The NptII protein is produced by several prokaryotes widely found in the environment, both in aquatic and terrestrial habitats, as well as in human and animal intestinal microflora. The NptII protein inactivates aminoglycoside antibiotics such as neomycin, gentamicin, geneticin, paromycin and kanamycin A, B and C using adenosine triphosphate (ATP) to phosphorylate them, thus preventing them from causing injury to cells when exposed to the mentioned antibiotics. This mechanism allows its use as a marker for selecting transformed plants. In a preferred aspect of the present invention, the NptII protein has the sequence SEQ ID NO 35.

**[0083]** The use of selection marker genes, such as the *nptII* gene, is important for selecting cells transformed in the process of genetic modification (HORSCH et al., 1985). The objective of inserting the *nptII* gene in the event of the present invention was, therefore, the selection of cells transformed with the cry1Ac gene.

**[0084]** In addition to the expression cassettes described, additional expression cassettes may also be used in event CTC75064-3.

**[0085]** The first and second expression cassettes comprised in event CTC75064-3 may be introduced into the plant on the same or on different plasmids. If the first and second expression cassettes are located on the same plasmid and are introduced into the plant by an *Agrobacterium-mediated* transformation method, they may be present within the same or different regions of T-DNA. In one embodiment of the present invention, the first and second expression cassettes are present in the same region as the T-DNA.

**[0086]** More particularly, the event of the present invention was obtained by *Agrobacterium tumefasciens-mediated* transformation with a genetic construct comprising a DNA fragment (T-DNA) containing the cry1Ac and *nptII* gene expression cassettes. Preferably, the genetic construct of the present invention comprises the nucleotide of sequence SEQ ID NO:1.

**[0087]** The event of the present invention was obtained by *Agrobacterium tumefasciens*-mediated transformation containing the T-DNA fragment as defined above (SEQ ID NO:1).

**[0088]** This T-DNA fragment was inserted into a binary plasmid that contains in its host spectrum the bacteria *Escherichia coli* and *Agrobacterium tumefaciens.* Specific genetic elements and the origins of the components of the original binary plasmid of the present invention are shown in Figure 4. The binary plasmid comprising the construct of the present invention is depicted in Figure 5.

**[0089]** In a preferred embodiment, the genetic construct of the present invention comprises the sequence of SEQ ID NO:14.

**[0090]** Said construct is transferred to an *Agrobacterium tumefaciens* (vector) strain by techniques known to one of ordinary skill in the art, such as electroporation or thermal shock, among others.

**[0091]** In an even more preferred embodiment, the vector is an *Agrobacterium tumefaciens* strain EHA105.

**[0092]** A method for producing the event of interest is further described. In a preferred embodiment, the said method comprising the steps of:

a) introducing a genetic construct into an *Agrobacterium* strain;
b) obtaining embryogenic callus from immature leaf rolls or top stalks of sugarcane *(Saccharum* spp.);
c) co-cultivating embryogenic callus with a culture of *Agrobacterium*;
d) selecting transformed cells containing the functional fragment in culture medium containing aminoglycoside antibiotics; and
e) regenerating transformed sugarcane plants.

**[0093]** In one embodiment, the step a) of the method of producing a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3 comprises introducing a genetic construct comprising SEQ ID NO: 20 and SEQ ID NO: 21 into an *Agrobacterium* strain. In another embodiment, the step d) comprises selecting transformed cells containing the functional fragment in culture medium containing geneticin. Additionally, step e) of said method comprises regenerating transformed sugarcane plants, wherein the genetically modified sugarcane plants comprise SEQ ID NO: 20 and SEQ ID NO: 21. The invention also contemplates, a plant part, plant cell, plant tissue, or seed of the genetically modified sugarcane plants produced by the method described herein.

**[0094]** Those skilled in the art are familiar with the composition of suitable culture media for the generation of embryogenic callus (stage b), as well as the means of the co-cultivation stages (stage c: co-cultivation + rest), selection (stage d), and regeneration (stage e; regeneration + elongation). Preferably, the culture media used are based on compositions comprising ingredients such as MS salts (Murashige and Skoog, 1962), sucrose, and vitamins B5. Optionally, the following can also be added: amino acids selected from the group comprising proline and asparagine; casein hydrolysate; citric acid; mannitol; copper sulfate; glycine; gelling agent; auxins; antibiotics; acetosyringone; and selection agents. The use of auxins is especially important in embryogenic callus generation, co-cultivation and selection, as well as aminoglycoside antibiotics, preferable geneticin, in the selection medium.

**[0095]** The "co-cultivation" step refers to the incubation of plant tissue that has been infected or contacted with *Agrobacterium* to allow the transfer of *Agrobacterium* T-DNA to plant cells. This stage corresponds to the period from the moment immediately after inoculation (contact of *Agrobacterium* with plant tissue) until the moment the bacterium is removed or inactivated.

**[0096]** Inoculated tissue may be co-cultured for about 1 to 30 days, preferably from 1 to 20 days, or more preferably from 1 to 10 days.

**[0097]** During the co-cultivation step, the temperature may be any suitable temperature known in the art for the target plant. Illustratively, for sugarcane, the temperature may range from about 15 °C to about 30 °C and from about 16 °C to about 29 °C. In some embodiments, the co-cultivation step occurs in the absence of light.

**[0098]** Following co-cultivation with *Agrobacterium,* the medium is removed, and the cells are transferred to a culture medium lacking *Agrobacterium.* The cells are then incubated in the dark at a temperature between about 20 °C and about 26 °C for a period of 1 to 20 days.

**[0099]** The method provided herein further includes selecting cells comprising at least one copy of the genetic sequence of interest. "Select" as used herein means the situation in which a selective agent is used for transformants, wherein said selective agent will allow preferential growth of plant cells containing at least one copy of the marker gene positioned within the T-DNA. Whereas, those cells that were not transformed will not contain the marker gene that permits survival in the selective agent. As indicated above, any suitable selection marker may be used. Preferably, the selection marker gene used is the *nptII*gene, which encodes an enzyme that confers resistance to aminoglycoside antibiotics, such as geneticin.

**[0100]** In some embodiments, an agent that inhibits *Agrobacterium* growth is also added.

**[0101]** Selection may occur under light or dark conditions, depending on the plant species being transformed and on the genotype, for example. In some cases, embryogenic callus or other tissues undergoing transformation may be sub-cultured at regular or irregular intervals in the same medium. In the case of callus transformation, individual calluses can be kept separate to ensure that only one plant is regenerated by each callus (thus ensuring that all regenerated plants are derived from independent transformation events). In a preferred embodiment, the selection step occurs in the dark using geneticin as a selection agent for about 1 to 10 weeks. More preferably the selection step occurs for about 2 to 5 weeks.

**[0102]** After the selection period, plant tissue that has continued to grow in the presence of the selection agent, and has therefore been genetically modified, can be manipulated and regenerated by placing it in suitable culture media and growth conditions. The transgenic plants thus obtained can be tested for the presence of the DNA of interest. For the purpose of this invention, the term "regenerate" refers to the formation of a plant comprising both an aerial part and roots. Regenerated plants can be planted on suitable substrate (such as soil) and transferred to the greenhouse. As used herein, "genetically modified" or "transgenic" or "stably transformed" means a plant cell, plant part, plant tissue, or plant that comprises a DNA sequence of interest that is introduced into its genome by transformation.

**[0103]** In one embodiment, the bacterium is of the genus *Agrobacterium.*

**[0104]** In a more preferred embodiment, the bacterium is *Agrobacterium tumefaciens.*

**[0105]** In an even more preferred embodiment, the bacterium is an *Agrobacterium tumefaciens* strain EHA105.

**[0106]** The present invention also relates to the characterization of the selected event (CTC75064-3) and methods of detecting plant material derived therefrom. Analytical methods for detection and characterization of transgenic plants include indirect methods (protein-based detection methods) or direct methods (DNA-based detection methods).

**[0107]** The definition of the T-DNA stable integration site in the host cell genome and the characterization of its flanking sequences is necessary for the development and validation of methodologies for the unambiguous identification and characterization of the event.

**[0108]** To identify the flanking regions at the ends of the T-DNA insert in event CTC75064-3, several DNA amplification and sequencing experiments were performed. Inverse PCR (iPCR) assays were performed at both ends of the T-DNA to isolate and clone the flanking regions of the insert. Subsequently, the fragments obtained and isolated were sequenced using the Sanger method to validate the results obtained by iPCR. The genetic insertion map present in event CTC75064-3 resulting from the data generated by these experiments is shown in Figure 3 and SEQ ID NO: 2. The flanking sequences of event CTC75064-3 are shown in SEQ ID NO: 23 and SEQ ID NO: 24.

**[0109]** According to one aspect of the invention, a polynucleotide comprising at least 14 contiguous nucleotides of the 26-nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a polynucleotide comprising at least 15 contiguous nucleotides of the 26-nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a polynucleotide comprising at least 16 contiguous nucleotides of the 26-nucleotides of SEQ ID NO: 18 is provided. In one embodiment, said polynucleotide comprises at least 17 contiguous nucleotides of the 26 - nucleotides of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 18 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 19 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 20 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 21 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 22 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 23 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 24 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises at least 25 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, said polynucleotide comprises SEQ ID NO: 18. In one further aspect of the invention, said polynucleotide comprises SEQ ID NO: 13.

**[0110]** According to one aspect of the invention, a polynucleotide comprising at least 14 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO 19 is provided. In one embodiment, a polynucleotide comprising at least 15 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO 19 is provided. According to one aspect of the invention, a polynucleotide comprising at least 16 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO 19 is provided. In one embodiment, a polynucleotide comprising at least 17 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 18 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 19 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 20 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 21 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 22 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 23 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising at least 24 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. According to one aspect of the invention, a polynucleotide comprising at least 25 contiguous nucleotides of the 26 - nucleotide of SEQ ID NO: 19 is provided. In one embodiment, a polynucleotide comprising SEQ ID NO: 19 is provided. In one aspect of the invention, a polynucleotide comprising SEQ ID NO: 12 is provided.

**[0111]** In a further aspect of the present invention a polynucleotide comprising the sequence of SEQ ID NO: 5 is provided. In still another aspect of the invention, a polynucleotide comprising the sequence SEQ ID NO: 22 is provided.

**[0112]** According to one aspect of the invention, there is provided a plant comprising at least 14 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, a plant comprising at least 15 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. According to one aspect of the invention, there is provided a plant comprising at least 16 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18. In one embodiment, a plant comprising at least 17 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 18 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 19 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 20 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 21 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 22 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 23 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 24 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising at least 25 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 18 is provided. In one embodiment, a plant comprising SEQ ID NO: 18 is provided. In an additional embodiment, a plant comprising SEQ ID NO: 13 is provided.

**[0113]** According to one aspect of the invention, there is provided a plant comprising at least 14 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19. In one embodiment, a plant comprising at least 15 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 16 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 17 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 18 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19. In one embodiment, a plant comprising at least 19 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 20 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 21 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 22 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 23 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 24 contiguous nucleotides of the 26 - nucleotide of sequence SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 24 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising at least 25 contiguous nucleotides of the 26 - nucleotide sequence of SEQ ID NO: 19 is provided. In one embodiment, a plant comprising SEQ ID NO: 19 is provided. In an additional embodiment, a plant comprising SEQ ID NO: 12 is provided.

**[0114]** In one embodiment of the present invention, said plant is a genetically modified sugarcane *(Saccharum* spp.) plant. Additionally, said plant is insect resistant and comprises the sequence SEQ ID NO: 5. Still in a further aspect, the insect resistant plant of the present invention comprises SEQ ID NO: 22. In a further embodiment, said plant is an insect-resistant sugarcane plant of event CTC75064-3 or a plant derived therefrom.

**[0115]** In one aspect of the invention, event CTC75064-3 is a sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 5. In a further aspect, event CTC75064-3 comprises SEQ ID NO: 22.

**[0116]** In other embodiment a specific method for detection and identification of CTC75064-3 event is provided.

**[0117]** According to the present invention, there is provided a method of detecting plant material derived from genetically modified sugarcane of event CTC75064-3 comprising the steps of:

a) obtaining a plant material sample for analysis;

b) extracting DNA from the sample;

c) providing primer pairs comprising at least a forward and a reverse primer;

d) amplifying a region between the primer pairs; and

e) detecting the presence of a product from amplification.

**[0118]** In one embodiment, primer pairs (step c) according to the detection method described are designed to bind to a polynucleotide comprising contiguous nucleotides of SEQ ID NO 2.

**[0119]** In another embodiment, the primer pairs in step c) are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one pair of primers comprises contiguous nucleotides sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31.

**[0120]** In one embodiment, the primer pairs above (step c) are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair comprises at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31. In one embodiment, the primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair comprises at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23,SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31. In addition, the primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair comprises at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31.

**[0121]** Additionally, primer pairs according to the detection method described are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, where at least one primer pair consists of a first primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and a second primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36.

**[0122]** In one embodiment, the primer pairs according to the detection method described are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair consists of a first primer comprising at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and a second primer comprising at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36. In an additional embodiment, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair consists of a first primer comprising at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and a second primer comprising at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36. In addition, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one primer pair consists of a first primer comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23,SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and a second primer comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36.

**[0123]** In one embodiment, primer pairs according to the detection method described are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one pair of primers comprises contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39. In one embodiment, primer pairs according to the detection method described are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair comprises at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39. In one embodiment, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair comprises at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39. In addition, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair comprises at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:

38 and SEQ ID NO: 39.

**[0124]** In one embodiment, primer pairs according to the detection method described are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair consists of a first primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39 and a second primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36. In one embodiment, primer pairs, according to the detection method described, are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair consists of a first primer comprising at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39 and a second primer comprising at least 3 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36. In one embodiment, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 36 and SEQ ID NO: 37, wherein at least one primer pair consists of a first primer comprising at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4 SEQ ID NO: 38 and SEQ ID NO: 39 and a second primer comprising at least 7 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO 36. Additionally, primer pairs are designed to bind to a polynucleotide comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one primer pair consists of a first primer comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39 and a second primer comprising at least 14 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36.

**[0125]** It is well known, especially to those skilled in the art, that the DNA molecule (or DNA) is made up of two strands (of nucleotides) that are held together by hydrogen bridges between the nucleotide bases. Pairing occurs according to the complementarity of the bases, following the general rule of: Adenine with Thymine and Cytosine with Guanine. Thus, although representation of nucleotide sequences is performed for only one of the strands, their complementary strand or complementary sequence is included within the scope of this invention and is considered for the definition of primers and probes described herein.

**[0126]** Methods for obtaining samples for DNA extraction are widely known to one of ordinary skill in the art and include the collection of any plant material derived from the CTC75064-3 transgenic event such as stalks, roots, and leaves. Preferably, the samples are obtained from intact leaves. Plant DNA extraction methods include, without limitation, those based on the use of CTAB detergent (Alianabi et al., 1999), (optionally) followed by further sample purification with cesium chloride or ammonium acetate, as well as other commercially available methods.

**[0127]** Primer pairs suitable for use in this detection method may be designed using parameters well known to those skilled in the art of molecular biology now that SEQs ID Nos: 2, 3, 4, 5, 22, 23, 24, 29, 30, 31, 36, 37, 38 and 39 have become available. For example, one or both primers of the pair may be designed to be construct-specific, trait gene-specific, promoter-specific, sequence-specific to the junction between inserted DNA and genomic DNA, and/or flanking sequence-specific.

**[0128]** There are many amplification methods that can be used in accordance with this aspect of the invention. One of the most common amplification techniques known to those skilled in the art, is the polymerase chain reaction (PCR). The amplification product of a PCR reaction can be visualized by staining the nucleotide chain with a fluorescent tag such as ethidium bromide and then exciting it with UV light (typically after size separation using agarose gel electrophoresis).

**[0129]** One embodiment of the present invention employs variations of the PCR principle such as quantitative real-time PCR, nested PCR, inverse PCR (iPCR), digital PCR, Long PCR, Touchdown PCR, Hot Start PCR, Multiplex PCR, among others. The amplification product can also be detected by different methodologies which are contemplated in the present invention, such as the SYBR Green ™ system which emits fluorescence when this reagent binds to double stranded DNA and the Taqman® system where detection is based on the interaction of fluorescent probes. The Taqman® methodology uses a probe that is complementary to the intended PCR product segment located between the reaction primers. During the hybridization stage of the PCR cycle the probe is bound to the target DNA, and during Taq polymerase extension, through its 5'-exonuclease activity, it removes the probe, releasing the fluorochrome and emitting fluorescence. Additional embodiments of this aspect of the present invention include but are not limited to: loop-mediated isothermal amplification (LAMP), capillary gel electrophoresis (CGE), microarray technology Luminex, "DNA walking" and Next Generation Sequencing (NGS), Sanger method, Illumina, among others.

**[0130]** The present invention describes a specific detection methodology based on the quantitative real-time PCR (qPCR) technique known as "Plus-Minus" or "Presence - Absence," presenting two variations of the methodology: SYBR GREEN ™ and Taqman ® technology.

**[0131]** In one embodiment of the present invention, primer pairs are provided wherein the forward primer consists of

SEQ ID NO: 6 and the reverse primer consists of SEQ ID NO: 7 and/or the forward primer is SEQ ID. NO: 8 and the reverse primer is SEQ ID NO: 9.

**[0132]** In an additional embodiment, the primer pairs used in step c) of the method of detecting plant material from genetically modified sugarcane of event CTC75064-3 comprise forward primer consists of SEQ ID NO: 6 and the reverse primer consists of SEQ ID NO: 7 and/or the forward primer is SEQ ID NO: 8 and the reverse primer is SEQ ID NO: 9. In addition, the amplicon (product from amplification) produced by the primers of SEQ ID NO: 6 and SEQ ID NO: 7 is viewed through a labeled probe of SEQ ID NO: 10. Alternatively, the amplicon produced by the primers of SEQ ID NO: 8 and SEQ ID NO: 9 is visualized through a labeled probe of SEQ ID NO 11. Thus, it is an aspect of the present invention that detection of the of the product from amplification obtained by the use of primers SEQ ID NO: 6 and SEQ ID NO: 7 and/or SEQ ID NO: 8 and SEQ ID NO: 9 is performed through hybridization of a probe comprising SEQ ID NO: 10 or SEQ ID NO: 11.

**[0133]** In one embodiment of the present invention, the region amplified by said method (the amplicon or product from amplification) is between 80 and 1000 base pairs in length. In an additional embodiment, the amplicon is between 100 and 300 base pairs in length. In one preferred embodiment, the amplicon obtained using the primers SEQ ID NO: 6 and SEQ ID NO: 7 is 107 base pairs in length, as defined by SEQ ID NO: 12. In another preferred embodiment, the amplicon obtained through the use of primers SEQ ID NO: 8 and SEQ ID NO: 9 is 135 base pairs in length, as defined by SEQ ID NO: 13.

**[0134]** Figures 6 (event-specific detection reaction of the invention via Taqman®) and 7 (SYBR GREEN ™ assay) represent the validation of both Methods.

**[0135]** Primers and probes described in the present invention may be used in combination to detect the CTC75064-3 event. Thus, a further embodiment of the present invention involves the use of multiplex PCR to identify plant material from the CTC75064-3 event.

**[0136]** Alternative primers and probes for the detection and characterization of the CTC75064-3 event are included in the invention. These and other variations may be used with but are not limited to any of the direct detection methods described above.

**[0137]** Additionally, the CTC75064-3 event can be detected from plant material by hybridizing DNA samples to the probes. Specifically, the present invention describes a method of detecting material from the genetically modified sugarcane event CTC75064-3 which comprises the steps of:

a) obtaining a plant material sample for analysis;

b) DNA or RNA extraction from the sample;

c) providing a probe or a combination of probes designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 29, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38 and SEQ ID NO 39, wherein the polynucleotide is single stranded;

d) hybridizing said probe or a combination of probes with the sample, and

e) detecting the actual hybridization of the probe or a combination of probes.

**[0138]** In preferred embodiment, the present invention describes a method of detecting material from the genetically modified sugarcane event CTC75064-3 which comprises the steps of:

a) obtaining a plant material sample for analysis;

b) DNA or RNA extraction from the sample;

c) providing a probe designed to bind to a polynucleotide comprising 14 or more contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33, wherein the polynucleotide is single stranded;

d) hybridizing said probe with the sample, and

e) detecting the actual hybridization of the probe.

**[0139]** According to one aspect of the invention, a probe designed to bind to a polynucleotide comprising at least 15 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33 is provided. In one embodiment, a probe designed to bind to a polynucleotide comprising at least 16 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33 is provided. According to one aspect of the invention, a probe designed to bind to a polynucleotide comprising at least 17 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33 is provided. In one embodiment, said polynucleotide comprises at least 18 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 19 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 19 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO : 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 20 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 21 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 22 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 23 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. In one embodiment, said polynucleotide comprises at least 24 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33. According to one aspect of the invention, a polynucleotide comprising at least 25 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33 is provided. According to one aspect of the invention, a polynucleotide comprising at least 26 contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 32 and SEQ ID NO: 33 is provided.

**[0140]** The probe may be, for example, a PCR product or a restriction digest fragment. In a further embodiment, the probe as described herein may be labeled with a fluorescent, radioactive, enzymatic, or other label suitable to enable hybridization to be detected. The person skilled in the art will now know how to design suitable probes given the advantage of the present disclosure.

**[0141]** In an additional embodiment, a probe hybridization method is provided to the sample under stringent conditions (high specificity). Stringent hybridization conditions are well known to those skilled in the art. Examples of stringent conditions include: hybridization at a temperature of approximately 65 ° C in a solution containing 6x SSC, 0.01% SDS and 0.25% skimmed milk powder followed by washing at the same temperature in a solution containing 0.2 x SSC and 0.1% SDS.

**[0142]** Suitable techniques for detecting plant material derived from event CTC75064-3 based on the hybridization principle include, but are not limited to, Southern Blots and *in situ* hybridization. One of skill in the art is familiar with such techniques.

**[0143]** Typically, these techniques involve incubating a probe with a sample, washing to remove the unbound probe, and detecting whether the probe has hybridized. Said detection method is dependent upon the type of label attached to the probe. For example, a radio-labelled probe can be detected by exposure to and development of X-ray film. Alternatively, an enzymatically labeled probe may be detected by converting a substrate to effect a color change.

**[0144]** Additionally, another aspect of the invention contemplates a method for detecting plant material derived from event CTC75064-3, which comprises: obtaining a sample for analysis; providing an antibody designed to bind to a Cry or NptII protein contained within a plant comprising at least 14 contiguous nucleotides of SEQ ID NO: 18 and/or SEQ ID NO: 19; incubating said antibody with the sample; and detecting whether the antibody bound. In one embodiment of the present invention, said Cry protein is encoded by nucleotide sequence SEQ ID NO: 20 and said NptII protein is encoded by nucleotide sequence SEQ ID NO: 21. In an additional embodiment, said Cry protein comprises SEQ ID NO: 34 and said NptII protein comprises SEQ ID NO: 35.

**[0145]** Suitable methods for detecting plant material derived from the CTC75064-3 event based on said antibody binding include (but are not limited to): western blots, ELISA (Enzyme-Linked ImmunoSorbent Assays), and mass spectrometry (e.g. surface-enhanced laser desorption/ionization (SELDI)). One of skill in the art is familiar with these immunological techniques. Typical steps include incubating a sample with an antibody that binds to the Cry or NptII protein, washing for removal of unbound antibody, and detecting whether the antibody has bound. Many such detection methods are based on enzymatic reactions: for example, the antibody may be linked with an enzyme such as peroxidase and upon application of a suitable substrate, a color change is detected. Such antibodies may be monoclonal or polyclonal.

**[0146]** Another aspect the invention contemplates a method for detecting plant material derived from event CTC75064-3, which comprises: obtaining a sample for analysis; providing a protein extract from the sample; providing test strips designed to detect the presence of a Cry or NptII protein in a plant comprising at least 14 contiguous nucleotides of SEQ ID NO: 18 and/or SEQ ID NO: 19; incubating the test strips with the sample; and detecting. In one embodiment of the present invention, said Cry protein is encoded by nucleotide sequence SEQ ID NO: 20 and the NptII protein is encoded by nucleotide sequence SEQ ID NO: 21. In an additional embodiment, said Cry protein comprises SEQ ID NO: 34 and said NptII protein comprises SEQ ID NO: 35.

**[0147]** In one embodiment of the invention there is provided a method for detecting plant material derived from the CTC75064-3 event, said method comprising: obtaining a sample derived from the CTC75064-3 event and a sample from a non-transgenic sugarcane variety for analysis (control); subjecting one or more insects of the species *Diatraea saccharallis* (susceptible to Cry1Ac) to the samples; detecting an insecticidal effect on the insects. In this aspect of the invention, "insecticide" refers to any inhibitory effect on the insect (including but not limited to): reduced feeding, retarded growth, reduced fecundity, paralysis, and death.

**[0148]** The method of detecting plant material from event CTC75064-3 includes, but is not limited to, biological leaf feeding assays where a leaf or other suitable part of the plant of event CTC75064-3, or any plant material derived from event CTC75064-3, is infested with one or more insect pests. Measurement of said detection can include assessing leaf or plant damage after adjusted time periods, assessing mortality or assessing other insecticidal effects. Such biological assays may be performed in the field or greenhouses and may entail either natural or artificial insect infestation.

**[0149]** In another aspect of the invention, a kit for detecting in a plant sample the presence of event CTC75064-3 is provided, said kit comprising: a means for detecting the presence of a polynucleotide comprising at least 14 contiguous nucleotides of the sequence of SEQ ID NO: 18 and/or SEQ ID NO: 19 and/or a pesticidal crystal protein (Cry). In one embodiment of the present invention, said kit may comprise DNA amplification detection technology such as PCR, qPCR, or Taqman®. In a further embodiment of the present invention, said kit may comprise probe hybridization detection technology such as Southern Blots or *in situ* hybridization. In one aspect, the means to detect material from transgenic sugarcane comprising a Cry1Ac protein (event CTC75064-3) comprises primer pairs designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one pair of primers comprises contiguous nucleotides sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31. Additionally, the means comprise primer pairs, wherein the forward primer comprises SEQ ID NO: 6 and the reverse primer comprises SEQ ID NO: 7, or the forward primer comprises SEQ ID NO: 8 and the reverse primer comprises SEQ ID NO: 9. In a further embodiment, the means to detect material from transgenic sugarcane comprising a CrylAc protein (event CTC75064-3) comprises a probe comprising SEQ ID NO: 10 or SEQ ID NO: 11. In another embodiment of the present invention, said kit may comprise antibody binding detection technology such as western blots, ELISAs, mass spectrometry (SELDI) or test strips. In a further embodiment of the present invention, said kit may comprise detection technology by biological insect testing such as leaf feeding biological assays or biological mortality assays. In a further embodiment of the present invention, said kit may comprise any combination of the detection technologies mentioned above.

**[0150]** The transgenic event as described in the present invention affect insects of one or more species of the group comprising insects of the order Lepidoptera. As a result, a reduced number of insecticidal sprays is required during cultivation of said plant compared with a non-transgenic sugarcane plant of the same variety.

**[0151]** The present invention is not itself bound to event CTC75064-3; rather, it is further extended to include any plant material derived therefrom, including seed, provided they contain at least one of the polynucleotides sequences of the present invention. In one embodiment, the present invention comprises a plant part, plant cell, plant tissue, or seed from the genetically modified sugarcane *(Saccharum* spp.) plant, wherein said plant part, plant cell, plant tissue, or seed comprises SEQ ID NO: 18 or SEQ ID NO: 19. In other embodiment, the invention contemplates plant part, plant cell, plant tissue, or seed comprising SEQ ID NO: 12 or SEQ ID NO: 13. Additionally, the invention includes a plant part, plant cell, plant tissue, or seed comprising SEQ ID NO: 5 or SEQ ID NO: 22. The present invention also includes, but is not limited to, plants that are derived from crossbred lineages with the CTC75064-3 event or a derivative thereof by conventional or other crossbreeding methods; thus, one embodiment of the present invention relates to the use of a plant, plant cell, plant part, or seed from the genetically modified sugarcane *(Saccharum* spp.) plant as described herein, which is used for regenerating a plant, planting, cultivating a field of plants, or producing a plant product. The present invention also contemplates a tissue culture of a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 18 or SEQ ID NO: 19. In other embodiment, the invention includes a tissue culture of a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 12 or SEQ ID NO: 13. Also contemplate in the present invention is a tissue culture of a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 5 or SEQ ID NO: 22. Additionally, a genetic modified sugarcane *(Saccharum* spp.) plant regenerated from the tissue culture describe above is also included in the present invention, wherein the regenerate plant comprises SEQ ID NO: 18 or SEQ ID NO: 19. Examples of plant cells and plant parts include but are not limited to: suspension cells, callus, somatic embryos, meristematic tissue, top stalks, stalks, leaf, leaf discs, tiller, shoots. Another aspect contemplates a method for producing an insect-resistant sugarcane *(Saccharum* spp.) plant, comprising crossing a first sugarcane plant with a second sugar cane plant, wherein the second sugar cane plant is a plant comprising event CTC75064-3, and producing offspring sugarcane plants therefrom. The plant comprising event CTC75064-3 is a genetically modified sugarcane

*(Saccharum* spp.) plant comprising SEQ ID NO: 18 or SEQ ID NO: 19. The present invention also contemplates a sugarcane *(Saccharum* spp.) plant and plant parts, plant cells, plant tissues, or seeds therefrom produced by the method for producing an insect-resistant sugarcane described above.

**[0152]** In a further embodiment, the present invention provides a commodity product, produced from a sugarcane plant comprising event CTC75064-3. Thus, the invention includes a commodity product, produced from a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 18 or SEQ ID NO: 19. In one embodiment, the invention contemplates a commodity product, produced from a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 12 or SEQ ID NO: 13. Additionally, the invention includes a commodity product, produced from a genetically modified sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 5 or SEQ ID NO: 22. Examples of commodity products include but are not limited to: bagasse, sugarcane juice, syrup, first generation ethanol (produced from sugarcane juice), second generation ethanol (cellulosic ethanol; produced from biomass), biomass, sugar, raw sugar, refined sugar, molasses, vinasse, and fiber.

**[0153]** The present invention further provides a plant material from CTC75064-3 event comprising additional polynucleotide sequences, modified or smaller than CTC75064-3, or exhibit other phenotypic characteristics. For example, the plant material CTC75064-3 event could be transformed to produce a new event comprising additional characteristics, such as, a second insect resistance gene. This process is known as gene stacking. Such second insect resistance gene codes, for example, insecticidal lectins, insecticidal protease inhibitors and other insecticidal proteins derived from *Bacillus thuringiensis.*

**[0154]** The present invention further provides an insect control method comprising providing plant material derived from the CTC75064-3 event at a location where said insects feed. The invention further provides an insect control method comprising providing the CTC75064-3 derived plant material at the site where said insects feed and applying other agrochemical reagents to said plant material (e.g. herbicides, fungicides, and the like).

