**Expression of *Bacillus thuringiensis* Cytolytic Toxin (Cyt2Ca1) in Citrus
Roots to Control *Diaprepes abbreviatus* larvae.**

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Abstract

Diaprepes abbreviatus (L.) is an important pest of citrus in the USA. Currently, no effective management strategies of *D. abbreviatus* exist in citriculture, and new methods of control are desperately sought. To protect citrus against *D. abbreviatus* a transgenic citrus rootstock expressing *Bacillus thuringiensis* Cyt2Ca1, an insect toxin protein, was developed using *Agrobacterium*-mediated transformation of 'Carrizo' citrange [*Citrus sinensis* (L) Osbeck *Poncirus trifoliata* (L) Raf]. The transgenic citrus root stock expressed the cytolytic toxin Cyt2Ca1 constitutively under the control of a 35S promoter in the transgenic Carrizo citrange trifoliata hybrid including the roots that are the food source of larval *D. abbreviatus*. The engineered citrus was screened by Western blot and RT-qPCR analyses for *cyt2Ca1* and positive citrus identified. Citrus trees expressing different levels of *cyt2Ca1* transcripts were identified (Groups A-C). High expression of the toxin in the leaves (10^9 transcripts/ng RNA), however, retarded plant growth. The transgenic plants were grown in pots and the roots exposed to 3 week old *D. abbreviatus* larvae using no-choice plant bioassays. Three *cyt2Ca1* transgenic plants were identified that sustained less root damage belonging to Group B and C. One plant caused death to 43 % of the larvae that fed on its roots expressed 8×10^6 *cyt2Ca1* transcripts/ng RNA. These results show, for the first time, that Cyt2Ca1 expressed in moderate amounts by the roots of citrus does not retard citrus growth and can protect it from larval *D. abbreviatus*.

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60 Keywords: *Diaprepes abbreviatus* ; transgenic citrus rootstock; *cyt2Ca1*; larval
61 control; root resistance; genetic transformation

62 Abbreviations:

63 *Bt*, *Bacillus thuringiensis*; Cq, quantification Cycle; Cry, Crystal; Cyt, Cytolytic; *E.*
64 *coli*, *Escherichia coli*; IRR, Infested Root Ratio ; qPCR, quantitative Polymerase
65 Chain Reaction; RDI, Root Damage Index; RT-qPCR, Reverse Transcriptase-
66 qPCR; rRNA, ribosomal RNA; TMOF, Trypsin Modulating Oostatic Factor; *wt*,
67 wild type; URR, Uninfested Root Ratio

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1. Introduction

Diaprepes abbreviatus (L.) is an invasive pest of citrus and other economically relevant crops in the USA. Accidentally introduced from the Caribbean circa 1960 [1], *Diaprepes abbreviatus* subsequently established itself as an important pest of citrus in many areas across Florida [2]. The larvae of *D. abbreviatus* spend most of their life burrowed in the soil, causing direct damage by feeding on citrus roots and by facilitating the entry of plant pathogens. After emergence from the subterranean pupal chamber, adults feed and lay eggs on leaves [3]. However, it is the larvae that cause the most damage to citrus by consuming and pruning the roots and, in extreme cases, completely girdling the main root resulting in tree death [4]. Larval feeding also allows *Phytophthora* spp. to invade the roots causing disease and death, and in some cases larval overfeeding causes girdling of the roots and subsequent citrus death [5,6]

In citriculture, no satisfactory control for *D. abbreviatus* is available. Heavy reliance on chemical insecticides is the mainstay of management of *D. abbreviatus* infestations. However, due to the subterranean location of the larvae, achieving effective control with conventional chemical insecticides is not practical. Additionally, dependence on chemical insecticides is deleterious to the environment, toxic to beneficial and non-target insects, and harmful to human and animal health. The use of adulticides, however, is ineffective and expensive [7].

A strategy of pest insect control that has been widely exploited for insect control in agriculture involves the use of the *Bt* insecticidal proteins, from the parasporal

inclusion bodies of the soil bacterium, *Bacillus thuringiensis* (*Bt*) [8-10]. The *Bt* insecticidal proteins include the crystal (Cry) and cytolytic (Cyt) proteins that disrupt insect gut integrity, leading to water and ion imbalance and death. The Cry toxins bind specific receptors and result in pore formations in the gut epithelium, whereas the Cyt toxins act in a detergent-like manner to destroy the integrity of the gut epithelium [11,12]. Numerous toxins from several *Bt* strains show specific activity towards Diptera, Lepidoptera, or Coleoptera. Whereas the Cyt toxins predominantly show dipteran toxicity [13], a few Cyt toxins (Cyt1Aa and Cyt2Ca) affect several coleopteran species [14], including Cyt2Ca1, which is active against *D. abbreviatus* [15]. Formulations of *Bt* are used effectively as spray applications in horticulture, however, rapid environmental degradation and the subterranean feeding habit of larval *D. abbreviatus*, makes *Bt* surface treatments impractical for controlling citrus root infestations.

Significant advancement in the use of *Bt* as a bio-control agent has been commercially achieved using insect-resistant transgenic crops expressing a variety of Cry toxins [9]. Rapid adoption of several transgenic crop varieties by farmers [16] provide a convincing proof of their resilience against destruction from feeding damage by pests. However, this approach has yet to be exploited in citrus. For managing larvae of *D. abbreviatus* in citrus, a standard commercial citrus cultivar could be grafted onto a transgenic rootstock plant expressing a *Bt* toxin. Thus, citrus rootstocks can be engineered for resistance against *D. abbreviatus*, whereas the fruit bearing scion remains devoid of transgenic genes. With this in mind, we engineered the citrus rootstock 'Carrizo' (a hybrid of *Citrus*

sinensis and *Ponirrus trifoliata*), to express a recombinant gene product of *cyt2Ca1*, a Cyt toxin expressed by *Bt*. This *cyt* isolate was previously reported to be active against *D. abbreviatus* neonates using artificial diet bioassays [15]. We report here for the first time that a transgenic citrus rootstock transformed with *cyt2Ca1* can successfully withstand aggressive feeding by larval *D. abbreviatus* opening the door for future control of this pest insect.

