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RESEARCH PAPER

Stability and tissue-specific Cry10Aa overexpression improves cotton resistance to the cotton boll weevil

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Abstract The cotton boll weevil (CBW, *Anthonomus grandis*) is the most destructive cotton insect pest affecting cotton crops. To overcome this problem, CBW-resistant genetically modified cotton plants overexpressing *Bacillus thuringiensis* entomotoxins were successfully obtained. Previous results showed that the overexpression of Cry10Aa protoxin led to high mortality of the CBW larvae in greenhouse conditions. In this study, we advanced three more generations (T2 to T4), with several cotton events constitutively overexpressing the Cry10Aa protoxin, and the transgene stability and agronomic performance were investigated. In addition, stable transgenic cotton overexpressing the Cry10Aa active (Cry10Aa protoxin lacking the α-helix N-terminal) driven by cotton flower bud-specific promoters were generated and characterized. Cotton events constitutively or tissue-specifically overexpressing the Cry10Aa protein

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(protoxin or active) represented mortality percentages of the CBW larva of up to 85 % in plants under greenhouse conditions. Events overexpressing the Cry10Aa active under control of the flower bud-specific promoter showed higher protein accumulation in stamens and carpels compared to the events with constitutive expression. Our findings suggested that the high stability of the Cry10Aa transgene and the elevated expression level and protein accumulation in flower bud tissues (primarily in stamen and carpels) contribute to improved resistance to CBW larvae. Finally, some notable events were selected with potential for future field trials in different cotton-producing regions of Brazil. Therefore, cotton events overexpressing high levels of the Cry10Aa protein in flower bud tissue may have a strong potential for commercial use in the integrated management of CBW.

Introduction

Cotton (*Gossypium hirsutum*) is one of the most socioeconomically important crops cultivated worldwide and is the main source of fiber for the textile industry and seed oil for biofuel (ABRAPA, 2018; CONAB, 2018; USDA, 2018). This crop is constantly challenged by several insect pest species (Trapero, Wilson, Stiller, & Wilson, 2016). The cotton boll weevil (CBW), *Anthonomus grandis* (Coleoptera: Curculionidae), is considered the major insect pest in South America and exhibits the highest incidence during the transition period from flowering to fructification in cotton (Santos, Marcellino, Monnerat, & Gander, 2003; Artico, Lambret-Frotté et al., 2014). CBW adults feed on and lay eggs inside the reproductive structures of the cotton plant, causing fruit abortion and fall (Showler, 2004, 2005). In addition, the larvae, which are endophytic, cause damage to flower buds, impacting fiber quality and leading to yield losses of up to 100 % (Santos et al., 2003; Ribeiro et al., 2017). The high reproductive capacity, plasticity and genetic variability of this pest have helped to increase its incidence, density and geographical distribution (Santos et al.,2003; Martins,Ayres, & Lucena, 2007; Stadler & Buteler, 2007; Pimenta et al., 2016).

In addition to having high cost and environmental impacts, pest management based on chemical control has been demonstrated to be inefficient (Santos et al., 2003; Papa & Celoto, 2016). Similarly, additional control methods such as pheromones (Fernandes, Carvalho, & Habib, 2001), biological control (Araujo, Braga Sobrinho, & Queiroz, 1999), integrated pest management, the removal of infested flower buds, and the use of earlier or more tolerant varieties have not been sufficient for CBW control (Santos et al., 2003; Sujii & Pires, 2015; Trapero et al., 2016). Thus, the RNA interference strategy (Almeida Garcia et al., 2017; Macedo et al., 2017) and entomotoxic crystal-forming proteins (termed endotoxins or Cry proteins) encoded by *Bacillus thuringiensis* (Martins, Praça et al., 2007; Ribeiro et al., 2017) are currently the major tools used to overcome these limitations and efficiently control several insect pest species of cotton crops. In addition, the pyramiding (gene stacking) of two or more Cry entomotoxins targeting the same insect pest species is an interesting strategy for insect resistance management (Carrière, Crickmore, & Tabashnik, 2015).

These Cry proteins are biologically inactive when in their native form (termed protoxins). However, after ingestion, these proteins are solubilized and enzymatically processed in the insect gut, giving rise to an active soluble toxin capable of binding with midgut epithelial receptors (Pardo-Lopez, Soberon, & Bravo, 2013; Adang,Crickmore, & Jurat-Fuentes, 2014). Although receptor binding is not required for some Cry proteins, for others this binding triggers the formation of pores in the gut epithelial membranes of juvenile insects. These pores disrupt the membrane selectivity, causing epithelial lysis and, mostly, the death of the insect (Bravo, Gill, & Soberon, 2007). Until now, around 308 Cry proteins have been characterized and organized into 75 classes according to amino acid sequence similarities (Crickmore et al., 1998, 2016). In addition, each of these classes is considered entomotoxic for few insect families or orders. For example, the Cry1 class is entomotoxic to the Lepidoptera order, while the Cry1, Cry3, Cry8, Cry22, Cry34, and Cry35 classes are active against Coleopteran species (Latham & Love, 2017; 2014). Furthermore, Cry1Ba6 (Martins et al., 2010), Cry8Ka5 (Farias, Peijnenburg, Grossi-de-Sa, & Carvalho, 2015), Cry1Ia (Martins et al., 2008; Silva et al., 2016), Cry1Ia12 (Grossi-de-Sa et al., 2007; de Oliveira et al., 2016) and Cry10Aa (Aguiar, Martins, Ribeiro, & Monnerat, 2012) were confirmed as entomotoxic to the CBW by *in vitro* bioassays using purified recombinant protein from *Escherichia coli* or extracts from recombinant virus-infected insects.

Genetically modified (GM) plants overexpressing Cry proteins (*e.g.*, maize, potato, tomato, soybean, and cotton) have been commercially adopted in several agricultural countries to improve resistance to insect pests (Sanahuja, Banakar, Twyman, Capell, & Christou, 2011; Latham & Love, 2017; Lucena et al., 2014). GM cotton overexpressing Cry10Aa (Ribeiro et al., 2017), Cry1Ia12 (de Oliveira et al., 2016d), and Cry1Ia (Silva et al., 2016) protoxins showed higher resistance to the CBW. Recently, Ribeiro et al. (2017) generated GM cotton (cultivar BRS372) constitutively overexpressing Cry10Aa protoxin (GenBank accession: AAA22614.1). These transgenic plants showed a yield of 3–14 (in the T_0 generation) and 4.05–19.57 (in the T_1 generation) μg of Cry10Aa per gram of fresh tissue. Bioassays showed an entomotoxic effect, and a high level of mortality was observed in CBW adults and larvae fed the leaves and flower buds of these plants in greenhouse conditions, when flower buds were inoculated with fertilized eggs.

The predicted tertiary structure of the Cry10Aa protein based on the Cry1Ac crystal (PDB ID: 4W8J) revealed the presence of three typical Cry domains (I, II, and III). The depicted α-helix in the C-terminal portion suggests that

the Cry10Aa belongs to the Cry protoxin class. In addition, Cry10Aa toxin has a typical Cry 3D-delta-endotoxin (three-domain) conformation, which is typical of pore-forming toxins, with seven helixes in domain I, three beta-sheets in domain II, and a beta-sandwich in domain III (Ribeiro et al., 2017). Previous studies suggest that this α-helix N-terminal is cleaved in the insect gut, making the protein soluble (active) and capable of binding on receptors (Pardo-Lopez, Soberon, & Bravo, 2013; Adang,Crickmore, & Jurat-Fuentes, 2014). These features suggest that α-helix removal from the N-terminal portion may make the Cry10Aa protoxin more accessible for intestinal uptake and delivery into CBW cells.

In the present work, the T_2 to T_3 generations were advanced with several cotton events constitutively overexpressing the Cry10Aa protoxin, and the transgene stability and agronomic performance were investigated. In addition, stable transgenic cotton overexpressing the Cry10Aa active (Cry10Aa protoxin lacking the α-helix N-terminal) driven by cotton flower bud-specific promoter were generated by agrolistic methods and characterized by molecular, serological, and *in planta* bioassays using flower buds. Our findings showed that the high stability of the Cry10Aa transgene and the higher expression level and protein accumulation in flower buds (stamen and carpels) tissue can improve the resistance to CBW larvae in greenhouse conditions. Finally, some notable events were selected with potential for future field trials in different cotton-producing regions of Brazil.

