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ORIGINAL ARTICLE

Transgenic soybean expressing Cry1Ab-Vip3A fusion protein confers broad-spectrum resistance to lepidopteran pest

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Abstract

Key message **Transgenic soybean event CAL-16 expressing fusion *Bt* protein Cry1Ab-Vip3A was developed for conferring broad-spectrum resistance to lepidopteran pests**

Abstract Lepidopteran insect species are important soybean pests causing significant yield loss and quality degradation worldwide. Transgenic soybeans expressing crystal (Cry) insecticidal proteins have been utilized for insect pest management. However, the efficacy of currently adopted insect-resistant soybean is challenged by insect resistance evolution. Vegetative insecticidal proteins (Vips) are highly active against a broad spectrum of lepidopteran insects. They differ from Cry in modes of action, and show great potential for lepidopteran pest management. Here, we report the creation and characterization of a transgenic soybean event CAL-16 which expresses a fusion protein of Cry1Ab and Vip3A. CAL-16 is a single copy T-DNA insertion transgenic event highly resistant to a broad-spectrum of lepidopteran insects. Insect bioassays demonstrated that CAL-16 caused 100% mortality to neonates of *Helicoverpa armigera*, *Spodoptera litura*, *Agrotis ipsilon*, *Spodoptera exigua* and *Spodoptera frugiperda*. Field trial also demonstrated its excellent resistance to *Leguminivora glycinivorella*, a severe pest feeding on soybean seeds. The expression of the fusion protein was found to be constitutively high in CAL-16 throughout developmental stages, and highly stable over 12 generations. Moreover, there was no statistical difference in agronomic traits between CAL-16 and its non-transgenic recipient control plants in field trial. In conclusion, CAL-16 is an elite soybean event with high efficacy toward major lepidopteran pests. It is expected to be released for commercial cultivation in the near future in China as it has been deregulated in China in 2023.

Keywords Transgenic soybean · Insect resistance · Cry1Ab · Vip3A · Fusion protein

Introduction

Soybean (*Glycine max L.*) is a major protein and oil source for food and feed, and considered one of the most important crops in the world. Lepidopteran pests are the major pests of soybean. (Osaki et al. 2021; Musser et al. 2022). In China, where soybean originated and is vastly cultivated, insects, such as *Spodoptera litura*, *Spodoptera exigua*, *Agrotis ipsilon*, *Helicoverpa armigera, Spodoptera frugiperda* and *Leguminivora glycinivorella* are considered as the major lepidopteran soybean pests (Yu et al. 2013; Gao et al. 2018; Wang et al. 2020). The insecticidal crystal proteins, produced by *Bacillus thuringiensis* during sporulation (Bulla et al. 1980) have been extensively expressed in transgenic insect-resistant (IR) crops for lepidopteran pest control with great success. In soybean, transgenic event MON87701 expressing Cry1Ac was first developed for control of soybean lepidopteran insects. The insect-resistant trait of

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MON87701 was stacked with herbicide tolerant trait of event MON89788. This stacked soybean event is highly resistant to *Anticarsia gemmatalis*, *Heliothis virescens*, *Helicoverpa. armigera*, and *Pseudoplusia includens* (Bernardi et al 2014a, 2012; Dourado et al. 2016; Yano et al. 2016), but has only limited activity against *S. litura, S. exigua and S. frugiperda*, and almost no activity against *A. ipsilon* (Yu et al. 2013; Bernardi et al. 2014b). Moreover, Cry1Ac resistant insect pests were reported to be widely distributed as transgenic *Bt*-Cry1Ac crops were heavily planted since 1996 (Singh et al. 2021; Santiago González et al. 2022; Guan et al. 2023).