2. Material and Methods

2.1 Genetic Engineering

cry29ET (GenBank accession AY030096.1) classified as *cyt2Ca1* [17] sequence was optimized for maximal citrus protein coding expression. Codon bias for four highly expressed genes in citrus was evaluated using two constitutive high expressed genes encoding: chlorophyll a-b binding protein 3C from *Citrus sinensis*, (GenBank accession: XM_006467451), Rubisco small subunit from *C. sinensis*, (GenBank accession: XM_006482191) and two inducible highly expressed genes encoding: metallothionein from *Citrus unshiu* (GenBank accession: AF320905) and miraculin-like from *C. sinensis* (GenBank accession: XM_006478073). The DNA coding sequence of *cyt2Ca1* was then modified reflecting highly used codons for each amino acid using the four highly expressed citrus genes and the modified optimized sequence was deposited in GenBank (accession number KT592537). The optimized sequence was checked for cryptic RNA processing signals by GenScript (Piscataway, NJ). The resulting citrus optimized sequence of *cyt2Ca1* was then synthesized by GenScript and cloned into plasmid, pUC57 that was named pDRW- Cyt2Ca1. The cloned

synthetic sequence contains an optimal Kozak sequence (CCGCCACC) preceding the ATG start sequence for an efficient translation initiation [18].

The optimized *cyt2Aa* sequence was then cloned into a Gateway entry vector, pCRTM8/GW/TOPO, using manufacturer's protocols (Life Technologies, Grand Island, NY) and transferred into a Gateway Agrobacterium binary vector, pMDC32, using a LR (Left, Right) recombination method [19] forming a plasmid (pMDC32- *cyt2Ca1*) with a *cyt2Ca1* sequence under the control of the constitutive dual 35S promoters. After cloning, the plasmid was sequenced using Sanger sequencing and the BigDye® Terminator v3.1 Cycle Sequencing kit (Life Technologies, Grand Island, NY) to verify the sequence and plasmid (pMDC32-*cyt2Ca1*) was electroporated into *A. tumefaciens* strain LBA4404 [20] and used for citrus transformation. Transgenic citrus plants were produced from seedling epicotyl explants using *Agrobacterium*-mediated transformation methods [21] and hygromycin selection (10 mg/L) to select transformed cells. Regenerated plantlets were micro-grafted onto Volkamer lemon seedlings. Transgenic plants utilized in this study exhibited a normal phenotype and growth pattern.

A total of 75 transgenic citrus cohorts, prepared from transgenic scions were grafted onto non-engineered rootstocks (batch 1) to accelerate plant growth. Leaf samples from these plants were screened by RT-qPCR for *cyt2Ca1*. Cuttings from positive qPCR parental transgenic citrus (Batch 1) were rooted (Batch 2) and *cyt2Ca1* transcript expressed in leaves and roots was analyzed by RT-qPCR. Plants were divided into three groups (A, B and C)

based on transcript abundance and multiple vegetative clones were produced from cuttings of these transgenic trees (Batch 3) and multiple replicate plants were produced from each category and used in potted-plant bioassays.

2.2 Primers

All primers used in q-PCR and RT-qPCR amplifications are listed in Table 1 and were purchased from IDT[®] (San Diego, CA). Primer pair BtCyt-f and BtCyt-r were used to amplify *cyt2Ca1* to follow transcript expression in citrus. Two other primer pairs CitCD-f and CitCD-r were used to amplify *Citrus sinensis* dehydrin (*cd*) (GenBank: XM_006473689.1; qPCR reference genes), and Cit18S-f and Cit18S-r were used to amplify 18S RNAs (GenBank: AF206997.1) to normalize the RT-qPCR analyses.

2.3 Nucleic Acid Extractions

2.3.1 DNA extraction:

Leaf samples randomly obtained from batch-1 to rapidly identify trees that carried *cyt2Ca1*, after analysis, leaf and root samples were obtained from batch-2 and 3. Transgenic citrus plants were cleaned with RNase[™] AWAY (Thermo Scientific, Waltham, MA). Leaves and roots were then thoroughly rinsed with de-ionized water, and excess water was blotted off. Leaf midribs were excised and roots were finely chopped using a new blade for each sample to prevent DNA cross-contamination and genomic DNA was extracted using the FastDNA[®] Green SPIN Kit (mpBIO, Solon, OH) following manufacturer's protocol. The purity and concentration of the DNA was quantified by NanoDrop[™] 1000 (Thermo Scientific).

2.3.2 RNA extraction

Leaf samples from batch-1 and leaf and root samples from batch-2 and 3 *cyt2Ca1* transgenic citrus were obtained and cleaned as described above, and the whole leaf including the midrib was used. Whole leaves were placed in zipper-sealed sample bags and flash frozen in liquid nitrogen. The samples were stored at -80 °C until ready to be used. RNA was extracted from leaf (batch-1) and leaf and root samples (batch 2 and 3) cohorts in TRI Reagent® (Sigma-Aldrich, St Louis, MO) using manufacturer extraction protocol. RNase AWAY (Thermo Scientific) was used to destroy contaminating RNase in plastic and glassware. After freezing, leaf and root samples were ground to a fine powder in the presence of liquid nitrogen with a mortar and pestle. Pulverized leaves (150 mg) were placed into screw-cap tubes (2 mL). TRI Reagent® (1 mL) was added to each tube and the mixture vortexed and incubated at room temperature for 5 min. After incubation, chloroform (2 mL) was added and the mixture vortexed and incubated for 10 min at room temperature. The mixture was then centrifuged at 12,000 rpm for 10 min at 4 °C in a refrigerated centrifuge (Eppendorf 5702 R), the aqueous phase was transferred into an Eppendorf tube (1.5 mL) free of DNA and RNA. Isopropanol (500 µL) was added, the tube briefly vortexed, and incubated for 10 min. After incubation, the solution was centrifuged at 12,000 rpm and the supernatant discarded. The pellet was washed in cold 75% ethanol (1 mL) by gently inverting the tube, the ethanol was removed by centrifugation at 12,000 rpm for 5 min and the RNA pellet air-dried for 10 min at room temperature. The pellet was then re-suspended in nuclease-free water (80 µL)