**[0155]** In other embodiment, the invention describes, a method of making a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3, comprising introducing a genetic modification to a sugarcane *(Saccharum* spp.) plant comprising SEQ ID NO: 5 or SEQ ID NO: 22 to produce a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3, wherein the genetically modified sugarcane *(Saccharum* spp.) plant has improved insect resistance as compared to a sugarcane *(Saccharum* spp.) plant without the genetic modification. In one additional embodiment, the invention provides a method of cultivating a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3, comprising growing a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3 comprising SEQ ID NO: 5 or SEQ ID NO: 22 under conditions comprising insect infestation, wherein the genetically modified sugarcane *(Saccharum* spp.) plant has an increase in insect resistance as compared to a sugarcane *(Saccharum* spp.) plant without the genetic modification grown under the same conditions. The invention also provides, a genetically modified sugarcane *(Saccharum* spp.) plant of event CTC75064-3 comprising SEQ ID NO: 5 or SEQ ID NO: 22.

## **Description of Transgenic Sugarcane** 'CTC75064-3'

**[0156]** Transgenic hybrid sugarcane 'CTC75064-3' plants are genetically substantially equal and phenotypically similar to the recipient (host) of the recombinant molecule, the parental plant variety' RB 867515, a commercial Brazilian sugarcane variety (Plant Variety Protection/PVP - protocol number - 21806.000439/2000-45; PVP Certificate № 271), but with a new and particular feature (Cry1Ac expression), which guarantees the insect resistance to sugarcane borer *D. saccharalis* (Lepidoptera). As its parental variety 'RB 867515, 'CTC75064-3' is a modern sugarcane hybrid that holds several desirable agronomic characteristics such as, genetic potential for high ratoon cane sprouting vigor, high cane yield, excellent ratooning, medium-late maturity, high sucrose content and is tolerant to leaf scald, smut, rust diseases and sugarcane mosaic.

**[0157]** Briefly, 'CTC75064-3' plants are characterized by purple stalks with purple hues when exposed to the sun and by purple-green stalks where not exposed to sun. 'CTC75064-3' plants exhibit medium-long size curved internodes and medium width yellow greenish growth rings. Smooth appearance of internodes and absent of growth crack, with a medium waxiness. 'CTC75064-3' plants exhibit an obovate bud shape with an apical position of the pubescence on the bud. Leaf architecture is erect with curved tips curvature. The average auricle shape is lanceolate, presenting crescent-shaped ligule.

**[0158]** The average mature stalk height (measured at 330 Harvest Day after Planting - DAP, except for Juazeiro, measured at 210 DAP; measured from the crown until the insertion of leaf +1), stalk diameter (measured at 330 Harvest Day after Planting - DAP, expect for Juazeiro, measured at 210 DAP), number of tillers (at 30, 60, 90, 120, 150, 180, 210, 240, 270, 300 and 330 DAP; not measured in Juazeiro-BA), weight (measured at 330 DAP, expect for Juazeiro, measured at 210 DAP), sugar content (BRIX %; measured at 330 DAP, expect for Juazeiro, measured at 210 DAP; extracted juice), flowering (observations during all developmental cycle until 330 DAP, expect for Juazeiro, data collected until 210 DAP) and tons of pol% (% of sucrose in the sugarcane juice) per hectare (TPH; measured at 330 Harvest Day after Planting - DAP, expect for Juazeiro, measured at 210 DAP) were evaluate in six different locations in comparison with the parental variety 'RB867515'. TPH is calculated according to the formulae:

$$TPH = (Pol\,\%\,Cana\;x\;TCH)/100$$

**[0159]** For each data set, data across all sites were combined for statistical analysis. Combined site analysis was done using the following statistical model:

$$y_{ijk} = \mu + G_k + (S)_i + B(S)_{ij} + (SG)_{ik} + \varepsilon_{ijk},$$

,wherein $y_{ijk}$ is the measurement of replicate *j* on site *i* for treatment *k*; $\mu$ is the overall mean; $S_i$ is the effect of site *i* (*i* = 1 to 6); $B_j$ is the effect of replicate *j* (j = 1 to 4); $B(S)_{ij}$ is the effect of replicate *j* on site *i* (j = 1 to 4); $G_k$ is the effect of the treatment *k* (k = 1 to 7); $(SG)_{ik}$ is the interaction between site *i* and treatment *k*; $\varepsilon_{ijk}$ is the experimental residual error on site j and treatment k.

**[0160]** The main effects analysis and model interaction were performed as described by Kuznetsova et al. (2017). All data were analyzed using mixed linear model by package lme4 (Bates et al., 2015).

**[0161]** The results of the agronomic and phenotypic characteristics analysis are shown in the table below and corroborate the conclusion that 'CTC75064-3' is similar to its parental variety ('RB867515').

Table 01. Average of agronomic and phenotypic characteristics for 'CTC75064-3' and 'RB867515' parental variety. Combined analysis for the Barrinha-SP, Piracicaba-SP, Valparaíso-SP, Quirinópolis-GO, Mandaguaçu-PR and Juazeiro-BA locations. Data from Juazeiro-BA collected at 210 DAP and other locations at 330 DAP.

| | Parameter | Mean | | SE | Range** | |
|---|---|---|---|---|---|---|
| | | CTC75064-3 | RB867515 | | Min | Max |
| Combined analysis | Height (m) | 2.38* | 2.63 | 0.18 | 2.07 | 3.52 |
| | Diameter (cm) | 23.94* | 27.97 | 0.57 | 23.02 | 32.59 |
| | Tillers (330 DAP) † | 64.08 | 60.75 | 12.07 | 30 | 88.5 |
| | Weight (Kg) | 10.24* | 15.32 | 0.87 | 8.71 | 21.88 |
| | BRIX (%) | 17.53* | 15.66 | 0.66 | 13.03 | 20.4 |
| | TPH | 6.01 | 6.05 | 0.98 | 6.61 | 8.69 |
| | Flowering (Total No. of Panicles) | 0 | 0 | - | 0 | 0 |

* Significant difference between the transgenic event and the parental variety by the t test at the level of 5% ($p \leq 0.05$). SE: Standard Error; **Range: Average minimum and maximum values observed in 3 commercial reference varieties. † Not evaluated in Juazeiro (BA).

**[0162]** Other compositional studies have been made and also demonstrated that 'CTC75064-3' is highly similar to its parental variety ('RB 867515') [compositional parameters related to nutrition and the use of sugarcane in the diet, as defined by the OECD Guidance Document (OECD, 2011)]. Based on the results of combined data analysis (six representative locations in the Brazilian sugarcane growing regions), the data suggest that the presence of proteins Cry1Ac and NPTII contained in samples of whole plant and stalk does not significantly interfere in the bromatological composition of the event CTC75064-3 compared to the parental variety RB 867515, and that the compositional equivalence observed for the 11 parameters evaluated corroborates with the premise of a high similarity between the event CTC75064-3, and the parental variety RB 867515.

Table 02. Mean values of compositional parameters measured in the genetically-modified event 'CTC75064-3' and its conventional counterpart 'RB 867515'.

| | Analyte | Mean | | SE | Range** | |
|---|---|---|---|---|---|---|
| | | CTC75064-3 | RB867515 | | Min | Max |
| Combined analysis | Dry matter | 22.65* | 20.44 | 0.92 | 16.15 | 24.61 |
| | Moisture | 75.05 | 77.51 | 1.36 | 65.69 | 82.26 |
| | Crude fiber[1] | 27.19* | 29.85 | 1.11 | 22.25 | 33.23 |
| | NDF[1] | 49.29* | 52.35 | 1.61 | 41.36 | 56.65 |
| | ADF[1] | 31.69* | 35.25 | 1.16 | 26.29 | 38.41 |
| | Crude protein[1] | 3.26 | 3.19 | 0.21 | 2.19 | 3.91 |
| | Crude fat[1] | 0.8 | 1.1 | 0.12 | 0.66 | 1.48 |
| | Ash[1] | 3.38 | 3.73 | 0.36 | 1.83 | 4.99 |
| | Sucrose[2] | 10.04* | 8.49 | 0.57 | 4.99 | 13.49 |
| | Glucose[2] | 0.89* | 1.03 | 0.12 | 0.53 | 1.82 |
| | Fructose[2] | 0.71 | 0.8 | 0.1 | 0.44 | 1.33 |

[1]Results are expressed on dry weight basis; [2]Values expressed sugarcane stalk basis; *Significant difference between the transgenic event and the parental variety by the t test at the 5% level (p 0.05). EP: Standard Error; **Range: Minimum and maximum average values observed in 3 commercial reference varieties observed in the individual analyzes.

## Examples

### Example 1. CTC75064-3 event Generation - *Agrobacterium* transformation

**[0163]** Event CTC75064-3 was obtained by *Agrobacterium tumefasciens*-mediated genetic transformation of the RB 867515 cultivar.

**[0164]** The RB 867515 cultivar is a commercial hybrid and is the donor genotype of the CTC75064-3 event (genetic background); that is, it represents the untransformed counterpart of the CTC75064-3 event. This cultivar has medium-late maturation and has been planting specially on Brazilian Center-South region. As with other commercial sugarcane hybrids, it is high-ploidy material with numerous chromosomes derived from its two parental varieties: S. *officinarum* and *S. spontaneum* (DANIELS and ROACH, 1987; SREENIVASAN et al., 1987).

**[0165]** The CTC75064-3 event has the *cry1Ac* gene, which confers resistance to *Diatraea saccharalis,* and the *nptII* gene, used as a selection marker. The purpose of the development of the CTC75064-3 event is to provide a proactive control of the sugarcane borer. It is expected that, after the hatching of the pest eggs on the leaves of the CTC75064-3 event, the young larvae begin to feed and, when ingesting the CrylAc protein, are controlled before they penetrate the culms of the CTC75064-3 event, avoiding if so the economic damage of the pest to the crop. The expression of the *cry1Ac* gene is regulated by a promoter region comprising doubled enhanced promoter CaMV 35s (2xCaMV35s), wheat leader sequence L-Cab, OsACT1 intron and Kosak sequence 5' upstream from the translational start site. The expression of *nptII* gene is regulated by the maize ubiquitin gene promoter UBI-1, which has an endogenous intron. CrylAc expression cassette uses CaMV 35S terminator and the *nptII* cassette uses the *Agrobacterium tumefaciens* nopaline synthase (nos) terminator.

### 1.1 Construct development using *cry1Ac* and *nptII* genes (Figure 5; SEQ ID NO 14).

**[0166]** Conventional gene cloning techniques using commercial bacterial plasmids, restriction enzyme digestion, and fragment ligation (with ligases) were used to develop the construct of the present invention (Figure 5).

**[0167]** The construct of the present invention was developed by joining the 2xCaMV35s-cry1Ac-T35s and UBI-*nptII*-TNOS cassettes. T-DNA containing both cassettes was transferred from a cloning plasmid to the base plasmid (Figure 4: binary plasmid vector, which contains in its host spectrum the bacteria *Escherichia coli* and *Agrobacterium tumefaciens*) using restriction enzymes, generating the construct of the present invention (Figure 5; SEQ ID NO: 14).

**[0168]** After the final cloning step, the construct (SEQ ID NO: 14) was inserted into *Escherichia coli* strain TOP10 using heat shock. An isolated colony containing the construct was inoculated into liquid LB medium supplemented with

150 μg/ml spectinomycin and incubated at 37°C while shaking at 250 rpm for a period of 16 hours. Stocks were then prepared containing bacterial suspension and 10% (v/v) glycerol, which were stored in an ultrafreezer at -80°C.

**[0169]** The construct of the present invention was then transferred from *E. coli* to *Agrobacterium tumefaciens* strain EHA105 by isolation and purification of plasmid DNA and transformation of *Agrobacterium* by electroporation. As with the E. *coli* strain, stocks containing the bacterial suspension of Agrobacterium and 10% (v/v) glycerol were stored in an ultrafreezer at -80°C.

1.2 *Agrobacterium*-mediated plant transformation

**[0170]** To obtain embryogenic callus, young RB 867515 sugarcane leaf rolls, grown in the field or greenhouse for up to 12 months, were collected for isolation of the initial explants.

**[0171]** After surface disinfection, transverse sections about 0.05-5mm thick were cut from above the meristem under aseptic conditions. The sections were placed on the surface of the callus induction culture medium [MS - Murashige and Skoog, 1962; sucrose, vitamins B5, amino acids selected from the group comprising proline, casein hydrolyzate, citric acid, mannitol, copper sulfate, glycine, gelling agent, 2,4D]. The cultures were kept in the dark at 26 ± 2 °C and sub-cultured every 15 days for three to five cycles of 7-28 days each. One week before transformation, calli were again selected for embryogenic characteristics (nodular, compact, opaque and slightly yellowish).

**[0172]** *Agrobacterium* culture, comprising strain EHA105 transformed with the construct of the present invention, was started from a glycerol stock and kept in the dark at 28°C for two to three days. The *Agrobacterium* suspension was prepared by resuspending the culture in MS liquid medium plus acetosyringone, adjusting to a final $OD_{600}$ of 0.1-1.0 (MS salts, sucrose, and vitamins B5) for infection of calli.

**[0173]** The calli with embryogenic characteristics were visually selected and directly transferred to the *Agrobacterium* suspension, where they remained for 30 minutes in the dark with constant agitation at 50 rpm.

**[0174]** After this period, calli were separated from the *Agrobacterium* suspension and excess suspension was removed. After, calli were cultured for 1-5 days in semi-solid medium (MS salts, sucrose, vitamins B5, citric acid, gelling agent, 2,4D and acetosyringone) at 22 °C in the dark.

**[0175]** After co-cultivation, callus was transferred to DT rest medium (MS salts; sucrose, B5 vitamins, amino acids selected from the group comprising proline and asparagine, casein hydrolyzate, citric acid, copper sulfate, glycine, gelling agent, 2,4D, timentin) and kept for 5-14 days at 26 °C in the dark.

**[0176]** Transformed cells were selected by successive sub-cultures in selection culture medium containing phytoregulators and the selective agent geneticin. (Selection medium with geneticin: MS salts, sucrose, vitamins B5, amino acids selected from the group comprising proline and asparagine, casein hydrolyzate, copper sulfate, glycine, gelling agent, 2,4D, timentin). The calli remained in this condition for 21 days at 26 °C in the dark, then the calli were transferred to the regeneration medium (equivalent to selection medium without 2,4D) and then to elongation medium (MS salts, sucrose, B5 vitamins, casein hydrolyzate, gelling agent, timentin). The calli were exposed to 16-hour photoperiod at 4,000 lux in the presence of the selective agent, then they were multiplied, rooted, and acclimatized before transfer to the greenhouse. This process was used to generate the clone that eventually created the event CTC75064-3.

Example 2. Molecular Characterization of Event CTC75064-3

2.1 DNA extraction.

**[0177]** Approximately 10 mg of leaf tissue from event CTC75064-3 was used. Genomic DNA extraction was performed on the BioSprint 96 Nucleic Acid Extractor (Quiagen, GER) with the BioSprint 96 DNA Plant Kit Extraction Kit (Quiagen, GER) according to the manufacturer's instructions. The DNA was normalized to a concentration of 10 ng/μL in a Multiskan GO spectrometer (Thermo Scientific, USA).

2.2 Determination of the number of transgene copies inserted into the host plant germplasm.

**[0178]** The copy number of *cry1Ac* and *nptII* genes inserted into CTC75064-3 event was initially evaluated by quantitative Taqman® PCR (qPCR/Taqman®), and the results were confirmed via Southern blot and/or sequencing.

**[0179]** The Taqman® real time PCR reactions were realized with 7500 Real-Time PCR System (Applied Biosystems, EUA) in the Fast mode. The primer pairs and probes used are shown at Table 03. As endogenous control of the *cry1Ac* and *nptII* reactions to confirm the presence and quality of the used DNA, as well as, effectiveness of the reaction, it was used the sugarcane polyubiquitin gene (forward primer: 5 'ACCATTACCCTGGAGGTTGAGA 3 ' (SEQ ID NO: 15); reverse primer: 5 'GTCCTGGATCTTCGCCTTCA 3' (SEQ ID NO: 16); probe: VIC -5 'CTCTGACACCATCGAC 3'-MGB (SEQ ID NO:17) in multiplex mode.

Table 03: Primers and probes (Taqman®) used to determine copy number via qPCR.

| Assay | ***Primers* / probes** | **Sequence 5' - 3'** | ***Amplicon* (pb)** |
|---|---|---|---|
| cry1Ac - 0 | pCTC001_Cry1Ac.F | GAGGTGCCAATCCACTTCCC SEQ ID NO 40 | 82 |
| | pCTC001_Cry1Ac.R | GAGTTACCCCAGTTCACGTTGAG SEQ ID NO 41 | |
| | Cry1Ac_probe | FAM-ACCTCCACCAGGTACAG-MGB SEQ ID NO 42 | |
| cry1Ac - 1 | cry1AcF.1_SB | CATCCCATACAACTGCCTGAG SEQ ID NO 43 | 103 |
| | cry1AcR.1_SB | CTGGGTCAAGGACAGGGAGAT SEQ ID NO 44 | |
| | sondal cry1Ac_SB | CCGGTTACACCCCC SEQ ID NO 45 | |
| cry1Ac - 2 | cry1AcF.2_SB | CTCTACCAAATCTACGCCGAGA SEQ ID NO 46 | 96 |
| | cry1AcR.2_SB | CATGTCGTTGAATTGAATGCG SEQ ID NO 47 | |
| | sonda2_cry1Ac_SB | TCTCCGCGAGGAAA SEQ ID NO 48 | |
| cry1Ac - 3 | cry1AcF.3_SB | CACCGTGCTGGACATTGTGT SEQ ID NO 49 | 77 |
| | cry1AcR.3_SB | TGAGTTGGGACACGGTGC SEQ ID NO 50 | |
| | sonda3_cry1Ac_SB | CTCTTCCCGAACTACGACT SEQ ID NO 51 | |
| cry 1Ac-4 | cry1AcF.4_SB | ACCCTGTCCTCCACCCTGTA SEQ ID NO 52 | 107 |
| | cry1AcR.4_SB | AGGTTGGAGGAGGTGCCGTA SEQ ID NO 53 | |
| | sonda4_cry1Ac_SB | CCTTCAACATCGGTATCA SEQ ID NO 54 | |
| nptII - 0 | nptII.F | TGCCGAATATCATGGTGGAA SEQ ID NO 55 | 55 |
| | nptII.R | CGGCCACAGTCGATGAATC SEQ ID NO 56 | |
| | nptII.probe | FAM-TGGCCGCTTTTCT-MGB SEQ ID NO 57 | |
| nptII - 1 | nptIIF.1_SB | ATGACTGGGCACAACAGACAATC SEQ ID NO 58 | 99 |
| | nptIIR.1-SB | CGGACAGGTCGGTCTTGA SEQ ID NO 59 | |
| | sondal_nptII_SB | CTGCTCTGATGCCGC SEQ ID NO 60 | |

(continued)

| Assay | *Primers* / probes | Sequence 5' - 3' | *Amplicon* (pb) |
|---|---|---|---|
| nptII - 2 | nptIIF.2_SB | CTGCCGAGAAAGTATCCATCATG SEQ ID NO 61 | 93 |
| | nptIIR.2_SB | GATGTTTCGCTTGGTGGTCG SEQ ID NO 62 | |
| | sanda2_nptII_SB | CTGATGCAATGCGGCGGC SEQ ID NO 63 | |
| c-StaA (*backbone*) | c-StaA.F | GCCGCTTGTAGCCTTCCA SEQ ID NO 64 | 64 |
| | c-StaA.R | CCGCCGACCTGGTGGA SEQ ID NO 65 | |
| | c-StaA_BB_probe | FAM-CGTGACCTCAATGCG-MGB SEQ ID NO 66 | |
| aad-A (*backbone*) | aad-A_BB_F | CACAATCGTCACCTCAACCG SEQ ID NO 67 | 75 |
| | aad-A_BB_R | ATCAACGACCTTCTGGAAACG SEQ ID NO 68 | |
| | aad-A_BB_probe | NED-CGCAGGATTTCGCTCTCG-MGB SEQ ID NO 69 | |

**[0180]** The qPCR reactions used IX TaqMan® Fast PCR Master Mix II (Applied Biosystems, USA), 300 nM from each primer and 200 nM from the corresponding probes. The cycling used was: a 50 °C cycle for 2 minutes for uracil N-glycosylase activation, a 95 °C cycle for 20 seconds for DNA polymerase activation, 40 cycles of 95 °C for 3 seconds (denaturation), and 60 °C for 30 seconds (annealing and extension).

**[0181]** Data analysis was performed by manually entering the threshold at the exponential phase of the amplification curve. For *cry1Ac* and *nptII* genes, the copy number was inferred from DeltaCt (dCt) analysis, in which the Ct (cycle at which the fluorescence signal emitted by the amplification product reaches the threshold) of the endogenous gene is subtracted from the Ct of the target gene. In this type of analysis, the number of copies is assumed to double every Ct and the reference number of control copies of the same variety whose value is known is taken as a reference.

**[0182]** As a result, both assays pointed to the presence of 1 copy for the *cry1Ac* gene in the CTC75064-3 event genome. The same copy number (i.e., 1 copy) was detected for the *nptII* gene, the assay of which is based on detection of the gene promoter.

**[0183]** For the Southern blot, 10 μg of genomic DNA from the CTC75064-3 event were digested separately with distinct restriction enzymes (*Eco*RV, *Hind*III *and Shp*I), following the cold probe tagging procedure (Digoxigenin - DIG). Briefly, genomic DNAs of the event CTC75064-3 and the parental variety RB 867515 were extracted using the *NucleoSpin® Plant II* kit in tubes, following the recommendations of the manufacturer (*Macherey-Nagel GmbH & Co. KG,* Germany). The quality of the extracted DNA was checked on a 1% agarose gel in TAE IX buffer (Tris-Acetate EDTA) and 10 μg of DNA were digested using 100 units of restriction enzyme (10 U/μg of gDNA) per sample, in a final volume of 400 μl of reaction.

**[0184]** The enzymatic reaction was carried out following the manufacturer's recommendations (Thermo Fisher, USA). The digestion product was precipitated with 2 volumes of ethanol and 10% 7.5 M ammonium acetate, and incubated for 48 h at -20 °C. The precipitated DNA was centrifuged at 14,000 x g for 30 min and the pellet formed was resuspended in 35 μl of milli-Q water until complete dissolution. The quality of digestion was visualized on a 1% agarose gel with IX TAE buffer (Tris-Acetate - EDTA). The *EcoRV* and *Hind*III enzymes were used to detect the cassettes containing the *cry1Ac* and *nptII*genes (Figure 23 A). *Sph*I enzyme was used to detect vector backbone (Figure 23 B).

**[0185]** Probes were design to detect *cry1Ac* gene (probe CrylAc - 1,079 bp), *nptII*gene (*nptII* probe 579 bp), and CaMV 35s promoter region (CaMV 35s promoter and the OsACT1 intron; 35S probe - 904 bp), presented in the CTC75064-3 event. The probes for detecting fragments of the vector (backbone) were designed throughout the vector, covering ~ 98% of the backbone (BB1 probe - 1,875 bp; BB2 probe -2,305 bp; BB3 probe -2,282 bp; Figure 23 B). The probes were prepared and used to detect targets using the *PCR DIG Probe Synthesis Kit* reagents (Roche, cat # 11636090910, Switzerland). About 100 pg of the linearized vector were used as a template for the amplification of the regions of interest and incorporation of the digoxigenin molecule (DIG) by PCR and its use as a probe. The primers used for the production of the probes, the hybridization temperatures (Thyb) and the expected size of each amplicon are listed in Table 04.

**[0186]** DNA from RB 867515 variety was used as a negative control. As a positive control, plasmid DNA containing the construction used to obtain event CTC75064-3was added to RB 867515 genomic DNA. Plasmid DNA was previously linearized with the restriction enzyme used in each assay.

Table 04. List of primers used in the synthesis of probes marked with DIG.

| Probes | Enzyme | Sequences 5'→ 3' | Target | Tm | Amplicon | Thyb |
|---|---|---|---|---|---|---|
| Cry | *Eco*RV/*Hind*III | CAACAACCCAAACATCAACG SEQ ID NO 105 | T-DNA | 58 °C | 1.079 bp | 50 °C |
| | | GGGTCCTGTAGACACCCTGA SEQ ID NO 106 | | | | |
| nptII | *Eco*RV/*Hind*III | GCTCACCCTGTTGTTTGGTGTT SEQ ID NO 107 | T-DNA | 58 °C | 679 bp | 50°C |
| | | CGGCCACAGTCGATGAATC SEQ ID NO 108 | | | | |
| 35S | *Eco*RV/*Hind*III | TGAAAAGAAAAACTACCGATGAA SEQ ID NO 109 | T-DNA | 58 °C | 904 bp | 50 °C |
| | | TGATGTGATGGTCCGATTGA SEQ ID NO 110 | | | | |
| *Backbone* 1 | *Sph*I | TCCCCTCGGGATCAAAGTA SEQ ID NO 111 | Vector | 57 °C | 1.875 bp | 50 °C |
| | | TAGCGTGTTTGTGCTTTTGC SEQ ID NO 112 | | | | |
| *Backbone* 2 | *Sph*I | GGCCGCTGAAATTAAATCAA SEQ ID NO 113 | Vector | 58 °C | 2.305 bp | 50 °C |
| | | GAGTCAGTGAGCGAGGAAGC SEQ ID NO 114 | | | | |
| *Backbone* 3 | *Sph*I | CGGTGAAAACCTCTGACACA SEQ ID NO 115 | Vector | 57 °C | 2.282bp | 50 °C |
| | | ATACAGGCAGCCCATCAGTC SEQ ID NO 116 | | | | |

**[0187]** The transfer of the digested DNA to the *nylon* membrane was carried out according to well-established protocols. Briefly, electrophoresis of the digested DNA in 1% agarose gel with TAE IX buffer was performed at 40 V for approximately 20 hours, using *SYBR Safe DNA gel stain* (Invitrogen # S33102, USA) as an intercalant agent. Then, the agarose gel was treated with four different solutions in the following order:

- Depurination solution (1.1% HCL); 10-15 min;
- Denaturation solution (0.5 N NaOH; 1.5 M NaCl); 30-40 min;
- Neutralization solution (1.5 M NaCl; 0.5 M Tris); 30-40 min;
- SSC 20x (Saline Sodium Citrate); 20 min

**[0188]** The DNA transfer to the *nylon* membrane was performed using *TurboBlotter Transfer System* 20 x 25 (Sigma # WHA10416324, USA) according manufacturer instructions. The membrane with the transferred DNA samples was placed in the *UV Crosslinker* equipment (UVP- Analytik-Jena, Germany) for DNA fixation (2 cycles of 700 x 100 (μJ/cm$^2$).

Prehybridization and hybridization were then performed.

**[0189]** The Prehybridization treatment consists of incubation of the membrane with *DIG Easy Hyb* solution (Roche # 11603558001, Switzerland) at40 °C for 3 hours, with denatured salmon sperm DNA in the final concentration of 100 $\mu g.mL^{-1}$ and constant rotation (0.5 x g). After, Hybridization was performed with the same Pre-hybridization solution *(DIG Easy Hyb* with salmon sperm DNA) added to the probe marked with DIG and denatured. The concentration of the probes used was 65 $\mu g.mL^{-1}$. The hybridization temperature was 50 °C.

**[0190]** Hybridization was carried out in a hybridization oven for approximately 16 hours with constant rotation (0.5 x g). The hybridized membranes were washed and blocked. After the blocking solution was discarded and the membrane was covered with a new blocking solution with the *Anti-Digoxigenin AP fragments* (Roche # 11093274910, Switzerland) diluted in a 1:20,000 ratio. The membrane was then incubated for 30 min at room temperature and slightly shaken. The antibody solution was discarded, and the membrane was washed and incubated with the *Detection buffer* (Roche # 11585762001, Switzerland) plus *CDP-Star ready to use* (Roche # 12041677001, Switzerland) for a final concentration of IX according to manufacturer instructions. A photographic film was added to detect chemiluminescence (Roche # 11666657001, Switzerland) in contact with the membrane for at least 16 hours and then immersed 1x Developing Solution (Kodak # 1900943, USA) for a visualization of the bands. The 1x Fixing Solution (Kodak # 1901875, USA) was used to fix the band images detected.

**[0191]** In hybridization with the Cry1Ac and 35S probe, for the materials digested with *Hind*III, a single band with about 8.5 kb was observed for all samples from CTC75064-3 event (Figure 24 left; Figure 25 right). For the same probes, the samples digested with *Eco*RV present a fragment with about 2.79 kb in all samples CTC75064-3 event (Figure 24, right; Figure 25 left). For hybridization with the *nptII* probe, in the materials digested with *Hind*III a fragment with the expected size of ~ 3.5 kb was observed for all samples from CTC75064-3 event (Figure 26 left). For the *Eco*RV enzyme, a fragment> 12 kb was observed (Figure 26 right).

**[0192]** The results of the Southern blot, using the enzymes *Eco*RV and *Hind*III in combination with the probes that recognize the *cry1Ac, nptII* genes and 35s promoter, confirm the results found using qPCR, which indicate that the CTC75064-3 event has only one integration of T-DNA with one copy of the *cry1Ac* gene and one copy of the *nptII* gene in the genome. In addition, the presence of the same fragments in all generations of the CTC75064-3 event from samples collected in consecutive vegetative generations (T0 - T3; Figures 24, 25 and 26), confirm the genetic stability of the insertion in the event over 4 generations of vegetative multiplication. Sequences of the vector backbone was not detected in the event CTC75064-3 (Figure 27).

2.3 Definition of flanking sequences.

**[0193]** To isolate the flanking regions at the ends of the T-DNA insert present in event CTC75064-3, several DNA sequencing experiments were performed. The map of the genetic insertion of event CTC75064-3 generated from the data of these experiments is shown in FIGURE 3.

**[0194]** Inverse PCR (iPCR) assays were performed for both ends of the T-DNA to isolate and clone the flanking regions of the insert. The iPCR methodology is based on genomic DNA digestion using enzymes that cleave the T-DNA sequence and a random event genome sequence. The cleavage products are circularized and subjected to multiple nested PCR cycles using primers for known T-DNA regions (Table 05). The isolated fragments were then isolated, cloned and sequenced by Sanger methodology. Finally, a consensus sequence of the flanking regions was assembled (SEQ ID NO: 23 and SEQ ID NO: 24).