More recently, transgenic soybeans expressing two *Bt* crystal toxins were developed for enhanced insect resistance and better management of resistance development of target pests. DAS-81419–2 developed by Dow AgroSciences, expressing both Cry1Ac and Cry1F, is highly active to *A. gemmatalis*, *C. includes*, *H. virescens*, *S. cosmioides*, *E. lignosellus* and *H. armigera*, but has only moderate activity to *A. ipsilon* (Marques et al. 2016, 2017). However, studies have revealed that Cry1F and Cry1Ac share a common receptor in multiple species, including *H. armigera*, *H. zea* and *S. exigua*, suggesting they may share the same mode of action (Hernández and Ferré 2005). Another transgenic soybean event, MON87751, expressing two crystal proteins Cry1.105 and Cry2Ab, was developed by Monsanto. MON 87701 × MON 89788 × MON 87751 × MON 87708 soybean that expresses Cry1A.105, Cry2Ab2, and Cry1Ac insecticidal proteins caused high mortality of *Spodoptera* species insensitive to Cry1Ac soybean, including S. *cosmioides*, *S. albula*, and *S. eridania* (Bernardi et al. 2014b; Barcellos et al. 2023). However, field-evolved resistance of the corn earworm *H. zea* to Cry1A.105 and Cry2Ab2 proteins has been widely distributed in the southeastern United States (Yu et al. 2021), as these two proteins were expressed in transgenic corn MON89034, which has been widely planted in USA. Therefore, it is valuable to develop IR soybean utilizing insecticidal proteins with different modes of action and a broader spectrum of lepidopteran activity.

Vegetative insecticidal proteins (Vips) are produced by *Bacillus thuringiensis* during its vegetative growth stage (Estruch et al. 1996). Vips show no significant sequence similarity with any crystal proteins and potentially have different modes of action to insect pests (Lee et al. 2003; Gupta et al. 2021). Vips are classified into four families according to the amino acid identity. Among them, members of the Vip3 family display high activity toward a broad spectrum of lepidopteran pests, some of which are less sensitive or insensitive to Cry proteins. Particularly, they are potent to pests of the genera of *Agrotis* and *Spodoptera* (Donovan et al. 2001; Zhu et al. 2019), many of which are important soybean pests. Vip3A has been utilized in transgenic corn and cotton, providing control of a broad spectrum of pests, especially *Spodoptera* pests (Ahmad et al. 2021; Wen et al. 2023). To manage the evolution of pest resistance to *Bt* crops, pyramiding insecticidal genes with different modes of action have been implemented, as the probability of a pest developing resistance to all insecticidal proteins at the same time is extremely rare (Zhao et al. 2003; Tabashnik et al. 2013, 2023). Also, the combination of Cry and Vip may provide a synergistic effect (Chakrabarty et al. 2020).

In this study, we created transgenic soybean expressing a fusion protein of Cry1Ab and Vip3A, both of which have not yet been utilized in transgenic insect-resistant soybean. By expressing the two types of insecticidal proteins in transgenic soybean simultaneously, a broad insecticidal spectrum can be achieved. No Vip3A soybean has been commercially planted so far, and Vip3A differs from Cry proteins used in currently adopted *Bt* soybeans in insecticidal mode of action, so the development of IR soybean expressing fusion protein Cry1Ab-Vip3A will be beneficial for pest resistance management either used alone or stacked with current commercialized IR soybean events. An elite single copy T-DNA insertion event CAL-16 was selected and characterized. CAL-16 demonstrated high resistance to a broad spectrum of important lepidopteran pests and without significant change in agronomic traits. CAL-16 has been awarded with Certificate of Safety in China, and its commercial release in the near future is expected.

Materials and methods

Construction of a binary vector containing the Cry1Ab-Vip3A gene

Binary vector pCAMBIA1300 was modified by replacing the marker gene hygromycin with a plant codon optimized glyphosate-tolerate gene *G10-epsps* originating from *Deinococcus radiodurans*. The fusion protein Cry1Ab-Vip3A was created by fusing the N terminus 1–649 AA of Cry1Ab and 2–789 AA of Vip3A via a 8-AA linker: GGAGGAGG. The fusion gene was codon optimized and synthesized along with nopaline synthase (NOS) gene terminator sequence at 3' end. A *Bam*H I site was introduced at the 5' end and a *Kpn* I site was added to the 3' end of the synthetic sequence. CsVMV promoter was synthesized with *Hin*d III site at 5' end and *Bam*H I site at 3' end. The synthetic gene fragment was digested with *Bam*H I and *Kpn* I, the CsVMV promoter fragment was digested with *Hin*d III and *Bam*H I, and the vector p1300-G10 was digested with *Hin*d III and *Kpn* I, respectively. The digested vector and fragments were ligated and transformed into chemical compotent cell *E. coli* TG1. The resulting binary vector was named pCAL. All the synthesized sequences were synthesized by Sangon Biotech (Shanghai, China).