and stored at -80 °C. The RNA suspension was treated with RQ1 DNase (Promega, Madison, WI) to remove DNA contamination. RQ1 DNase and 10X DNase buffer (1:1, 20 µL) was added to the RNA suspension. The mixture was incubated at 37 °C for 1 h. Following incubation, 200 µl nuclease-free water and 300 µl phenol (pH 4.3) were added and the RNA suspension vortexed, and centrifuged at 12,000 rpm for 10 min. The upper aqueous phase was collected into an Eppendorf tube and an equal volume (0.6 mL) of phenol: chloroform was added, the tube vortexed, and centrifuged at 12,000 rpm for 10 min. The upper phase was collected into a new clean tube and sodium acetate (3 M, pH 5.2, 60 µL), and 100% ethanol (1.5 mL) were added, the sample gently mixed by inversion and the RNA precipitated overnight at -80 °C. The RNA precipitate was pelleted at 12,000 rpm or 20 min and the supernatant discarded. The pellet was washed with cold 70% ethanol (300 µL) by gently inverting the tube, the ethanol discarded and the pellet re-suspended in nuclease-free water (50 µl). RNA concentration and, purity was determined in a NanoDrop™ 1000 (Thermo Scientific) instrument and the RNA integrity by agarose gel (1.2%) electrophoresis using DEPC-treated TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8) .

2.4 qPCR

qPCR were run using SensiMix HRM™ Kits (Bioline, Taunton, MA) with in a reaction mixture of 12.75 µl containing template DNA (150 ng) and with primer pair, BtCyt-f and BtCyt-r to amplify a 234 bp region of *cyt2Ca1*, and primer pair CitCD-f and CitCD-r to amplify a 240 bp region of a control gene, citrus

dehydrin (*cd*) for normalization. Amplifications of *cyt2Ca1* and *cd* (control) were done in triplicate for each transgenic citrus. CAS1200 Liquid Handling System (Corbett Robotics- Qiagen, Valencia, CA) was used to aliquot multiple reactions simultaneously, and Rotor-Gene 6000 (Corbett Robotics) was used to run the qPCR. Amplification cycles were done at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 sec and 60 °C for 30 sec and 72 °C for 30 sec. Amplification cycles immediately followed by a melt curve analysis at 70-90 °C of the amplified product. The presence of the amplified product was determined by the number of amplification cycles needed to reach a common fixed threshold C_q (quantification cycle) during the exponential phase of the PCR cycle.

2.5 RT-qPCR

RT-qPCR (12.5 µl) were run with QuantiTect® SYBR® Green (Qiagen) kit following manufacturer's recommendations. Transcript (*cyt2Ca1*) expression data were normalized with a reference gene that was stably expressed across the root tissues. Three reference genes, ubiquitin, actin, and 18S RNA, were assayed using RNA extracted from 12 transgenic citrus expressing *cyt2Ca1* and 1 non-engineered citrus (control). RNA templates (100 ng) were first reverse transcribed and ssDNAs of *cyt2Ca1*, *cd* and 18S RNA were amplified with primer pairs BtCyt-f and BtCyt-r (234 bp), CitCD-f and CitCD-r (240 bp) and Cit18S-f and Cit18S-r (153 bp) (1 µM), respectively (Table 1). RT-qPCR were run in triplicate and compared with qPCR alone (control) to check for DNA contamination. A second control was run with nuclease free water and without RNA template, to check for cross-contamination. A CAS1200 Liquid Handling

System was used to perform multiple reactions simultaneously and Rotor-Gene 6000 was used to run the reactions. The following thermal cycling conditions were used: 50 °C for 30 min, 95 °C for 15 min, followed by 35 cycles at 94 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. Amplification reactions were followed with melt curve analyses at 70-90 °C to verify the identity of the amplified DNA. Expression levels were assessed based on the number of amplification cycles C_q (quantification cycles) needed to reach a common fixed threshold during the exponential phase of the PCR amplification cycles. The relative level of the *cyt2Ca1* transcript in transgenic citrus plants was normalized with the 18S RNA transcript expressed as $\Delta Cq = Cq_{18S \text{ rRNA}} - Cq_{Cyt2Ca1}$.

To quantify the number of transcripts of *cyt2Ca1* in the roots and leaves a calibration curve of Ct values was plotted against the ng of *cyt2Ca1* insert tested in the qPCR. The amount of expressed *cyt2Ca1* transcripts/ng RNA tested in the roots and leaves of batch 2 and 3 transgenic citrus was then determined from a calibration curve [22].

2.6 Potted-Plant Bioassays

The transgenic plants were divided into three groups A, B and C using ΔCq levels in the leaves of batch-1 transgenic citrus. For example, $\Delta Cq > 1$ (Group A), $-1 < \Delta Cq < 1$ (Group B) and $\Delta Cq < -1$ (Group C) (Fig. 1). Cuttings were made from these plants (batch-2 and 3). The growth of cuttings was uneven so we used for the potted plant bioassays only plants that were fairly uniform in height. Seven *Cyt2Ca1* transgenic citrus plants were selected from the three groups. From Group A (H-3A, H-U2, H-417), from Group B (M-413, M-414) and

from Group C (L-A3, L-422). Using an experimental design described by Lapointe and Bowman [23], 10 cohorts of each selected transgenic citrus (from 8-month-old cuttings) in addition to 10 cohorts of the wild type (*wt*) Carrizo control were used for the potted plant bioassays. The transgenic citrus plants were challenged with three-week-old *D. abbreviatus* larvae to determine their resistance to root wounding as a result of feeding injury and to assess larval weight gain and mortality after feeding on the transgenic plant roots. Citrus transgenic and wild type (*wt*) control plants (10 each) initially grown in small containers (D x H, 3.4 x 12.4 cm) were transplanted into 2-L pots (D x H, 11 x 25 cm) with drain holes containing white, sterile sand. To prevent escape of larvae from infested pots, two pots were nested into each other and a plastic screen was placed between them with the inner pot drain holes covered by the screen. After removing the plants from the small containers and just prior to transplanting them, the root-volume of each plant was measured by water displacement. Each root system was immersed into a volumetric cylinder containing a known volume of water. The volume of water displaced was recorded as the starting volume of the seedling roots. After transplanting into a pot containing sand, plants were placed in a greenhouse and maintained on a fertilizer mix (N: P: K, 20:10:20) diluted in water at a rate of 150 mg/L/week. Temperature during the experimental period in the greenhouse fluctuated between 35 and 15 °C, with relative humidity of 34 to 93% and no supplemental light was provided. Three-week-old *D. abbreviatus* larvae were obtained from a colony maintained on artificial diet at USDA-USHRL at Ft. Pierce FL [24]. Larvae were placed in 40