Materials and methods

Cotton events constitutively overexpressing Cry10Aa protoxin or tissue-specific Cry10Aa active

Binary vectors were synthesized by the company Epoch Life Science (Sugar Land, TX, USA) and subsequently transformed into the *E. coli* or *A. tumefaciens* strain LBA4404. Cotton meristematic cells (cultivar BRS372) were transformed by the biolistic method using a minimal expression cassette (Cry10Aa protoxin events) according to Ribeiro et al. (2017) or the agrolistic method (Cry10Aa active events) mediated by *A. tumefaciens*. The Cry10Aa protoxin was engineered under the control of the strong and constitutive *UceA1.7* promoter (Viana et al., 2011) (Fig. 1A). In contrast, the Cry10Aa active was driven by the tissue-specific promoter *GhFS1* (Artico, Lambret-Frotté et al., 2014), which enhances the expression in cotton flower buds (Fig. 1B). For both, the synthetic *acetohydroxyacid synthase* (*ahas*) gene was used as a selectable marker for *in vitro* selection using the herbicide Imazapyr ARSENAL® NA (BASF®, Germany). Previously, Ribeiro et al. (2017) generated and characterized T_0 and T_1 events by molecular, serological, and feeding assays using CBW larvae and adult insects maintained *in house* with a standard rearing diet. In this study, we advanced more than three generations (T_2 to T_4 events) with 12 independent or segregating Cry10Aa protoxin events. In addition, cotton events tissue-specific overexpressing the Cry10Aa active were generated. For this, cotton seeds (cultivar BRS372) were sterilized and pre-germinated in sterile water, and embryos were isolated and used in the agrolistic-mediated transformation, according to Rech, Vianna, and Aragao (2008) and using the *A. tumefaciens* LBA4404 strain. Non-transformed plants were cultivated as negative controls. Imazapyr-resistant seedlings were selected and acclimated in a greenhouse. The T_0 to T_3 generations were advanced, and molecular, serological, and insect-resistance assays under greenhouse conditions were performed with adult plants.

Molecular screening of the transgenic events

Genomic DNA from young leaves was isolated according to Dellaporta, Wood, and Hicks (1983)) or using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was estimated using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA) and integrity was evaluated with 1 % agarose gel electrophoresis. Transgene insertion into the cotton genome was confirmed by conventional PCR using specific primers (Supplemental Table 1) and the QIAGEN Multiplex PCR Kit (Cat No./ID: 206143, Qiagen). In addition, events overexpressing the Cry10Aa active, carrying the *bar* selectable marker gene, were also screened using a QuickStix for PAT/bar in cotton leaf & seed kit (EnviroLogix, Portland, Oregon, USA).

Copy number estimation of the transgene

Genomic DNA from cotton events was isolated from young leaves using the DNeasy Plant Mini Kit (Qiagen, Netherlands) according to the manufacturer's instructions. The concentration was determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA), and integrity was evaluated with 1 % agarose gel electrophoresis. The copy number of the transgene integrated into the plant genome was estimated according to Yang et al. (2012) using the qPCR-based $2^{-\Delta\Delta Ct}$ method. The pBSK-ahas-ubc1 plasmid containing a fragment of the cotton *ubc1* gene (which is a single-copy gene) (Zhang et al., 2003) and the selectable marker *ahas* gene (present in T-DNA) (Ribeiro et al., 2017) was used, based on the standard curves previously determined. The absolute quantification of the *ubc1* and *ahas* genes was performed according to Ribeiro et al. (2017) using specific primers (Supplemental Table 1). The copy number of the *ahas* transgene was obtained by dividing the absolute concentration of the *ahas* transgene by the concentration of the endogenous *ubc1* gene in the respective sample.

Identification of the transgene insertion sites

Insertion sites of Cry10Aa protoxin transgene were identified using a Universal Genome Walker™ 2.0 Kit (Clontech Laboratories, Takara Bio, Kyoto, Japan) by nested PCR and Sanger sequencing according to the manufacturer's instructions. Genomic DNA was isolated from young leaves using a NucleoSpin Tissue kit (Macherey-Nagel, Bethehem, Philadelphia, USA) or DNeasy Plant Mini Kit (Qiagen, Germany). The DNA concentration was estimated using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA), and integrity was evaluated with 1 % agarose gel electrophoresis plus staining with ethidium bromide. Approximately 2.5 μg of highly pure genomic

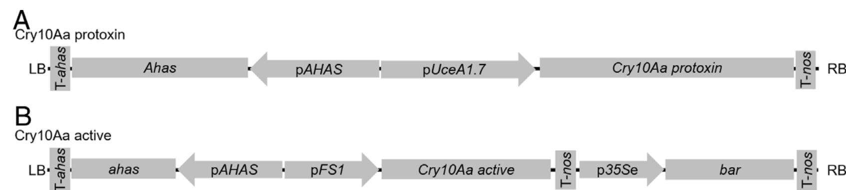

Fig. 1 Overview of the binary vectors for constitutive **(A)** and tissue-specific **(B)** overexpression of Cry10Aa protein used in the plant transformation mediated by biolistic and agrolistic, respectively. The Cry10Aa protoxin was driven by the *UceA1.7* promoter (Viana et al., 2011), while Cry10Aa active was driven by the flower specific (*GhFS1*) promoter (Artico, Lambret-Frotté et al., 2014). The synthetic *ahas* gene was used as a selectable marker gene for *in vitro* selection, while the *bar* gene was used for greenhouse screening.

DNA was digested with *Dra*I, *Eco*RV, *Pvu*II or *Stu*I restriction enzyme overnight or for 16 h. Then, digested DNA samples were purified using a NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) and ligated to an adapter provided by the Genome Walker kit using T4 DNA ligase at 16 °C overnight to produce six Genome Walker libraries. Three gene-specific reverse primers (GSP1, GSP, and GSP3) were designed from the 5′ end of the Cry10Aa T-DNA (targeting the left border of the T-DNA and the beginning of the *AHAS* promoter sequence) to amplify the upstream fragment with two adaptor primers (AP1 and AP2) by nested PCR (Supplemental Table 1). After primary PCR and two nested PCRs using an Advantage 2 PCR Kit (Clontech Laboratories, Inc.), purified PCR products were ligated into pGEMT easy vector (Promega, Madison, USA) and multiplied in *E. coli* DH5α. The plasmidial DNA from positive clones was purified using a QIAprep® Spin Miniprep Kit (QIAGEN) and sequenced using T7 and SP6 universal primers by the Sanger method by Macrogen Service (Geumcheon-gu, Seoul, South Korea). Analyses of the chromatograms were carried out using Geneious R10 software (Kearse et al., 2012). Contigs (Supplemental Information 1) were used to perform local alignment searches with the BLASTn tool (NCBI) (Altschul, Gish, Miller, Myers, & Lipman, 1990) using the *Gossypium hirsutum* v1.1 genome (Zhang et al., 2015) available from the Phytozome v.12.1 database as a reference (Goodstein et al., 2012).

Expression level of *cry10Aa* mRNA in different developmental stages or flower-bud tissues

The expression level of *cry10Aa* mRNA was evaluated in leaves at three different developmental stages or flower buds of 3-, 6-, or 9-mm diameters with or without egg oviposition by the CBW (5–7 days after egg oviposition) from plants constitutively overexpressing the Cry10Aa protoxin. Four biological replicates composed of three to four plants each were used (one young and whole leaves or two to three flower buds per plant were sampled). In addition, events constitutively or tissue-specific overexpressing the Cry10Aa protein were evaluated for the mRNA expression level or Cry10Aa protein accumulation in stamen and carpels from flower buds measuring 6-mm in diameter using both RT-qPCR and ELISA (enzyme-linked immunosorbent assay). Three biological replicates composed of three plants each and two flower buds per plant were used. Flower buds were individually harvested, dissected in a stereomicroscope, samples immediately frozen in liquid nitrogen, and stored at −80 °C. Total RNA isolation was performed using Concert Plant RNA Reagent (Invitrogen, Carlsbad, California, USA) supplemented with Polyvinylpyrrolidone (PVP-40, Sigma, Cat. N. 9003-39-8) or using an InviTrap Spin Plant RNA Mini Kit (Stratec Molecular GMBH, Berlin, Germany) according to the manufacturer's instructions. In addition, the protein extract from these same samples was purified for serological assays, as described in the item below. The RNA concentration was estimated using a spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Massachusetts, USA), and integrity was evaluated with 1 % agarose gel electrophoresis. RNA samples were treated with RNase-free RQ1 DNase I (Promega) according to the manufacturer's instructions. Then, 2 μg of DNase-treated RNA was used as a template for cDNA synthesis using Oligo-$(dT)_{20}$ primer and SuperScript III RT (Life Technologies, Carlsbad-CA, USA), according to the manufacturer's instructions. The cDNA was quantified by spectrophotometry and diluted with nuclease-free water to 200 ng/μl. RT-qPCR assays were performed with 7500 Fast Real-Time PCR System (Applied Biosystems, Massachusetss, USA) using 400 ng of cDNA, 0.2 μM each gene-specific primer (Supplemental Table 1) and GoTaq® qPCR Master Mix (Promega). All samples were carried out in technical triplicate reactions. Primer efficiencies and target-specific amplification were confirmed by a single and distinct peak in melting curve analysis. The relative expression (fold-change) of *cry10Aa* or endogenous *uceA1.7* mRNA (Gohir.A11G023700) was calculated using the $2^{-\Delta\Delta CT}$ method (Rao, Huang, Zhou, & Lin, 2013) with *GhUBQ14* or *GhPP2A1* as endogenous reference genes (Artico, Nardeli, Brilhante, Grossi-de-Sa, & Alves-Ferreira, 2010).