Table 05. Restriction enzyme and primer nucleotide sequences used to perform iPCR (inverse PCR) to identify flanking regions.

| T-DNA Border | Restriction Enzyme | Reaction | Oligonucleotide sequence |
|---|---|---|---|
| Left border | *PaeI* | Nested PCR1 | 5' - ACGGAACCGTTGAAGAGGAA - 3' - - SEQ ID NO 70<br>5' - ATGTGTGAGTAGTTCCCAGATAAG - 3' - SEQ ID NO 71 |
| | | Nested PCR2 | 5' - GATCCAGGAGAACATCGGAG - 3' - SEQ ID NO 72<br>5' - CTATAGGGTTTCGCTCATGTGTTG - 3' - SEQ ID NO 73 |

(continued)

| T-DNA Border | Restriction Enzyme | Reaction | Oligonucleotide sequence |
|---|---|---|---|
| Right border | *Bgl II* | Nested PCR1 | 5' - AATTATACATTTAATACGCG - 3' - SEQ ID NO 74<br>5' - CGCGGTGTCATCTATGTTAC - 3' - SEQ ID NO 75 |
| | | Nested PCR2 | 5' - CGCGGTGTCATCTATGTTAC - 3' - SEQ ID NO75<br>5' - TCAAGAAGGCGATAGAAGGCGATG - 3' - SEQ ID NO 76 |
| Right border | *pstI* | Nested PCR1 | 5' - AATTATACATTTAATACGCG - 3' - SEQ ID NO 74<br>5' - AATAACGTCATGCATTACATG - 3' - SEQ ID NO 77 |
| | | Nested PCR2 | 5' - CGCGGTGTCATCTATGTTAC - 3' - SEQ ID NO 75<br>5' - GATAATCATCGCAAGACCGG - 3' - SEQ ID NO 78 |
| | | Nested PCR3 | 5' - TCGTCGACTCTAGACTCGAG - 3'- SEQ ID NO 79<br>5' - GATAATCATCGCAAGACCGG - 3' - SEQ ID NO 78 |
| Right border | *EcoRV* | Nested PCR1 | 5' - AATTATACATTTAATACGCG - 3' - SEQ ID NO 74<br>5' - AATAACGTCATGCATTACATG - 3' - SEQ ID NO 77 |
| | | Nested PCR2 | 5' - CGCGGTGTCATCTATGTTAC - 3' - SEQ ID NO 75<br>5' - GATAATCATCGCAAGACCGG - 3' - SEQ ID NO 78 |

| Cycle Number | Denaturation | Ringing | Extension |
|---|---|---|---|
| **1st** | 94 °C, 5 min | - | - |
| **2-36th** | 94 °C, 30 sec | 50 °C 45 sec | 72 °C, 3 min |
| **37th** | - | - | 72 °C, 7 min |

**[0195]** In parallel, as there is currently no fully sequenced genome that could be used as a reference for RB 867515 germplasm, a capture sequencing methodology was adopted as an additional effort to isolate the T-DNA inserted into the event CTC75064-3 and its flanking regions. In this strategy, small overlapping polynucleotide fragments (probes) were developed to cover the entire T-DNA sequence. These probes were hybridized to the fractionated genomic DNA of both RB 867515 and CTC75064-3 cultivars, and hybrid sequences were isolated. Isolated fragments were then sequenced using Illumina® technology according to standard protocol. The data obtained were aligned with the T-DNA sequence present in the transformation vector and, together with the iPCR data mentioned above, the complete T-DNA consensus sequence (SEQ ID NO: 2) of the CTC75064-3 event and its flanking sequences (SEQ ID NO: 22, SEQ ID NO: 5, SEQ ID NO: 23, SEQ ID NO: 24) were obtained.

2.4 Method for the detection and characterization of event CTC75064-3 (event-specific assay)

**[0196]** For the validation of the methodology, both non-GMO (WT) plants and other genetically-modified events having the same construct were used as experimental controls. DNA extraction occurred as described above.

**[0197]** Real-time PCR assays for identification of the CTC75064-3 event were designed and validated based upon the molecular characterization of the T-DNA insertion genomic flanking sequences. For the development of specific detection methodology, the real-time PCR (qPCR) technique known as "Plus-Minus" or "Presence - Absence" was chosen, validating the two variations of the methodology: via SYBR GREEN™ and via Taqman® technology. Specific primer pairs have been designed to generate information about the insertion of T-DNA in both methodologies, such that one primer binds in the construct and the second primer binds in the host genome. For the use of Taqman® technology, specific probes were designed between the primers.

**[0198]** In a preferred embodiment, the primers designed are the primers as defined on the SEQ ID Nos: 6 to 9, wherein the primer of SEQ ID NO: 6 is a forward primer of the right border, the primer of SEQ ID NO: 7 is a reverse primer of the right border, the primer of SEQ ID NO: 8 is a forward primer of the left border and the primer of SEQ ID NO: 9 is a reverse primer of the left border (Table 06).

**[0199]** In a preferred embodiment, the probe employed in Taqman® PCR technology consists of SEQ ID NO: 10 in the RB border and/or SEQ ID NO: 11 in the LB border.

**[0200]** Taqman® real-time PCR reactions were performed using the 7500 Real-Time PCR System (Applied Biosystems, USA) in its Fast mode.

**[0201]** The sugarcane poly-ubiquitin gene (endogenous gene) was used as an internal reaction control to confirm the presence and quality of the DNA used. The following reagents were multiplexed with the assay developed for the event: forward primer (SEQ ID NO: 15); reverse primer (SEQ ID NO: 16); probe (SEQ ID NO: 17).

Table 06. List of primers and probes used in Real-time PCR assays.

| | Primer/Probe | Oligonucleotide sequence | Amplicon |
|---|---|---|---|
| **Detection of left board (LB)** | Forward<br>Probe LB<br>Reverse | CATGACCAAAATTGCTAATATTTCAC - SEQ ID NO 08<br>FAM-CTTAATAACACATTGCGGACGT-MGB - SEQ ID NO 11<br>AATCCAGTACTAAAATCCAGATCCC - SEQ ID NO 09 | 135 bp |
| **Detection of right board (RB)** | Forward<br>Probe RB<br>Reverse | CAAGGGCGAATTCCAGCAC - SEQ ID NO 06<br>FAM-CCGTTACTAGTGGATCGA-MGB-SEQ ID NO 10<br>GTTCTTGTCAGTTTTCTGTCCAAA - SEQ ID NO 07 | 107 bp |
| **Endogenous control (pUBI)** | Forward<br>Probe RB<br>Reverse | ACCATTACCCTGGAGGTTGAGA- SEQ ID NO 15<br>VIC - CTCTGACACCATCGAC - MGB-SEQ ID NO 17<br>GTCCTGGATCTTCGCCTTCA - SEQ ID NO 16 | |

**[0202]** qPCR reactions used IX TaqMan® Fast PCR Master Mix II (Applied Biosystems, USA), 150nM from each event-specific primer and 100nM from the corresponding probe, 300nM from the primers for the endogenous poly-ubiquitin gene and 200nM of its probe, 100-200 ng of DNA and enough water to complete the 20 μL volume. The following PCR program was used: a 50 °C cycle for 2 minutes for uracil N-glycosylase activation, a 95 °C cycle for 20 seconds for DNA polymerase activation, 40 cycles of 95 ° C for 3 seconds (denaturation) and 60 °C for 30 seconds (annealing and extension).

**[0203]** qPCR reactions using SYBR GREEN™ were also performed using the 7500 Real-Time PCR System (Applied Biosystems, USA) in its standard mode for this type of assay and QuantStudio 6 Flex da Applied biosystems. Reactions were performed using 1 X QuantiFast SYBR Green™ PCR Kit (QIAGEN ™), 150 nM forward primer and 150 nM reverse primer, 20 ng DNA and sufficient water for a final volume of 25 μL. The reactions were performed using the event of the invention, the other events transformed with the same construct as the event of the invention (negative controls), wild type sugarcane (WT; non transformed) samples, and experimental controls (extraction and reaction blank).

**[0204]** The following PCR program was used: a DNA denaturation cycle at 95°C for 5 minutes, 35 cycles for primer annealing cycles and amplification at 95°C for 15 seconds and 60°C for 1 minute and a dissociation cycle for generation melting peak (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec). The reaction with SYBR safe does not allow for the use of multiplex; therefore, it is necessary to prepare a separate endogenous gene amplification reaction, using the same DNA, to eliminate false negatives.

**[0205]** As a result, it was possible to validate the event-specific detection reaction of the invention via high-accuracy Taqman®, as illustrated in Figure 6. Taqman® technology probes specifically bind to DNA and are released during DNA amplification, thus generating the fluorescence signal captured by the equipment during this process.

**[0206]** Samples corresponding to the event of the invention showed specific amplification: having well-defined amplification curve formation and characteristic sigmoidal shape; whereas samples from other events, WT, and extraction and reaction blanks did not show event-specific amplification. As expected, the endogenous control presented amplification for all samples except extraction and reaction blanks, demonstrating both the quality of the DNA used in the reaction, as well as the quality of the reaction and cycling.

**[0207]** The SYBR GREEN™ assay showed specific amplification of the event samples at the expected melting temperature. Samples of the other events, as well as WT and blanks did not peak at this temperature (Figure 7).

Example 3. Event CTC75064-3 Generation - Genome editing (GE)

**[0208]** The event of the invention, CTC75064-3, is generated using a genome editing (GE) approach, thus recreating the event generated using the preferred *Agrobacterium-mediated* transformation methods described herein.

**[0209]** In this way, the event CTC75064-3 is recreated with the insertion of the *cry1Ac* gene into the same location of the genome as the CTC75064-3 event. The *cry1Ac* gene expression is regulated by a promoter or a promoter region and a terminator capable to drive the CrylAc protein expression at levels sufficient to control infestation of the target pest. Additionally, a marker gene or selection system is also inserted (transiently or stably) to enable event selection. Preferably, the T-DNA of the claimed invention (SEQ ID NO: 2) is inserted into the same location of the genome as the CTC75064-3 event. Thus, the event CTC75064-3 is recreated with the insertion of the *cry1Ac* gene, which expresses a toxin to control *D. saccharalis,* and the *nptII* gene. The expression of the *cry1Ac* is regulated by a promoter region comprising doubled enhanced promoter CaMV35s, wheat leader sequence L-Cab, OsACT1 intron and Kosak sequence 5' upstream from the translational start site. The expression of *nptII* genes is regulated by the maize ubiquitin gene promoter UBI-1, which has an endogenous intron. The *cry1Ac* expression cassette has CaMV35s terminator (T35s) and *nptII* expression cassette uses the *Agrobacterium tumefaciens* nopaline synthase (nos) terminator.

**[0210]** In the case that the aforementioned genome editing approaches to generating event CTC75064-3 result in low-efficiency integration of the T-DNA at the target site, developmental genes or other regulatory elements could be delivered in conjunction with the GE reagents in order to improve the integration efficiency.

3.1 Constructs development.

**[0211]** Conventional gene cloning techniques using commercial plasmids, restriction enzyme digestion, fragment ligation (with ligases) and other known methodologies are used to develop the constructs (plasmids) of the present invention.

**[0212]** The GE reagents can be delivered on multiple plasmids, each one comprised of an element of the enzymatic complex (endonuclease, crRNA or guide RNA, and the homologous recombination (HR) template; Figure 19).

**[0213]** In one embodiment, the HR template constructs comprises the T-DNA (SEQ ID NO: 2) of the claimed invention flanked by DNA homologous to the flanking sequences described for CTC75064-3 (SEQ ID Nos: 23 and 24) located on either side of the T-DNA. In a preferred embodiment, the HR template constructs comprises the SEQ ID NO: 26 (Figure 21). The invention also comprises a second construct comprising an endonuclease expression cassette. In a preferred embodiment, the endonuclease expression cassette comprises a Cas9 endonuclease sequence. In more preferred embodiment the Cas9 sequence is codon optimized for sugarcane expression. In one embodiment, the Cas 9 construct comprises additionally the guide/ crRNA sequence. Preferably, the Cas 9 construct comprises the crRNA sequence SEQ ID NO: 28. In a more specific embodiment, the Cas 9 construct comprises the SEQ ID NO: 27 (Figure 20). A third construct comprising the guide RNA expression cassette alone is also contemplated in the present invention and comprises SEQ ID NO: 28.

**[0214]** In one additional embodiment the genome editing construct comprises HR template, the nuclease and the guide RNA expression cassettes, delivering all the GE reagents in a single construct. In one embodiment, the single construct comprises the T-DNA (SEQ ID NO: 2) of the claimed invention flanked by DNA homologous to the flanking sequences described for CTC75064-3 (SEQ ID Nos: 23 and 24) located on either side of the T-DNA. In a preferred embodiment, the construct comprises the SEQ ID NO: 25 (Figure 22).

**[0215]** Optionally, the constructs also comprise fluorescent/selection markers and/or other genetic engineering systems to remove marker genes and/or nucleases cassettes, such as a Cre/loxP recombination system from the bacteriophage PI. In this case, the marker/nuclease gene cassette, which should be deleted, is flanked by the loxP regions, while Cre recombinase removes this fragment during a transient expression.

3.2 Direct Delivery

**[0216]** In one embodiment, the event of the invention is generated using methodologies for direct delivery of proteins, RNA or plasmids. The methodologies for direct delivery are selected from the group consisting of particle bombardment, electroporation, lipofection and protoplast transfection; however, other delivery methodologies known to those of skill in the art could also be utilized.

3.2.1 Direct delivery - RNP approach

**[0217]** In one embodiment, the event of the invention is generated using ribonucleoprotein (RNP) delivery. The preferred methodologies for RNP delivery are selected of the group consisting of particle bombardment of sugarcane calli and protoplast transfection. Moreover, other sugarcane cell types can also be used for transformation including (but not limited to): leaf disc, meristem, and calli-derived suspension cells.

**[0218]** Using the RNP approach to genome editing, the endonuclease and crRNA or guide RNA are delivered in RNP form, separate from the HR template, which is delivered via plasmid.

**[0219]** The guide RNA may be preliminarily produced by in vitro transcription or be chemically synthesized as riboo-ligonucleotide, while the corresponding nuclease may be produced in vivo with further purification (bacterial expression) or purchased from any manufacturer of such products. In a preferred embodiment, the guide RNA comprises SEQ ID NO: 28. In another embodiment the nuclease is Cas9 nuclease.

**[0220]** A ready ribonucleoprotein (RNP) complex consisting of the corresponding nuclease and guide RNA, and the HR template plasmid are sorbed on golden particles and direct delivery to the cells or tissue.

3.2.2 Direct delivery - Plasmid

**[0221]** In another embodiment of the invention, the event of the invention is generated using plasmid delivery, wherein the GE reagents will be expressed in a transient manner, thus achieving the site-directed integration of CTC75064-3 without the integration of additional transgenes associated with the GE approach.

**[0222]** Methodologies for plasmid delivery is selected from the group consisting of particle bombardment (biolistic) of sugarcane calli or polyethylene glycol transformation of protoplasts; however, other methodologies known to those of skill in the art could also be utilized. Moreover, other sugarcane cell types can also be used for transformation including (but not limited to): protoplast, leaf disc, meristem, and calli-derived suspension cells.

**[0223]** In yet another embodiment of the invention, the event of the invention is generated using plasmid delivery, where the GE reagents are stably expressed, thus requiring the excision of the integrated GE reagents and selectable marker using, for example, a Cre/Lox system. Using this approach, LoxP sites will remain in the genome of the event CTC75064-3 plant. Other DNA excision approaches known to those skilled in the art may also be used to remove the GE reagent DNA from the genome of the event CTC75064-3 plant.

3.3 Indirect Delivery

**[0224]** In one embodiment, the event of the invention is generated using methodologies for indirect delivery of plasmids, *as_Agrobacterium* transformation. *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* can be used. Plant viruses also can be used for indirect delivery of plasmids to plant cells and tissues. For example, genetically modified plant geminiviruses make it possible to achieve higher transformation efficiency, specially without stable insertion into a genome. Moreover, different tissue and cell types can also be used for transformation including (but not limited to): calli, protoplast, leaf disc, meristem, and calli-derived suspension cells.

**[0225]** In a preferred embodiment the *Agrobacterium* transformation is performed as describe at Example 1.

**[0226]** In another preferred embodiment, the event of the invention is generated using plasmid delivery by *Agrobacterium* transformation, wherein the GE reagents will be expressed in a transient manner, thus achieving the site-directed integration of CTC75064-3 without the integration of additional transgenes associated with the GE approach.

**[0227]** The GE reagents can be delivered on multiple plasmids, but preferably on a single plasmid. In a preferred embodiment, the constructs is SEQ ID NO: 25 and comprises a selectable marker, a nuclease, crRNA or guide RNA, and homologous recombination (HR) template (Figure 22). The HR template comprises T-DNA (SEQ ID NO: 2) of the claimed invention flanked by the DNA homologous to the flanking sequences described for CTC75064-3 (SEQ ID Nos: 23 and 24) located on either side of the expression cassettes.

**[0228]** In a preferred embodiment, the event of the invention is generated using plasmid delivery, where the GE reagents are stably expressed, thus requiring the excision of the integrated GE reagents and selectable marker using, for example, a Cre/Lox system. Using this approach, LoxP sites will remain in the genome of the event CTC75064-3 plant. Other DNA excision approaches known to those skilled in the art may also be used to remove the GE reagent DNA from the genome of the event CTC75064-3 plant.

**[0229]** With all the transformation processes described above, the resulting transformed cells will be regenerated to form a plant containing the event of the invention.

3.4 Molecular characterization.

**[0230]** The event CTC75064-3, generated using genome editing, is evaluated for accurate insertion of the T-DNA into the target site of the genome using the primers of the invention (SEQ ID NO: 6-9) as is described in the specification herein.

**[0231]** Additionally, pair of primers designed to amplify the outermost sequences in the flanking sequences of event CTC75064-3 are validated and could be used to evaluate the integrity of the recombination site (Table 07, Figure 28).

Table 07. Pair of primers designed for Flanking sequences of CTC75064-3.

| Position | Name | ***Primer Forward 5'→ 3***<br>***Primer Reverse* 5 → 3** | **Amplicon (bp)** |
|---|---|---|---|
| 5' Flanking region | 75_064 FSLB 1 FW 1<br>T35S Ter RV 2 | AACTTTATTCTAGGATAAAGCTATGT -SEQ ID NO 80<br>CAGATAAGGGAATTAGGGTTCCTAT-SEQ ID NO 81 | 683 |
| | 75_064 FSLB 2 FW 1<br>T35S Ter RV 2 | ACATACAACATCACAATCAAGCAGT-SEQ ID NO 82<br>CAGATAAGGGAATTAGGGTTCCTAT-SEQ ID NO 81 | 576 |
| | 75_064 FSLB 3 FW 1<br>T35S Ter RV 2 | CTCAAAACAACGCTAGATACGGTAG-SEQ ID NO 84<br>CAGATAAGGGAATTAGGGTTCCTAT-SEQ ID NO 81 | 448 |
| 5' Flanking region + T-DNA | 75_064 FSLB 1 FW 1<br>ZmUBI RV 2 | AACTTTATTCTAGGATAAAGCTATGT-SEQ ID NO 80<br>AGAGTCCTGTTGTCAAAATACTCAA-SEQ ID NO 83 | 4079 |
| | 75_064 FSLB 2 FW 1<br>ZmUBI RV 2 | ACATACAACATCACAATCAAGCAGT-SEQ ID NO 82<br>AGAGTCCTGTTGTCAAAATACTCAA-SEQ ID NO 83 | 3988 |
| | 75_064 FSLB 3 FW 1<br>ZmUBI RV 2 | CTCAAAACAACGCTAGATACGGTAG-SEQ ID NO 84<br>AGAGTCCTGTTGTCAAAATACTCAA-SEQ ID NO 83 | 3860 |
| 3' Flanking region | 75_064 FSRB 1 RV 1<br>NOS FW1 | GCATCTTCATGATTGTTGCCTTATG-SEQ ID NO 85<br>ATGATTAGAGTCCCGCAATTATACA-SEQ ID NO 86 | 807 |
| | 75_064 FSRB 2 RV 2<br>NOS FW1 | CTTGAATAGGAAATGCTTGAGAAAA-SEQ ID NO 87<br>ATGATTAGAGTCCCGCAATTATACA-SEQ ID NO 86 | 674 |
| | 75_064 FSRB 3 RV 1<br>NOS FW1 | GATCTGAATGTTTGGTTTGTTTGAC-SEQ ID NO 89<br>ATGATTAGAGTCCCGCAATTATACA-SEQ ID NO 86 | 548 |
| | 75_064 FSRB 4 RV 1<br>NOS FW1 | GTTTGTCCTTGTTAGCCGTAGAAT-SEQ ID NO 90<br>ATGATTAGAGTCCCGCAATTATACA-SEQ ID NO 86 | 350 |
| 3' Flanking region + T-DNA | 75_064 FSRB 1 RV 1<br>CAB FW1 | GCATCTTCATGATTGTTGCCTTATG-SEQ ID NO 85<br>CTTGAGTGTGTGGAAGATGGTTCTA-SEQ ID NO 88 | 4454 |
| | 75_064 FSRB 2 RV 2<br>CAB FW1 | CTTGAATAGGAAATGCTTGAGAAAA-SEQ ID NO 87<br>CTTGAGTGTGTGGAAGATGGTTCTA-SEQ ID NO 88 | 4321 |
| | 75_064 FSRB 3 RV 1<br>CAB FW1 | GATCTGAATGTTTGGTTTGTTTGAC-SEQ ID NO 89<br>CTTGAGTGTGTGGAAGATGGTTCTA-SEQ ID NO 88 | 4195 |
| | 75_064 FSRB 4 RV 1<br>CAB FW1 | GTTTGTCCTTGTTAGCCGTAGAAT-SEQ ID NO 90<br>CTTGAGTGTGTGGAAGATGGTTCTA-SEQ ID NO 88 | 3997 |

**[0232]** PCR reactions use 0.2 μM of primers (Table 7), 20ng of DNA, IX Dream Taq- Thermo Fisher buffer (Thermo Fisher Scientific Inc - USA), Taq polymerase (final volume 20 μL). Reaction conditions are: one cycle at 94°C for 1 min;

35 cycles at 94°C for 30 seconds, 65°C for 45 seconds and 72°C for 3 min; and one cycle of extension at 72°C for 7 minutes. Amplicons with more than 2kb, the reactions are performed with 1U de Takara LA Taq enzyme - Takara (Takara Bio Inc-USA), 1 X LA PCR Buffer (Takara Bio Inc - USA), 2.5 mM MgCl2, 2.5mM dNTP, 0.5 $\mu$M of primers and 200 ng of DNA (final volume 50 $\mu$L).

**[0233]** Amplicons generated by PCR reactions with a combination of "T-DNA" and "Flanking regions" primers from Table 07, could be further sequencing by Sanger, using the pair of primers described at Table 08 (pair of primers designed to anneal to the T-DNA sequence of CTC75064-3 event) to confirm integrity of the insert.

Table 08. Pair of primers designed to T-DNA of the event CTC75064-3.

| ***Primers*** | **Sequence (5' → 3')** | **Element** |
|---|---|---|
| CLNG P04 FW | TGATGCATATACAGAGATGCTTTTT - SEQ ID NO 91 | UBI |
| CLNG P01 FW | TTCATCCATTTTATTAGTACATCCA - SEQ ID NO 92 | UBI |
| CLNG P03 FW | ACGGATGCGACCTGTACG - SEQ ID NO 93 | UBI |
| CLNG P07 RV | TCAGTAAAACCCACATCAAC - SEQ ID NO 94 | UBI |
| CLNG P08 RV | GACCACATCATCACAACCAAG - SEQ ID NO 95 | UBI |
| CLNG P09 RV | GCTCCGAACAACACGAGGTTG - SEQ ID NO 96 | UBI |
| CLNG P10 RV | ATGAAGTATTATATAGGTGAAG - SEQ ID NO 97 | UBI |
| CLNG P19 FW | AGAGGCTATTCGGCTATGAC - SEQ ID NO 98 | nptII |
| CLNG P20 FW | CAGCCGAACTGTTCGCCAGG - SEQ ID NO 99 | nptII |
| CLNG P21 RV | AGCACGAGGAAGCGGTCAGC - SEQ ID NO 100 | nptII |
| CLNG P22 RV | TGAGATGACAGGAGATCCTG - SEQ ID NO 101 | nptII |
| CLNG P65 FW | CAACAGCTCCGTGAGCATCATC - SEQ ID NO 102 | Cry1Ac |
| CLNG P66 FW | CCAGCGACTTCGGTTACTTC - SEQ ID NO 103 | Cry1Ac |
| CLNG P70 RV | CTCTCGGCGTAGATTTGGTAG - SEQ ID NO 104 | Cry1Ac |

Example 4. Evaluation of the gene expression product inserted in the event of the invention.

**[0234]** The gene expression product in the event of the present invention has been characterized in detail using ELISA and Western blot to determine the concentration of CrylAc and NptII proteins and to confirm the identities of these heterologous proteins.

**(ELISA) Enzyme-Linked Immunosorbent Assay**

**[0235]** To evaluate *crylAc* and *nptII* gene expression via ELISA, different sugarcane tissues were studied at different stages of crop development. To produce tissue samples of the event of the invention and the parental control, the experimental tests conducted was AGRO/PHENO.

**[0236]** "AGRO/PHENO" trials were conducted at six representative sites of the parental control cultivation area, three in the state of São Paulo (Barrinha, Piracicaba and Valparaiso), one in the state of Goiás (Quirinópolis), one in the state of Bahia (Juazeiro), and on in the state of Paraná (Mandaguaçu) (Table 09). The experiments were conducted in a randomized complete block design with 4 replications. The plots were composed of four 8 meters rows and the spacing between rows was 1.5 meter.

Table 09. *Assay* information used for sample collection for analysis of *cry1Ac* and *nptII* gene expression produced by the event of the invention. (Number of Days After Planting (DAP) represents time of sample collection for analysis.)

| **Assay Type** | **Location** | **Tissue** | **DAP** |
|---|---|---|---|
| | Barrinha-SP | leaf | 100, 200, 300,330 |
| | | stalk | 330 |
| | | root | 330 |

(continued)

| Assay Type | Location | Tissue | DAP |
|---|---|---|---|
| AGRO/PHENO | Piracicaba -SP | leaf<br>stalk<br>root | 100, 200, 300,330<br>330<br>330 |
| | Valparaiso-SP | leaf<br>stalk<br>root | 100, 200, 300,330<br>330<br>330 |
| | Quirinópolis-GO | leaf<br>stalk<br>root | 100, 200, 300,330<br>330<br>330 |
| | Juazeiro (JZ) | leaf<br>stalk<br>root | 100, 200, 300,210<br>210<br>210 |
| | Mandaguaçu (MG) | leaf<br>stalk<br>root | 100, 200, 300,330<br>330<br>330 |

**[0237]** The expression analysis of CrylAc and NptII proteins produced by the event CTC75064-3 was investigated at different periods of sugarcane plant development. The conditions evaluated were:

- Expression of heterologous proteins in leaves over a cultivation cycle of the event of CTC75064-3 (100, 200 and 300 DAP);
- Expression of heterologous proteins in leaves, stalk and roots at 330 DAP of a first harvest sugarcane. In Juazeiro at 210 DAP.

**[0238]** Leaf samples were collected on experimental treatment plots (CTC75064-3 and parental control - RB 867515) in the AGROPHENO assays at 100, 200, 300 and 330 DAP. Stalk and root samples were collected only at 330 DAP. 330 DAP samples were collected at 210 DAP in Juazeiro. After collection, the samples were sent for ELISA analysis to determine Cry1Ac and NptII protein expression levels.

**[0239]** Leaf Samples: 30 cm of tissue were collected from the tip of 5 to 10 "diagnostic" leaves on zigzag lines 2 and 3 avoiding diseased leaves. After removal of the central rib, the leaves were chopped into pieces, homogenized and packed in previously identified ziplock bags.

**[0240]** Stalk Samples: 10 whole sugarcanes were collected. After removing the dried leaves and pointers, the canes were cut into small tails, homogenized, and packed into labelled packages.

**[0241]** Root Samples: A representative clump from rows 2 and 3 of the experimental plot was collected. The soil was crushed, and the roots were washed with clean water to remove excess soil. The clean roots were then minced into pieces, homogenized, and packaged into labelled plastic bags.

**[0242]** All samples (from leaf, stalk, and root tissues) were transferred to dry ice in a Styrofoam box within 15 min of sampling. The genetic identity of all clumps sampled was confirmed by event-specific assay as described above.

**[0243]** For the analysis of the Cry1Ac protein 30 $\pm$ 1 mg of leaf, 200$\pm$ 1 mg of stalks, and 20 $\pm$ 1 mg of root tissue were macerated using TissueLyser equipment. To the macerated leaf tissue was added 750 $\mu$L saline phosphate extraction buffer (PBS) supplemented with Tween 20 (0.138 M NaCl; 0.027 mM KCl; 0.05% Tween 20, pH 7.4) diluted according to manufacturer's instructions (Envirologix ™, USA). For stalk 375 $\mu$L of the same buffer was used and for root samples, 1,500 $\mu$L were added.

**[0244]** For the analysis of the NptII protein in leaves, 40 $\pm$ 1 mg were weighted and macerated and 200 $\pm$ 1 mg were weighted for stalk and root samples and macerated. To the macerated leaf tissue samples, 750 $\mu$L of the extraction buffer were added; while in the stalk and root samples, 1,500 $\mu$L of the same buffer were added. The extraction buffer used in this case was PEB1 1x (pH 7.0), and the dilution was made according to manufacturer's instruction (PathoScreen® Kit for NPTII,Agdia, USA).

**[0245]** After buffer addition, vortex homogenization was performed and then centrifugation for 20 minutes at maximum speed. The resulting supernatant was collected, and total protein was quantified using the Bradford assay (CrylAc) and BCA (NptII).

**[0246]** The standards used for obtaining the calibration curve were the already-diluted commercial BSA (Bovine Serum Albumin) standards supplied with the kit described above. The 2000, 1000, 500, 250, 125, and 0 μg/mL calibrators (prepared in PBST buffer) were used. 10 μL of each standard calibrator was added in triplicate to plate wells. In total, 6 curves were generated from independent dilutions. For the samples, 10μL of the 3 individual protein extractions were used in each well. Then 200μL of Coomassie Plus Reagent Solution was added to each well containing the calibrators and samples. The plates were covered and incubated for 5 minutes at room temperature. Absorbance was read at 595 nanometers (nm) using SoftmaxPro 7.0 software (Molecular Device, US).

**[0247]** Total proteins were obtained in triplicate for each sample studied. After the total protein quantification of each replicate, the sample with the smallest variation of the median quantification value was chosen for ELISA analysis. After quantification of total proteins, leaf samples were diluted as follows: leaf samples for CrylAc analysis were diluted 2,500x, except by 100DAP samples that were 3,500x diluted; stalk samples for CrylAc analysis were diluted 2,500x; while root samples for CrylAc analysis were diluted 200x. All samples (leaf, root and stalk) for NptII analysis were diluted 8x..