containers with seedlings and in five pots containing sterile soil without plants as starvation control. Five potted seedlings without larvae were used as controls. Six larvae weighing 20-35 mg each were placed individually into holes made with a pencil (10 cm deep) in the soil at a distance of 2 cm from the stem of each test plant. Larvae and root volumes were examined 28 days after infestation. Root volume of each plant (with and without larvae) was determined using the water displacement method as described above. The change in root-volume for each plant was calculated as a ratio of the final root volume divided by the initial root volume ($V_{\text{final}} / V_{\text{initial}}$). From this, an average root volume was determined for each uninfested cohort as the V_{URR} (uninfested root ratio). Similarly, the V_{IRR} (infested root ratio) was calculated for infested potted plants. The ratio of $V_{\text{IRR}} / V_{\text{URR}}$ for each plant was then used to standardize the change in root volumes of individual transgenic citrus infested (IF) and non-infested (NIF) with *D. abbreviatus* as compared with wild type (*wt*) citrus. A root damage index (V_{RDI}) was calculated for each citrus plant as the difference between V_{URR} and V_{IRR} ($V_{\text{RDI}} = V_{\text{URR}} - V_{\text{IRR}}$). Mortality (M) and weights of live larvae were measured at 28 days after infestation. Abbott's formula [25] was used to adjust for mortality not associated with Cyt2Ca1 larval toxicity by subtracting the number of dead larvae fed on untransformed citrus (*wt*) roots from the number of dead larvae fed on transgenic citrus ($M_{\text{specific}} = M_{\text{Cyt2Ca1}} - M_{\text{wt}}$). Corrected percentage mortality was transformed with the angular transformation (arcsine) to normalize the data for statistical analysis. Data were statistically analyzed using GraphPad Prism 5 (La Jolla, CA) with Dunnett's multiple comparison tests to compare differences

between *cyt2Ca1* and *wt* citrus plants. The results were also compared with larvae that were placed in sterile sand to show that potted sand did not cause mortality.

2.7. Molecular Modeling of *Cy2Ca1*

Homology modeling of the inactive (dimer) form of *Cyt2Ca1* *Bt* insecticidal proteins (Fig. 8A) was employed using the YASARA structure program [26] and a 2.53 GHz Intel core duo Macintosh computer. Eleven different models of *Cyt2Ca1* were built from the X-ray coordinates of *CytB* insecticidal protein from *Bacillus thuringiensis* subsp. *kyushuensis* (PDB code 1CBY) [27], *Cyt2Ba* insecticidal protein from *Bacillus thuringiensis* subsp. *israelensis* (PDB code 2RCI) [28], and *Cyt1Aa* insecticidal protein from *Bacillus thuringiensis* subsp. *israelensis* (PDB code 3RON) [17] that were used as templates. A hybrid model of the inactive form of *Cyt2Ca1* was built using eleven previous models. Initially seven 3-dimensional (3D) models of the active form of *Cyt2Ca1* were built as templates by homology modeling, using *CytB* (1CBY), *Cyt2Ba* (2RCI), and *Cyt1Aa* (3RON) as templates. A final hybrid model of the active form of *Cyt2Ca1* was then built using the seven 3D models. The geometric quality of both 3D models was assessed by PROCHECK [29]. All of the residues in our 3D model were correctly assigned in the allowed regions of the Ramachandran plot, except for H225 of the inactive form of *Cyt2Ca1*, and L40, S90 and D198 of the active form of *Cyt2Ca1*, all are located in the non-allowed region of the plot. The model was evaluated by ANOLEA [30] indicating that only 4 amino acid residues from over 450 residues of the inactive dimer and 3 amino acid residues from over 205

in the active monomer, exhibited energy higher than the allowable threshold value. These amino acid residues are mainly located in the loop regions that connect α -helices and β -sheets. The calculated QMEAN6 score of the models is 0.56 and 0.50 for the inactive dimer and the active monomer forms, respectively [31,32]. UCSF Chimera [33] was used to superpose the active form of Cyt2Ca1, Cyt1Aa *Bti* insecticidal proteins (PDB code 3RON) [17], and the fungal VVA2 volvatoxin (PDB code 1PPO) [34] (Fig. 6C). The electrostatic potentials of the models were estimated and mapped on the molecular surfaces with PyMol (<http://www.delanoscientific.com>).

2.8. SDS PAGE and Western blot analyses

Protein was extracted from the roots and leaves of transgenic citrus [35, 36], and protein quantity determined using a Qubit assay kit (Life Technologies, Grans Island, NY). The extracted protein (20 μ g) was separated by gradient SDS-PAGE (4-12%) on NuPAGE® Novex® Bis-Tris protein gels using a XCell SureLock® Mini-Cell (Life Technologies) and the gel stained with Coomassie brilliant blue R250. For Western blot the SDS PAGE resolved proteins were transferred onto a nitrocellulose membrane with the XCell II™ Blot Module (Life Technologies) and Cyt2Ca1 was detected using chicken raised polyclonal antibody using the Pierce ECL Plus Western Blotting system (Thermo Scientific, Rockford, IL) followed by incubation with rabbit anti chicken IgG-horse radish peroxidase, and visualized by adding a chemiluminescence reagent (Perkin Elmer) and developed in a Kodak Digital Science™ Image Station 440CF (IS440CF).