Serological assays for Cry10Aa immunodetection

A quantitative indirect ELISA was performed in microplates using anti-Cry10Aa polyclonal antibody produced in rabbit by GenScript© (New Jersey, USA) from a synthetic peptide (VSSDSKIVKGPGHT), secondary goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated antibody (1706515, Bio-Rad® Laboratories, Hercules, California, USA) and 3,3′,5,5′-tetramethylbenzidine dihydrochloride hydrate (T8768, Sigma-Aldrich, ST. Louis, Missouri, USA). Protein crude extracts were obtained from fresh and young leaves, flower buds, or tissue-specific from flower buds according to Ribeiro et al. (2017). Total protein samples were previously quantified using Bradford reagent (BioRad® Laboratories, Hercules, California, USA) according to Bradford

(1976). Recombinant Cry10Aa protein expressed and purified from the *E. coli* BL21(DE3) strain was used to obtain a standard curve ranging from 0 to 300 μg of Cry10Aa protein. Protein extracts from non-transgenic plants were used as a negative control. ELISA assays were carried out in biological and technical triplicate. The absorbance of the developed color was measured at 450 nm. The standard curve was used to estimate the protein concentration in cotton tissues. In addition, Cry10Aa protein accumulation in flower buds was also evaluated by Western blot assay according to Ribeiro et al. (2017).

In planta feeding bioassays

Feeding bioassays were performed under greenhouse conditions according to Ribeiro et al. (2017), manually placing a fertilized CBW female on each individualized flower bud. Flower buds of approximately 6-mm in diameter were used as the standard. Populations of CBW were maintained *in house* (EMBRAPA Genetic Resources and Biotechnology, Brasília-DF, Brazil) with a standard rearing diet or adult CBW insects were harvested from Brazilian cotton fields (Rondonópolis-MT, Brazil). The experiments were carried out using at least thirty flower buds per independent event, inoculating a maximum of five flower buds at a time. Non-transgenic plants were used as a negative control for the bioassays. Daily monitoring was performed to detect oviposition, CBW females were removed, and the oviposited flower buds were identified for tracking. Flower buds were evaluated approximately 20 days after oviposition, and the mortality rate of larvae, hatching rate, and flower bud drop rate were measured. The CBW mortality rate was estimated using the Schneider-Orelli's formula (Schneider-Orelli, 1947): mortality rate % = (CBW mortality rate in Cry10Aa events / CBW mortality rate in NT plants) / (100 - CBW mortality rate in NT plants) x 100. All data were statistically analyzed by ANOVA, and the means were compared by Student's t-test with a 0.05 probability.

Field simulation for evaluation of event resistance to CBW

Transgenic plants grown in large pots were randomly distributed in a greenhouse room surrounded by a thin screen. Fertilized CBW females harvested from Brazilian cotton fields (Embrapa Cotton, Goiânia-GO, Brazil) were placed freely in the room. The ratio of four insects per plant was used, while flower buds of different sizes (3- to 9-mm) were sampled. Daily monitoring was performed to detect oviposition, and then flower buds were coated and identified for tracking. The experiments were carried out using at least twenty flower buds per independent event, whereas non-transgenic plants were used as a negative control. Oviposited flower buds were evaluated after 20–30 days, and the emergence rate of larvae was measured using Schneider-Orelli's formula, as described above.

Agronomic performance and yield of the Cry10Aa protoxin events

For ecophysiological analysis, seeds from Cry10Aa protoxin events were germinated on Germitest® paper, and seedlings were transplanted and cultivated in soil bags containing 7 kg of soil mixed with organic fertilizer and periodically supplemented with Osmocote Classic® 14-14-14 (ICL Brazil Ltda). Sixteen plants of the T_3 generation from 9.4.3, 82.14.6, 14.13.4, 8.11.7, 5.1.8, 4.15.3 and NT plants maintained in the greenhouse were consistently irrigated at field capacity until reaching the booting stage. Then, the water deficit (soil Ψw of −0.85 to −0.95 MPa) and field capacity (soil Ψw of −0.03 MPa) treatments were applied. The soil moisture was systematically monitored using a WP4C Dewpoint Potentiometer psychrometer (Decagon WP4, Pullman, WA, USA), and the water levels in the soil were adjusted every 8–10 hours. The ecophysiological analysis was performed four days after all plant groups achieved the desired level of water deficit. The trials were carried out in a randomized design with eight replicates for each treatment and one plant per replicate. Gas exchange measurements were carried out using a portable conventional infrared gas analyzer system with a 6.25 cm^2 automatic leaf chamber (LCpro-SD; ADC BioScientific Ltd., UK). The photosynthetic photon flux density (PPFD) was fixed at 1400 $\mu mol\, m^{-2} s^{-1}$ using a red-blue LED light source built into the leaf cuvette. The net photosynthetic rate (A_N), intercellular CO_2 concentration (Ci), leaf transpiration rate (E), and stomatal conductance (g_s) parameters were measured. The leaf chlorophyll content (SPAD index) was determined using a CCM-200 Plus Chlorophyll Content Meter (Opti-Sciences, Inc., USA), and the SPAD index values were the averages of three readings per leaf/plant. The efficiency of photosystem II (PSII) was evaluated using a MINI-PAM II (Walz, Germany). All measurements were performed in young and fully expanded leaves from the top of plants. The data were statistically analyzed by ANOVA, and the means were compared by Tukey's test at a 5 % probability.

For flower buds, seed and fiber yield, plants from several events (12 segregation events or six independent events) maintained at field capacity or submitted to water deficit were evaluated for the number of flower buds per plant at 30 and 40 days (segregation or independent events, respectively) after the beginning of flowering. Additionally, fiber weight (without seeds), the number of seeds per cotton boll and the weight of ten seeds were determined from two cotton bolls per plant (n = eight to nine plants per event). After the water deficit assay, the plants were again watered thoroughly and periodically fertilized with Osmocote. Five cotton bolls per plant were maintained, and the others were systematically removed. All data were statistically analyzed by ANOVA, and the means were compared by Tukey's test at a 5 % probability.

Results

Successful generation of cotton events constitutively overexpressing Cry10Aa protoxin or tissue-specific the Cry10Aa active

Twelve independent events constitutively overexpressing Cry10Aa protoxin in the T_0 and T_1 generations were previously characterized by molecular (conventional PCR, Southern blotting and quantitative real-time PCR) and serological (indirect ELISA and Western blotting) assays. In addition, bioassays (from the leaves and flower buds) were performed using direct inoculation of CBW eggs (eggs previously fertilized were manually inoculated into 6-mm flower buds) or inoculation mediated by adult CBW insects (females previously fertilized) that were multiplied *in house* and maintained on an artificial diet or CBW insects collected from cotton plants under field conditions (Ribeiro et al., 2017). From these assays, 12 segregation events were selected for advancing the T_2 and T_4 generations based on the higher Cry10Aa protoxin protein accumulation in leaves and flower buds and contrasting with the percent of CBW larvae mortality. In contrast, from 5456 inoculated embryos, 54 independent events overexpressing the Cry10Aa active were generated. Transgene insertion was confirmed by conventional PCR using specific primers for *bar* selectable marker and *Cry10Aa* genes (data not shown) and from the serological test using PAT/bar strip. Seeds from transgenic events were germinated on Germitest® paper, transplanted in pots containing approximately 15 kg of soil, and managed in the greenhouse until all analyses and seed harvesting were performed.