**[0248]** Results were obtained by 96-well plate spectrometry reading at two different wavelengths: 450 nm and 630 nm on a SpectraMax Plate reader (Molecular Devices, USA). For CrylAc the Envirologix AP003 CRBS kit is used for protein detection and quantification. For NptII the *PathoScreen® Kit for NPT II* (Agdia, USA) kit was used. In all cases, the manufacturers recommendations were followed.

**[0249]** The analysis was based on the association of the absorbance values of the test samples with the predicted values in an equation estimated by measuring the absorbance of a standard curve. Synthetic proteins were diluted to desired concentrations in PBST buffer. Analyzes were performed in experimental duplicate for each sample. CrylAc and NptII protein concentrations were presented based on Total Protein (μg/mg), Fresh Tissue (μg/g) and Dry Tissue (μg/g).

**[0250]** The results of the statistical analyzes for the expression of the proteins CrylAc and *nptII* are found in Tables 10 and 11, respectively. The protein expression data were obtained from the average of four biological replicates (experimental plots) in duplicate. Table 10 presents the results of the individual and Combined analyzes by location for CrylAc protein expression data, in sugarcane leaves collected over a year of cultivation (100, 200 and 300 DAP). Table 11 shows the results of the analyzes for the NptII protein, under the same conditions.

Table 10. Comparison of the means of expression of Cry1Ac in leaves of the event CTC75064-3 over a year of cultivation (100, 200 and 300 DAP; DAP = Days After Planting), Individual and Combined statistical analysis for the 6 tested sites. Data not available in Juazeiro-BA at 300 DAP. Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). SE: standard error.

| Location | DAP | μg CrylAc / g Fresh Tissue | SE | μg CrylAc / g Dry Tissue | SE |
|---|---|---|---|---|---|
| Barrinha-SP | 100 | 90.54 a | 7.89 | 290.42 a | 26.12 |
| | 200 | 159.80 b | 7.89 | 513.01 b | 26.12 |
| | 300 | 130.45 c | 7.89 | 454.28 b | 26.12 |
| Piracicaba -SP | 100 | 86.84 a | 8.20 | 269.25 a | 26.41 |
| | 200 | 127.90 b | 8.20 | 399.67 b | 26.41 |
| | 300 | 199.40 c | 8.20 | 700.38 c | 26.41 |
| Valparaiso-SP | 100 | 79.16 a | 6.31 | 317.40 a | 23.96 |
| | 200 | 103.98 b | 6.31 | 356.70 a | 23.96 |
| | 300 | 87.20 ab | 6.31 | 351.40 a | 23.96 |
| Quirinópolis-GO | 100 | 111.23 a | 12.95 | 356.77 a | 41.03 |
| | 200 | 138.51 ab | 12.95 | 408.88 ab | 41.03 |
| | 300 | 153.10b | 12.95 | 515.85 b | 41.03 |
| Mardaguaçu-PR | 100 | 94.88 a | 8.21 | 361.46 a | 28.26 |
| | 200 | 136.83 b | 8.21 | 456.10 b | 28.26 |
| | 300 | 153.97 b | 8.21 | 545.60 b | 28.26 |

(continued)

| Location | DAP | µg CrylAc / g Fresh Tissue | SE | µg CrylAc / g Dry Tissue | SE |
|---|---|---|---|---|---|
| Juazeiro-BA | 100 | 62.67 a | 10.05 | 236.01 a | 31.70 |
| | 200 | 80.11 a | 10.05 | 249.37 a | 31.70 |
| | 300 | NA | - | NA | - |
| Combined | 100 | 87.55 a | 12.07 | 305.22 a | 37.62 |
| | 200 | 124.52 b | 12.07 | 397.29 ab | 37.62 |
| | 300 | 140.89 b | 12.97 | 509.35 b | 41.14 |

Table 11. Comparison of the averages of NptII expression in leaves of the CTC75064-3 event over a year of cultivation (100, 200 and 300 DAP). Individual and Combined statistical analysis for the 6 tested sites. Data not available in Juazeiro-BA at 300 DAP in individual and combined analyzes. Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$), SE: standard error.

| Location | DAP | µg NptII / g Fresh Tissue | SE | µg NptII / g Dry Tissue | SE |
|---|---|---|---|---|---|
| Barrinha-SP | 100 | 1.69 a | 0.06 | 7.22 a | 0.27 |
| | 200 | 1.24 b | 0.06 | 5.32 b | 0.27 |
| | 300 | 1.78 a | 0.06 | 8.26 c | 0.27 |
| Piracicaba-SP | 100 | 1.20 a | 0.04 | 4.96 a | 0.17 |
| | 200 | 0.86 b | 0.04 | 3.57 b | 0.17 |
| | 300 | 1.49 c | 0.04 | 6.97 c | 0.17 |
| Valparaiso-SP | 100 | 1.68 a | 0.12 | 9.01 a | 0.62 |
| | 200 | 2.00 a | 0.12 | 9.13 a | 0.62 |
| | 300 | 1.64 a | 0.12 | 8.80 a | 0.62 |
| Quirinópolis-GO | 100 | 1.32 a | 0.07 | 5.63 a | 0.28 |
| | 200 | 0.45 b | 0.07 | 1.76 b | 0.28 |
| | 300 | 1.25 a | 0.07 | 5.61 a | 0.28 |
| Mandaguaçu -PR | 100 | 1.35 a | 0.1 | 6.88 a | 0.49 |
| | 200 | 1.46 a | 0.1 | 6.51 a | 0.49 |
| | 300 | 1.55 a | 0.1 | 7.32 a | 0.49 |
| Juazeiro - SP | 100 | 1.41 a | 0.13 | 7.07 a | 0.6 |
| | 200 | 1.10 a | 0.13 | 4.57 b | 0.6 |
| | 300 | NA | - | NA | - |
| Combined Analysis | 100 | 1.44 a | 0.14 | 6.80 a | 0.74 |
| | 200 | 1.18 a | 0.14 | 5.14 b | 0.74 |
| | 300 | 1.53 a | 0.15 | 7.37 a | 0.77 |

**[0251]** Cry1Ac expression data (leaves; µg/g fresh and dry tissue) from the combined analysis of the 6 locations of the event of the invention over a year of cane cultivation (Table 10) are shown in Figure 8 (in µg protein/g fresh tissue) and Figure 9 (in µg protein/g dry tissue). NptII expression data (leaves; µg/g fresh and dry tissue) from the combined analysis of the 6 locations of the event of the invention over a year of cane cultivation (Table 11) are shown in Figure11 (in µg protein/g fresh tissue) and Figure 12 (in µg protein/g dry tissue).

**[0252]** The average expression of CrylAc protein in leaves from CTC75064-3 event over a sugarcane cycle in all locations (with exception of Juazeiro at 300DAP - no data) was 125.18 μg/g of fresh tissue and 423.75 μg/g of dry tissue. For NptII, the average expression in leaves from CTC75064-3 event over a sugarcane cycle in all locations (with exception of Juazeiro at 300DAP - no data) was 1.401 μg/g of fresh tissue e de 6.370 μg/g of dry tissue.

**[0253]** Cry1Ac protein expression data in leaves from the event CTC75064-3 harvested at 330 DAP (210 DAP for Juazeiro) are shown in Table 12 and in Figure 10 (in μg protein/g of fresh and dry tissue).

Table 12. Comparison of average CrylAc expression in leaves of the event of the invention harvested at 330 DAP (* 210 DAP). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | μg Cry1Ac / g Fresh Tissue | SE | μg Cry 1Ac / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 205.95 b | 5.71 | 678.48 b | 19.21 |
| Piracicaba -SP | 135.37 b | 5.94 | 420.73 b | 18.69 |
| Valparaiso-SP | 113.71 b | 3.31 | 383.13 b | 11.44 |
| Quirinópolis-GO | 119.45 b | 4.64 | 421.19 b | 16.45 |
| Mardaguaçu-PR | 144.72 b | 6.59 | 491.33 b | 22.55 |
| Juazeiro-BA * | 80.11 b | 8.14 | 249.37 a | 25.67 |
| Combined Analysis | 133.22 b | 10.13 | 440.70 b | 34.84 |

**[0254]** NptII protein expression data in leaves from the event CTC75064-3 harvested at 330 DAP (210 DAP for Juazeiro) are shown in Table 13 and Figure 13 (in μg protein/g of fresh and dry tissue).

Table 13. Comparison of average NptII expression in leaves of the event of the invention harvested at 330 DAP (*210 DAP). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | Fresh Tissue | SE | μg NptII / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 1.83 b | 0.01 | 7.83 b | 0.05 |
| Piracicaba -SP | 1.23 b | 0.03 | 5.15 b | 0.14 |
| Valparaiso-SP | 1.42 b | 0.04 | 6.48 b | 0.16 |
| Quirinópolis-GO | 1.28b | 0.03 | 5.01 b | 0.11 |
| Mardaguaçu-PR | 1.41 b | 0.05 | 6.28 b | 0.2 |
| Juazeiro - BA* | 1.10 b | 0.08 | 4.57 b | 0.33 |
| Combined Analysis | 1.38 b | 0.06 | 5.89 b | 0.3 |

**[0255]** CrylAc protein expression data in mature stalks from the event CTC75064-3 harvested at 330 DAP are shown in Table 14 and Figure 10 (μg/g fresh and dry tissue).

Table 14. Comparison of average CrylAc expression in mature stalk of the event of the invention harvested at 330 DAP (*210 DAP). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | μg Cry1Ac / g Fresh Tissue | SE | μg Cry1Ac / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 57.81 a | 5.71 | 248.56 a | 19.21 |
| Piracicaba -SP | 42.01 a | 5.94 | 179.60 a | 18.69 |
| Valparaiso-SP | 42.82 a | 3.31 | 154.33 a | 11.44 |
| Quirinópolis-GO | 30.63 a | 4.64 | 128.95 a | 16.45 |
| Mardaguaçu-PR | 41.44 a | 6.59 | 156.26 a | 22.55 |
| Juazeiro - BA* | 41.21 a | 8.14 | 163.82 a | 25.67 |

(continued)

| Location | μg Cry1Ac / g Fresh Tissue | SE | μg Cry1Ac / g Dry Tissue | SE |
|---|---|---|---|---|
| Combined Analysis | 42.65 a | 10.13 | 171.92 a | 34.84 |

**[0256]** NptII protein expression data from mature stalks of the event of the invention collected at 330 DAP are shown in Table 15 and Figura 13 (μg/g fresh and dry tissue)..

Table 15. Comparison of average NptII expression from mature stalks of the event of the invention (μg/g fresh and dry tissue) harvested at 330 DAP (*210 DAP). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | Fresh Tissue | SE | μg NptII / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 0.17 a | 0.01 | 0.74 a | 0.05 |
| Piracicaba -SP | 0.16 a | 0.03 | 0.68 a | 0.14 |
| Valparaiso-SP | 0.14 a | 0.04 | 0.51 a | 0.16 |
| Quirinópolis-GO | 0.05 a | 0.03 | 0.22 a | 0.11 |
| Mardaguaçu-PR | 0.17 a | 0.05 | 0.64 a | 0.2 |
| Juazeiro - BA* | 0.14 a | 0.08 | 0.56 a | 0.33 |
| Combined Analysis | 0.14 a | 0.06 | 0.56 a | 0.3 |

**[0257]** CrylAc protein expression data in roots from the event CTC75064-3 harvested at 330 DAP are shown in Table 16 and and Figure 10 (μg/g fresh and dry tissue).

Table 16. CrylAc protein expression values in root tissues of the event of the invention (μg/g fresh and dry tissue) harvested at 330 DAP(*210 DAP). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | μg Cry1Ac / g Fresh Tissue | SE | μg Cry1Ac / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 10.85 c | 5.71 | 42.55 c | 19.21 |
| Piracicaba -SP | 5.86 c | 5.94 | 28.00 c | 18.69 |
| Valparaiso-SP | 9.92 c | 3.31 | 54.61 c | 11.44 |
| Quirinópolis-GO | 12.13 c | 4.64 | 40.76 c | 16.45 |
| Mardaguaçu-PR | 12.31 c | 6.59 | 65.56 c | 22.55 |
| Juazeiro - BA* | 6.10 c | 8.14 | 29.60 b | 25.67 |
| Combined Analysis | 9.53 c | 10.13 | 43.51 c | 34.84 |

**[0258]** NptII protein expression data in roots of the event of the invention collected at 330 DAP are shown in Table 17 and and Figure 13 (μg/g fresh and dry tissue).

Table 17. NptII protein expression values in root tissues of the event of the invention (μg/g fresh and dry tissue). Means followed by the same letter do not differ by t test at the 5% level ($p \leq 0.05$). (SE: standard error).

| Location | Fresh Tissue | SE | μg NptII / g Dry Tissue | SE |
|---|---|---|---|---|
| Barrinha-SP | 0.07 c | 0.01 | 0.27 c | 0.05 |
| Piracicaba -SP | 0.08 a | 0.03 | 0.37 a | 0.14 |
| Valparaiso-SP | 0.12 a | 0.04 | 0.66 a | 0.16 |
| Quirinópolis-GO | 0.10 a | 0.03 | 0.32 a | 0.11 |

(continued)

| Location | Fresh Tissue | SE | μg NptII / g Dry Tissue | SE |
|---|---|---|---|---|
| Mardaguaçu-PR | 0.09 a | 0.05 | 0.49 a | 0.2 |
| Juazeiro - BA | 0.10 a | 0.08 | 0.47 a | 0.33 |
| Combined Analysis | 0.09 a | 0.06 | 0.43 a | 0.3 |

**[0259]** The average expression of CrylAc protein in stalks from CTC75064-3 event in all locations was 42.94 μg/g of fresh tissue and 173.54 μg/g of dry tissue. For NptII, the average expression in stalks from CTC75064-3 event in all locations was 0.138 μg/g of fresh tissue e de 0.558 μg/g of dry tissue.

**[0260]** The average expression of CrylAc protein in roots from CTC75064-3 event in all locations was 10.21 μg/g of fresh tissue and 46.30 μg/g of dry tissue. For NptII, the average expression in roots from CTC75064-3 event in all locations was 0.092 μg/g of fresh tissue e de 0.422 μg/g of dry tissue.

**[0261]** The results obtained for leaf, stem, and roots indicate that the event of the invention has CrylAc protein expression levels much higher than NptII expression.

**[0262]** The average concentration of CrylAc from leaves of the event of the invention at 100 DAP is lower than the average concentration at 200 and 300 DAP. For NptII the average expression levels in leaves throughout the sugarcane cycle remained constant.

**[0263]** Cry1Ac and NptII protein expression levels from CTC75064-3 event at 330 DAP were much higher in leaves than in stalk and roots. Expression data of CrylAc protein also showed significant differences between stalk and roots, with roots demonstrating the lowest protein expression. For NptII, the average expression of protein in stalks and roots did not differ significantly.

**[0264]** It is therefore concluded that the expression levels of CrylAc and NptII proteins from the event of the invention was characterized at different times, tissues and planting sites representative of its cultivation in Brazil. CrylAc protein expression levels, especially in the leaves of the event of the invention, remain high throughout the cultivation cycle, ensuring the intended effect of resistance to *Diatraea saccharalis.*

**Western blot**

**[0265]** For identification of the heterologous proteins expressed by the event of the invention, 50mg (±0,5 mg) of leaf frozen in liquid nitrogen was used for CryAc and 300 (±0,5 mg) for NptII. Maceration was performed in the TissueLyser equipment for 10 minutes at 25Hz, with the addition of three steel beads (3mm - Qiagen, DEU). To the macerated tissue, 500μl of Tween 20-supplemented saline phosphate extraction buffer (PBS) was added (0.138 M NaCl; 0.027 mM KCl; 0.05% Tween 20, pH 7.4) diluted according to manufacturer's instructions (Envirologix™, USA). After buffer addition, vortex homogenization was performed, and the mixture was centrifuged for 20 minutes at 4,000 RPM at 4°C. The resulting supernatant was collected and total protein was quantified. For NptII, the protein extract was concentrated before proceeding (Amicon® Ultra-0.5 Centrifugal Filter Devices (3000 NMWL).

**[0266]** Quantitation adopted for analysis of CrylAc and *NptII* proteins was performed according to the recommendations of ThermoScientific™ Coomassie Plus (Bradford) Protein Assay Kit (23236) - Microplate Procedure and BCA. Thus, the standards used for obtaining the calibration curve were the already-diluted commercial BSA (Bovine Serum Albumin) standards supplied with the kit described above. The 2000, 1000, 500, 250, 125, and 0 μg/ml calibrators prepared in PBST buffer were used. 10 μL of each standard calibrator was added to the plate wells in triplicate. The plates were covered and incubated for 5 minutes at room temperature. Absorbance was read at 595 nanometers (nm) using Soft-maxPro 7.0 software (Molecular Device, USA). Once the extraction of total proteins was carried out, 3 μg of protein extract were mixed with the 2X Laemmli Sample Buffer (Bio-Rad, USA) and denatured by heating at 100°C for 5 minutes to identify the CrylAc protein present in the sample. In the case of *nptII* detection and identification, 40 μg of the total protein extract was mixed with the sample buffer (2X Laemmli Sample Buffer) and denatured.

**[0267]** As a negative control of the presence of heterologous proteins, 3 μg of protein extract from the conventional parental variety (WT - RB 867515) was used for CryAc experiments and 40μg for NptII assays. In addition, positive controls were prepared to detect CrylAc and *NptII* proteins. The first positive control was prepared using either 0,5ng of CrylAc (~69 kDa, GenScript, USA) or NptII (~29 kDa, Bon Opus Biosciences, USA), diluted in total protein solution extracted from conventional parental variety (WT - RB 867515) leaves. The second positive control was made by diluting 1ng of purified CrylAc protein or 5ng of *NptII* protein in PBST extraction buffer.

**[0268]** Samples were denatured and applied on 4-20% polyacrylamide gel (Mini-PROTEAN® TGXTM Precast Gel) submerged in Tris/glycine/SDS running buffer (Bio-Rad, USA) and separated by electrophoresis at 50V for 5 minutes and then at 120V for approximately 90 minutes. Next, the polyacrylamide gels were equilibrated in Tris/Glycine Transfer

Buffer (Bio-Rad, USA), which was added with 20% methanol for 10-15 minutes. The PVDF membrane was treated with absolute methanol. The transfer system was mounted in a container filled with the cold Transfer Buffer for immersion transfer ("wet transfer") at a constant voltage of 50V for 3 hours. Upon completion of the transfer, the membrane was blocked for 16 hours at 4 °C, under constant agitation, in blocking solution [5% skimmed milk powder (Bio-Rad, USA) and TBS/T (20mM Tris, 150mM NaCl, 1% Tween20)] to prevent possible nonspecific membrane binding.

**[0269]** In the next step, the membrane was incubated with the primary antibody for 90 minutes to detect and confirm the presence and integrity of the CrylAc and *NptII* proteins. The polyclonal antibodies used in this assay were rabbit Anti-CrylAb (Fitzgerald, USA), which bind both CrylAc and CrylAb proteins; and rabbit Anti- *NptII*(Rhea, BRA IM0770-18088),), which reacts with *NptII*protein, diluted 1: 500 in TBS/T (v/v).

**[0270]** The membrane was washed 3 times per 5 minute (3x5) in TBS/T and incubated with goat-produced HRP-conjugated secondary Anti-Rabbit antibody (Sigma Aldrich, USA) at a concentration of 1: 20,000 or at a concentration of 1:5,000 - v/v (Fitzgerald, USA) for 60 minutes. After incubations, the membrane was again washed with TBS/T (3x5 minutes), and the enzyme-linked immunoassay was verified on Amersham Hyperfilm ECL X-ray films (GE Healthcare, USA) by Clarit Western ECL Substrate Kit substrate reaction (Biorad, USA) according to the manufacturer's instructions. X-ray film exposure to membrane ranged from 15 seconds to 3 minutes.

**[0271]** The results revealed that the expression profile of CrylAc protein appears as one immunoreactive bands of ~66 kDa (Figure 14). All samples (R1-R4) are biological replicates of the event of the invention, obtained from four experimental plots at the Piracicaba site. As expected, the negative control (WT) showed no immunoreactivity. The negative control consisted of total protein samples extracted from the parental variety. The positive control (WT+CP) where the Cry1Ac protein was added to the total protein extracted from the parental cultivar, presented two immunoreactive bands, from approximately 69 and 66kDa. In this case (WT+CP),it is commonly known as doublet and accepted as a product of intracellular proteolytic breaks of Cry proteins in plant leaves generally produced by removing of terminal amino acid residues by proteases released during plant tissue processing, as well as other bands with smaller molecular weights (~20 and 10 kDa) visualized in the samples.

**[0272]** The protein encoded by the *nptII*selection gene was also detected by western blot assay. Even though *NptII* is expressed at lower levels than the CrylAc protein, western blot assays demonstrated the presence of an immunoreactive band at the expected size of 29kDa in all used biological replicates of the event of the invention (Figure 15).

**[0273]** Two positive and one negative control were added to the membrane. The first positive control (CP) corresponds to 0.5ng of purified *NptII*protein (~29 kDa, Bon Opus Biosciences, USA), and the second positive control (WT+CP) corresponds to 0.5ng of purified *NptII* protein diluted in protein extract of the parental cultivar. Negative control correspond to protein extract of the parental cultivar. The diagnostic band corresponding to the *NptII* protein is present in both controls at the expected weight and is identical to the band present at the event of the invention, confirming its identity.

**[0274]** It is therefore concluded that the identity of the Cry1Ac and *NptII* proteins expressed by the event of the invention was confirmed by western blot. The proteins expressed by the event of the invention are of the expected size, and no evidence of truncated/fused proteins being expressed by said event was found.

Example 5. Biological tests: Susceptibility to the sugarcane borer *(D. saccharalis)*

**[0275]** Biological Assays (bioassays) with target pest *D. saccharalis* (cane borer) can also be used for detection and characterization of event CTC75064-3, demonstrating the efficacy on the pest control provided by the expressed insecticidal protein Cry1Ac. Different bioassays may be contemplated within the scope of the present invention: for example, Leaf Disk Assay, Screenhouse bioassays, Tissue Dilution Assays, among others.

**[0276]** For leaf disc assay, leaves of event CTC75064-3 plants were collected, cut into discs of 16 mm$^2$ and distributed in bioassay plates containing gelled agar. Each well from culture plates was infested with *D. saccharalis* (0-24h old) neonate and incubated at 27 $\pm$ 1°C, relative humidity 60 $\pm$ 10%, and photoperiod 12:12h (light: dark) for a period of 7 days. At the end of incubation, larval mortality and inhibition of larval development of surviving individuals was evaluated, and the relative efficacy was calculated by the following formula:

$$\text{LDA Relative efficacy} = 100 - \left(\frac{\text{\%live larvae of the event}}{\text{\%live larvae of the control}}\right) \text{x } 100$$

**[0277]** The surviving larvae were submitted to image analysis by Digimizer software (v 4.6.1) for assessment of larval stage based on width of cephalic capsule. The larvae that did not reach the first instar were considered dead. Non-transgenic sugarcane varieties that are genetically very similar to the evaluated transgenic event can be used as assay controls.

**[0278]** To characterize event efficacy in controlling target pest *D. saccharalis* in laboratory, leaf disc assays were performed with plant tissue from CTC75064-3 event (30DAP). Non-transgenic sugarcane RB 867515 was used as control (CTC75-TC). The experimental design was completely randomized with four replicates per treatment. An average of 98.2% of mortality rate was observed when comparing the conventional variety (CT75-TC) and CTC75064-3 event after 7 days feeding with leaf discs. Also, based on measurement of cephalic capsule width, it was observed that 100% of the surviving individuals did not develop beyond the first instar, evidencing high suppression in the development of *D. saccharalis* after feeding with the transgenic event (Figure 18).

**[0279]** For tissue dilution assays, the leaves of the CTC75064-3 event were sampled in the Agro/pheno fields at Piracicaba, Valparaiso, Barrinha (SP) and Quirinópolis (GO), with collection points in 100, 200 and 300 DAP. These samples were chopped, dehydrated in a freeze dryer and then macerated to obtain a uniform green powder. To prepare the bioassay plates, each quadrant of 16 cells received the sample diluted 25x with MS diet (Multiple Species), filling approximately 1 mL per cell. For infestation, 2 neonates larvae (0-24h of age) were transferred in each cell (32 caterpillars per quadrant). The plates were identified and incubated at a temperature of 27 ± 1 ° C, relative humidity 60 ± 10% and a 12:12 photoperiod (light: dark), for a period of 10 days. At the end of the incubation, each quadrant was evaluated for effective mortality and average mass of larvae. Effective mortality was calculated from the equation:

$$\text{Effective mortality} = \frac{(\text{n dead larvae} + \text{n larvae on 1st instar})}{\text{total number of live larvae}} \text{x } 100$$

**[0280]** Relative effectiveness for dilution tests were calculated using the equation:

$$\text{Relative effectiveness in dilution} = 100 - \left(\frac{\text{\%Live larvae of the event}}{\text{\%Live larvae of the control}}\right) \text{x } 100$$

**[0281]** Through the analysis of tissue dilution from the fields of Agro/pheno, it was possible to verify that the effectiveness, both for effective mortality and for mass reduction of larvae, is 51 and 94%, respectively.

**[0282]** For screenhouse bioassays, seedlings of the transgenic event are planted in a screened nursery, where the plants are planted in the soil similar to natural environmental conditions, but in controlled environment to prevent the occurrence of natural infestations. At least 5 infestations are performed, containing 20-35 *Diatraea saccharalis* eggs per tiller. The evaluation occurs when all infected stalks are harvested and cutting longitudinally to quantify the damage. Infestation Intensity is calculated dividing the number of internodes with damage by the total number of internodes, and the result was multiplied by 100 (Infestation Intensity). Percentage of Effective Damage was calculated, considering the total of internodes with damage caused by the insect divided by the total number of stalks evaluated in the plot.

**[0283]** Screenhouse trials were performed to characterize the efficacy of CTC75064-3 event in controlling borer attack in comparison to its parental variety RB 867515 (WT;non-transgenic) in a randomized block design, with 4 replications. Each experimental plot was composed by eight clumps of cane-plant that received 10 artificial infestations with approximately 30 eggs of *D. saccharalis* every 15 days. After eight months, the Infestation Intensity (II) and the effective Damage for both varieties were calculated. Relative efficacy was calculated according to the formula below:

$$relative\ efficacy\ (\%) = 1 - \left(\frac{\text{(average I.I.\% of the event)}}{\text{average I.I.\% of the controls}}\right)$$

**[0284]** Under the artificial infestation the event CTC75064-3 presented relative efficacy in controlling infestation by *D. saccharalis* of 98.8% and in controlling stalk damage (length) superior to 99.9% in relation to its non-transgenic parental variety RB 867515 (Control). The damage caused by *D. saccharalis* in CTC75064-3 stalks was visibly lower than in the conventional sugarcane RB 867515. There were statistically differences (t-test, $P < 0.05$) between CTC75064-3 and the non-transgenic variety RB 867515 in both parameters evaluated ($df = 16$; $P < 0.0001$), showing that under massive infestation with *D. Saccharalis* the event suppressed the damages caused by the pest (Figure 16).

**[0285]** On sugarcane conventional production *D. saccharalis* is considered controlled when the infestation intensity is lower than 3% (Gallo et al., 2002). For CTC75064-3, the intensity of infestation was lower than 0,01% reinforcing the event effectiveness to control its main target pest.

**[0286]** In addition to employing bioassays that use artificial infestations to observe the degree of infestation and damage caused by *D. saccharalis,* observations of infestation and damage percentage can also be made based on information collected directly from the fields where the event CTC75064-3 is grown. The II (infestation intensity), for example, can be calculated for natural infestation evaluation by defining an experimental area for stalk sampling cutting and quantification the number of internodes with and without damages) to obtain the infestation intensity (II).

**[0287]** In the tests performed for the event of the invention, the infestation intensity (II) was calculated in four experimental areas: Piracicaba, Barrinha, and Valparaiso (SP) and Quirinopolis (GO). The conventional variety RB 867515 (parental; non transgenic) was used as control. This assay illustrates the resistance of the event CTC75064-3 to *D. saccharalis* infestation compared to the parental variety (RB 867515): it was observed a lower intensity of infestation for CTC75064-3 plants in comparison to the parental variety (RB 867515) in all the four experimental areas (Combined analysis; Figure 17). Event CTC75064-3 presented relative efficacy in controlling infestation by *D. saccharalis* of 100% in all locations, except for Valparaiso, which showed 99.1% effectiveness, very close to 100%. Thus, it appears that the event CTC75064 was, on average in the 4 locations, 99.7% more effective than conventional materials, for controlling *Diatraea saccharalis* (Figure 17).

**[0288]** Having described examples of preferred embodiments, it should be understood that the scope of the present invention encompasses other possible variations and is limited only by the content of the appended claims, including the possible equivalents thereof.