3. Results

3.1. Molecular Transformation and Selection of *cyt2Ca1* Citrus

After *A. tumefaciens* transformations, 75 *cyt2Ca1* citrus plants (batch 1) were identified as possible transformed plants by antibiotics resistance. Using qPCR analysis 31 citrus plants were positively identified by amplification of a *cyt2Ca1* amplicon exhibiting Cqs very close to that detected for the citrus reference gene indicating nuclear integration and by melt analyses showing that the correct fragment was amplified (data not shown). Trees representing these *cyt2Ca1*-positive transgenic citrus plants were then analyzed by RT-qPCR and *cyt2Ca1* transcript levels were determined in the 31 transgenic citrus plants from batch-2. The expression levels of *cyt2Ca1* were normalized with ribosomal RNA (18s rRNA) and the difference between the Cq of 18S RNA and *cyt2Ca1* for each analyzed plant was then plotted allowing visualization of differences in *cyt2Ca1* expression level among plants. This approach allowed us to omit the analysis of a reference gene [37], because the 18S rRNA proportion of the total RNA used in this analysis is sufficient in normalizing *cyt2Ca1* expression by RT-qPCR [38]. Using this approach we selected three groups of transgenic plants (A, B, and C) and tested 2-3 different citrus plants from each group. In Group A, H-3A, H-U2 and H-417 (ΔCq of 3.2, 3.0 and 2.8, respectively), in Group B, M-413 and M-414 (ΔCq of -0.4 and -0.3, respectively) and in Group C, L-422 and L-A3 (ΔCq of -2.8 and -2.8, respectively) (Fig. 1).

3.2 Potted-Plant Bioassays

Above ground plant growth from batch 2 cuttings was much slower in plants of group A that expressed 3.3 and 12-fold higher levels of *cyt2Ca1* transcript in their leaves as compared with plants from group B and C (Table 2 and Fig. 2). These results suggest that very high amounts of *cyt2Ca1* transcript (10^9) in the leaves inhibit normal growth and development whereas plants from batch 2 of group B that on the average produced 3-fold less *cyt2Ca1* transcripts in their leaves (Table 2) grew normally. After a third selection (batch 3) of plant 414 cuttings produced an average of 1.7×10^7 and 2.2×10^6 *cyt2Ca1* transcripts/ng RNA in their leaves and roots, respectively. These plants were highly resistant to feeding damage by larval *D. abbreviatus* when compared with *wt* plants (Figs. 3 and 6). The infested root ratio (IRR) divided by the non-infested root ratio of transgenic citrus of Groups A (H-3A, H-U2 and H-417), B (M-413, M-414) and Group C (L-A3, L-422) shows that the growth of transgenic citrus roots of transgenic citrus tree M-414 was significantly less impacted in the presence of larval *D. abbreviatus* as compared with *wt* control (0.7 and 0.22, respectively) (Fig. 4A) A ratio of 1.0 indicates no impact on root growth in the presence of *D. abbreviatus*. A root damage index (V_{RDI}) assuming that if Cyt2Ca1 stops larval feeding on the roots, the V_{RDI} should be close to zero indicating root resistance to larval feeding damage (Fig. 4B). A large V_{RDI} on the other hand, indicates that the seedlings are highly susceptible to larval attack. This evaluation showed that the damage to the root of the transgenic plant M-414 was significantly less severe than the wild type citrus plant (Figs. 3 and 4B). The V_{RDI} of the transgenic plant M-414 (0.50 ± 0.20) (Fig. 4B) was lower than determined in *wt*

controls (2.53 ± 0.47) but not significantly different from V_{RDI} of transgenic plants M-413 (0.61 ± 0.16) and L-422 (1.06 ± 0.17) from Groups B and C, respectively as compared with transgenic plants from Group A (H-3A, H-U2, H-417) and *wt* control. The V_{RDI} of *wt* citrus (2.53 ± 0.47) is significantly higher than in citrus expressing *cyt2Ca1* in Groups B and C (Fig. 4B). These results show that larval feeding on the roots has a greater impact on wild type citrus than on citrus expressing *cyt2Ca1* in groups B and C.

The mean weight gain of larvae recovered from Citrus expressing *cyt2Ca1* was not significantly different from *wt* Citrus control. Larvae that fed on plant roots from Group A (H-417 and H-U2) or *wt* control citrus gained 1.8-fold more weight than larvae that fed on citrus from Group B (M-414 and M-413) (Fig 5A) that showed low root damage index (Fig. 4B). Larvae infesting the roots of citrus M-414 exhibited significantly higher mortality compared with larvae infesting wild type seedlings (43.2 ± 11.9 and $8.4 \pm 5.1\%$, respectively; $t = 3.617$, $df = 4$, $p = 0.0024$) (Fig. 5B). RT-qPCR analyses of citrus from Group A, B and C show that the transcript levels of *cyt2Ca1* in the roots are between 3.6×10^4 to $6.5 \times 10^6/\text{ng}$ RNA (Fig. 6), indicating that the *cyt2Ca1* transcript level is important in protecting the seedlings against larval *D. abbreviatus*.

3.3. Western blot analysis

Extraction of proteins from *cyt2Ca1* transgenic citrus leaves and roots detected a protein band on SDS PAGE that migrated with the same expected kDa of Cyt2Ca1 standard (Fig. 7A,C, respectively). Western blot analysis of the putative Cyt2Ca1 protein band using poly clonal antibodies raised in chickens

436 identified the protein as Cyt2Ca1 in Groups A, B and C of the transgenic citrus.

437 On the other hand, when the leaves and roots of control (*wt*) were extracted, and
438 separately resolved by SDS PAGE and analyzed by Western blot, Cyt2Ca1 was
439 not found (Fig. 7B, D, respectively). These results indicate that only transgenic
440 citrus synthesize Cyt2Ca1 in their leaves and roots.