Estimation of Cry10Aa protoxin transgene copy number and identification of the transgene insertion sites

All Cry10Aa protoxin events were obtained from the biolistic method using a minimal transgene expression cassette, which ranged from 1–2 copies for T_0 and T_1 events (Ribeiro et al., 2017). From T_2 to T_4 events, we again evaluated transgene copy number to monitor the segregation of the multi-copies and then estimate the transgene stability. Our analysis showed that almost all T_2 events presented a single-copy transgene, while all T3 events presented as single-copy (Supplemental Table 2). Then, we successfully identified the transgene insertion sites in the six independent events using the genome walking strategy. All insertion sites identified were mapped to large intergenic regions upstream or downstream of the operons, apparently not causing knockout or knockdown of endogenous genes (Supplemental Table 3; Supplemental Information 1). With respect to cotton events overexpressing the Cry10Aa active, which were all obtained using the agrolistic method, previous assays with some T_0 to T_2 events showed transgene insertion in a single-copy rate (Supplemental Table 4).

Expression level of *cry10Aa* mRNA

The *cry10Aa* protoxin gene expression was controlled by the constitutive *UceA1.7* promoter, which was isolated from cotton and showed enhanced expression (primarily in flower buds) in transgenic *Arabidopsis thaliana* (Viana et al., 2011). In this study, we evaluated the expression stability of *cry10Aa* and endogenous *uceA1.7* mRNA in leaves and flower buds. For this, we selected the 4.15.3 event from the T_3 generation because it presented a single copy of the transgene and stable accumulation of Cry10Aa protoxin. Three plant developmental stages (Fig. 2A) and three flower bud sizes with or without egg oviposition by the CBW (Fig. 2B) were screened by RT-qPCR assays. The relative expression of *cry10Aa* mRNA in young leaves showed no significant differences among the three developmental stages. Similarly, *uceA1.7* mRNA also did not show significant differences among the three developmental stages, and the expression level was similar to that of NT plants (Fig. 2C). In contrast, the expression levels of *cry10Aa* mRNA in 6 and 9-mm diameter flower buds were 1–2 times higher than in 3-mm flower buds in both cotton events constitutively or tissue-specific overexpressing Cry10Aa. In addition, the average level of *cry10Aa* expression in flower buds (3- to 9-mm in diameter) was higher than that in leaves, while the expression of *uceA* mRNA again was stable (Fig. 2C to E). Similarly, 6-mm flower buds with or without egg oviposition by the CBW showed similar *cry10Aa* and *uceA1.7* expression levels (Fig. 2E). Thus, our data indicate that the *cry10Aa* gene is more highly expressed in 6- and 9-mm diameter flower buds and that its expression level in 6-mm flower buds is unchanged after CBW oviposition. Additionally, the use of the cotton *UceA1.7* promoter to drive constitutive Cry10Aa expression apparently does not alter the expression level of the endogenous *uceA gene* in either leaves or flower buds.

Serological assays for Cry10Aa immunodetection

Total protein extract was purified from young leaves, stalks, stamen, carpels, and 3- to 9-mm diameter flower buds from adult plants. ELISA indirect results showed different levels of Cry10Aa protoxin accumulation in leaf and flower buds among the 12 cotton events from T_0 to T_3 generations (Fig. 3A and B). A lower level of Cry10Aa protoxin accumulation was observed in T_0 (Ribeiro et al., 2017), while in T_2 and $T_{3,}$ we observed higher and stable accumulation levels. In addition, higher Cry10Aa accumulation was observed in flower buds (approximately 18–20 μg/g of fresh leaf weight; LFW) than in leaf tissues (approximately 15 μg/g of LFW). The 4.15.3 to 4.15.9 segregation events in the T_2 and T_3 generations showed the greatest accumulation of Cry10Aa protein (16.02–20.09 and 17.63–20.27 μg, respectively). In addition, we also observed that the accumulation of Cry10Aa protoxin in the leaves and flower buds of clone plants of each transgenic event ranged (standard deviation) from 2.5 to 3.2 and 2.1–2.9 μg/g LFW, respectively

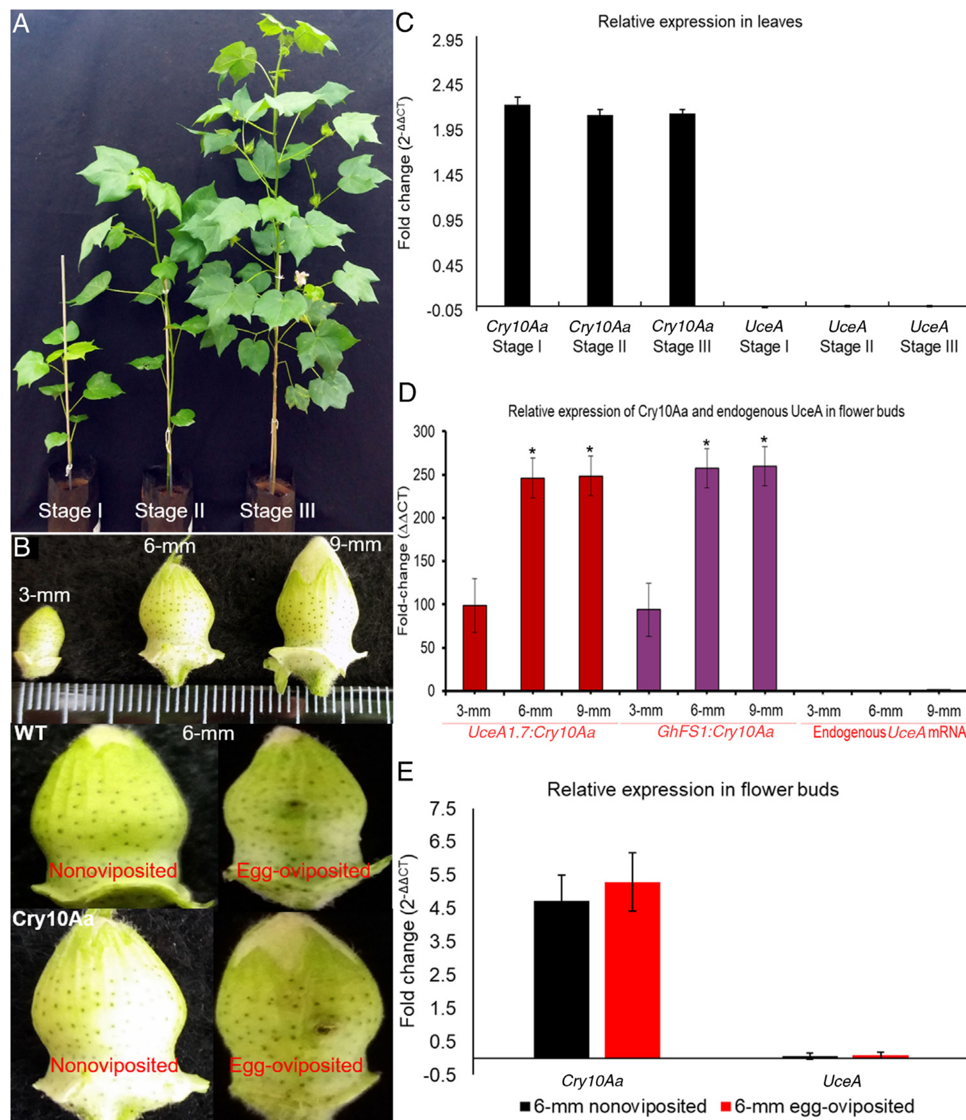

Fig. 2 Expression level of *cry10Aa* protoxin or endogenous *uceA* mRNA in different developmental stages or plant tissues (leaf and flower bud) from cotton events constitutively or tissue-specific overexpressing Cry10Aa. **(A)** Vegetative developmental stages (I, II and III) of T_3 plants of the 4.15.3.1 event; **(B)** 3-, 6-, and 9-mm diameter flower buds at 5–7 days after egg oviposition by the CBW from 4.15.3.1 event; **(C)** Relative expression level of *cry10Aa* or endogenous *uceA* mRNA in cotton leaves at three developmental stages from 4.15.3.1 event; **(D)** Relative expression level of *cry10Aa* and endogenous *uceA* mRNA (*uceA* gene corresponding to *UceA1.7* promoter) in 3-, 6-, and 9-mm diameter flower buds from cotton events constitutively (4.15.3.1 event) or tissue-specific (Event 181.3.1) overexpressing the Cry10Aa protein. The *uceA* expression level in flower buds was evaluated only in events constitutively overexpressing Cry10Aa; **(E)** Relative gene expression level of *cry10Aa* or endogenous *uceA1.7* in non-oviposited and egg-oviposited flower buds. All fold-change values were calculated using the $2^{-\Delta\Delta CT}$ method using leaf and flower buds from wild-type plants as a reference. Error bars represent standard deviations of 4 (from leaves) and 3 (from flower buds) biological replicates. Asterisks indicate significant differences between expression levels in 3-, 6-, and 9-mm diameter flower buds based on Tukey's test at 5 %.