**SEQUENCES**

**> SEQ ID 01 - Genetic construct comprising Cry1Ac and nptII genes**

TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTA
GTACTGATATCGGTACCTTAATTCGGGGGATCTGGATTTTAGTACTGGATTTTGGTTTTAGGAATTAGAAATTTTATTGATAG
AAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGAGCGAAACCCTATAGGAACCCTAATTCC
CTTATCTGGGAACTACTCACACATTATTATGGAGAAACTCGAGCTTGTCGATCGATCACTCAGCCTCGAGGGTGGCGGTCACT
GGGATGAACTCGAACCTGTCGATGATCACGCCGGCGGTGCCGCTGAAGTTCCTCACGCCCACGATGTTACCGAGGGAGGAGGT
GAAAGCGTTGGCGCTCTCGAAGTAACCGAAGTCGCTGGATTGGAGGTTGTCCAGGGAGGTAGCGGTAGCTGGCACGGTGTTGG
AGAAGATGGAGGAGTTACCCCAGTTCACGTTGAGGTGGATCGGGGTCACGGAAGCGTACCTCACGCGCACCCTGTACCTGGTG
GAGGTGGATGGGAAGTGGATTGGCACCTCGATGTAGCCCCTGTTCTGGATGTTGTTGCCGCTGCTGTTGAGCCTCACGAGGTC
GCCACCGGTGAAGCCTGGGCCGGAAATGACGGAACCGTTGAAGAGGAAGTTGCCCTTCACGGCCGGGATTTGGGTGATGCTGT
CGGAGGCGATGATGTTGTTGAACTCAGCGCTGCGGTGGATCCAGGAGAACATCGGAGCCCTGATGATGCTCACGGAGCTGTTG
CTGAAGCCGGAGCGGAACATGGACACGTGGCTCAGCCTGTGGGAGAAGCCTTGCCTGGGTGGCACGTTGTTGTTCTGTGGTGG
GATCTCGTCCAGGGAGTCCACGGTGCCGCTCTTCCTGTAGACAGCGGATGGCAGGTTGGAGGAGGTGCCGTAGGCGAACTCGG
TGCCGTCGAGCACGGACAGTTGCTGGTTGTTGATACCGATGTTGAAGGGCCTCCTGTACAGGGTGGAGGACAGGGTCCTGTAG
ACACCCTGACCCAGTTGAGCCACGATGCGTTGCTGTGGAGCGGCGTTGCCCATGGTGCCGTAGAGCGGGAAGGTGAACTCGGG
GCCGCTGAAGCCCACTGGGGAGGCCATGATCTGGTGGCCGGACCAGTAGTACTCGCCCCTGTGAGCGTCGGTGTAGATGGTGA
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**> SEQ ID 02 (T-DNA fragment event CTC75064)**

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**>SEQ ID NO 3 – Junction nucleotide sequence between 5' region of insert and sugarcane genome of event CTC75064.**

GTGATCCTAGACTTTCTAGAAATCTTAGGAAGATTACTACACTTTAGTTATTCATACCAAAAACTAATTCATAGCTTAACATG
ACCAAAATTGCTAATATTTCACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAGTA
CTGATATCGGTACCTTAATTCGGGGGATCTGGATTTTAGT

**>SEQ ID NO 4 – Junction nucleotide sequence between 3' region of insert and sugarcane genome of event CTC75064**

TCATCTATGTTACTAGATCGGCGCGCCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCGAGCTCGTCGACTC
TAGACTCGAGGGCGCGCCGATACCAATTTGGACAGAAAACTGACAAGAACTTCGAAAATACATAATTGGAGTTCTAGAGACAC
AACAAAAGTGATCCTTAACTTTATGTAAAGCTTATTAAGT

**>SEQ ID NO 5 - Event CTC75064: flanking sequences and T-DNA**

GTGATCCTAGACTTTCTAGAAATCTTAGGAAGATTACTACACTTTAGTTATTCATACCAAAAACTAATTCATAGCTTAACATG
ACCAAAATTGCTAATATTTCACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTAGTA
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TTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGCTCTAGCCGTTCCGCAGACGGGATCGATTTCATGATTTTT
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TTAAGT

**>SEQ** ID **NO 6** - **primer forward (Right Border)**
CAAGGGCGAATTCCAGCAC
**> SEQ** ID **NO 07** - primer reverse (Right border)
GTTCTTGTCAGTTTTCTGTCCAAA
**> SEQ** ID **NO 08** - - primer forward (Left Border)
CATGACCAAAATTGCTAATATTTCAC
**> SEQ** ID **NO 09** - - primer reverse (Left Border)
AATCCAGTACTAAAATCCAGATCCC
**> SEQ ID NO 10** - probe (Right border)
CCGTTACTAGTGGATCGA
**> SEQ ID NO 11** - - probe (Left border)
CTTAATAACACATTGCGGACGT

**> SEQ ID NO 12** - Amplicon Event CTC75064 (Right Border)

CAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCGAGCTCGTCGACTCTAGACTCGAGGGCGCGCCGATACCAA
TTTGGACAGAAAACTGACAAGAAC

**> SEQ ID NO 13 –** Amplicon Event CTC75064 (Left Border)

CATGACCAAAATTGCTAATATTTCACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATT
AGTACTGATATCGGTACCTTAATTCGGGGGATCTGGATTTTAGTACTGGATT

> **SEQ ID 14 - Binary plasmid comprising cry1Ac and nptII genes**

TGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGAATTA
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**>SEQ ID NO 15 - primer forward: sugarcane poly-ubiquitin gene**
ACCATTACCCTGGAGGTTGAGA
**>SEQ ID NO 16 - primer reverse: sugarcane poly-ubiquitin gene**
GTCCTGGATCTTCGCCTTCA
**>SEQ ID NO 17 - probe sugarcane poly-ubiquitin gene**
CTCTGACACCATCGAC
**>SEQ ID NO 18 - Junction sequence between 5'region of insert and sugarcane genome of event CTC75064**
TTGCTAATATTTCACAAATTGACGCT
**>SEQ ID NO 19 - Junction sequence between 3'region of insert and sugarcane genome of event CTC75064**
GAGGGCGCGCCGATACCAATTTGGAC

**>SEQ ID 20 - Sugarcane optimized truncate cry1Ac gene (B. thurigiensis).**

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TACGACTCCAGGACCTACCCGATCCGCACCGTGTCCCAACTCACCAGGGAGATCTACACCAACCCAGTGCTGGAGAACTTCGA
CGGTAGCTTCCGCGGTTCCGCCCAGGGTATCGAGGGCTCCATCAGGAGCCCACACCTGATGGACATCCTGAACAGCATCACCA
TCTACACCGACGCTCACAGGGGCGAGTACTACTGGTCCGGCCACCAGATCATGGCCTCCCCAGTGGGCTTCAGCGGCCCCGAG
TTCACCTTCCCGCTCTACGGCACCATGGGCAACGCCGCTCCACAGCAACGCATCGTGGCTCAACTGGGTCAGGGTGTCTACAG
GACCCTGTCCTCCACCCTGTACAGGAGGCCCTTCAACATCGGTATCAACAACCAGCAACTGTCCGTGCTCGACGGCACCGAGT
TCGCCTACGGCACCTCCTCCAACCTGCCATCCGCTGTCTACAGGAAGAGCGGCACCGTGGACTCCCTGGACGAGATCCCACCA
CAGAACAACAACGTGCCACCCAGGCAAGGCTTCTCCCACAGGCTGAGCCACGTGTCCATGTTCCGCTCCGGCTTCAGCAACAG
CTCCGTGAGCATCATCAGGGCTCCGATGTTCTCCTGGATCCACCGCAGCGCTGAGTTCAACAACATCATCGCCTCCGACAGCA
TCACCCAAATCCCGGCCGTGAAGGGCAACTTCCTCTTCAACGGTTCCGTCATTTCCGGCCCAGGCTTCACCGGTGGCGACCTC
GTGAGGCTCAACAGCAGCGGCAACAACATCCAGAACAGGGGCTACATCGAGGTGCCAATCCACTTCCCATCCACCTCCACCAG
GTACAGGGTGCGCGTGAGGTACGCTTCCGTGACCCCGATCCACCTCAACGTGAACTGGGGTAACTCCTCCATCTTCTCCAACA
CCGTGCCAGCTACCGCTACCTCCCTGGACAACCTCCAATCCAGCGACTTCGGTTACTTCGAGAGCGCCAACGCTTTCACCTCC
TCCCTCGGTAACATCGTGGGCGTGAGGAACTTCAGCGGCACCGCCGGCGTGATCATCGACAGGTTCGAGTTCATCCCAGTGAC
CGCCACCCTCGAGGCTGAGTGA

**> SEQ ID NO 21- Sugarcane optimized nptII gene (B. thurigiensis)**

TCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGT
CAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGG
CCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACGAGATC
CTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCT
GATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGA
TCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTG
CCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCG
TGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGG
CGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTC
CACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCCACAT

**>SEQ ID NO 22 - CTC75064 event: Flanking sequences and T DNA fragment**

AACGAGGTGACAACTAGGGCACTACCTAGATAACAAACACACATACAACATCACAATCAAGCAGTCATTTGAGAAAATTCAAA
CAAATGGGCTTAAGCAACCAACACAAGACACAAGGCTCACCCAAAATAAGCATTTATCTCAAGCATGAACTAACTACAAGTTT
AGCTCAAAACAACGCTAGATACGGTAGCCAACTTAGCATGTGCCTAGAATCTGGACAGCAACGCAGTAGTTACTTGTTTCAAC
CATAACTGAGGTTATAGACATCCCACAAAGGTGATCCTAGACTTTCTAGAAATCTTAGGAAGATTACTACACTTTAGTTATTC
ATACCAAAAACTAATTCATAGCTTAACATGACCAAAATTGCTAATATTTCACAAATTGACGCTTAGACAACTTAATAACACAT
TGCGGACGTTTTTAATGTACTGAATTAGTACTGATATCGGTACCTTAATTCGGGGGATCTGGATTTTAGTACTGGATTTTGGT
TTTAGGAATTAGAAATTTTATTGATAGAAGTATTTTACAAATACAAATACATACTAAGGGTTTCTTATATGCTCAACACATGA
GCGAAACCCTATAGGAACCCTAATTCCCTTATCTGGGAACTACTCACACATTATTATGGAGAAACTCGAGCTTGTCGATCGAT
CACTCAGCCTCGAGGGTGGCGGTCACTGGGATGAACTCGAACCTGTCGATGATCACGCCGGCGGTGCCGCTGAAGTTCCTCAC
GCCCACGATGTTACCGAGGGAGGAGGTGAAAGCGTTGGCGCTCTCGAAGTAACCGAAGTCGCTGGATTGGAGGTTGTCCAGGG
AGGTAGCGGTAGCTGGCACGGTGTTGGAGAAGATGGAGGAGTTACCCCAGTTCACGTTGAGGTGGATCGGGGTCACGGAAGCG
TACCTCACGCGCACCCTGTACCTGGTGGAGGTGGATGGGAAGTGGATTGGCACTCGATGTAGCCCCTGTTCTGGATGTTGTTG
CCGCTGCTGTTGAGCCTCACGAGGTCGCCACCGGTGAAGCCTGGGCCGGAAATGACGGAACCGTTGAAGAGGAAGTTGCCCTT
CACGGCCGGGATTTGGGTGATGCTGTCGGAGGCGATGATGTTGTTGAACTCAGCGCTGCGGTGGATCCAGGAGAACATCGGAG
CCCTGATGATGCTCACGGAGCTGTTGCTGAAGCCGGAGCGGAACATGGACACGTGGCTCAGCCTGTGGGAGAAGCCTTGCCTG
GGTGGCACGTTGTTGTTCTGTGGTGGGATCTCGTCCAGGGAGTCCACGGTGCCGCTCTTCCTGTAGACAGCGGATGGCAGGTT
GGAGGAGGTGCCGTAGGCGAACTCGGTGCCGTCGAGCACGGACAGTTGCTGGTTGTTGATACCGATGTTGAAGGGCCTCCTGT
ACAGGGTGGAGGACAGGGTCCTGTAGACACCCTGACCCAGTTGAGCCACGATGCGTTGCTGTGGAGCGGCGTTGCCCATGGTG
CCGTAGAGCGGGAAGGTGAACTCGGGGCCGCTGAAGCCCACTGGGGAGGCCATGATCTGGTGGCCGGACCAGTAGTACTCGCC
CCTGTGAGCGTCGGTGTAGATGGTGATGCTGTTCAGGATGTCCATCAGGTGTGGGCTCCTGATGGAGCCCTCGATACCCTGGG
CGGAACCGCGGAAGCTACCGTCGAAGTTCTCCAGCACTGGGTTGGTGTAGATCTCCCTGGTGAGTTGGGACACGGTGCGGATC
GGGTAGGTCCTGGAGTCGTAGTTCGGGAAGAGGGACACAATGTCCAGCACGGTGAGGGTCAACTCCCTCCTGAACTGGTTGTA
CCTGATCCAGTCCCTGGAGTCCGGACCCCAGACGCGCTCCAGGCCGGTGTTGTACCAGCGCACAGCGTGGTCGGTGTAGTTGC

CAATCAGCCTGGTCAGGTCGTTGTAGCGGCTGTTGATGGTGGCAGCATCGAAGCCCCACCTTTGGCCGAACACGCTCACGTCG CGCAGCACGCTGAGGTGCAGGTTAGCGGCTTGCACGTACACGGACAGGAGCGGCACTTGGTAGTTCTGGACGGCGAACAGTGG GATAGCGGTGGTCAGGGCGCTGTTCATGTCGTTGAATTGAATGCGCATTTCCTCGCGGAGAGCTGGGTTGGTCGGGTCGGCCT CCCACTCCCTGAAGCTCTCGGCGTAGATTTGGTAGAGGTTGCTCAGGCCCTCCAGCCTGGAGATGGCCTGGTTCCTGGCGAAC TCTTCGATCCTCTGGTTGATCAGCTGCTCGATTTGCACCAGGAAGGCGTCCCATTGGGATGGACCGAAGATACCCCAGATGAT GTCCACCAGGCCGAGCACGAAGCCAGCACCTGGCACGAACTCGCTGAGCAGGAACTGGGTCAAGGACAGGGAGATGTCGATGG GGGTGTAACCGGTCTCGATGCGCTCGCCACCCAGCACCTCCACCTCTGGGTTGCTCAGGCAGTTGTATGGGATGCACTCGTTG ATGTTTGGGTTGTTGTCCATGGCGGGGTTGATCAGGTTGATCACTTCTACCTACAAAAAAGCTCCGCACGAGGCTGCATTTGT CACAAATCATGAAAAGAAAAACTACCGATGAACAATGCTGAGGGATTCAAATTCTACCCACAAAAAGAAGAAAGAAAGATCTA GCACATCTAAGCCTGACGAAGCAGCAGAAATATATAAAAATATAAACCATAGTGCCCTTTTCCCCTCTTCCTGATCTTGTTTA GCACGGCGGAAATTTTAAACCCCCCATCATCTCCCCCAACAACGGCGGATCGCAGATCTACATCCGAGAGCCCCATTCCCCGC GAGATCCGGGCCGGATCCACGCCGGCGAGAGCCCCAGCCGCGAGATCCCGCCCCTCCCGCGCACCGATCTGGGCGCGCACGAA GCCGCCTCTCGCCCACCCAAACTACCAAGGCCAAAGATCGAGACCGAGACGGAAAAAAAAAACGGAGAAAGAAAGAGGAGAGGG GCGGGGTGGTTACCGGCGGCGGCGGAGGCCTCCCTTGGATCTTATGGTGTGTTGTCCCTGTGTGTTCTCCAATAGTGTGGCTT GAGTGTGTGGAAGATGGTTCTAGAGGATCTGCTAGAGTCAGCTTGTCAGCGTGTCCTCTCCAAATGAAATGAACTTCCTTATA TAGAGGAAGGGTCTTGCGAAGGATAGTGGGATTGTGCGTCATCCCTTACGTCAGTGGAGATATCACATCAATCCACTTGCTTT GAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACCACTGTCGGCAGAGGC ATCTTCAACGATGGCCTTTCCTTTATCGCAATGATGGCATTTGTAGGAGCCACCTTCCTTTTCCACTATCTTCACAATAAAGT GACAGATAGCTGGGCAATGGAATCCGAGGAGGTTTCCGGATATTACCCTTTGTTGAAAAGTCTCAATCGGACCATCACATCAA TCCACTTGCTTTGAAGACGTGGTTGGAACGTCTTCTTTTTCCACGATGCTCCTCGTGGGTGGGGGTCCATCTTTGGGACCACT GTCGGCAGAGGCATCTTCAACGATGGCCTTTCCTTTATCGCAATGATGGCATTTGTAGGAGCCACCTTCCTTTTCCACTATCT TCACAATAAAGTGACAGATAGCTGGGCAATGGAATCCGAGGAGGTTTCCGGATATTACCCTTTGTTGAAAAGTCTCAATCGGA CCTGTTTAAACCCTGAAGCTTACGCGTATGCCTGCAGTGCAGCGTGACCCGGTCGTGCCCCTCTCTAGAGATAATGAGCATTG CATGTCTAAGTTATAAAAAATTACCACATATTTTTTTTGTCACACTTGTTTGAAGTGCAGTTTATCTATCTTTATACATATAT TTAAACTTTACTCTACGAATAATATAATCTATAGTACTACAATAATATCAGTGTTTTAGAGAATCATATAAATGAACAGTTAG ACATGGTCTAAAGGACAATTGAGTATTTTGACAACAGGACTCTACAGTTTTATCTTTTTAGTGTGCATGTGTTCTCCTTTTTT TTTGCAAATAGCTTCACCTATATAATACTTCATCCATTTTATTAGTACATCCATTTAGGGTTTAGGGTTAATGGTTTTTATAG ACTAATTTTTTTAGTACATCTATTTTATTCTATTTTAGCCTCTAAATTAAGAAAACTAAAACTCTATTTTAGTTTTTTTATTT AATAATTTAGATATAAAATAGAATAAAATAAAGTGACTAAAAATTAAACAAATACCCTTTAAGAAATTAAAAAAACTAAGGAA ACATTTTTCTTGTTTCGAGTAGATAATGCCAGCCTGTTAAACGCCGTCGACGAGTCTAACGGACACCAACCAGCGAACCAGCA GCGTCGCGTCGGGCCAAGCGAAGCAGACGGCACGGCATCTCTGTCGCTGCCTCTGGACCCCTCTCGAGAGTTCCGCTCCACCG TTGGACTTGCTCCGCTGTCGGCATCCAGAAATTGCGTGGCGGAGCGGCAGACGTGAGCCGGCACGGCAGGCGGCCTCCTCCTC CTCTCACGGCACGGCAGCTACGGGGGATTCCTTTCCCACCGCTCCTTCGCTTTCCCTTCCTCGCCCGCCGTAATAAATAGACA CCCCCTCCACACCCTCTTTCCCCAACCTCGTGTTGTTCGGAGCGCACACACACACACAACCAGATCTCCCCCAAATCCACCCGTC GGCACCTCCGCTTCAAGGTACGCCGCTCGTCCTCCCCCCCCCCCCCCTCTCTACCTTCTCTAGATCGGCGTTCCGGTCCATGGT TAGGGCCCGGTAGTTCTACTTCTGTTCATGTTTGTGTTAGATCCGTGTTTGTGTTAGATCCGTGCTGCTAGCGTTCGTACACG GATGCGACCTGTACGTCAGACACGTTCTGATTGCTAACTTGCCAGTGTTTCTCTTTGGGGAATCCTGGGATGGCTCTAGCCGT TCCGCAGACGGGATCGATTTCATGATTTTTTTTGTTTCGTTGCATAGGGTTTGGTTTGCCCTTTTCCTTTATTTCAATATATG CCGTGCACTTGTTTGTCGGGTCATCTTTTCATGCTTTTTTTTGTCTTGGTTGTGATGATGTGGTCTGGTTGGGCGGTCGTTCT AGATCGGAGTAGAATTCTGTTTCAAACTACCTGGTGGATTTATTAATTTTGGATCTGTATGTGTGTGCCATACATATTCATAG TTACGAATTGAAGATGATGGATGGAAATATCGATCTAGGATAGGTATACATGTTGATGCGGGTTTTACTGATGCATATACAGA GATGCTTTTTGTTCGCTTGGTTGTGATGATGTGGTGTGGTTGGGCGGTCGTTCATTCGTTCTAGATCGGAGTAGAATACTGTT TCAAACTACCTGGTGTATTTATTAATTTTGGAACTGTATGTGTGTGTCATACATCTTCATAGTTACGAGTTTAAGATGGATGG AAATATCGATCTAGGATAGGTATACATGTTGATGTGGGTTTTACTGATGCATATACATGATGGCATATGCAGCATCTATTCAT ATGCTCTAACCTTGAGTACCTATCTATTATAATAAACAAGTATGTTTTATAATTATTTTGATCTTGATATACTTGGATGATGG CATATGCAGCAGCTATATGTGGATTTTTTTAGCCCTGCCTTCATACGCTATTTATTTGCTTGGTACTGTTTCTTTTGTCGATG CTCACCCTGTTGTTTGGTGTTACTTCTGCAGGTCGACTCTAGAATGTGGATTGAACAAGATGGATTGCACGCAGGTTCTCCGG CCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCA GCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATC GTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCG AAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTG CATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGG TCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGC CCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTC ATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGG CGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACG AGTTCTTCTGAGATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATA TAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAG AGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCAT CTATGTTACTAGATCGGCGCGCCAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAGTGGATCGAGCTCGTCGACTCTAGA CTCGAGGGCGCGCCGATACCAATTTGGACAGAAAACTGACAAGAACTTCGAAAATACATAATTGGAGTTCTAGAGACACAACA AAAGTGATCCTTAACTTTATGTAAAGCTTATTAAGTTGTCTATAACTTTCTTATTATCATATTAAGATGATTCTACGGCTAAC AAGGACAAACATAGCAAAGAAAACATCTCTGCCGGATTTGGACAGATTCTGTCATTCTACTTTGCAAGCTCATAACTAGAGAT TTAGACCATCACAATTAGTGCTATAGGACATTTTGGAAAGCTTAGAAAAAGTACTACACTTCTTCTATTATCTCTAAGACACG ATTCCATATGCACACAGGTCAAACAAACCAAACATTCAGATCTATTCAGAACATTGACAAAACCTGACCGTAAACTTTTAAAA TTCATAACTCTAAGCTCAAGCATGTAGTCACACTTATTTCTAGTAAAGCTAAGCACACATTTTTCTCAAGCATTTCCTATTCA

AGCAACATGGAACAAAGCATTCTTGTTTGACTCCACACAAGGAAGATAGGATACCACATCACTCACAAATTACTCATCATTTG AACAAGGGTGAGAGGAGCATAGCTATGCATAAGGCAACAATCATGAAGATGC

**>SEQ ID NO 23 - CTC75064 event: Flanking sequence - Left border (5')**

AACGAGGTGACAACTAGGGCACTACCTAGATAACAAACACACATACAACATCACAATCAAGCAGTCATTTGAGAAAATTCAAAC
AAATGGGCTTAAGCAACCAACACAAGACACAAGGCTCACCCAAAATAAGCATTTATCTCAAGCATGAACTAACTACAAGTTTA
GCTCAAAACAACGCTAGATACGGTAGCCAACTTAGCATGTGCCTAGAATCTGGACAGCAACGCAGTAGTTACTTGTTTCAACC
ATAACTGAGGTTATAGACATCCCACAAAGGTGATCCTAGACTTTCTAGAAATCTTAGGAAGATTACTACACTTTAGTTATTCA
TACCAAAAACTAATTCATAGCTTAACATGACCAAAATTGCTAATATTTC

**> SEQ ID NO 24 - CTC75064 event: Flanking sequence - Right border (3')**

TACCAATTTGGACAGAAAACTGACAAGAACTTCGAAAATACATAATTGGAGTTCTAGAGACACAACAAAAGTGATCCTTAACT
TTATGTAAAGCTTATTAAGTTGTCTATAACTTTCTTATTATCATATTAAGATGATTCTACGGCTAACAAGGACAAACATAGCA
AAGAAAACATCTCTGCCGGATTTGGACAGATTCTGTCATTCTACTTTGCAAGCTCATAACTAGAGATTTAGACCATCACAATT
AGTGCTATAGGACATTTTGGAAAGCTTAGAAAAAGTACTACACTTCTTCTATTATCTCTAAGACACGATTCCATATGCACACA
GGTCAAACAAACCAAACATTCAGATCTATTCAGAACATTGACAAAACCTGACCGTAAACTTTTAAAATTCATAACTCTAAGCT
CAAGCATGTAGTCACACTTATTTCTAGTAAAGCTAAGCACACATTTTTCTCAAGCATTTCCTATTCAAGCAACATGGAACAAA
GCATTCTTGTTTGACTCCACACAAGGAAGATAGGATACCACATCACTCACAAATTACTCATCATTTGAACAAGGGTGAGAGGA
GCATAGCTATGCATAAGGCAACAATCATGAAGATGC

**>SEQ ID 25 Cas9/crRNA/HR template construction**

CTTAGAATAACGGATATTTAAAAGGGCGTGAAAAGGTTTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCC
CTCGGGATCAAAGTACTTTGATCCAACCCCTCCGCTGCTATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCTTCTGAA
AACGACATGTCGCACAAGTCCTAAGTTACGCGACAGGCTGCCGCCCTGCCCTTTTCCTGGCGTTTTCTTGTCGCGTGTTTTAG
TCGCATAAAGTAGAATACTTGCGACTAGAACCGGAGACATTACGCCATGAACAAGAGCGCCGCCGCTGGCCTGCTGGGCTATG
CCCGCGTCAGCACCGACGACCAGGACTTGACCAACCAACGGGCCGAACTGCACGCGGCCGGCTGCACCAAGCTGTTTTCCGAG
AAGATCACCGGCACCAGGCGCGACCGCCCGGAGCTGGCCAGGATGCTTGACCACCTACGCCCTGGCGACGTTGTGACAGTGAC
CAGGCTAGACCGCCTGGCCCGCAGCACCCGCGACCTACTGGACATTGCCGAGCGCATCCAGGAGGCCGGCGCGGGCCTGCGTA
GCCTGGCAGAGCCGTGGGCCGACACCACCACGCCGGCCGGCCGCATGGTGTTGACCGTGTTCGCCGGCATTGCCGAGTTCGAG
CGTTCCCTAATCATCGACCGCACCCGGAGCGGGCGCGAGGCCGCCAAGGCCCGAGGCGTGAAGTTTGGCCCCCGCCCTACCCT
CACCCCGGCACAGATCGCGCACGCCCGCGAGCTGATCGACCAGGAAGGCCGCACCGTGAAAGAGGCGGCTGCACTGCTTGGCG
TGCATCGCTCGACCCTGTACCGCGCACTTGAGCGCAGCGAGGAAGTGACGCCCACCGAGGCCAGGCGGCGCGGTGCCTTCCGT
GAGGACGCATTGACCGAGGCCGACGCCCTGGCGGCCGCCGAGAATGAACGCCAAGAGGAACAAGCATGAAACCGCACCAGGAC
GGCCAGGACGAACCGTTTTTCATTACCGAAGAGATCGAGGCGGAGATGATCGCGGCCGGGTACGTGTTCGAGCCGCCCGCGCA
CGTCTCAACCGTGCGGCTGCATGAAATCCTGGCCGGTTTGTCTGATGCCAAGCTGGCGGCCTGGCCGGCCAGCTTGGCCGCTG
AAGAAACCGAGCGCCGCCGTCTAAAAAGGTGATGTGTATTTGAGTAAAACAGCTTGCGTCATGCGGTCGCTGCGTATATGATG
CGATGAGTAAATAAACAAATACGCAAGGGGAACGCATGAAGGTTATCGCTGTACTTAACCAGAAAGGCGGGTCAGGCAAGACG
ACCATCGCAACCCATCTAGCCCGCGCCCTGCAACTCGCCGGGGCCGATGTTCTGTTAGTCGATTCCGATCCCCAGGGCAGTGC
CCGCGATTGGGCGGCCGTGCGGGAAGATCAACCGCTAACCGTTGTCGGCATCGACCGCCCGACGATTGACCGCGACGTGAAGG
CCATCGGCCGGCGCGACTTCGTAGTGATCGACGGAGCGCCCCAGGCGGCGGACTTGGCTGTGTCCGCGATCAAGGCAGCCGAC
TTCGTGCTGATTCCGGTGCAGCCAAGCCCTTACGACATATGGGCCACCGCCGACCTGGTGGAGCTGGTTAAGCAGCGCATTGA
GGTCACGGATGGAAGGCTACAAGCGGCCTTTGTCGTGTCGCGGGCGATCAAAGGCACGCGCATCGGCGGTGAGGTTGCCGAGG
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ACCGTTCTTGAATCAGAACCCGAGGGCGACGCTGCCCGCGAGGTCCAGGCGCTGGCCGCTGAAATTAAATCAAAACTCATTTG
AGTTAATGAGGTAAAGAGAAAATGAGCAAAAGCACAAACACGCTAAGTGCCGGCCGTCCGAGCGCACGCAGCAGCAAGGCTGC
AACGTTGGCCAGCCTGGCAGACACGCCAGCCATGAAGCGGGTCAACTTTCAGTTGCCGGCGGAGGATCACACCAAGCTGAAGA
TGTACGCGGTACGCCAAGGCAAGACCATTACCGAGCTGCTATCTGAATACATCGCGCAGCTACCAGAGTAAATGAGCAAATGA
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CCCGAGGAATCGGCGTGACGGTCGCAAACCATCCGGCCCGGTACAAATCGGCGCGGCGCTGGGTGATGACCTGGTGGAGAAGT
TGAAGGCCGCGCAGGCCGCCCAGCGGCAACGCATCGAGGCAGAAGCACGCCCCGGTGAATCGTGGCAAGCGGCCGCTGATCGA
ATCCGCAAAGAATCCCGGCAACCGCCGGCAGCCGGTGCGCCGTCGATTAGGAAGCCGCCCAAGGGCGACGAGCAACCAGATTT
TTTCGTTCCGATGCTCTATGACGTGGGCACCCGCGATAGTCGCAGCATCATGGACGTGGCCGTTTTCCGTCTGTCGAAGCGTG
ACCGACGAGCTGGCGAGGTGATCCGCTACGAGCTTCCAGACGGGCACGTAGAGGTTTCCGCAGGGCCGGCCGGCATGGCCAGT
GTGTGGGATTACGACCTGGTACTGATGGCGGTTTCCCATCTAACCGAATCCATGAACCGATACCGGGAAGGGAAGGGAGACAA
GCCCGGCCGCGTGTTCCGTCCACACGTTGCGGACGTACTCAAGTTCTGCCGGCGAGCCGATGGCGGAAAGCAGAAAGACGACC