Soybean transformation

The binary vector pCAL was electroporated into *Agrobacterium tumefaciens* EHA105 by electroporator 2510 (Eppendorf). The soybean variety Tianlong 1, a cultivar adapted to the Yangtze River region in China, was used for the transformation. Before transformation, seeds were surface-sterilized by chlorine gas in a tightly sealed vacuum dryer for 16 h. Cotyledonary explant isolation and medium recipe was followed the method described by (Paz et al. 2006) After cotyledonary explants inoculated with *Agrobacterium* for 30 min, explants were co-cultivated at 25℃ in the dark for 5 days. After co-cultivation, the explants were transferred into the shoot initiation medium containing 25 mg/L glyphosate for selection for 3 weeks. Explants were subsequently transferred to the shoot elongation medium containing 10 mg/L glyphosate. Elongated explants were finally transferred to the rooting medium. Rooted plantlets were transplanted in peat moss soil in the greenhouse at 25 ℃ and 16L:8D photoperiod.

Screening of positive transgenic soybean lines and protein quantification of CAL

All T0 plants were planted and screened by enzyme-linked immunosorbent assay (ELISA). For the selected transgenic line CAL-16, spatiotemporal expression in different tissues among T1, T2, and T3 generations was detected. leaves were sampled at V4, V6, R2, and R5 stages; pods, stems, and root were sampled at the R6 stage; and seeds were sampled at the R8 stage. Thirty-mg plant tissue was ground to powder in a 2.0-ml tube by Tissue Lyser at 45 Hz for 45 s 1 ml PBS was added to each tube to extract soluble proteins. Samples were vigorously vortexed for 15 s and then incubated on ice for 15 min. After centrifugation at 4 ℃ for 15 min and appropriate dilution of the supernatants, ELISA was conducted using the QualiPlate Kit for Cry1Ab (Envirologix, Portland, ME, USA).

Molecular characterization

The junction sequences were amplified by high-efficiency thermal asymmetric interlaced polymerase chain reaction(hiTail-PCR) (Liu and Chen 2007). Genomic DNA was extracted from soybean leaves by CTAB method. The PCR products were sequenced and analyzed by BLAST at SoybeanGBD (https://venyao.xyz/soybeangdb/) (Li et al. 2023). For southern blot analysis, 300-ug genomic DNA of CAL-16 and Tianlong 1 was digested with *Eco*R I and *Kpn* I, respectively. The transformation vector pCAL, digested with *Hind* III, was used as control. Digested DNA fragments were separated by electrophoresis at 30 V for 10 h, then transferred onto the Hybond N + nylon membrane (Amersham, UK). The fragments were fixed by baking the membrane at 120℃ for 30 min. The probe against the whole length of the fusion protein cassette was prepared by PCR amplification, and labeling using DIG High Prime DNA Labeling and Detection Kit II (Roche, Germany). Primers used to amplify the fragments of probes was displayed in Table S1.

Polymerase chain reaction analsyis

Event-specific primers (Table S1) were designed according to the sequence at the junction between the soybean genome and the T-DNA. Gene-specific primers (Table S1) were designed according to the fusion gene Cry1Ab-Vip3A and selection marker gene G10-epsps. (event-specific primer T-DNA-RF / Gm-RR and T-DNA-LF / Gm-LR. Gene-specific primer CAL-ID-F /CAL-ID-R and G10-ID-F / G10-ID-R.)

The gDNA of CAL-16 and Tianlong 1 were used as templates.