(data not shown). Additionally, higher Cry10Aa protoxin accumulation was observed in 9-mm diameter flower buds, while Cry10Aa active showed higher accumulation in both flower buds of 6- and 9-mm diameter (Fig. 3C). In contrast, Cry10Aa active showed higher accumulation in stamen and carpels when compared to Cry10Aa protoxin (Fig. 3C). Finally, Cry10Aa protoxin or active accumulation in different tissues from constitutively or tissue-specific events was confirmed by Western blotting (Supplemental Fig. 1A to D).

In planta feeding bioassays against CBW larvae

Bioassays were conducted to evaluate the resistance level of events overexpressing constitutively or tissue-specific Cry10Aa. This evaluation was based on the mortality percentage of CBW larvae fed in 6-mm diameter flower buds. Flower buds from NT plants were used as a negative control. A lower mortality rate was observed for some T_0 events by Ribeiro et al. (2017), while T_1 to T_3 events showed larger and more homogeneous percentages (80–100%) (Fig. 4A). In

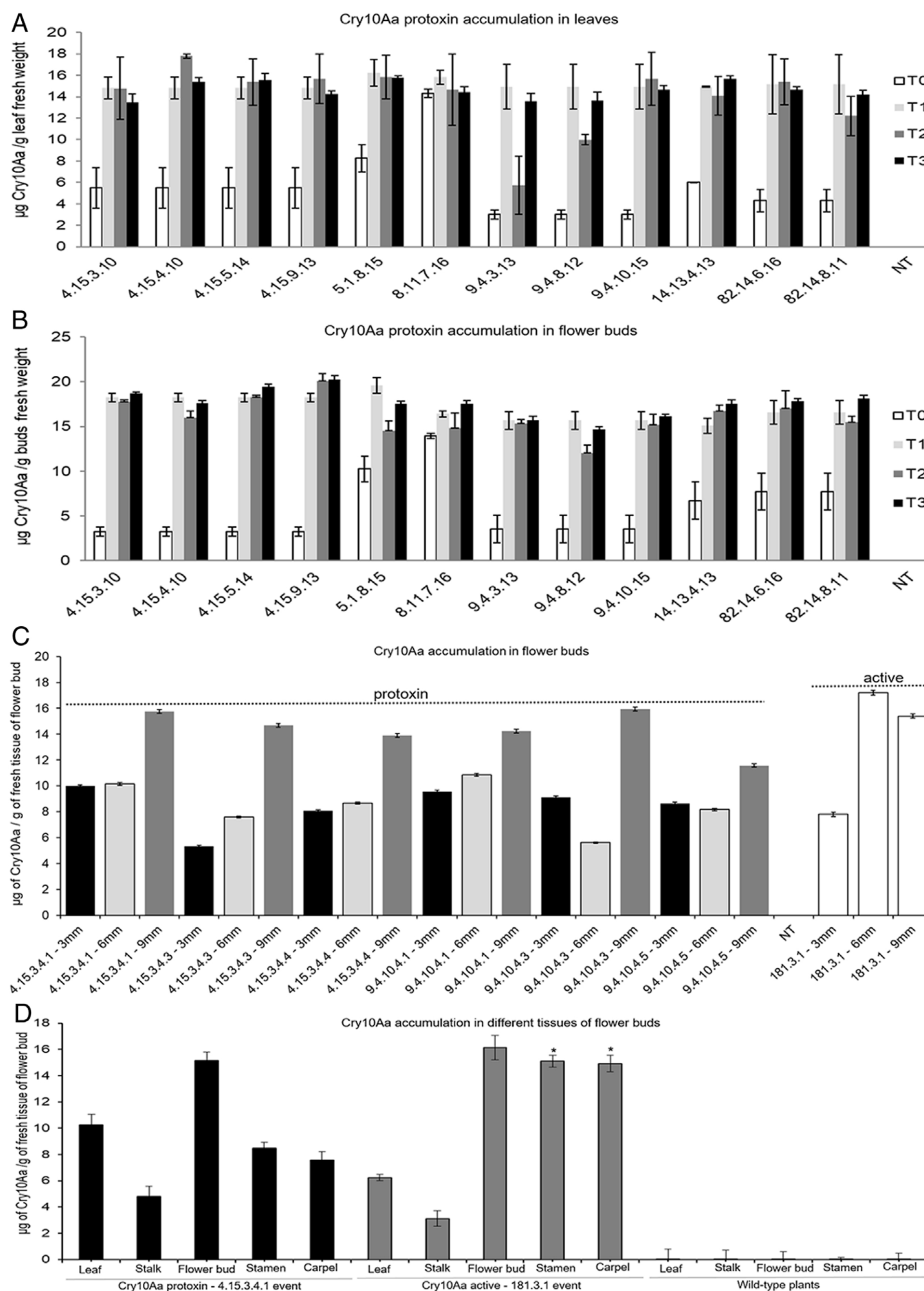

Fig. 3 Indirect ELISA assay using polyclonal antibody against Cry10Aa protein from **(A)** leaves and **(B)** flower bud samples of the T_0 to T_3 cotton events constitutively overexpressing Cry10Aa protoxin. **(C)** Cry10Aa protein accumulation in 3, 6, and 9-mm diameter flower buds from cotton events constitutively or tissue-specific overexpressing Cry10Aa protoxin or active. **(D)** Cry10Aa protein accumulation in leaf, stalk, 6-mm diameter flower buds, stamen, and carpels from cotton events constitutively or tissue-specific overexpressing Cry10Aa protoxin or active, respectively. Asterisks indicate significant differences in Cry10Aa protein accumulation in stamen and carpels among events constitutively or tissue-specific overexpressing Cry10Aa protoxin or active based on Tukey's test at 5 %. Leaf and flower buds from non-transgenic (NT) plants were used as a negative control. The amount of total protein was previously adjusted by the Bradford method and used 1.0 μg per plate wells for ELISA assays. Recombinant Cry10Aa protein expressed and purified from *E. coli* was used to obtain a standard curve of quantification and as a positive control of ELISA assays. All samples were carried out in technical triplicates. Error bars correspond to the standard deviation of biological triplicates (different plants from each event or different flower buds harvested in the same event).

addition to CBW insects multiplied and *in house*-maintained on artificial diets, we also used CBW insects collected from cotton plants under field conditions. In bioassays when inserting CBW eggs (from artificial diet) into flower buds, we observed resistance of up to 100 % for T_2 events, while field insects achieved a resistance of 80−95 % for T_2 and 83.2−92.1 % for T_3 events. The high and homogeneous mortality rate was linearly related to Cry10Aa protein accumulation (rang-