TGGTAGAAACCTGCATTCGGTTAAACACCACGCACGTTGCCATGCAGCGTACGAAGAAGGCCAAGAACGGCCGCCTGGTGACG
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AGCTGATTGGATGTACCGCGAGATCACAGAAGGCAAGAACCCGGACGTGCTGACGGTTCACCCCGATTACTTTTTGATCGATC
CCGGCATCGGCCGTTTTCTCTACCGCCTGGCACGCCGCGCCGCAGGCAAGGCAGAAGCCAGATGGTTGTTCAAGACGATCTAC
GAACGCAGTGGCAGCGCCGGAGAGTTCAAGAAGTTCTGTTTCACCGTGCGCAAGCTGATCGGGTCAAATGACCTGCCGGAGTA
CGATTTGAAGGAGGAGGCGGGGCAGGCTGGCCCGATCCTAGTCATGCGCTACCGCAACCTGATCGAGGGCGAAGCATCCGCCG
GTTCCTAATGTACGGAGCAGATGCTAGGGCAAATTGCCCTAGCAGGGGAAAAAGGTCGAAAAGGTCTCTTTCCTGTGGATAGC
ACGTACATTGGGAACCCAAAGCCGTACATTGGGAACCGGAACCCGTACATTGGGAACCCAAAGCCGTACATTGGGAACCGGTC
ACACATGTAAGTGACTGATATAAAAGAGAAAAAAGGCGATTTTTCCGCCTAAAACTCTTTAAAACTTATTAAAACTCTTAAAA
CCCGCCTGGCCTGTGCATAACTGTCTGGCCAGCGCACAGCCGAAGAGCTGCAAAAAGCGCCTACCCTTCGGTCGCTGCGCTCC
CTACGCCCCGCCGCTTCGCGTCGGCCTATCGCGGCCGCTGGCCGCTCAAAAATGGCTGGCCTACGGCCAGGCAATCTACCAGG
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GAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGG
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TGCGGCATCAGAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCA
TCAGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAG
GCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCG
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GTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTC
TTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGC
GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCC
AGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGC
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TCACGTTAAGGGATTTTGGTCATGCATTCTAGGTATTATTTGCCAACGACCTTCGTGATCTCGCCCTTGACATAGTGGACAAA
TTCTTCGAGCTGGTCGGCCCGGGACGCGAGACGGTCTTCTTCTTGGCCCAGATAGGCTTGGCGCGCTTCGAGGATCACGGGCT
GGTATTGCGCCGGAAGGCGCTCCATCGCCCAGTCGGCGGCGACATCCTTCGGCGCGATCTTGCCGGTAACCGCCGAGTACCAA
ATCCGGCTCAGCGTAAGGACCACATTGCGCTCATCGCCCGCCCAATCCGGCGGGGAGTTCCACAGGGTCAGCGTCTCGTTCAG
TGCTTCGAACAGATCCTGTTCCGGCACCGGGTCGAAAAGTTCCTCGGCCGCGGGGCCGACGAGGGCCACGCTATGCTCCCGGG
CCTTGGTGAGCAGGATCGCCAGATCAATGTCGATGGTGGCCGGTTCAAAGATACCCGCCAGAATATCATTACGCTGCCATTCG
CCGAACTGGAGTTCGCGTTTGGCCGGATAGCGCCAGGGGATGATGTCATCGTGCACCACAATCGTCACCTCAACCGCGCGCAG
GATTTCGCTCTCGCCGGGGGAGGCGGACGTTTCCAGAAGGTCGTTGATAAGCGCGCGGCGCGTGGTCTCGTCGAGACGGACGG
TAACGGTGACAAGCAGGTCGATGTCCGAATGGGGCTTAAGGCCGCCGTCAACGGCGCTACCATACAGATGCACGGCGAGGAGG
GTCGGTTCGAGGTGGCGCTCGATGACACCCACGACTTCCGACAGCTGGGTGGACACCTCGGCGATGACCGCTTCACCCATTTA
TTATTTCCTTCCTCTTTTCTACAGTATTTAAAGATACCCCAAGAAGCTAATTATAACAAGACGAACTCCAATTCACTGTTCCT
TGCATTCTAAAACCTTAAATACCAGAAAACAGCTTTTTCAAAGTTGTTTTCAAAGTTGGCGTATAACATAGTATCGACGGAGC
CGATTTTGAAACCGCGGTGATCACAGGCAGCAACGCTCTGTCATCGTTACAATCAACATGCTACCCTCCGCGAGATCATCCGT
GTTTCAAACCCGGCAGCTTAGTTGCCGTTCTTCCGAATAGCATCGGTAACATGAGCAAAGTCTGCCGCCTTACAACGGCTCTC
CCGCTGACGCCGTCCCGGACTGATGGGCTGCCTGTATCGAGTGGTGATTTTGTGCCGAGCTGCCGGTCGGGGAGCTGTTGGCT
GGCTGGTGGCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACT
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**>SEQ ID 26 HR_template construct**

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**>SEQ ID 27 Cas9/crRNA construction**

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TGTCCCTCGGCCTGACCCCCAACTTCAAGAGCAACTTCGACCTGGCCGAGGACGCCAAGCTCCAGCTGTCCAAGGACACCTAC
GACGACGACCTCGACAACCTGCTCGCCCAGATCGGCGACCAGTACGCCGACCTCTTCCTGGCCGCCAAGAACCTCTCCGACGC
CATCCTGCTCAGCGACATCCTGAGGGTGAACACCGAGATCACCAAGGCCCCGCTGTCCGCCAGCATGATCAAGCGCTACGACG
AGCACCACCAGGACCTCACTCTCCTGAAGGCCCTCGTCCGCCAGCAGCTGCCCGAGAAGTACAAGGAGATTTTCTTCGACCAG
AGCAAGAACGGCTACGCGGGCTACATCGATGGCGGCGCCTCCCAGGAAGAGTTCTACAAGTTCATCAAGCCTATCCTGGAGAA
GATGGACGGCACCGAGGAGCTGCTCGTGAAGCTGAACCGCGAGGACCTGCTCCGCAAGCAGAGGACCTTCGACAACGGCAGCA
TCCCTCACCAGATCCACCTGGGCGAGCTGCACGCTATCCTCCGCCGCCAGGAAGACTTCTACCCATTCCTGAAGGACAACCGC
GAGAAGATCGAGAAGATCCTCACCTTCCGCATCCCGTACTACGTGGGCCCCCTGGCCCGCGGCAACTCCAGGTTCGCCTGGAT
GACCAGGAAGAGCGAGGAGACCATCACCCCGTGGAACTTCGAGGAAGTGGTGGACAAGGGCGCCTCCGCTCAGAGCTTCATCG
AGCGCATGACCAACTTCGACAAGAACCTCCCTAACGAGAAGGTGCTGCCAAAGCACTCCCTGCTCTACGAGTACTTCACCGTC
TACAACGAGCTGACCAAGGTGAAGTATGTGACCGAGGGCATGAGGAAGCCCGCCTTCCTCAGCGGCGAGCAGAAGAAGGCCAT
CGTGGACCTGCTCTTCAAGACCAACCGCAAGGTGACCGTCAAGCAGCTGAAGGAAGACTACTTCAAGAAGATCGAGTGCTTCG
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AAGGACTTCCTCGACAACGAGGAGAACGAGGACATCCTGGAGGACATCGTGCTCACCCTGACCCTCTTCGAGGACCGCGAGAT
GATCGAGGAGAGGCTCAAGACCTACGCCCACCTGTTCGACGACAAGGTCATGAAGCAGCTGAAGAGGCGCAGGTACACTGGCT
GGGGCCGCCTCAGCAGGAAGCTGATCAACGGCATCAGGGACAAGCAGTCCGGCAAGACCATCCTGGACTTCCTCAAGAGCGAC
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CGGCCAGGGCGACAGCCTCCACGAGCACATCGCTAACCTGGCGGGCAGCCCTGCCATCAAGAAGGGCATCCTCCAGACCGTGA
AGGTGGTGGACGAGCTGGTGAAGGTCATGGGCCGCCACAAGCCAGAGAACATCGTCATCGAGATGGCCAGGGAGAACCAGACC
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ACCAGGAGCTGGACATCAACAGGCTGTCCGACTACGACGTGGACCACATCGTCCCTCAGTCCTTCCTCAAGGACGACAGCATC
GACAACAAGGTGCTGACCCGCAGCGACAAGAACAGGGGCAAGTCCGACAACGTCCCAAGCGAGGAAGTGGTCAAGAAGATGAA
GAACTACTGGCGCCAGCTGCTCAACGCCAAGCTCATCACCCAGCGCAAGTTCGACAACCTGACTAAGGCGGAGAGGGGCGGCC
TGTCCGAGCTGGACAAGGCTGGCTTCATCAAGCGCCAGCTCGTGGAGACCAGGCAGATCACCAAGCACGTCGCCCAGATCCTG
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CAAGACCGAGATCACCCTCGCCAACGGCGAGATCCGCAAGAGGCCACTGATCGAGACCAACGGCGAGACTGGCGAGATCGTGT
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GAGATCATCGAGCAGATCAGCGAGTTCAGCAAGCGCGTGATCCTCGCCGACGCCAACCTCGACAAGGTCCTGTCCGCCTACAA
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TCCATCACCGGCCTGTACGAGACTCGCATCGACCTCAGCCAGCTGGGCGGCGACCCGAAGAAGAAGCGCAAAGTCTGAGGGAC
CCTCGATCGACAAGCTCGAGTTTCTCCATAATAATGTGTGAGTAGTTCCCAGATAAGGGAATTAGGGTTCCTATAGGGTTTCG
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CGTCCCGCCGAGGGCGCGTGGCCGTGCTGTGCAGCAGCACGTCTAACATTAGTCCCACCTCGCCAGTTTACAGGGAGCAGAAC
CAGCTTATAAGCGGAGGCGCGGCACCAAGAAGCAAAGTCTAGGATCACCTTTGTGTTTTAGAGCTAGAAATAGCAAGTTAAAA
TAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTCCTCGAGGGGCGATAGGAACACGTACAA
CGGCCGTTGTACGTGTTCCTATCGCCGGTACCACTAGTATTAATTAAGTTTAAACGGCGCGCCAAGGGCGAATTCCAGCACAC
TGGCGGCCGTTACTAGTGGATCGAGCTCGTCGACTCTAGACTCGAGGGCGCGCCTGACAGGATATATTGGCGGGTAAAC

**>SEQ ID 28- Cas9 - crRNA**

AAGTCTAGGATCACCTTTGT

**>SEQ ID 29- Complementary sequence of SEQ ID NO 22 (reverse-complement)**

GCATCTTCATGATTGTTGCCTTATGCATAGCTATGCTCCTCTCACCCTTGTTCAAATGATGAGTAATTTGTGAGTGATGTGGT
ATCCTATCTTCCTTGTGTGGAGTCAAACAAGAATGCTTTGTTCCATGTTGCTTGAATAGGAAATGCTTGAGAAAAATGTGTGC
TTAGCTTTACTAGAAATAAGTGTGACTACATGCTTGAGCTTAGAGTTATGAATTTTAAAAGTTTACGGTCAGGTTTTGTCAAT
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CGGCCGCCAGTGTGCTGGAATTCGCCCTTGGCGCGCCGATCTAGTAACATAGATGACACCGCGCGCGATAATTTATCCTAGTT
TGCGCGCTATATTTTGTTTTCTATCGCGTATTAAATGTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACG
TCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGA
TTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAACGATCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTG
CGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAG
CCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATG
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GGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGA
TGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGAT
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GTATGACACACACATACAGTTCCAAAATTAATAAATACACCAGGTAGTTTGAAACAGTATTCTACTCCGATCTAGAACGAATG
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CATGTATACCTATCCTAGATCGATATTTCCATCCATCATCTTCAATTCGTAACTATGAATATGTATGGCACACACATACAGAT
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CAACCAAGACAAAAAAAAGCATGAAAAGATGACCCGACAAACAAGTGCACGGCATATATTGAAATAAAGGAAAAGGGCAAACC
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CAAACAAGTGTGACAAAAAAAATATGTGGTAATTTTTTATAACTTAGACATGCAATGCTCATTATCTCTAGAGAGGGGCACGA
CCGGGTCACGCTGCACTGCAGGCATACGCGTAAGCTTCAGGGTTTAAACAGGTCCGATTGAGACTTTTCAACAAAGGGTAATA
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CAAAGGGTAATATCCGGAAACCTCCTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGG
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CCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTCCACT
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ACGCTGACAAGCTGACTCTAGCAGATCCTCTAGAACCATCTTCCACACACTCAAGCCACACTATTGGAGAACACACAGGGACA
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CGATCCGCCGTTGTTGGGGGAGATGATGGGGGGTTTAAAATTTCCGCCGTGCTAAACAAGATCAGGAAGAGGGGAAAAGGGCA
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GAGGGCCTGAGCAACCTCTACCAAATCTACGCCGAGAGCTTCAGGGAGTGGGAGGCCGACCCGACCAACCCAGCTCTCCGCGA
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CGCTCCTGTCCGTGTACGTGCAAGCCGCTAACCTGCACCTCAGCGTGCTGCGCGACGTGAGCGTGTTCGGCCAAAGGTGGGGC
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CAACACCGGCCTGGAGCGCGTCTGGGGTCCGGACTCCAGGGACTGGATCAGGTACAACCAGTTCAGGAGGGAGTTGACCCTCA
CCGTGCTGGACATTGTGTCCCTCTTCCCGAACTACGACTCCAGGACCTACCCGATCCGCACCGTGTCCCAACTCACCAGGGAG
ATCTACACCAACCCAGTGCTGGAGAACTTCGACGGTAGCTTCCGCGGTTCCGCCCAGGGTATCGAGGGCTCCATCAGGAGCCC
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CGAATTAAGGTACCGATATCAGTACTAATTCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGCGTCAATTT
GTGAAATATTAGCAATTTTGGTCATGTTAAGCTATGAATTAGTTTTTGGTATGAATAACTAAAGTGTAGTAATCTTCCTAAGA
TTTCTAGAAAGTCTAGGATCACCTTTGTGGGATGTCTATAACCTCAGTTATGGTTGAAACAAGTAACTACTGCGTTGCTGTCC
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TGCTTATTTTGGGTGAGCCTTGTGTCTTGTGTTGGTTGCTTAAGCCCATTTGTTTGAATTTTCTCAAATGACTGCTTGATTGT
GATGTTGTATGTGTGTTTGTTATCTAGGTAGTGCCCTAGTTGCCACCTC

**>SEQ ID 30 - Complementary sequence of SEQ ID NO 23 (reverse-complement)**

GAAATATTAGCAATTTTGGTCATGTTAAGCTATGAATTAGTTTTTGGTATGAATAACTAAAGTGTAGTAATCTTCCTAAGATT
TCTAGAAAGTCTAGGATCACCTTTGTGGGATGTCTATAACCTCAGTTATGGTTGAAACAAGTAACTACTGCGTTGCTGTCCAG
ATTCTAGGCACATGCTAAGTTGGCTACCGTATCTAGCGTTGTTTTGAGCTAAACTTGTAGTTAGTTCATGCTTGAGATAAATG
CTTATTTTGGGTGAGCCTTGTGTCTTGTGTTGGTTGCTTAAGCCCATTTGTTTGAATTTTCTCAAATGACTGCTTGATTGTGA
TGTTGTATGTGTGTTTGTTATCTAGGTAGTGCCCTAGTTGCCACCTC

**>SEQ ID 31- Complementary sequence of SEQ ID NO 24 (reverse-complement)**

GCATCTTCATGATTGTTGCCTTATGCATAGCTATGCTCCTCTCACCCTTGTTCAAATGATGAGTAATTTGTGAGTGATGTGGT
ATCCTATCTTCCTTGTGTGGAGTCAAACAAGAATGCTTTGTTCCATGTTGCTTGAATAGGAAATGCTTGAGAAAAATGTGTGC
TTAGCTTTACTAGAAATAAGTGTGACTACATGCTTGAGCTTAGAGTTATGAATTTTAAAAGTTTACGGTCAGGTTTTGTCAAT
GTTCTGAATAGATCTGAATGTTTGGTTTGTTTGACCTGTGTGCATATGGAATCGTGTCTTAGAGATAATAGAAGAAGTGTAGT
ACTTTTTCTAAGCTTTCCAAAATGTCCTATAGCACTAATTGTGATGGTCTAAATCTCTAGTTATGAGCTTGCAAAGTAGAATG
ACAGAATCTGTCCAAATCCGGCAGAGATGTTTTCTTTGCTATGTTTGTCCTTGTTAGCCGTAGAATCATCTTAATATGATAAT
AAGAAAGTTATAGACAACTTAATAAGCTTTACATAAAGTTAAGGATCACTTTTGTTGTGTCTCTAGAACTCCAATTATGTATT
TTCGAAGTTCTTGTCAGTTTTCTGTCCAAATTGGTA

**>SEQ ID 32- Complemmentary sequence of SEQ ID NO 18 (reverse-complement)**
AGCGTCAATTTGTGAAATATTAGCAA
**>SEQ ID 33- Complementary sequence of SEQ ID NO 19 (reverse-complement)**
GTCCAAATTGGTATCGGCGCGCCCTC

**>SEQ ID NO 34 (Cry1Ac protein) - Truncate cry1Ac protein (B. thurigiensis)**

Met Asp Asn Asn Pro Asn Ile Asn Glu Cys Ile Pro Tyr Asn Cys Leu Ser Asn Pro Glu Val
Glu Val Leu Gly Gly Glu Arg Ile Glu Thr Gly Tyr Thr Pro Ile Asp Ile Ser Leu Ser Leu
Thr Gln Phe Leu Leu Ser Glu Phe Val Pro Gly Ala Gly Phe Val Leu Gly Leu Val Asp Ile
Ile Trp Gly Ile Phe Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile Glu Gln Leu Ile
Asn Gln Arg Ile Glu Glu Phe Ala Arg Asn Gln Ala Ile Ser Arg Leu Glu Gly Leu Ser Asn
Leu Tyr Gln Ile Tyr Ala Glu Ser Phe Arg Glu Trp Glu Ala Asp Pro Thr Asn Pro Ala Leu
Arg Glu Glu Met Arg Ile Gln Phe Asn Asp Met Asn Ser Ala Leu Thr Thr Ala Ile Pro Leu
Phe Ala Val Gln Asn Tyr Gln Val Pro Leu Leu Ser Val Tyr Val Gln Ala Ala Asn Leu His
Leu Ser Val Leu Arg Asp Val Ser Val Phe Gly Gln Arg Trp Gly Phe Asp Ala Ala Thr Ile
Asn Ser Arg Tyr Asn Asp Leu Thr Arg Leu Ile Gly Asn Tyr Thr Asp His Ala Val Arg Trp
Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly Pro Asp Ser Arg Asp Trp Ile Arg Tyr Asn Gln
Phe Arg Arg Glu Leu Thr Leu Thr Val Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr Asp Ser
Arg Thr Tyr Pro Ile Arg Thr Val Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn Pro Val Leu
Glu Asn Phe Asp Gly Ser Phe Arg Gly Ser Ala Gln Gly Ile Glu Gly Ser Ile Arg Ser Pro
His Leu Met Asp Ile Leu Asn Ser Ile Thr Ile Tyr Thr Asp Ala His Arg Gly Glu Tyr Tyr
Trp Ser Gly His Gln Ile Met Ala Ser Pro Val Gly Phe Ser Gly Pro Glu Phe Thr Phe Pro
Leu Tyr Gly Thr Met Gly Asn Ala Ala Pro Gln Gln Arg Ile Val Ala Gln Leu Gly Gln Gly
Val Tyr Arg Thr Leu Ser Ser Thr Leu Tyr Arg Arg Pro Phe Asn Ile Gly Ile Asn Asn Gln
Gln Leu Ser Val Leu Asp Gly Thr Glu Phe Ala Tyr Gly Thr Ser Ser Asn Leu Pro Ser Ala
Val Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu Asp Glu Ile Pro Pro Gln Asn Asn Asn Val
Pro Pro Arg Gln Gly Phe Ser His Arg Leu Ser His Val Ser Met Phe Arg Ser Gly Phe Ser
Asn Ser Ser Val Ser Ile Ile Arg Ala Pro Met Phe Ser Trp Ile His Arg Ser Ala Glu Phe
Asn Asn Ile Ile Ala Ser Asp Ser Ile Thr Gln Ile Pro Ala Val Lys Gly Asn Phe Leu Phe
Asn Gly Ser Val Ile Ser Gly Pro Gly Phe Thr Gly Gly Asp Leu Val Arg Leu Asn Ser Ser
Gly Asn Asn Ile Gln Asn Arg Gly Tyr Ile Glu Val Pro Ile His Phe Pro Ser Thr Ser Thr
Arg Tyr Arg Val Arg Val Arg Tyr Ala Ser Val Thr Pro Ile His Leu Asn Val Asn Trp Gly
Asn Ser Ser Ile Phe Ser Asn Thr Val Pro Ala Thr Ala Thr Ser Leu Asp Asn Leu Gln Ser
Ser Asp Phe Gly Tyr Phe Glu Ser Ala Asn Ala Phe Thr Ser Ser Leu Gly Asn Ile Val Gly
Val Arg Asn Phe Ser Gly Thr Ala Gly Val Ile Ile Asp Arg Phe Glu Phe Ile Pro Val Thr
Ala Thr Leu Glu Ala Glu

**> SEQ ID NO 35 (nptII protein)**

MWIEQDGLHAGSPAAWVERLFGYDWAQQTIGCSDAAVFRLSAQGRPVLFVKTDLSGALNELQDEAARLSWLATTGVPCAAVLD
VVTEAGRDWLLLGEVPGQDLLSSHLAPAEKVSIMADAMRRLHTLDPATCPFDHQAKHRIERARTRMEAGLVDQDDLDEEHQGL
APAELFARLKARMPDGEDLVVTHGDACLPNIMVENGRFSGFIDCGRLGVADRYQDIALATRDIAEELGGEWADRFLVLYGIAA
PDSQRIAFYRLLDEFF

**>SEQ ID 36- Complementary sequence of SEQ ID NO 02 (reverse-complement)**

TCGGCGCGCCCTCGAGTCTAGAGTCGACGAGCTCGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCGCCCTTGGCGCGC
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GTATAATTGCGGGACTCTAATCATAAAAACCCATCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGT
AATTCAACAGAAATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCAAATGTTTGAA
CGATCTCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAA
GCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCA
GCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCATCGCCATGGGTCACGACG
AGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATC
ATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAG
CCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGA
TCCTGCCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCC
CGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCCTGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAA
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CAAACAACAGGGTGAGCATCGACAAAAGAAACAGTACCAAGCAAATAAATAGCGTATGAAGGCAGGGCTAAAAAAATCCACAT
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**>SEQ ID 37- Complementary sequence of SEQ ID NO 05 (reverse-complement)**

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**>SEQ ID 38- Complementary sequence of SEQ ID NO 03 (reverse-complement)**

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**>SEQ ID 39- Complementary sequence of SEQ ID NO 04 (reverse-complement)**

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**>SEQ ID NO 41 - Artificial sequence**
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**>SEQ ID NO 42 - Artificial sequence**
ACCTCCACCAGGTACAG
**>SEQ ID NO 43 - Artificial sequence**
CATCCCATACAACTGCCTGAG
**>SEQ ID NO 44 - Artificial sequence**
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**>SEQ ID NO 45 - Artificial sequence**
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**>SEQ ID NO 51 - Artificial sequence**
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**>SEQ ID NO 53** - **Artificial sequence**
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**>SEQ ID NO 54** - **Artificial sequence**
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**>SEQ ID NO 55** - **Artificial sequence**
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**>SEQ ID NO 56** - **Artificial sequence**
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**>SEQ ID NO 57** - **Artificial sequence**
TGGCCGCTTTTCT
**>SEQ ID NO 58** - **Artificial sequence**
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**>SEQ ID NO 59** - **Artificial sequence**
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**>SEQ ID NO 60** - **Artificial sequence**
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**>SEQ ID NO 61** - **Artificial sequence**
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**>SEQ ID NO 64** - **Artificial sequence**
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**>SEQ ID NO 65** - **Artificial sequence**
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**>SEQ ID NO 66** - **Artificial sequence**
CGTGACCTCAATGCG
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**>SEQ ID NO 68** - **Artificial sequence**
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**>SEQ ID NO 101 -Artificial sequence**
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<160> 116

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<223> Junction nucleotide sequence between 3′ region of insert and sugarcane genome of event CTC75064

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<223> Event CTC75064: flanking sequences and T-DNA

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<220>
<223> primer forward (Right Border)

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<210> 7
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<212> DNA
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<220>
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<210> 8
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<223> primer forward (Left Border)

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<210> 9
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<211> 379
<212> DNA
<213> Artificial sequence

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gtgccctagt tgccacctc 379

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<211> 617
<212> DNA
<213> Artificial sequence

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<223> Complementary sequence of SEQ ID NO 24 (reverse-complement)

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<212> DNA
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<220>
<223> Complemmentary sequence of SEQ ID NO 18 (reverse-complement)

<400> 32
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<210> 33
<211> 26
<212> DNA
<213> Artificial sequence

<220>
<223> Complementary sequence of SEQ ID NO 19 (reverse-complement)

<400> 33
gtccaaattg gtatcggcgc gccctc 26

<210> 34
<211> 615
<212> PRT
<213> B. thurigiensis

<400> 34

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Tyr Thr Pro Ile Asp Ile Ser Leu Ser Leu Thr Gln Phe Leu Leu Ser
        35                  40                  45

Glu Phe Val Pro Gly Ala Gly Phe Val Leu Gly Leu Val Asp Ile Ile
    50                  55                  60

Trp Gly Ile Phe Gly Pro Ser Gln Trp Asp Ala Phe Leu Val Gln Ile
65                  70                  75                  80

Glu Gln Leu Ile Asn Gln Arg Ile Glu Glu Phe Ala Arg Asn Gln Ala
                85                  90                  95

Ile Ser Arg Leu Glu Gly Leu Ser Asn Leu Tyr Gln Ile Tyr Ala Glu
            100                 105                 110

Ser Phe Arg Glu Trp Glu Ala Asp Pro Thr Asn Pro Ala Leu Arg Glu
        115                 120                 125

Glu Met Arg Ile Gln Phe Asn Asp Met Asn Ser Ala Leu Thr Thr Ala
    130                 135                 140

Ile Pro Leu Phe Ala Val Gln Asn Tyr Gln Val Pro Leu Leu Ser Val
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Val Phe Gly Gln Arg Trp Gly Phe Asp Ala Ala Thr Ile Asn Ser Arg
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Tyr Asn Asp Leu Thr Arg Leu Ile Gly Asn Tyr Thr Asp His Ala Val
        195                 200                 205

Arg Trp Tyr Asn Thr Gly Leu Glu Arg Val Trp Gly Pro Asp Ser Arg
    210                 215                 220

Asp Trp Ile Arg Tyr Asn Gln Phe Arg Arg Glu Leu Thr Leu Thr Val
225                 230                 235                 240

Leu Asp Ile Val Ser Leu Phe Pro Asn Tyr Asp Ser Arg Thr Tyr Pro
                245                 250                 255

Ile Arg Thr Val Ser Gln Leu Thr Arg Glu Ile Tyr Thr Asn Pro Val
            260                 265                 270

Leu Glu Asn Phe Asp Gly Ser Phe Arg Gly Ser Ala Gln Gly Ile Glu
        275                 280                 285

Gly Ser Ile Arg Ser Pro His Leu Met Asp Ile Leu Asn Ser Ile Thr
    290                 295                 300

Ile Tyr Thr Asp Ala His Arg Gly Glu Tyr Tyr Trp Ser Gly His Gln
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Ile Met Ala Ser Pro Val Gly Phe Ser Gly Pro Glu Phe Thr Phe Pro
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Leu Tyr Gly Thr Met Gly Asn Ala Ala Pro Gln Gln Arg Ile Val Ala
            340                 345                 350

Gln Leu Gly Gln Gly Val Tyr Arg Thr Leu Ser Ser Thr Leu Tyr Arg
        355                 360                 365

Arg Pro Phe Asn Ile Gly Ile Asn Asn Gln Gln Leu Ser Val Leu Asp
    370                 375                 380

Gly Thr Glu Phe Ala Tyr Gly Thr Ser Ser Asn Leu Pro Ser Ala Val
385                 390                 395                 400

Tyr Arg Lys Ser Gly Thr Val Asp Ser Leu Asp Glu Ile Pro Pro Gln
                405                 410                 415
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Val Ser Met Phe Arg Ser Gly Phe Ser Asn Ser Ser Val Ser Ile Ile
435 440 445

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Ile Ile Ala Ser Asp Ser Ile Thr Gln Ile Pro Ala Val Lys Gly Asn
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485 490 495

Asp Leu Val Arg Leu Asn Ser Ser Gly Asn Asn Ile Gln Asn Arg Gly
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Tyr Ile Glu Val Pro Ile His Phe Pro Ser Thr Ser Thr Arg Tyr Arg
515 520 525

Val Arg Val Arg Tyr Ala Ser Val Thr Pro Ile His Leu Asn Val Asn
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545 550 555 560

Ser Leu Asp Asn Leu Gln Ser Ser Asp Phe Gly Tyr Phe Glu Ser Ala
565 570 575

Asn Ala Phe Thr Ser Ser Leu Gly Asn Ile Val Gly Val Arg Asn Phe
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Ser Gly Thr Ala Gly Val Ile Ile Asp Arg Phe Glu Phe Ile Pro Val
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Thr Ala Thr Leu Glu Ala Glu
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Ser Asp Ala Ala Val Phe Arg Leu Ser Ala Gln Gly Arg Pro Val Leu
        35                  40                  45

Phe Val Lys Thr Asp Leu Ser Gly Ala Leu Asn Glu Leu Gln Asp Glu
    50                  55                  60

Ala Ala Arg Leu Ser Trp Leu Ala Thr Thr Gly Val Pro Cys Ala Ala
65                  70                  75                  80

Val Leu Asp Val Val Thr Glu Ala Gly Arg Asp Trp Leu Leu Leu Gly
                85                  90                  95

Glu Val Pro Gly Gln Asp Leu Leu Ser Ser His Leu Ala Pro Ala Glu
            100                 105                 110

Lys Val Ser Ile Met Ala Asp Ala Met Arg Arg Leu His Thr Leu Asp
        115                 120                 125

Pro Ala Thr Cys Pro Phe Asp His Gln Ala Lys His Arg Ile Glu Arg
    130                 135                 140

Ala Arg Thr Arg Met Glu Ala Gly Leu Val Asp Gln Asp Asp Leu Asp
145                 150                 155                 160

Glu Glu His Gln Gly Leu Ala Pro Ala Glu Leu Phe Ala Arg Leu Lys
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Ala Arg Met Pro Asp Gly Glu Asp Leu Val Val Thr His Gly Asp Ala
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Cys Leu Pro Asn Ile Met Val Glu Asn Gly Arg Phe Ser Gly Phe Ile
        195                 200                 205

Asp Cys Gly Arg Leu Gly Val Ala Asp Arg Tyr Gln Asp Ile Ala Leu
    210                 215                 220

Ala Thr Arg Asp Ile Ala Glu Glu Leu Gly Gly Glu Trp Ala Asp Arg
225                 230                 235                 240

Phe Leu Val Leu Tyr Gly Ile Ala Ala Pro Asp Ser Gln Arg Ile Ala
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<223> Complementary sequence of SEQ ID NO 05 (reverse-complement)

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## Claims

1. A Polynucleotide comprising at least 14 contiguous nucleotides of SEQ ID NO: 18.

2. The polynucleotide of claim 1, comprising at least 15 contiguous nucleotides of SEQ ID NO: 18, preferably at least 16 contiguous nucleotides of SEQ ID NO: 18, wherein preferably the polynucleotide comprises SEQ ID NO: 18, and wherein more preferably the polynucleotide comprises SEQ ID NO: 13, 5 or 22.

3. A Polynucleotide, comprising at least 14 contiguous nucleotides of SEQ ID NO: 19.

4. The polynucleotide of claim 3 comprising at least 15 contiguous nucleotides of SEQ ID NO: 19, preferably comprising at least 16 contiguous nucleotides of SEQ ID NO: 19, wherein preferably the polynucleotide comprises SEQ ID NO: 19, and more preferably wherein the polynucleotide comprises SEQ ID NO: 12, 5 or 22.