441 3.4. Molecular Modeling of Cyt2Ca1

442 Cyt2Ca1 is a cytolytic *Bt* insecticidal protein, that is synthesized as an
443 inactive monomer that dimerizes by non-covalent association of the N-terminal
444 intertwining strands and the C-terminal ends (Fig. 8A). The dimer form is
445 activated by proteolysis at D15 and F214 (Fig. 9), removing the N- and C-
446 terminal ends of the monomeric dimers and liberating two active Cyt2Ca1
447 monomers. The active Cyt2Ca1 monomer exhibits α/β organization, made of 6
448 central strands of antiparallel β -sheets associated with 7 peripheral stretches of
449 α -helix (Fig. 8B) found in all of the cytolytic *Bt* insecticidal proteins. Cyt2Ca1 was
450 superposed on Cyt1Aa δ -endotoxin and the fungal volvatoxin VVA2 from
451 *Volvariella volvacea* [34] showing similar 3D folding (Fig. 8C). Several
452 hydrophobic residues (W121, I122, Y134, Y136, I138, F140, I142, A152, L154,
453 I156, F158, Y180, V182, and V184) (Fig. 8D, 9) jut out into the groove located
454 between the bundle of β -sheets and helices α 1 and α 2, and were identified in the
455 Cyt2Ca1 three-dimensional model as lipid-binding site allowing the anchorage of
456 the Cyt2Ca1 monomer to the plasma membrane of the larval midgut epithelial
457 cells (Fig. 8D). In addition, the key residues for the toxicity of Cyt1Aa are nicely
458 conserved in Cyt2Ca1 (E12, R41, K80, E118, K125, E126, K165, E166, D175,

N187, and D202) except for K203 and K225 in Cyt1Aa that have been replaced by E118 and N187 in Cyt2Ca1 (Fig. 9) [17]. In spite of similar folding and amino acids conservation, Cyt1Aa and Cyt2Ca1 exhibit a different electrostatic potentials mapping at their surfaces (Fig. 8E, F, respectively) indicating that different electrostatic distribution of charges play a role in binding of the toxin to the gut's plasma membrane. A homology modeling of Cyt2Ca1 was reported by Zhao et al. [39], however, these authors described the monomeric activated form of Cyt2Ca1 toxin and failed to describe the dimer form that is the inherent conformation of the toxin that is synthesized by *Bti* and by the roots and leaves of our transgenic citrus prior to activation in the insect's gut by proteolysis. This report describes, for the first time, a molecular modeling of the pre-activated dimer form of Cyt2Ca1.

4.0 Discussion

Genetic transformation for citrus scion and rootstock breeding has been reported in Spain, USA and Brazil [40-42]. Transgenic citrus plants have been produced with genes that can modify the level of resistance to *Citrus tristeza* virus [43], *Citrus psorosis* virus [44] and *Phytophthora* spp. [45]. A reduction in citrus canker susceptibility of Hamlin sweet orange to *X. axonopodis* pv. *citri* through the use of citrus transgenic technology was also reported by expressing the *attacin* A [46] and the rice *Xa21* gene to protect citrus against citrus canker [47]. Although transformations to develop transgenic plants are not always successful [48], we report that an increase in resistance to *D. abbreviatus* was achieved through transgenic expression of the *Bt* insecticidal toxin *cyt2Ca1* in the

482 roots of transgenic citrus (Fig. 6). We also show that plant growth partitioning
483 between the root and the scion appears to be altered by expression of this gene
484 and the level of disruption is related to the expression level of *cyt2Ca1* in the
485 seedlings. Thirty-one transgenic plants were divided into 3 groups (A, B and C)
486 expressing *cyt2Ca1* transcripts using quantitative cycle (Cq) data obtained by
487 RT-qPCR (Fig. 1). Using this approach, and uniformly growing transgenic citrus
488 we selected suitable transgenic plants to test for root damage and resistance
489 against larval *D. abbreviatus*.

490 Expressing *cyt2Ca1* in transgenic citrus allows better root growth in the
491 presence of *D. abbreviatus*, as indicated by calculating the V_{IRR}/V_{URR} (infested
492 root ratio/unifested root ratio) (Fig. 4A). One of the transgenic citrus (M-414 from
493 Group B) showed a significant higher ratio as compared with *wt* and, as well as,
494 high *cyt2Ca1* expression in the roots and leaves that did not retard canopy
495 growth (Fig. 6). The level of *cyt2Ca1* expressed in the leaves is important.
496 Young citrus cuttings from group A expressing very high amounts of *cyt2Ca1*
497 transcript in the leaves developed slower (Table 2 and Fig.2). Even though the
498 mechanism that causes this phenomenon is unknown, similar observation has
499 been reported by Manasherob et al. [49] these authors showed that *cyt1Aa*
500 expressed in *E. coli* killed the host bacterium. When *cyt1Aa* was expressed
501 together with the accessory protein P20 it protected the *E. coli* cells by
502 preventing the activation of the Cyt1Aa toxin by proteolysis in the bacterium
503 cells. Based on these observations we would like to suggest that very high
504 expression of *cyt2Ca1* in the leaves in Group A after the initial screening of the

transgenic plants (Table 2) caused activation of the Cyt2Ca1 dimers (Fig. 8A), by proteolysis, and the activated monomers bound the lipid moieties of the plant cell membranes causing pore formations and affected plant's growth (Fig. 1). RT-qPCR analyses of leaves and roots of transgenic citrus trees from Groups A, B and C (417, 414 and 422, respectively) show that citrus trees belonging to Group A synthesize similar amounts of *cyt2Ca1* molecules/ng RNA in the roots and leaves (10^5 to 2.9×10^5), whereas groups B and C transgenic citrus synthesize higher levels of Cyt2Ca1 molecules/ng RNA in the leaves (2×10^6 to 4×10^7) and lower in the roots (3×10^4 to 6×10^6) except for Group B citrus (414-11) that synthesizes similar amounts of Cyt2Ca1 molecules in both the leaves and roots (7×10^6 and 6×10^6 , respectively) (Fig. 6). Western blot analyses of plants from Groups A, B and C show that Cyt2Ca1 is expressed in the leaves and roots (Fig. 7B, D) except for citrus plant 3AE-4 from group A that synthesized very low amounts of Cyt2Ca1 Fig. 7 D. The plants that were screened after the initial transformation in group A synthesized 10^9 *cyt2Ca1* transcripts/ng RNA (Table 2). However, most of these trees did not survive. The trees that survived and were propagated in our green house from groups A, B and C did not synthesize more than 4×10^7 *cyt2Ca1* transcripts/ng RNA (Fig. 6). Indeed, genetic engineering of plants has been reported to cause gene silencing, plant toxicity by foreign proteins with serious pleiotropic effects, altered growth and development of rice plants, and inhibited photosynthesis and root growth of transgenic tobacco plants [50-52]. In this study we used a *Cauliflower mosaic virus* (CMV) promoter 35S that is not tissue specific and drives genes constitutively throughout our

transgenic citrus plants causing inhibition of canopy development at high transcript levels of *cyt2Ca1* transcripts (Fig. 1, Table 2). To avoid a toxic effect on canopy growth, in the future, root specific promoters such as the SLERO promoter that targets the transgene to the mature root cortex and RB7 promoter of tobacco that has been used to drive the *Arabidopsis thionin* (*Thi2.1*) gene in tomato roots [53] should be used.