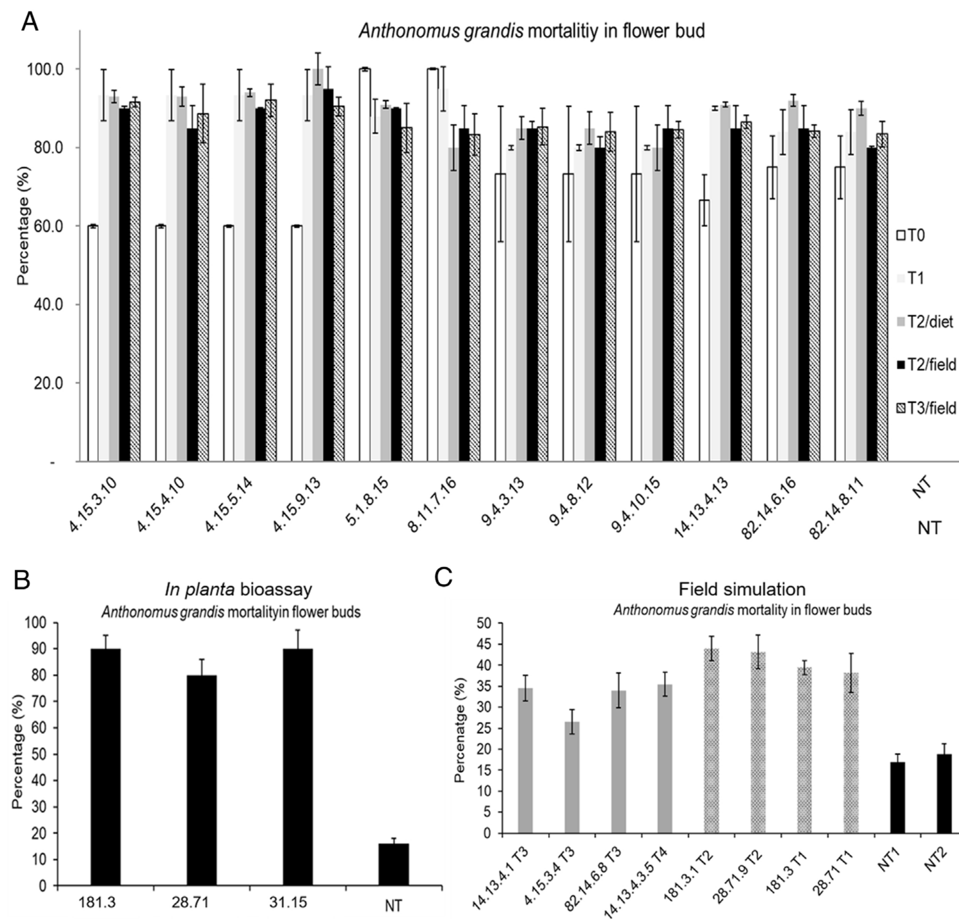

Fig. 4 *In planta* bioassays and field simulation in greenhouse conditions from cotton events constitutively or tissue-specific overexpressing Cry10Aa using cotton boll weevil (CBW) adult insects. **(A)** Percent of CBW mortality in T_0 to T_3 events (n = > 30 flower buds per event) submitted to *in planta* bioassays. **(B)** Percent of CBW mortality after *in planta* feeding bioassays using flower buds of T_1 events overexpressing Cry10Aa active under tissue-specific promoter control. **(C)** Percent of CBW mortality after bioassays of field simulation in events overexpressing Cry10Aa protoxin or active, under control of constitutive or tissue-specific promoters, respectively. In all bioassays, the n = > 30 flower buds per event.

ing from 16 to 20 µg/g of LFW) in flower buds of T_2 and T_3 events (*e.g.*, the 4.15.3–4.15.9 events). The CBW larvae fed transgenic flower buds showed a typical phenotype of toxicity (Supplemental Figure 3B3 to 13). In addition, malformations in nonemerged juvenile insects and dead insects were also observed in these flower buds (Supplemental Figure 3B14 to 17). Similarly, tissue-specific events overexpressing Cry10Aa active in T_1 generation presented CBW mortality of 80–90% (Fig. 4B). Lastly, a lower abortion rate of flower buds after egg oviposition by the CBW was observed for some T_2 and T_3 events, while higher mortality of CBW insects was observed in the pupal stage (Supplemental Fig. 2A to D).

Field simulation for evaluation of resistance to CBW

Bioassays under a condition similar to the field were conducted in the greenhouse from both events overexpressing constitutively or tissue-specific Cry10Aa. This evaluation was based on the mortality percentage of CBW larvae fed in any oviposited flower buds from transgenic events, while NT plants were used as a negative control. Compared to the *in planta* feeding bioassay, the field simulation showed lower resistance to the CBW of all evaluated events, while events overexpressing tissue-specific Cry10Aa active showed higher resistance (Fig. 4C).

Agronomic performance and yield potential of the Cry10Aa GM plants

Six independent events (4, 5, 8, 9, 14, and 82) from the T_3 generation were selected to evaluate the agronomic performance compared to NT plants under greenhouse conditions. Plants at the booting stage and maintained under well-irrigated (field capacity) or moderate water deficit (soil Ψw of -0.85 to -0.95 MPa) conditions were evaluated for gas exchange, leaf chlorophyll content, photochemical efficiency of PSII and yield (flower buds, seeds and fiber per plant). Transgenic events showed that the A_N, *Ci*, *E* and *gs* parameters were similar to those in NT plants, both in well-irrigated plants and in plants under water deficit (Fig. 5A and

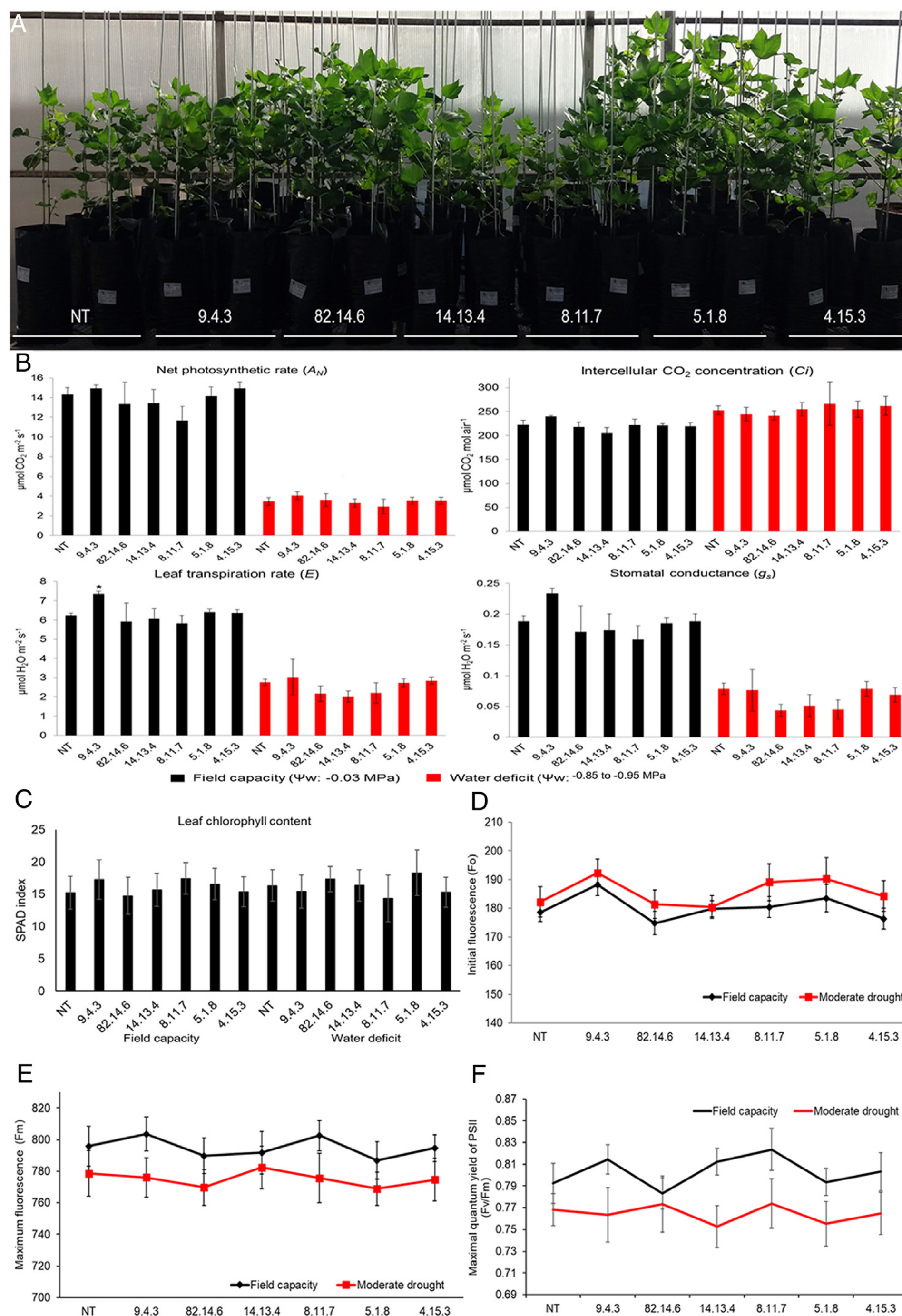

Fig. 5 Ecophysiological analysis of six different cotton events constitutively overexpressing Cry10Aa protoxin (T_3 generation) and non-transgenic (NT) cotton maintained under field capacity (soil Ψw: -0.03 MPa) or water deficit (soil Ψw: -0.85 to -0.95 MPa) conditions. **(A)** Phenotype of adult plants at the booting stage under field-capacity conditions. In general, plants cotton cv. BRS372 (both wild-type or NT and Cry10Aa events) show an uneven initial growth due to the genetic background, but their growth becomes uniform when they are adult plants.; **(B)** gas exchange analysis represented as the net photosynthetic rate (A_N), intercellular CO_2 concentration (*Ci*), leaf transpiration (*E*) and stomatal conductance (g_s); **(C)** leaf chlorophyll content represented as the SPAD index; and photosystem II (PSII) efficiency represented as **(D)** initial fluorescence (F_0), **(E)** maximum fluorescence (*Fm*), and **(F)** maximum quantum yield of PSII (*Fv/Fm*) of well-watered or water-deficit plants. Asterisks indicate statistical significance between NT and Cry10Aa events (Tukey's test at 5 %).