5. Primer pairs comprising forward and reverse primers wherein the forward primer comprises SEQ ID NO: 6 and the reverse primer comprises SEQ ID NO: 7, or the forward primer comprises SEQ ID NO: 8 and the reverse primer comprises SEQ ID NO: 9.

6. A method of detecting plant material from genetically modified sugarcane of event CTC75064-3, comprising the steps of:

a) obtaining a plant material sample for analysis;
b) extracting DNA from the sample;
c) providing primer pairs comprising at least a forward and a reverse primer;
d) amplifying a region between the primer pair; and
e) detecting the presence of a product from amplification;
or comprising the steps of

(a) obtaining a plant material sample for analysis;
(b) extracting DNA or RNA from the sample;
(c) providing a probe or a combination of probes designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO 2, SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO 22, SEQ ID NO 23, SEQ ID NO 24, SEQ ID NO 29, SEQ ID NO 30, SEQ ID NO 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO 36, SEQ ID NO 37, SEQ ID NO 38 and SEQ ID NO 39;
(d) hybridizing said probe with the sample; and
(e) detecting the actual hybridization of the probe.

7. The method of claim 6, wherein the primer pairs in step c) are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one pair of primers comprises contiguous nucleotides sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and optionally at least one primer pair consists of a first primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31 and a second primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36; or wherein the primer pairs in step c) are designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 5 and SEQ ID NO: 37, wherein at least one pair of primers comprises contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39; and optionally at least one primer pair consists of a first primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 38 and SEQ ID NO: 39 and a second primer comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 36; wherein more preferably the forward primer comprises SEQ ID NO: 6 and the reverse primer comprises SEQ ID NO: 7, or the forward primer comprises SEQ ID NO: 8 and the reverse primer comprises SEQ ID NO: 9.; and wherein preferably the product from amplification comprises SEQ ID NO: 12 or SEQ ID NO: 13, wherein optionally detection of the product from amplification is performed through hybridization of a probe comprising SEQ ID NO: 10 or SEQ ID NO: 11.

8. A kit for detecting material from transgenic sugarcane comprising a Cry1Ac protein (event CTC75064-3) comprising a means to detect the presence of a polynucleotide comprising, at least, 14 contiguous nucleotides of SEQ ID NO: 18 and/or of SEQ ID NO: 19 and/or a pesticidal crystal protein (Cry), wherein preferably the means comprise primer pairs designed to bind to a polynucleotide comprising contiguous nucleotides of sequences selected from the group consisting of SEQ ID NO: 22 and SEQ ID NO: 29, wherein at least one pair of primers comprises contiguous nucleotides sequences selected from the group consisting of SEQ ID NO: 23, SEQ ID NO: 24, SEQ ID NO: 30 and SEQ ID NO: 31, and wherein preferably the means comprise a probe comprising SEQ ID NO: 10 or SEQ ID NO: 11.

9. Genetic construct comprising SEQ ID NO: 1 or SEQ ID NO:2, preferably comprising SEQ ID NO: 14.

10. A genetically modified sugarcane (Saccharum spp.) plant or a plant part, plant cell, plant tissue, or seed thereof comprising SEQ ID NO: 18 or SEQ ID NO: 19; or comprising SEQ ID NO: 12 or SEQ ID NO: 13; or comprising SEQ ID NO: 5 or SEQ ID NO: 22, wherein the plant is insect-resistant.

11. A tissue culture of the genetically modified sugarcane (Saccharum spp.) plant of claim 10; and optionally a genetically modified sugarcane (Saccharum spp.) plant regenerated therefrom, wherein the regenerate plant comprises SEQ ID NO: 18 or SEQ ID NO: 19.

12. A commodity product produced from the genetically modified sugarcane (Saccharum spp.) plant of claim 10.

**13.** A method of producing a genetically modified sugarcane (Saccharum spp.) plant of event CTC75064-3, comprising the steps of:

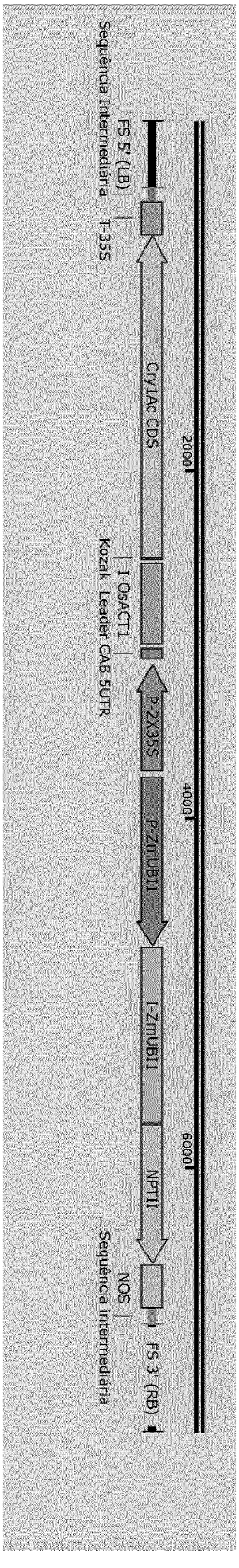
a) introducing a genetic construct comprising SEQ ID NO: 20 and SEQ ID NO: 21 into an Agrobacterium strain;
b) obtaining embryogenic callus from immature leaf rolls or top stalks of sugarcane (Saccharum spp.);
c) co-cultivating embryogenic callus with a culture of Agrobacterium;
d) selecting transformed cells containing the functional fragment in culture medium containing aminoglycoside antibiotics; and
e) regenerating transformed sugarcane plants, wherein the genetically modified sugarcane plants comprise SEQ ID NO: 20 and SEQ ID NO: 21.

**14.** A method of making a genetically modified sugarcane (Saccharum spp.) plant of event CTC75064-3, comprising introducing a genetic modification to a sugarcane (Saccharum spp.) plant comprising SEQ ID NO: 5 or SEQ ID NO: 22 to produce a genetically modified sugarcane (Saccharum spp.) plant of event CTC75064-3, wherein the genetically modified sugarcane (Saccharum spp.) plant has improved insect resistance as compared to a sugarcane (Saccharum spp.) plant without the genetic modification.

**15.** A method of cultivating a genetically modified sugarcane (Saccharum spp.) plant of event CTC75064-3, comprising growing a genetically modified sugarcane (Saccharum spp.) plant of event CTC75064-3 comprising SEQ ID NO: 5 or SEQ ID NO: 22 under conditions comprising insect infestation, wherein the genetically modified sugarcane (Saccharum spp.) plant has an increase in insect resistance as compared to a sugarcane (Saccharum spp.) plant without the genetic modification grown under the same conditions

Figure 1

Figure 2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NOS
Sequência intermediária
FS 3' (RB)

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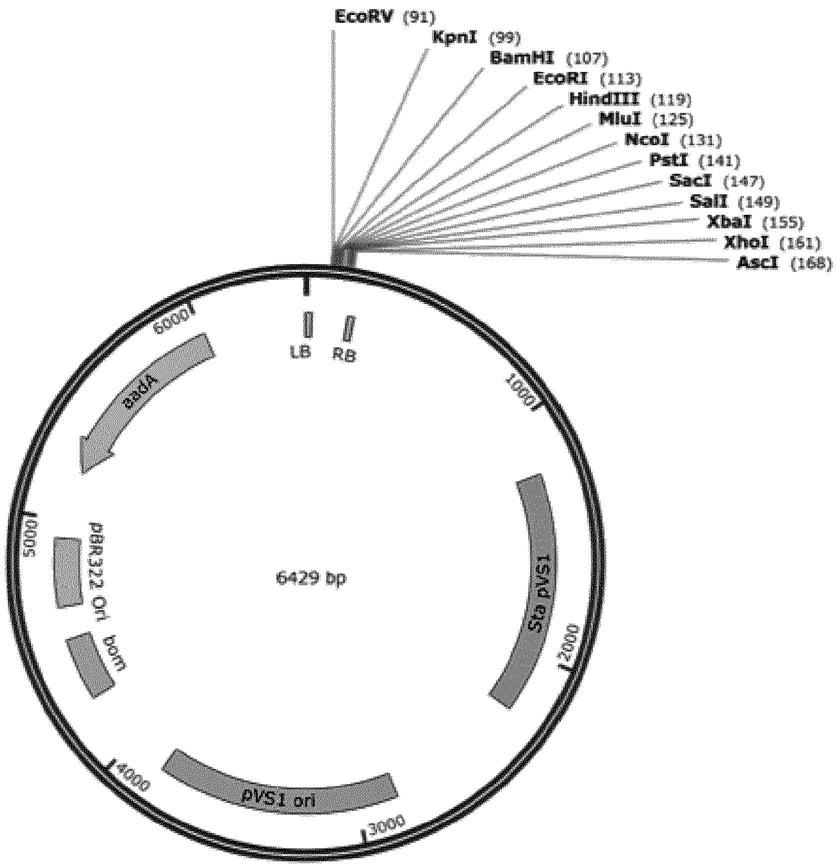
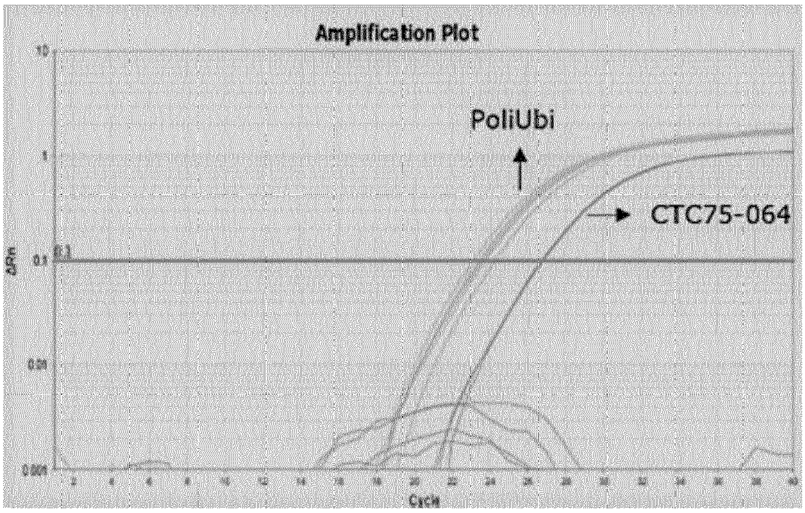



Figure 4


Figure 5

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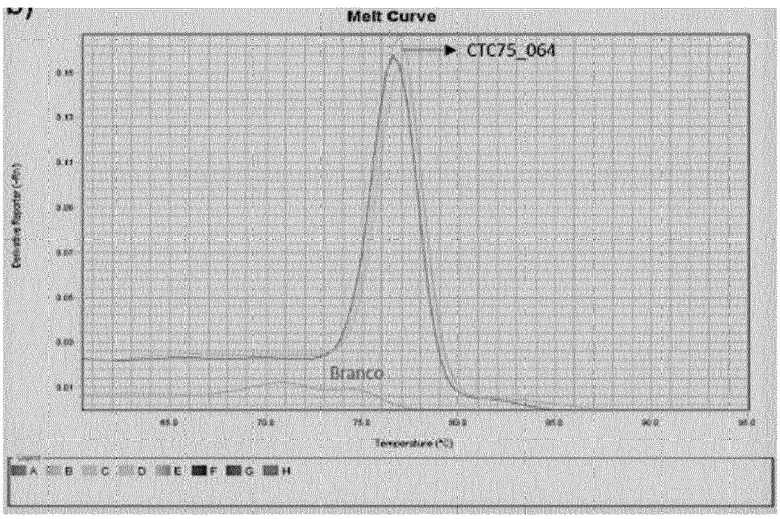


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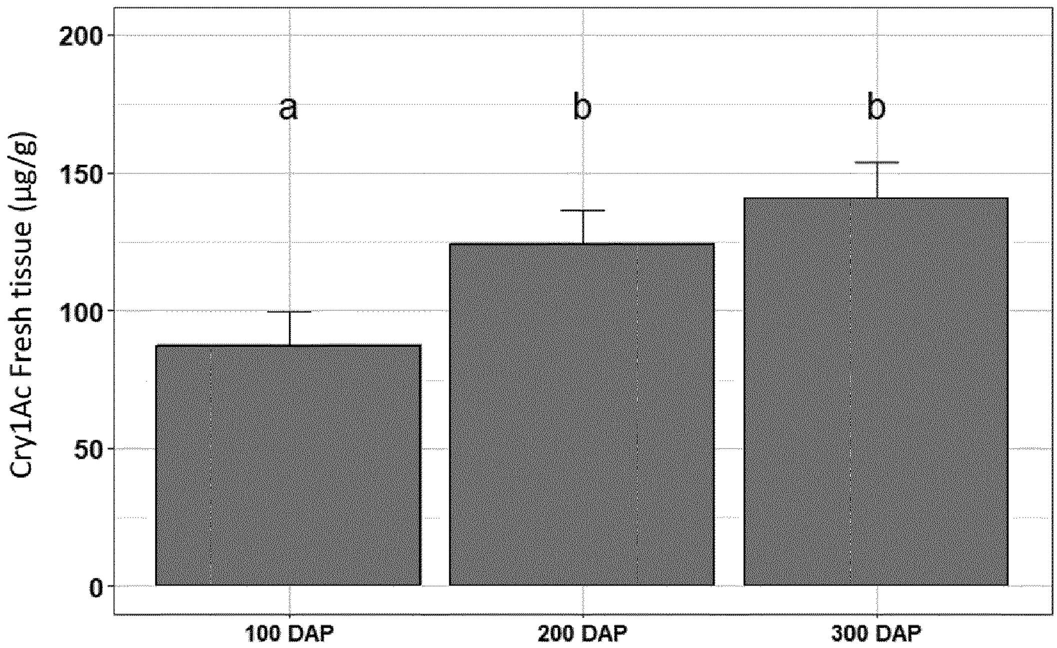
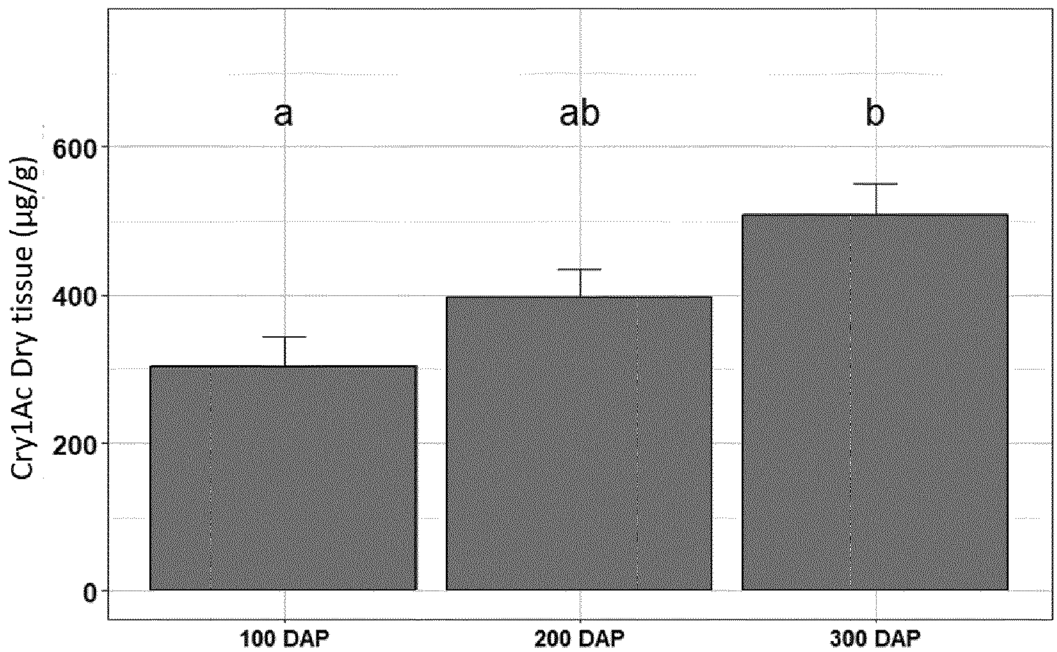



Figure 8

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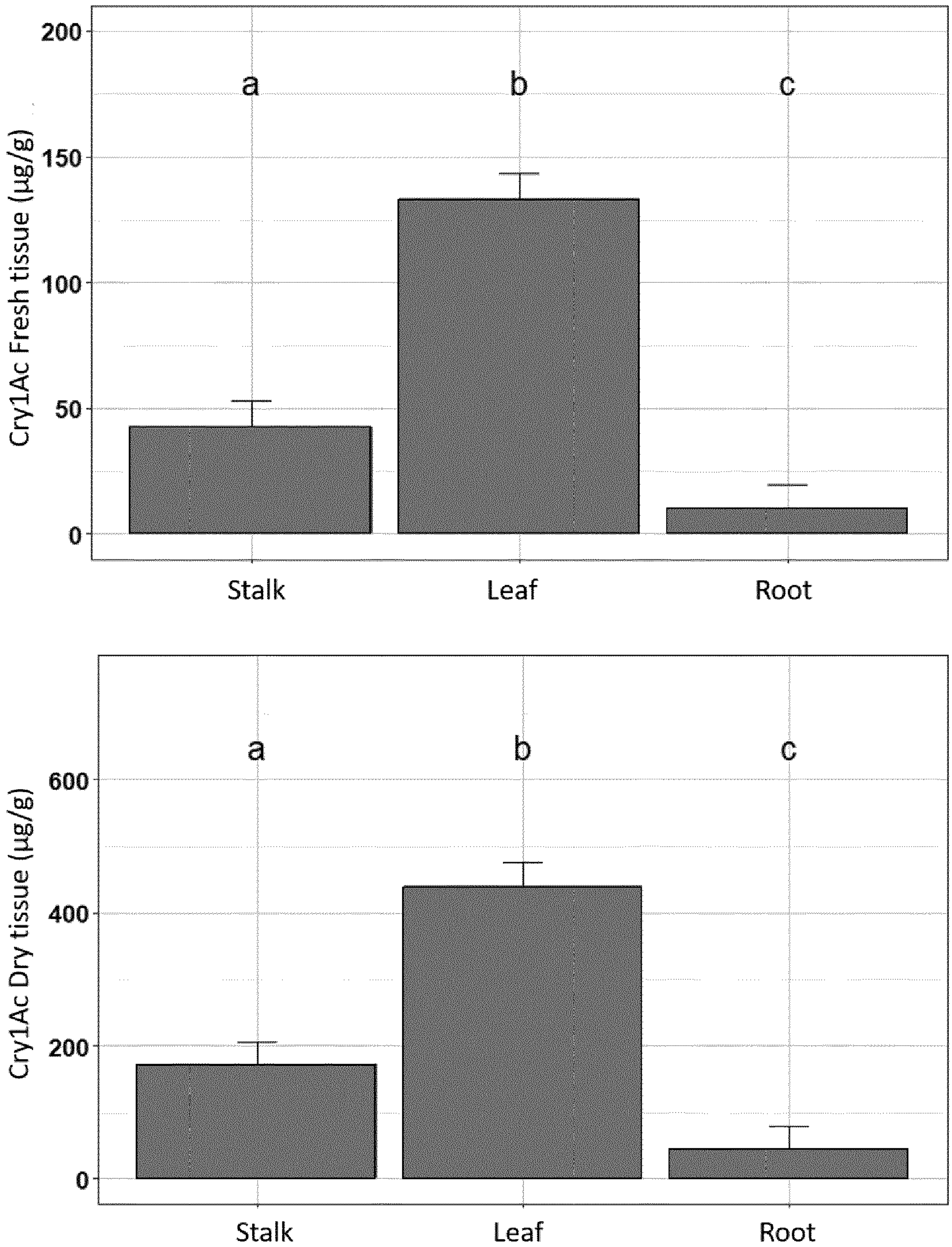
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100
50
0
Cry1Ac Fresh tissue (µg/g)
a
b
c
Stalk
Leaf
Root
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400
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0
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a
b
c
Stalk
Leaf
Root

Figure 10

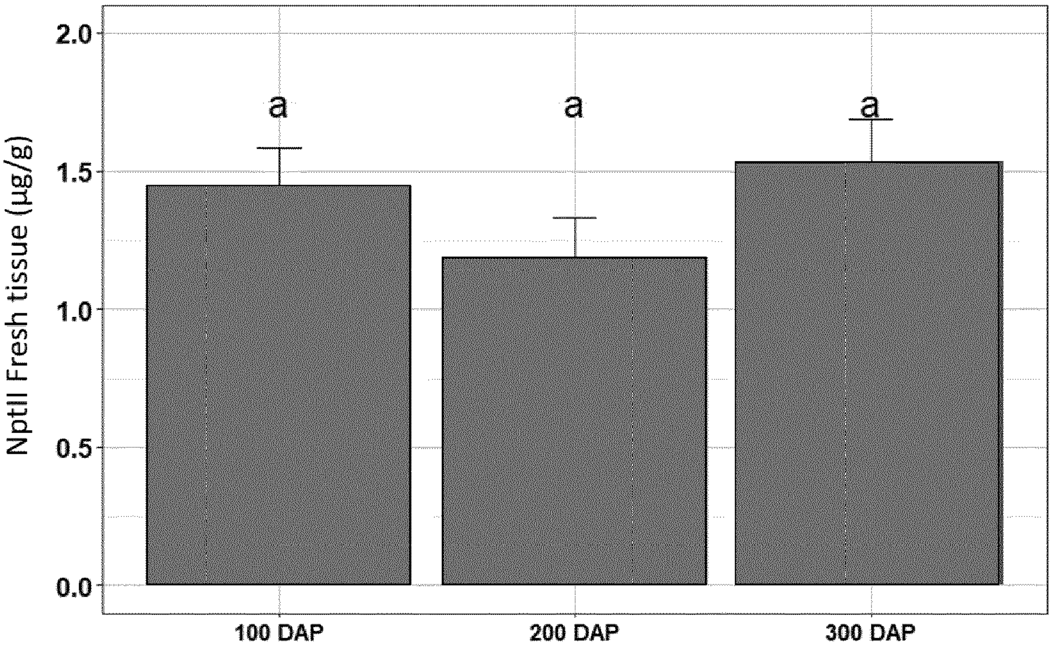



Figure 11

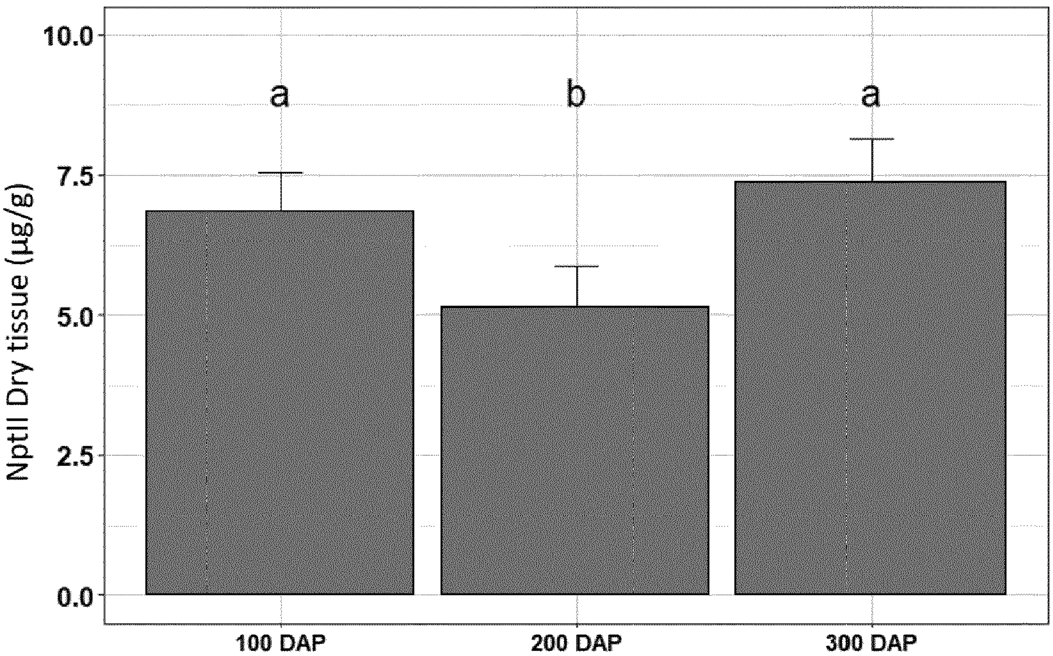
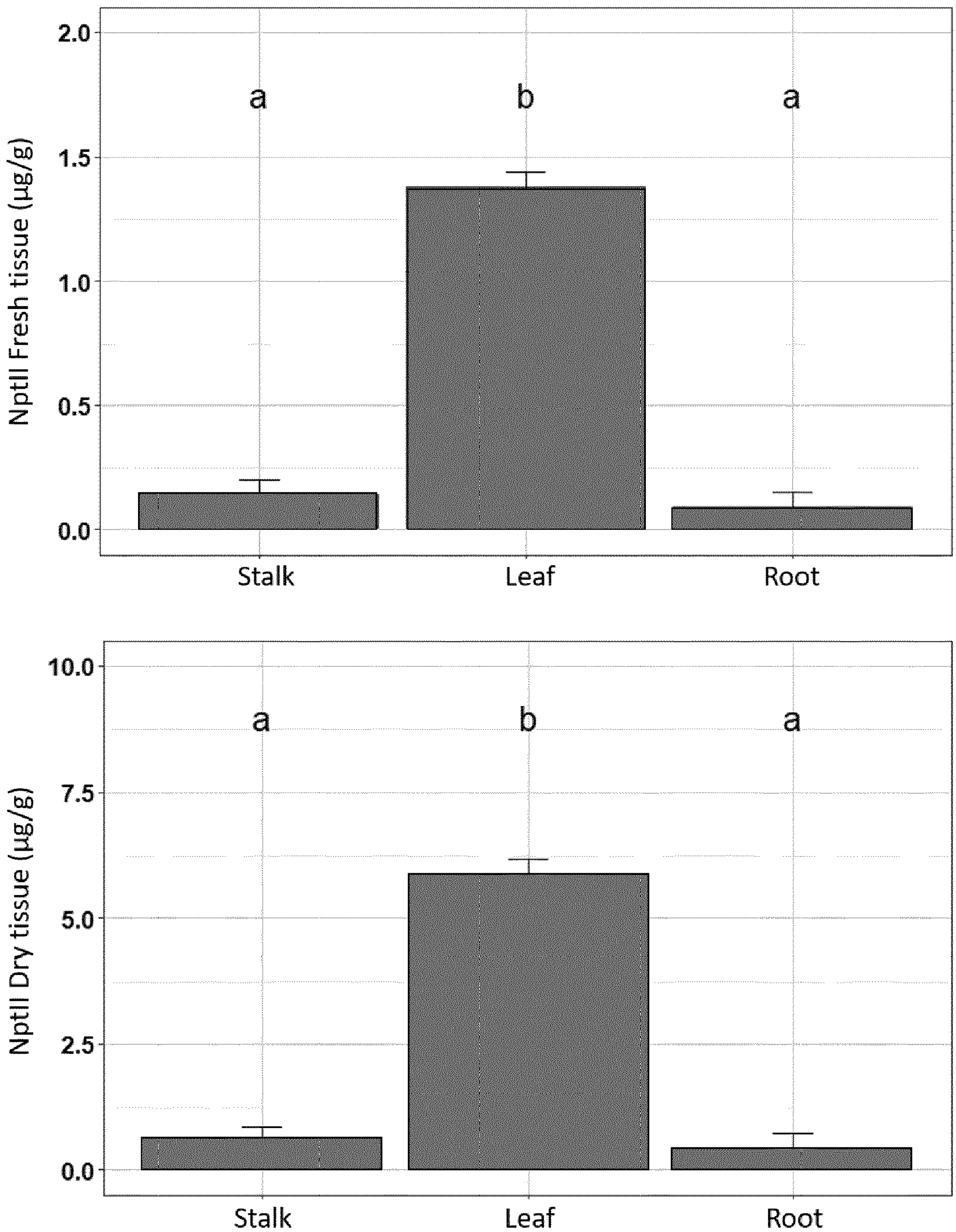



Figure 12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b
a
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2.5
0.0
NptII Dry tissue (µg/g)
a
b
a
Stalk
Leaf
Root