We observed a significant difference between the root growth of Groups B citrus plant 414 as compared to plant A and B and *wt* control in the presence and absence of *D. abbreviatus* larvae (Fig. 4A). We compared the volume of each plant grown in the presence of infecting larvae and normalized it with the volume of a similar plant grown in the absence of infecting larvae so the differences in the initial root volumes between groups will not affect the results (Fig. 4A). Our calculated root damage index (RDI) accounts for the intrinsic differences in root growth ratios that occurred among the different transgenic lines by normalizing the impact of feeding damage to roots and identifying transgenic plants from Groups B and C (M-413 , M-414 and L-422) that sustained significant lower root damage (Figs.3 and 4B). Significant larval mortality of *D. abbreviatus* and a low RDI are two important characteristics that show an increase in resistance to *D. abbreviatus* and are shared by the transgenic citrus line M-414 from Group B that also synthesizes high amounts of *cyt2Ca1* transcript/ng RNA (Fig. 5B, 6). Our results, thus, suggest that perhaps non-specific membrane interacting function of Cyt proteins as was reported for *E. coli* (Manasherob et al. 2001) is the reason for the observed effect on canopy growth.

Compared with crystalline (*Bt*) proteins, the cytolytic (Cyt) proteins are not recognized by specific plasma gut membrane receptors but interact directly with membrane lipids in the midgut epithelial cells of susceptible insects [27,54,55]. Our 3D model shows that hydrophobic residues jut out from a groove located between the bundle of β -sheets and helices $\alpha 1$ and $\alpha 2$, were identified as the lipid-binding site allowing the anchorage of the active Cyt1Aa monomer to the plasma membrane of the midgut epithelial cells in susceptible insects [17]. These hydrophobic residues are conserved or were replaced by equivalent hydrophobic residues (in bold letters) in the Cyt2Ca1 three-dimensional model, namely W121, I122, Y134, Y136, **I138**, F140, I142, **A152**, **L154**, **I156**, F158, Y180, V182, and **V184** (Figs. 8D, 9). Manceva et al. [12] reported that electrostatic forces play an important role in the Cyt1Aa – membrane interactions. These authors identified the amino acids that interact with the lipid head groups by mutagenesis showing that when the charged amino acids were replaced with neutral Ala residues the toxin lost its activity. Cyt2Ca1 as compared with Cyt1Aa is likewise charged (Fig. 8 E, F) and probably also interacts with the cell membrane lipid head groups using the charged K, D, R, and E (Fig. 9) allowing the toxin to bind to the cell membrane surface. After binding, Cyt2Ca1 changes its conformation exposing the hydrophobic amino acids that play an important role in the binding (Figs. 8D, 9) by hydrophobicity and van der Waals forces making the toxin lipid binding irreversible and thus, facilitating pore formation as was shown for Cyt1Aa [12]. It is conceivable that proteases that process the Cyt2Ca1 into a functional toxin are present at higher

levels in the plant scion or that the scion membranes are more susceptible than the root membranes. Further studies will have to carefully determine the amount of Cyt2Ca1 that is produced by these transgenic trees to find out the maximal amount of the synthesized toxin that does not cause damage to citrus and also effective in controlling larval *D. abbreviatus*.

Larval mortalities in this study never reached the high level (80.4%) that was reported by Weathersbee et al. [15] after feeding *D. abbreviatus* neonates lyophilized *Bacillus thuringiensis* isolate expressing Cyt2Ca (300 µg/ml). However, the plant with the least root damage (M414) expressing between 4×10^4 to 6×10^6 molecules of *cyt2Ca1*/ng RNA (Fig. 6) caused 2-fold lower mortality (43.2 %) to larval *D. abbreviatus* than what was reported earlier [15]. Since our larvae consumed transgenic roots from a plant it is very unlikely that the amount of Cyt2Ca1 expressed by the roots and consumed by the larvae is as high as the lyophilized Cyt2Ca1 powder that was fed to the larvae by Weathersbee et al. [15]. Several reports indicate that it is possible to enhance the potency of Cyt toxins by synergism with Cry toxins [14,56,57]. Thus, expressing several Cry toxins such as CryET33 and CryET43 [15] in citrus roots may enhance the insecticidal activity of Cyt2Ca1. To enhance the toxicity of Cyt2Aa against hemipterans Chougule et al. [58] inserted a 12 amino acid peptide into one of the 3 loops of Cyt2Aa that specifically binds the pea aphid gut and thus enhanced the insecticidal effect of Cyt2Aa against the pea aphid, *Acyrtosiphon pisum* Harris, and the green peach aphid, *Myzus persicae* Sulzer. Likewise, similar strategies could be employed to enhance the toxicity of Cyt2Ca1 in

transformed citrus roots using Trypsin Modulating Oostatic Factor (TMOF) that affects trypsin biosynthesis in the gut of *Diaprepes abbreviatus* [59]. TMOF could be expressed together with Cyt2Ca1 as was done with Cry4Aa to control *Ae. aegypti* larvae [60]. The transgenic citrus plant M-414 is an excellent candidate for further genetic manipulations and adding other candidate genes that affect *D. abbreviatus* with different mode of actions may enhance the toxic effect of Cyt2Ca1.

Acknowledgements

This work has been supported by TSTAR USDA and Florida Citrus Advanced Technology Program grants to D.B., C.P and R.S. One of us (D.B.) is an established scientist fellow at the Oak Ridge Institute for Science and Education (ORISE). This research was supported in part by an appointment to the Agricultural Research Service (ARS) Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture (USDA). ORISE is managed by ORAU under DOE contract number DE-AC05-06OR23100. All opinions expressed in this paper are the author's and do not necessarily reflect the policies and views of USDA, ARS, DOE, or ORAU/ORISE.