B). In addition, the chlorophyll content (Fig. 5C) and photochemical efficiency (Fig. 5D to F) were also similar to those of NT plants. Then, we evaluated the yield of events under well-watered or water deficit conditions compared to that of NT plants. Initially, we evaluated flower bud yield from all 12 segregating events maintained under well-irrigated conditions in a greenhouse. All transgenic events, except the 8.11.7 and 14.13.4 events, showed flower bud yields similar

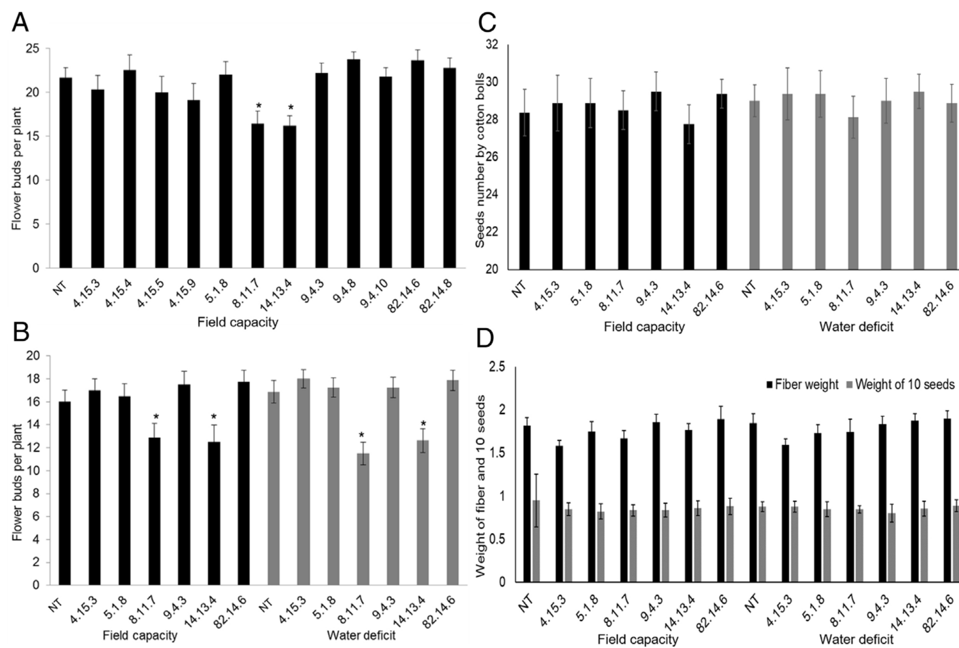

Fig. 6 Yield of cotton events constitutively overexpressing the Cry10Aa protoxin maintained in a greenhouse under well-watered (field capacity) or water-deficit conditions. **(A)** Number of flower buds per well-irrigated plant at 40 days after the beginning of flowering (n = 9 plants); **(B)** number of flower buds per plant under well-irrigated or water-deficit conditions at 30 days after the beginning of flowering; **(C)** seed number per cotton bolls from well-watered (field-capacity) and under water-deficit plants; and **(D)** weight of fiber and 10 seeds per cotton bolls from well-watered (field capacity) and under water-deficit plants (n = 8 plants; 2 cotton bolls per plant). Asterisks indicate significant differences between NT and Cry10Aa events (Tukey's test at 5 %).

to those of NT plants 30 days after the beginning of flowering (Fig. 6A and B). These two highlighted events showed less flowering precocity and consequently a lower flower bud yield when evaluated at 30 or 40 days. However, in later stages, these events were equivalent to the other events or NT plants (data not shown). In contrast, events submitted to water deficit or well-irrigated conditions showed a flower bud yield (Fig. 6B), the number of seeds per cotton bolls (Fig. 6C), and weights of fiber and ten seeds per cotton boll were similar to those in NT plants (Fig. 6D). Thus, our findings suggested that at least four independent events showed similar agronomic performance as an equivalence to NT plants.

Discussion

Cotton is an economically important crop for different countries and is the main source of fiber for the textile industry (Zhang et al., 2015; Pimenta et al., 2016). However, there are several field-related drawbacks to be overcome. CBW is one of the most important pests of this crop worldwide, mainly in major cotton-producing countries in the Americas (Grossi-de-Sa et al., 2007; de Lima, Degrande, Miranda, & dos Santos, 2013). The high incidence of this pest can lead to significant economic losses, with a reduction in cotton boll number of up to 100 % and a decrease in fiber yield (Foster, 2009). The occurrence of the CBW has been reported not only in the flower bud-producing stages but also in earlier stages of the plant, forcing producers to increase the number of agrochemical applications (in some cases, up to 30 applications per crop) (Neves, Colares, Torres, Santos, & Bastos, 2014), consequently increasing the cost of production, causing environmental damage and reducing the population sizes of non-target insects, which act on the biocontrol of other important insect pests. Cultural practices, reduced number of commercial cultivars of *G. hirsutum* that exhibit at least low CBW resistance, volunteer cotton plants that grow during the off-season period and alternative hosts are some of the primary factors responsible for the large sources of CBW inoculum and the failure of CBW management (Ribeiro et al., 2010; Magalhaes et al., 2012; Neves et al., 2014; Magalhaes et al., 2016; Pimenta et al., 2016). Thus, the search for new or additional alternatives to assist in management has become important for reducing its population size, decreasing economic losses and effectively controlling the CBW. However, new biotechnological tools (NBTs) based on the RNA interference (RNAi) strategy *in planta* for cross-talk control (Baum et al., 2007; Burand & Hunter, 2013), the RNAi strategy for topical application in cotton (Gillet et al., 2017; Macedo et al., 2017), and *in planta* overexpression of entomotoxic proteins (*Bt* proteins) (Grossi-de-Sa et al., 2007; Martins et al., 2008; Aguiar et al., 2012; Silva et al., 2016; de Oliveira et al., 2016; Ribeiro et al., 2017) are considered promising alternatives to assist in the management of the CBW in cotton crops.

Entomotoxic *Bt* proteins have been successfully tested for the control of various Coleopteran species, including the CBW. The Toxins Cry1Ba6, Cry8Ka5, Cry1Ia, Cry1Ia12, and Cry10Aa have already been shown to be entomotoxic to the CBW by *in vitro* screenings (Grossi-de-Sa et al., 2007; Martins et al., 2008; Aguiar et al., 2012; Silva et al., 2016; de Oliveira et al., 2016; Ribeiro et al., 2017). Furthermore,

Cry1Ia12, Cry1Ia, and Cry10Aa showed an entomotoxic effect on the CBW, as demonstrated by ecotypic expression in transgenic cotton (de Oliveira et al., 2016; Silva et al., 2016; Ribeiro et al., 2017). Previous studies have shown that the lethal dose of CryIa12 to *A. grandis* and *Spodoptera frugiperda* larvae is 230 and 5 μg/ml in artificial diet bioassays, respectively (Grossi-de-Sa et al., 2007). The CBW fed Cry1Ia12 GM cotton accumulating up to 2.56 μg/g of the leaf, showed up to a 60 % reduction in the emergence of pupae, while the surviving larvae and adult insects were weaker and significantly smaller than those fed NT plants (de Oliveira et al., 2016d). Similarly, the 50 % lethal concentration (LC_{50}) of Cry1Ia protein against *A. grandis* was 21.5 μg/ml (Martins et al., 2008). In contrast, the CBW adults and larvae fed on leaves and flower buds of Cry1Ia GM cotton, accumulating up to 2.7 μg/g of dry weight, showed mortality percentages of up to 85.7 and 60.7 %, respectively (Silva et al., 2016).