Figure 13

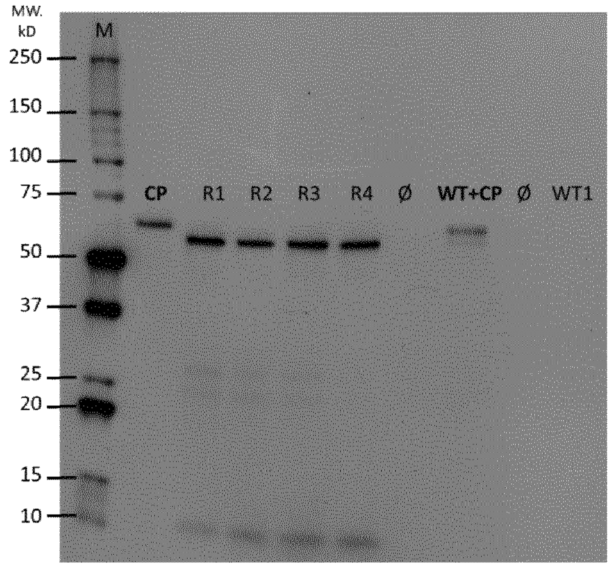


Figure 14

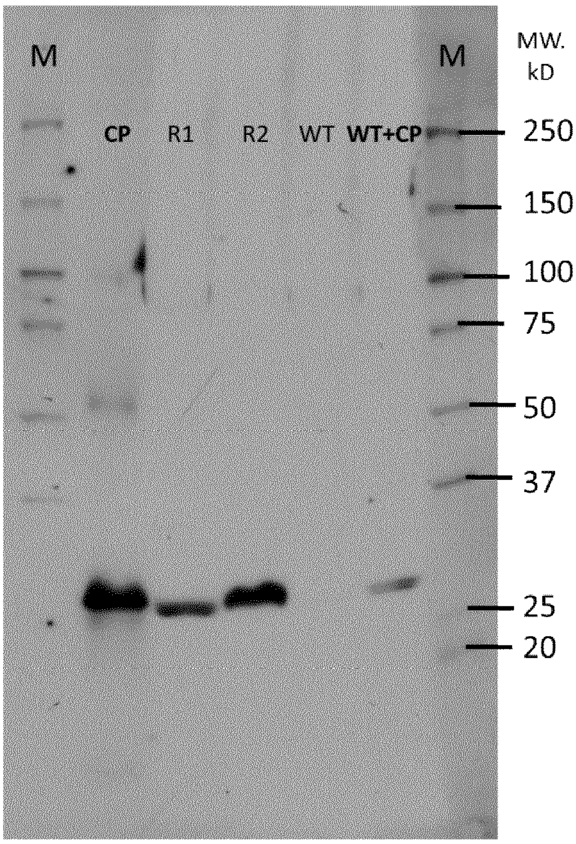


Figure 15

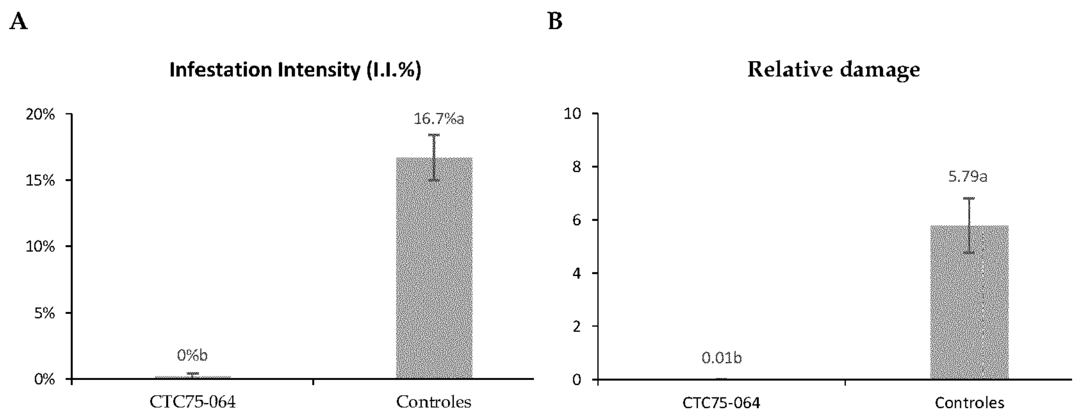


Figure 16

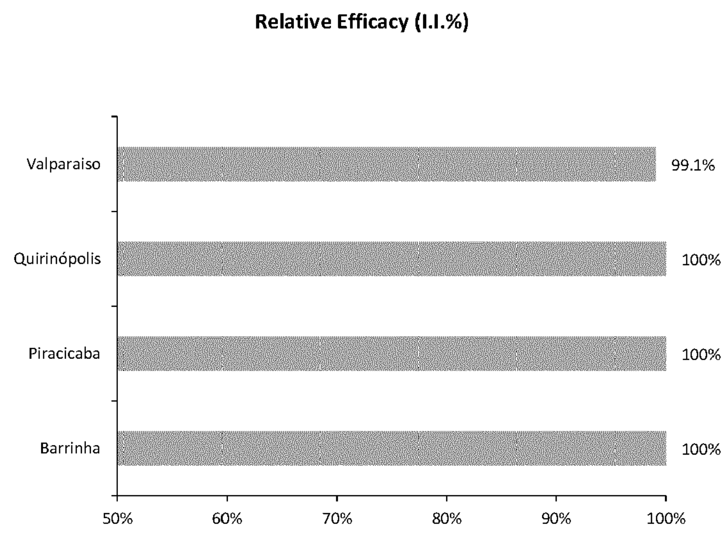


Figure 17

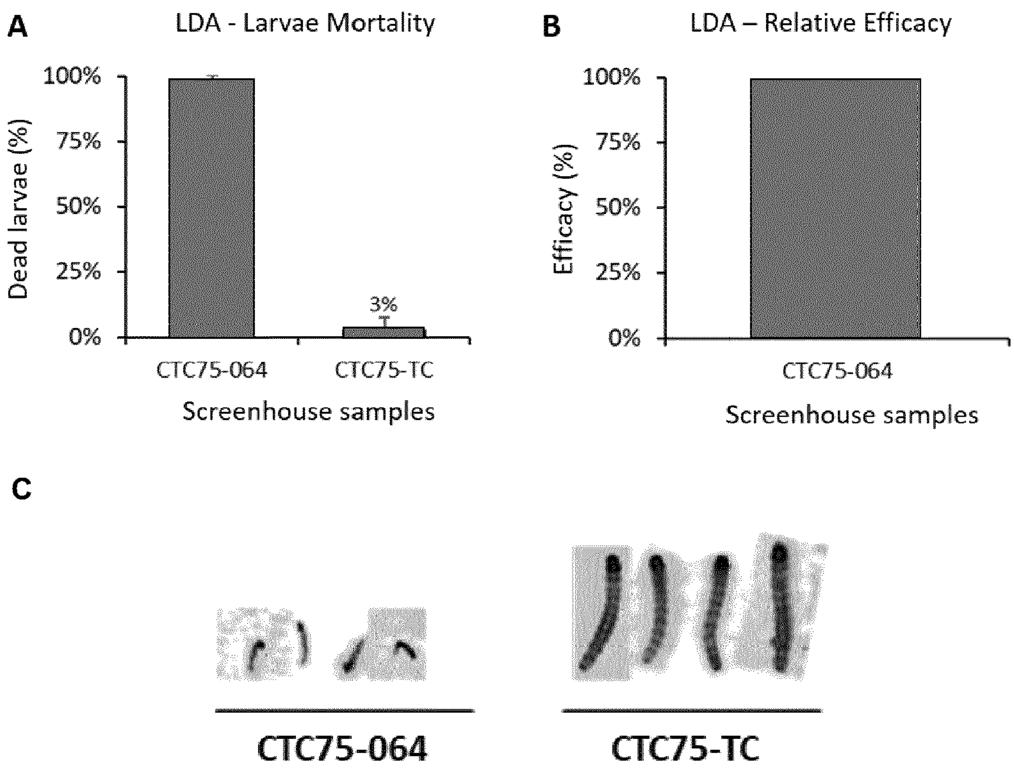


Figure 18

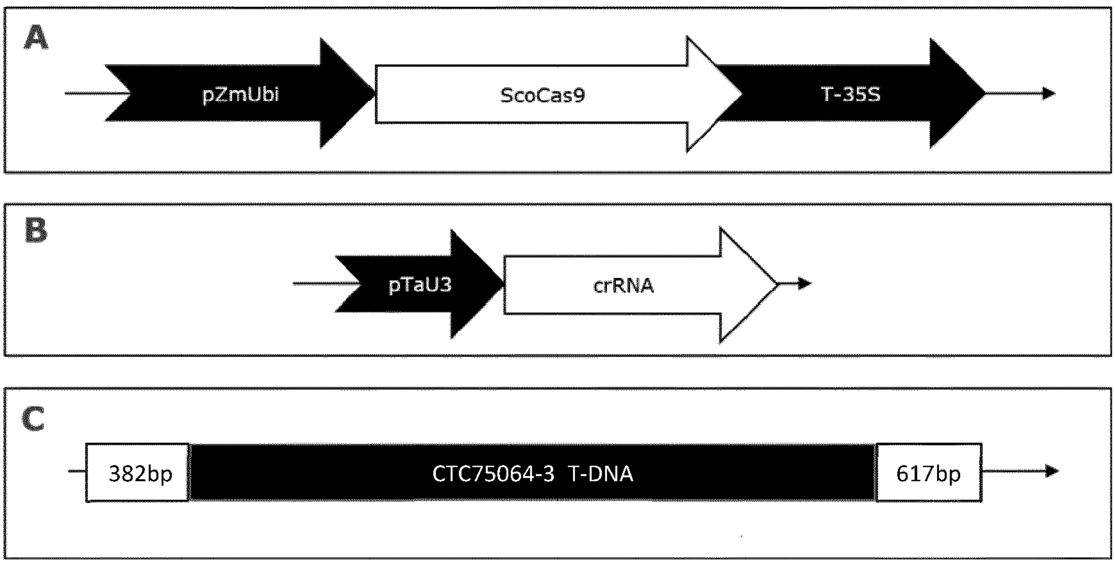


Figure 19

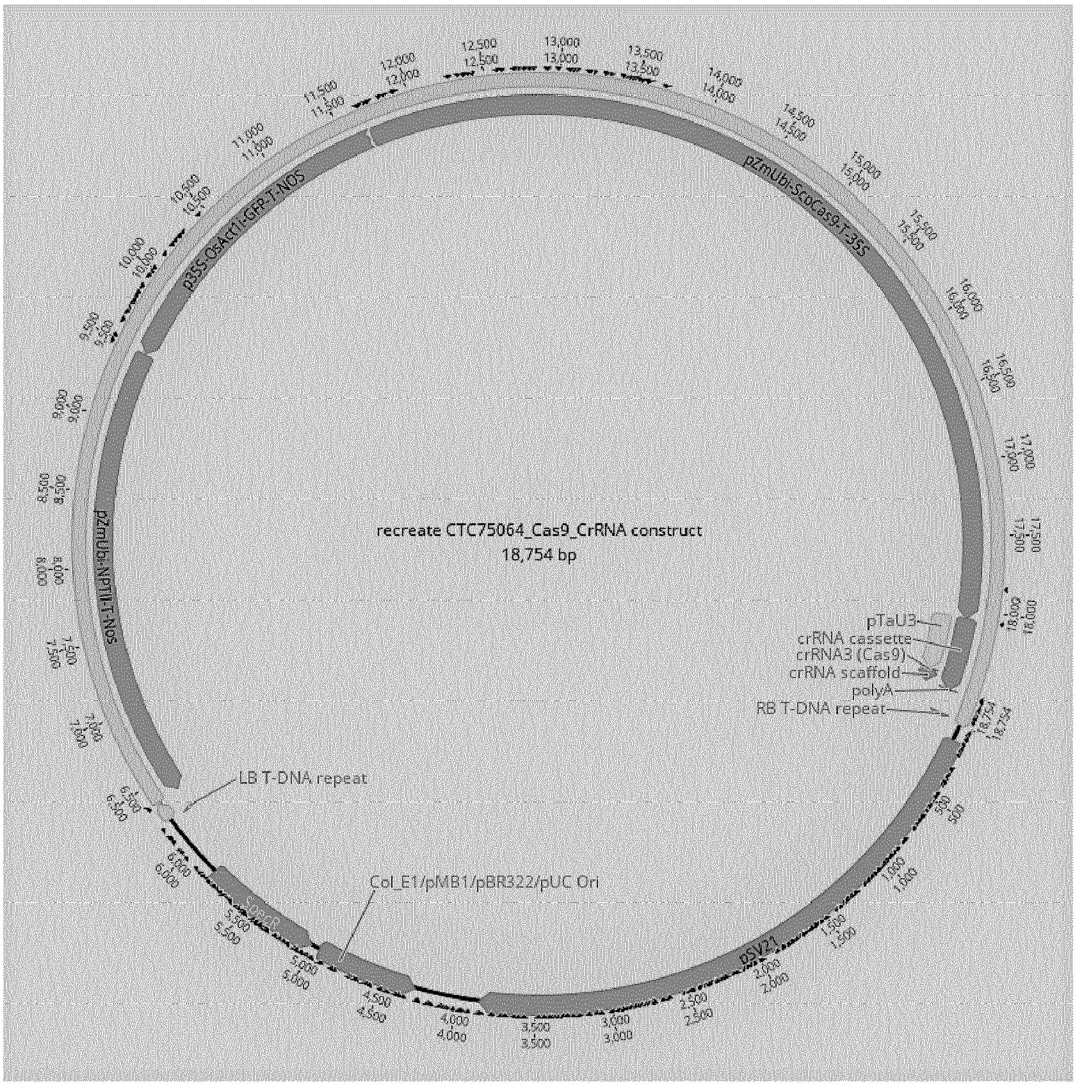
recreate CTC75064_Cas9_CrRNA construct
18,754 bp
pZmUbi-ScoCas9-T-35S
p35S-OsAct(1)-GFP-T-NOS
pZmUbi-NPTII-T-Nos
pTaU3
crRNA cassette
crRNA3 (Cas9)
crRNA scaffold
polyA
RB T-DNA repeat
LB T-DNA repeat
Col_E1/pMB1/pBR322/pUC Ori
pSV21

Figure 20

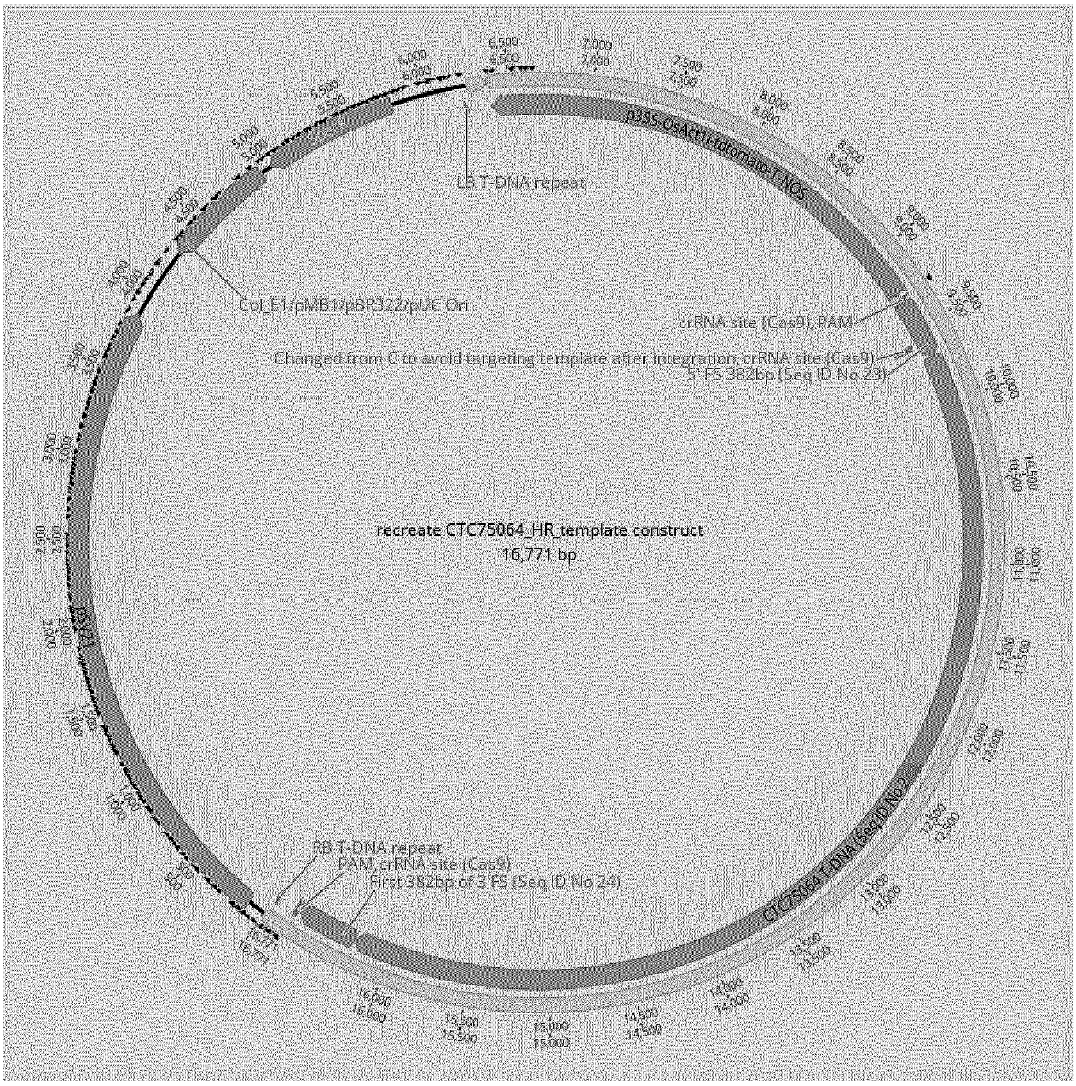
recreate CTC75064_HR_template construct
16,771 bp
p35S-OsAct1i-tdtomato-T-NOS
LB T-DNA repeat
SpecR
Col_E1/pMB1/pBR322/pUC Ori
crRNA site (Cas9), PAM
Changed from C to avoid targeting template after integration, crRNA site (Cas9)
5' FS 382bp (Seq ID No 23)
pSV2.1
RB T-DNA repeat
PAM, crRNA site (Cas9)
First 382bp of 3'FS (Seq ID No 24)
CTC75064 T-DNA (Seq ID No 2)

Figure 21

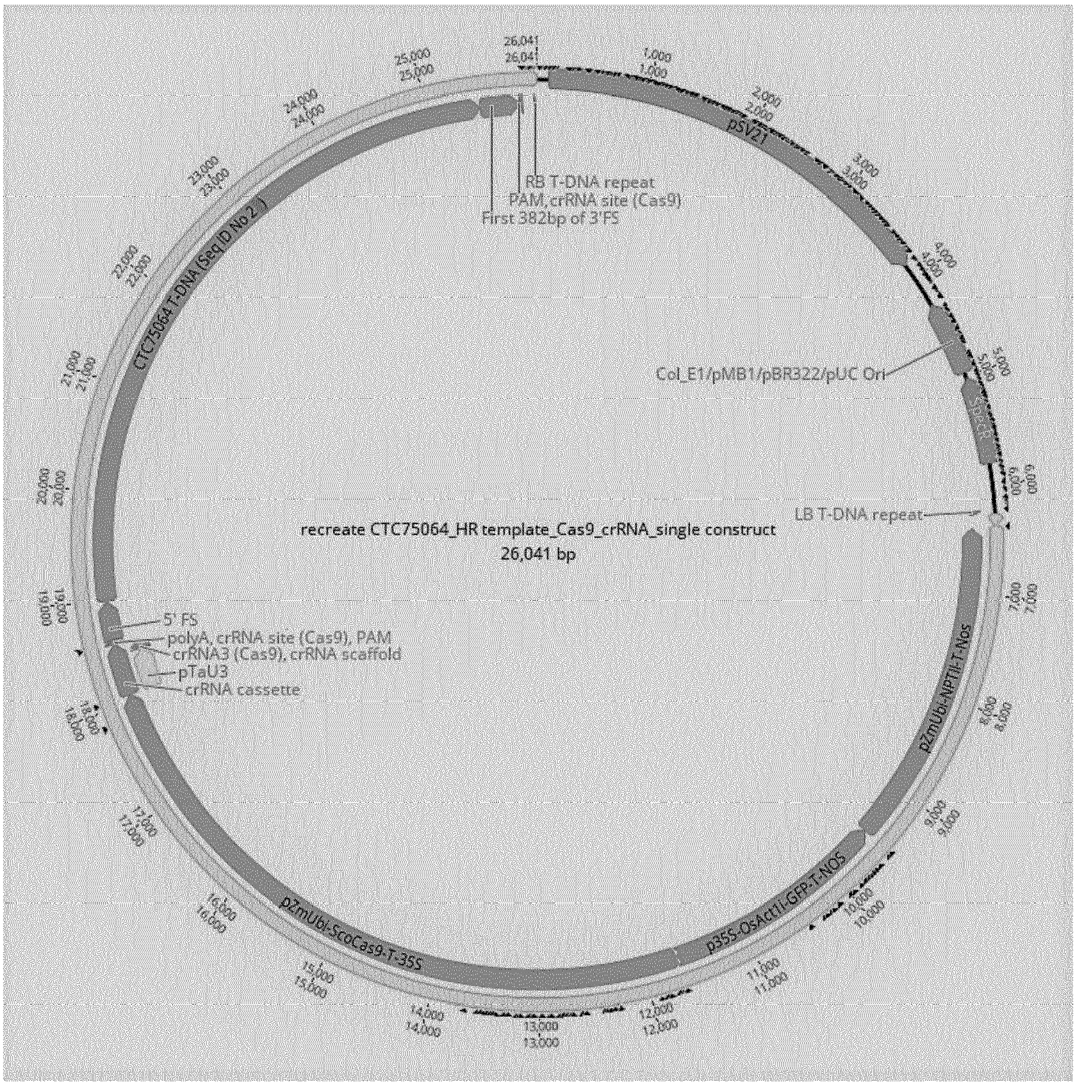
recreate CTC75064_HR template_Cas9_crRNA_single construct
26,041 bp
pSV21
RB T-DNA repeat
PAM,crRNA site (Cas9)
First 382bp of 3'FS
Col_E1/pMB1/pBR322/pUC Ori
LB T-DNA repeat
pZmUbi-NPTII-T-Nos
p35S-OsAct1I-GFP-T-NOS
pZmUbi-ScoCas9-T-35S
5' FS
polyA, crRNA site (Cas9), PAM
crRNA3 (Cas9), crRNA scaffold
pTaU3
crRNA cassette
CTC75064 T-DNA (Seq ID No 2 )

Figure 22

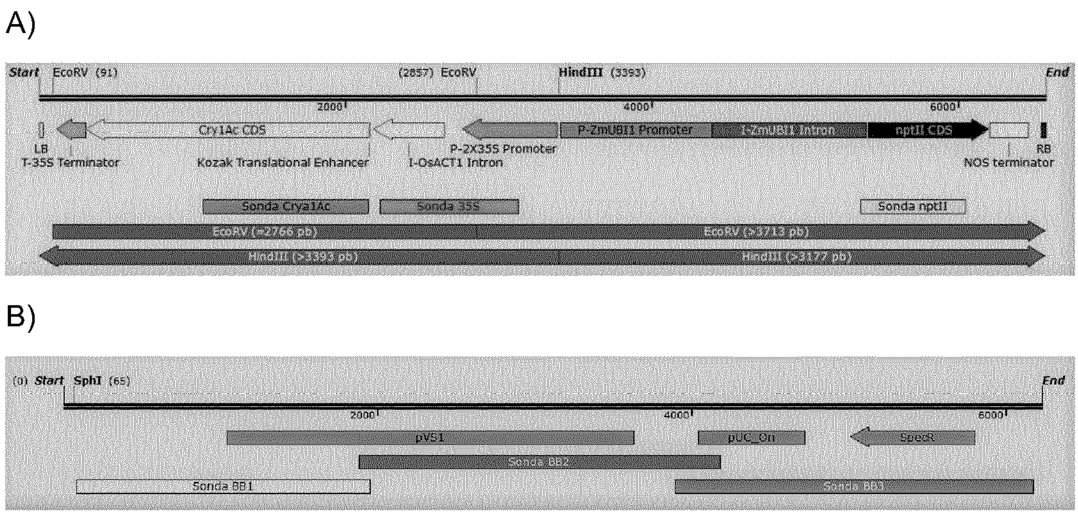


Figure 23

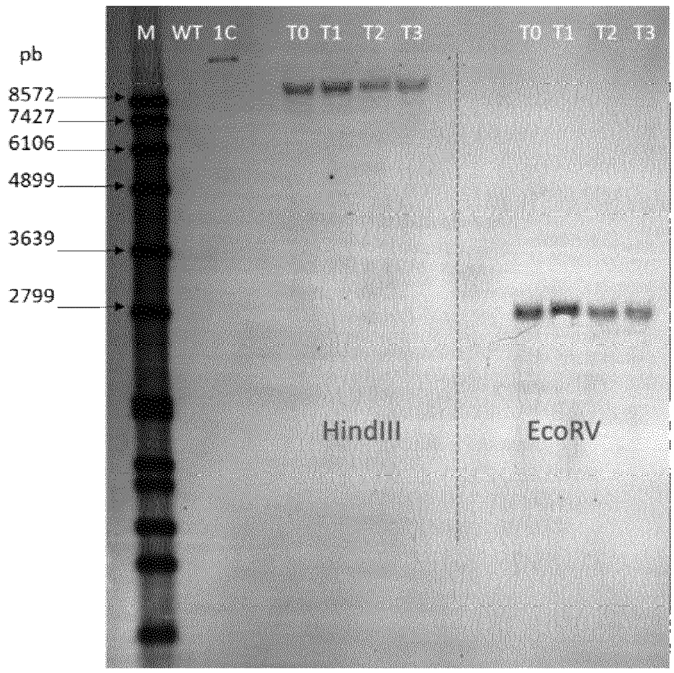


Figure 24

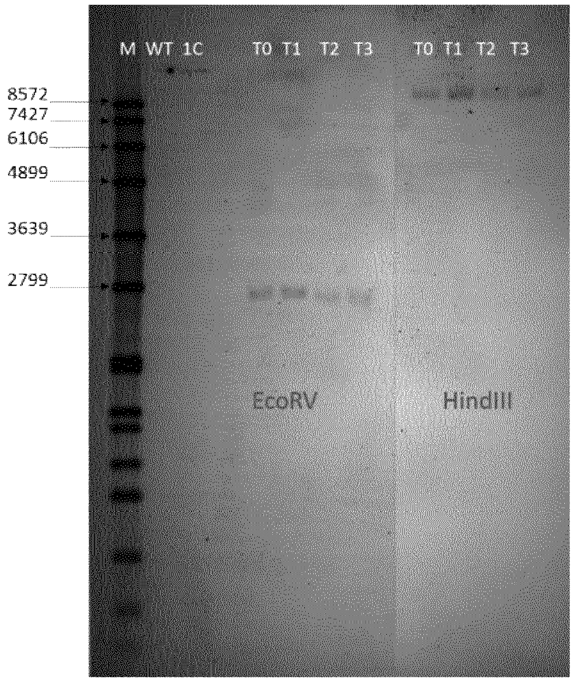


Figure 25

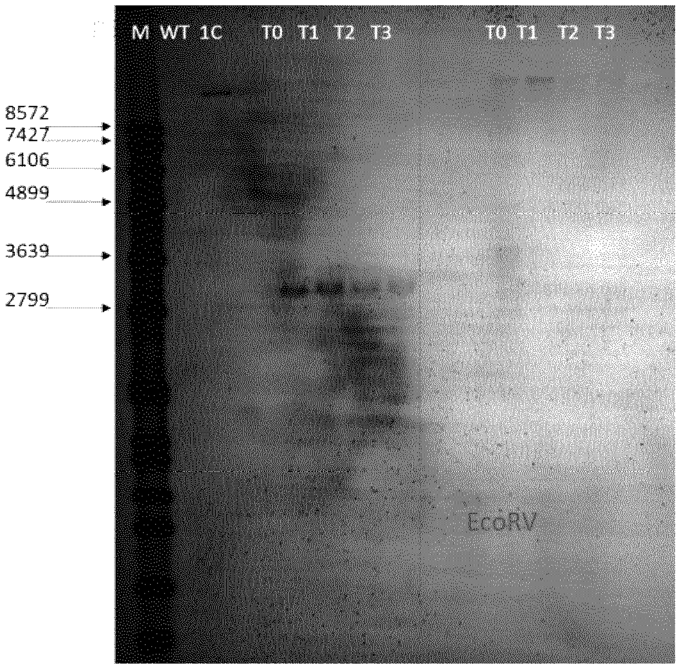


Figure 26

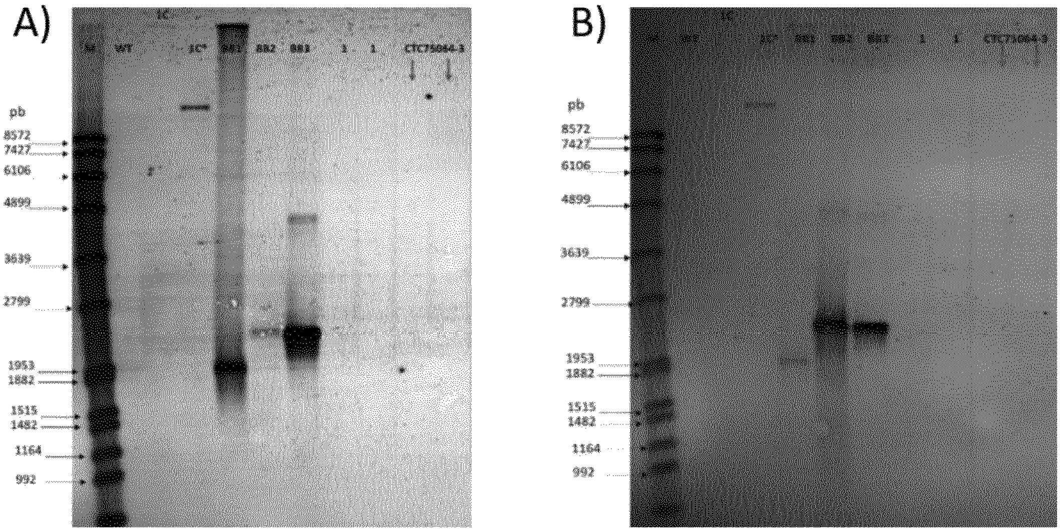


Figure 27

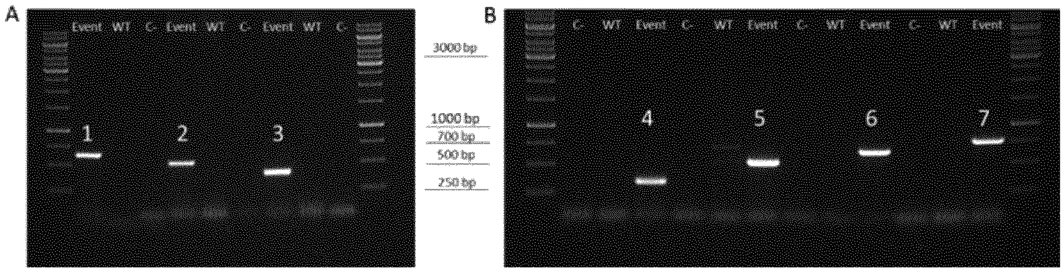


Figure 28

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# EUROPEAN SEARCH REPORT

Application Number
EP 20 20 6809

## DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document with indication, where appropriate, of relevant passages | Relevant to claim | CLASSIFICATION OF THE APPLICATION (IPC) |
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| X | GAO SHIWU ET AL: "Transgenic Sugarcane with a cry1Ac Gene Exhibited Better Phenotypic Traits and Enhanced Resistance against Sugarcane Borer", PLOS ONE, vol. 11, no. 4, 19 April 2016 (2016-04-19), page e0153929, XP055795869, DOI: 10.1371/journal.pone.0153929 | 1-15 | INV. C12N15/82 C07K14/325 |
| Y | * abstract * | 9,13 | |
| | ----- | | |
| X | ZHOU DINGGANG ET AL: "Foreign cry1Ac gene integration and endogenous borer stress-related genes synergistically improve insect resistance in sugarcane", BMC PLANT BIOLOGY, vol. 18, no. 1, 1 December 2018 (2018-12-01), XP055795875, DOI: 10.1186/s12870-018-1536-6 Retrieved from the Internet: URL:http://link.springer.com/content/pdf/10.1186/s12870-018-1536-6.pdf> | 1-15 | |
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| | ----- | | C12N C12R C07K |
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| Y | * abstract * | 9,13 | |
| | ----- | | |
| | -/-- | | |

The present search report has been drawn up for all claims

1

| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| Munich | 28 April 2021 | Mundel, Christophe |

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Application Number
EP 20 20 6809

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TECHNICAL FIELDS SEARCHED (IPC)

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| Place of search | Date of completion of the search | Examiner |
|---|---|---|
| Munich | 28 April 2021 | Mundel, Christophe |

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| CN 102250923 B | 16-10-2013 | NONE | |

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