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Tables

Table 1. Primers used for qPCR and RT-qPCR

Name	Sequence (5'- 3')	T _m (melting temperature) °C
BtCyt-f	ATGTTCTTCAACCGCGTTATC	54.0
BtCyt-r	TTAGCTGTTGCTGCAGATTTT	54.9
CitCD-f	TGAGTACGAGCCGAGTGTTG	56.7
CitCD-r	CTGGTGGATCGGTGAAGTTT	55.2
Cit18s-f	AAACGGCTACCACATCCAAG	55.0
Cit18s-r	CCTCCAATGGATCCTCGTTA	53.2

Table 2. *cyt2Ca1* transcripts synthesized by the leaves of transgenic citrus

Group	Transgenic citrus trees (N)	<i>cyt2Ca1</i> transcripts/ng RNA x 10 ⁷ (Average ± SEM)
A	11	100 ± 8
B	8	30 ± 7
C	10	8 ± 2

Leaves from groups (A, B and C) of citrus trees that were transformed with *cyt2Ca1* were analyzed by RT-qPCR and the number of *cyt2ca1* transcripts/ng RNA in each group were determined. Results are expressed as means of 8-11 determinations ± SEM.

Figure Legends

Fig. 1. Relative levels of *cyt2CA1* transcripts in transgenic citrus plants normalized with 18S RNA. Transgenic citrus were categorized into Three groups: A ($\Delta Cq \geq 1$; green bars), B ($1 > \Delta Cq > -1$; yellow bars), and C ($\Delta Cq \leq -1$; red bars) used in the potted plant bioassays. Transgenic trees represented by the blue bars were not used.

Fig. 2. Effect of *cyt2Ca1* transcript levels on citrus growth in the 3 groups tested. Plants that belong to Group A were severely affected by expression of *Cyt2Ca1* in their leaves, whereas plants in Group B and C were not affected

Fig. 3. Resistance of *cyt2Ca1* transgenic citrus roots to larval *D. abbreviatus* damage. (A). *cyt2Ca1* transgenic citrus plants M-414, (B). *wt* control citrus plants with a root mass excised (arrow) showing damage caused by larval *D. abbreviatus*.

Fig. 4. Effect of larval *D. abbreviatus* feeding on *cyt2Ca1* citrus plants from Groups A, B and C (H-3A, H-U2, H-417, M-413, M-414 and L-A3, L-422), respectively as compared with feeding on wild type (WT) citrus control plants. Six larvae (3-week old) were allowed to feed on citrus roots for 28 days (5 plants each). Controls were run with transgenic and WT citrus were grown for 28 days without larvae. Roots were analyzed for change in root volume (A) in infested (IF) and not infested (NIF) roots and the infested root ratio (IRR) was compared with the uninfested root ratio (URR) in individual transgenic citrus in Groups A, B and C and wild type (WT) control. (C). Root damage index (RDI) after comparing infested and uninfested potted plants. *Significantly different to WT control ($p < 0.05$) by one-way ANOVA and Dunnett's multiple comparison test.

Fig. 5. Effect of root feeding of *cyt2Ca1* transgenic citrus on larval *D. abbreviatus* on (A) weight, and (B) mortality using potted-plant bioassays. * Significantly different from WT control ($t = 3.617$, $df = 4$, $P = 0.0024$) by 2-tailed, one sample t test.

Fig. 6. Determination of *cyt2Ca1* transcript molecules/ng RNA in the roots and leaves of group A, B and C trees using RT-qPCR. Each determination is the average of two determinations with 3-5% variations between each determination.

Fig. 7. SDS PAGE and Western Blot analyses of transgenic citrus roots expressing Cyt2Ca1. Leaves and roots of transgenic citrus plants (Groups A, B and C) were extracted and the proteins separated by SDS PAGE (A, C leaves and roots, respectively), stained with Coomassie brilliant blue and analyzed by Western blot analyses (B ,D leaves and roots, respectively) using Cyt2Ca1 polyclonal antibodies. Cyt2Ca1 (1.5 μ g) was run as a positive control. The SDS PAGE of *w/d* proteins is not shown. The analysis was repeated 2 times with the same results.

Fig. 8. . A. Ribbon presentation of the inactive Cyt2Ca1 dimer (A). The Cyt2Ca1 monomers are colored olive green and red, respectively including the N- and C-terminal ends. (B). Three-dimensional folding of the active Cyt2Ca1 monomer. Strands of β -sheet ($\beta 1$ - $\beta 6$) and stretches of α -helix ($\alpha 1$ - $\alpha 6$), are numbered from the N- to the C-terminal ends and lack the N- and C-terminal coils found in the inactivated dimer. (C). Structural overlay of Cyt2Ca1 (red), Cyt1Aa (magenta), and VVA2 (green), showing a good overlapping alignment of the β -sheets (best) and α -helices. (D). An open contour plane of the active Cyt2Ca1 monomer showing the hydrophobic residues (yellow sticks) that form the lipid-binding groove of the cytolytic δ -endotoxin residing between the

central bundle of the β -sheets and the α -helices $\alpha 1$ and $\alpha 2$. Most of these hydrophobic residues are found on the central β -sheets bundle. Surface electrostatic potential maps of Cyt2Ca1 (E) and Cyt1Aa (F). Electropositive and electronegative areas are colored blue and red, respectively and neutral surfaces are colored white.

Fig. 9. Structural organization of Cyt2Ca1 inactive monomer. The N- and C-terminal ends (bold letters) of each inactive monomer in the Cyt2Ca1 dimer are cleaved by proteolytic enzymes (arrows). The active Cyt2Ca1 monomer is made up of β -sheets and α -helices (underlined) that are numbered $\beta 1$ - $\beta 6$, and $\alpha 1$ - $\alpha 7$, respectively. Hydrophobic residues forming the lipid-binding groove (gray boxes) and charged residues involved in the initial binding of Cyt2Ca1 to the lipid head groups are also shown (black boxes).

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