A previous *in vitro* bioassay revealed that Cry10Aa toxin showed an LC_{50} (the lethal dose that kills 50 % of the insect larvae) of 6.35 μg/ml against CBW larvae (Ribeiro et al., 2017). Similarly, Aguiar et al. (2012) performed bioassays with virus-infected insect extracts and observed high toxicity to CBW larvae, with an LC_{50} of 7.12 μg/ml. Recently, Ribeiro et al. (2017) showed that the Cry10Aa GM cotton accumulating up to 20 μg/g of flower buds fresh weight from T_0 and T_1 generation resulted in CBW mortality levels of up to 100 % when fed in leaves or flower buds of plants in greenhouse conditions. Thus, these LC_{50} values of Cry10Aa protein toxicity to CBW larvae are lower than those observed for other Cry proteins (Oliveira et al., 2011; de Oliveira et al., 2016). Previous studies have suggested that the *in planta* accumulation of entomotoxins for efficient control of insect pests in field conditions should be approximately 10–20 times greater than the LC_{50} (Wu & Tian, 2019).

In this work, 12 segregating events overexpressing the Cry10Aa protoxin were selected from the T_1 generation (based on high Cry10Aa protein accumulation in flower buds and a high mortality percentage of CBW larvae) obtained by Ribeiro et al. (2017). After advancing the T_2 and T_4 generations, it was possible to confirm the stability of the Cry10Aa protoxin transgene. The high Cry10Aa protoxin accumulation in leaves or flower buds (up to 18 and 20 μg/g of LFW, respectively) for the T_2 to T_3 events was correlated with the highest percent CBW mortality in greenhouse bioassays. In addition, the Cry10Aa accumulation and percentage of CBW mortality from the T_1 to T_3 generations were highly stable for each event, in contrast to the results for the T_0 generation, suggesting the stability of transgene expression. The variation in expression level and protein accumulation in different events can be explained primarily by different insertion sites of the transgene in the genome and the intrinsic specificity of *Cry* genes or proteins (de Oliveira et al., 2016d). Moreover, the low and variable Cry10Aa transgene expression during T_0 events, in contrast to that in more advanced generations, can be a consequence of the need for locus rearrangement due to the insertion of foreign DNA sequences (Kohli et al., 2006). The use of transcriptional promoter sequences with flower bud-specific activities or CBW-induced activity is an alternative to further enhance Cry10Aa expression and accumulation in flower bud tissues (Artico, Lambret-Frotté et al., 2014, 2014b; Lambret-Frotte et al., 2016). The cotton *UceA1.7* promoter used in this study was previously characterized as a strong and constitutive promoter with high expression levels in flower buds (Viana et al., 2011). In addition, the *UceA1.7* promoter drove up to 7-fold-higher levels of *uid*A transcripts than the CaMV 35S promoter in siliques of transgenic *A. thaliana* (Ribeiro et al., 2017). In this study, our findings also confirm the high and stable expression of *cry10Aa* mRNA driven by the *UceA1.7* promoter in leaves and both egg-oviposited and non-oviposited flower buds.

In this sense, cotton events tissue-specific overexpressing the Cry10Aa active showed higher accumulation of this protein in the stamen and carpels compared to events constitutively overexpressing the Cry10Aa protoxin, which can be explained by the use of *GhFS1* (flower-specific) promoter. CBW insects feed and oviposit on the flower bud stamen, while their emerging larvae feed initially on the stamen and later on the carpels (Ribeiro et al., 2017). Therefore, cotton events tissue-specific overexpressing the Cry10Aa active may be promising for CBW control compared to the events constitutively overexpressing the Cry10Aa protoxin. The results of the *in planta* feeding bioassays and field simulation confirmed that the higher accumulation of Cry10Aa active in stamen and carpels enhanced the cotton resistance to CBW. However, the low CBW resistance of events in field simulation assays highlighted the need to further increase the accumulation and availability of this protein in stamen and carpels. In addition to the higher accumulation, better pH-dependent availability of these entomotoxic proteins may be required for efficient CBW control. Recently, Hou et al. (2019) showed that fusion of Cry proteins with *E. coli* maltose-binding protein (MBP) enhanced its activity against Western Corn Rootworm, probably by increased solubility of the MBP-Cry8Hb fusion in the rootworm midgut.

A higher level of Cry toxin accumulation in transgenic plants than the LC_{50} for efficient control of the CBW or other insect species has already been suggested and discussed in other studies (Gould, 1998; de Oliveira et al., 2016). Therefore, this high protein accumulation can increase the efficiency of CBW control and reduce the probability of the emergence of resistant populations, while resistance management can ensure greater durability of this toxin as a biotechnological tool (Perlak et al., 2001). The gene stacking of two or more *cry* genes into one cultivar alone or associated with an *in planta* RNAi strategy is also considered a promising alternative to increase the durability of resistance (Christou, Capell, Kohli, Gatehouse, & Gatehouse, 2006; Yang, Chen, Tang, Hua, & Lin, 2011). However, the discovery of new toxins or the improvement of existing toxins is necessary. The Cry10Aa protein accumulates in plants and is ingested by the CBW as a protoxin, and endogenous proteases of insects cleave some domains and release the active Cry10Aa toxin (Bravo et al., 2007; Pardo-Lopez et al., 2013; Adang et al., 2014). This pattern suggests that the engineering of Cry10Aa or other Cry toxins, previously by removing some of these specific domains, may increase their entomotoxic effect (Pardo-Lopez et al., 2009). Another promising alternative is the transcriptional improvement of *cry10Aa* mRNA using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/dead Cas9 (dCas9) system guided by a guide RNA targeting the *UceA1.7* promoter (Gao, 2018).

Geno- and hepatotoxic assays using recombinant Cry10Aa protein revealed no apparent mutagenic effect in mice, highlighting its biosafety potential for use in transgenic crops (de Souza Freire et al., 2014). Given the entomotoxic potential for CBW control and safe use of Cry10Aa GM cotton, we evaluated the agronomic performance of this resource in a greenhouse. Six independent events with a low copy number and high and stable transgene expression were screened, and some features were measured. Our findings suggest that Cry10Aa expression in transgenic cotton does not cause apparent penalties in yield and stress tolerance (*e.g.*, water deficit). The 8.11.7 and 14.13.4 events showed a small delay in initial growth and, consequently, a smaller flower bud number 30 or 40 days after planting. However, in later stages, these plants presented equivalent vegetative development and yield. The transgene insertion site identified provided evidence of the possible absence of off-targets in these six Cry10Aa protoxin events. Thus, we consider these events to be good candidates for further evaluating the effectiveness of Cry10Aa protoxin in cotton fields for CBW management.

In conclusion, we highlight the potential of Cry10Aa toxin for CBW control and the need for new strategies to increase the tissue-specific accumulation and availability of this toxin. Currently, field trials are being conducted with several events, and in parallel, our research team is working with other NBTs to increase the accumulation of Cry10Aa protein in cotton, improving its activity in CBW adults or larvae, improving its pH-dependent availability in flower bud tissue, and associating the Cry10Aa entomotoxin with an RNAi strategy (by both *in planta* production and topical delivery) to improve the resistance of the transgenic cotton to the CBW. We believe that the Cry10Aa GM cotton may soon be part of CBW management strategies in cotton crops and contribute to reducing yield losses and improving the sustainability of agribusiness.

Consent for publication

Not applicable.

Authors' contributions

MFGS, LLPM, and TPR conceived the experiments. MFGS was the lead researcher for all the work and provided intellectual input and financial support. TPR, MFB, MHC, DMLS, CVM and OBON assisted by LLPM and WAL performed and analyzed the data from molecular, serological, bioassay, and agronomic equivalence experiments. ITLT measured the transgene copy number and helped with RT-qPCR for gene expression. MFB and ERCP performed mapping of the transgene insertion site. MFB and TPR wrote the draft manuscript, while MFGS and MCMS provided intellectual inputs. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare that the research was conducted in the absence of any commercial, financial, or non-financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biori.2019.12.003.

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