The T-DNA inserted along with its genomic flanking sequences was split in to five segments with overlap and PCR amplified with primers as listed in Table S1. (Gm-LR / G10-ID-F, CAL-ID-R3 /CAL-ID-F3, CAL-ID-R2 /CAL-ID-F2, CAL-ID-R1 /CAL-ID-F1, CAL-ID-R /Gm-RR).

Each reaction mixture contained 100 ng of gDNA, 25-μL Taq premix (Takara TaqTM Version 2.0 plus dye Takara, Kusatsu, Japan), 1 μL each of primers (10 μM), and add sterile distilled water up to 50 μL. The PCR procedure was conducted as follows: 1 cycle of 98 ℃ for 3 min, 33 cycles of 98 ℃ for 15 s, 58 ℃ for 15 s, 72 ℃ for 1 min/kb, and a final extension at 72 ℃ for 10 min.

Western blot

Western blot analysis was performed to detect the expression of the fusion protein in T10, T11 and T12 generation of CAL-16. The 2nd upper most trifoliate leaf was sampled at V4 stage. Thirty-mg soybean leaf tissue was ground to powder in a 2.0-ml tube by Tissue Lyser at 45 Hz for 45 s. Total protein was extracted by adding 500-ul PBS buffer to each tube, vigorous vortex. After centrifugation at 12,000 g for 5 min, the supernatant was collected, mixed with 5 × SDS-PAGE loading buffer, and boiled for 10 min. Ten ul of the denatured sample was loaded into each well of 4–20% polyacrylamide gel (GenScript, Nanjing, China), electrophoresed at 200 v for 25 min, and transferred to PVDF membrane. The blotted membrane was blocked, hybridized and detected. Polyclonal antisera, prepared from New Zealand white rabbits which were immunized with purified recombinant Cry1Ab-Vip3A fusion protein from *E. coli*, was used as the primary antibody. HRP conjugated goat anti-rabbit IgG (H + L) antibody (Huabio, HA1031, Hangzhou, China) was used as the secondary antibody. The bands were visualized

by M5 Hiper ECL Western HRP Substrate (Mei5bio, Beijing, China).

Insect bioassays

In the T0 generation, events with various expression levels were selected to evaluate the resistance to *Helicoverpa armigera and Spodoptera litura*. The second uppermost trifoliate leaf of the V4 stage was collected, soybean leaves were punched with a 12-mm diameter hole puncher and lay flat on 24-well tissue culture plates containing 700 µl of 1% agarose. Each event was inoculated with 48 neonates for one species, and each well was inoculated with two neonates. The plates were sealed with Breath-Easy sealing membrane and incubated at 28℃ under 8L:16D photoperiod.

To test the resistance of CAL-16 to major lepidopteran pests, neonates of five lepidopteran species: *Helicoverpa armigera*, *Spodoptera litura*, *Spodoptera exigua*, *Agrotis ipsilon and Spodoptera frugiperda* were tested. Ninety six neonates of each species were inoculated onto both Tianlong 1 and CAL-16. Three days post-inoculation, photos were taken, and mortality was recorded.

Field trial to test resistance to *Leguminivora glycinivorella*

Resistance to *L. glycinivorella* was evaluated in a field trial in 2019 under natural infestation condition in Sanya (18°14′35.02" N 109°30′18.00" E), Hainan Province, China. Non-transgenic Tianlong 1 and CAL-16 were planted in adjacent plots (10 m × 10 m) in triplicate. No insecticide was applied throughout the trial. At maturation, 30 soybean plants were randomly sampled from each plot to count the number of pods and damaged pods per plant. Hundred pods were randomly collected from each plot to count the number of soybean pod borers.

Field agronomic traits measurement

The gemination rate was analyzed by separately planting Tianlong1 and the transgenic line CAL-16 in pots filled with soil media in a greenhouse at Zhejiang University. Hundred seeds were placed in each pot, with four replicates. The number of germinated seedlings were counted 1 week later. The field trial was carried out at Changxing County, Huzhou City, China, with non-transgenic recipient Tianlong 1 planted as control. Three plots each of CAL-16 and Tianlong I were planted in the field, each plot was 12 m^2. Two plants were planted in each hole with 25-cm apart. Ten plants were randomly selected from each plot for agronomic traits test after maturation. Plant height was measured from the cotyledon mark to the apex of the main stem. First pod height was measured from the cotyledon mark to first pod insertion. Branch numbers was assessed by counting all plant branches. The number of nodes on the main stem was determined by counting the number of cotyledon nodes to the apex of the main stem. The number of pods per plant was calculated by counting the total number of viable pods. Hundred seeds weight was obtained by weighing the mass of 100 random seeds. Yield was determined at full maturity of each plot of 12 m^2. Seeds were cleaned and weighed.

Data analysis

The t test analysis was carried out using SPSS version 26.0 software (IBM Corp., NY), values of $P < 0.05$ ware considered significant.; Excel 2016 and PowerPoint 2016 (Microsoft Corp., WA) were used to draw the figures and three-line table.

Results

Generation of transgenic soybean plants

To generate insect-resistant transgenic soybean, a T-DNA was constructed to contain two expression cassettes, one for the *Cry1Ab-Vip3A* expression under the control of pCsVMV promoter, and the other for the expression of *G10-EPSPS* serving as a glyphosate selection marker (Fig. 1). The soybean cultivar "Tianlong 1" was used as the transgene recipient. Seventy-one independent transgenic lines were obtained

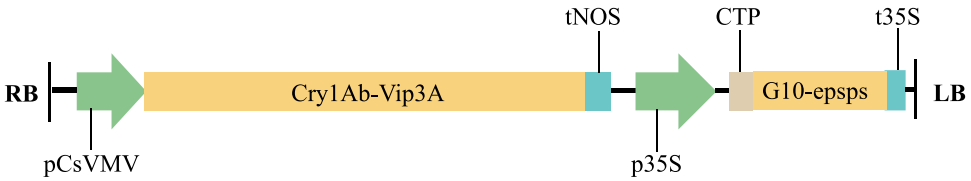

Fig. 1 Diagram of T-DNA for soybean transformation. RB, T-DNA right border; pCsVMV, Cassava vein mosaic virus promoter; Cry1Ab-Vip3A, the fusion *Bt* gene of Cry1Ab and Vip3A; tNOS, *Agrobacterium tumefaciens* nopaline synthase terminator; p35S, CaMV 35S promoter; CTP, chloroplast transit peptide from *Arabidopsis*; *G10-epsps*, glyphosate tolerance gene; t35S, CaMV 35S terminator; LB, T-DNA left border

via *Agrobacterium*-mediated transformation method and subsequently screened by both enzyme-linked immunosorbent assay (ELISA) of Cry1Ab and bioassays with neonates of *S. litura* and *H. armigera*. The concentration of Cry1Ab-Vip3A in leaves of transgenic lines ranged from 0 to 27.54 μg/g fw (fresh weight). The insecticidal activity bioassay results indicated that the events with Cry1Ab-Vip3A expression levels over 20 μg/g fw were highly resistant against both *S. litura* and *H. armigera*, while events with expression levels under 10 μg/g fw showed significantly lower resistance (Fig. S1). The transgenic events with expression levels over 20 μg/g fw were selected for molecular characterization and insect resistance evaluation. The single copy event CAL-16 with high expression was finally selected for regulatory study, including agronomic performance and stability of transgene expression across generations.

Molecular characterization of the T-DNA insertion of CAL-16

To determine the T-DNA copy number of CAL-16, the probe covering the full length of *cry1Ab-vip3A* gene expression cassette was used for southern blot analysis. Genomic DNA of CAL-16 and Tianlong 1 was digested by *Eco*R I and *Kpn* I, respectively, and then hybridized with the probe. One single band was observed for each digestion after hybridization (Fig. 2A), indicating that a single copy of *cry1Ab-vip3A* was inserted into the CAL-16 genome.

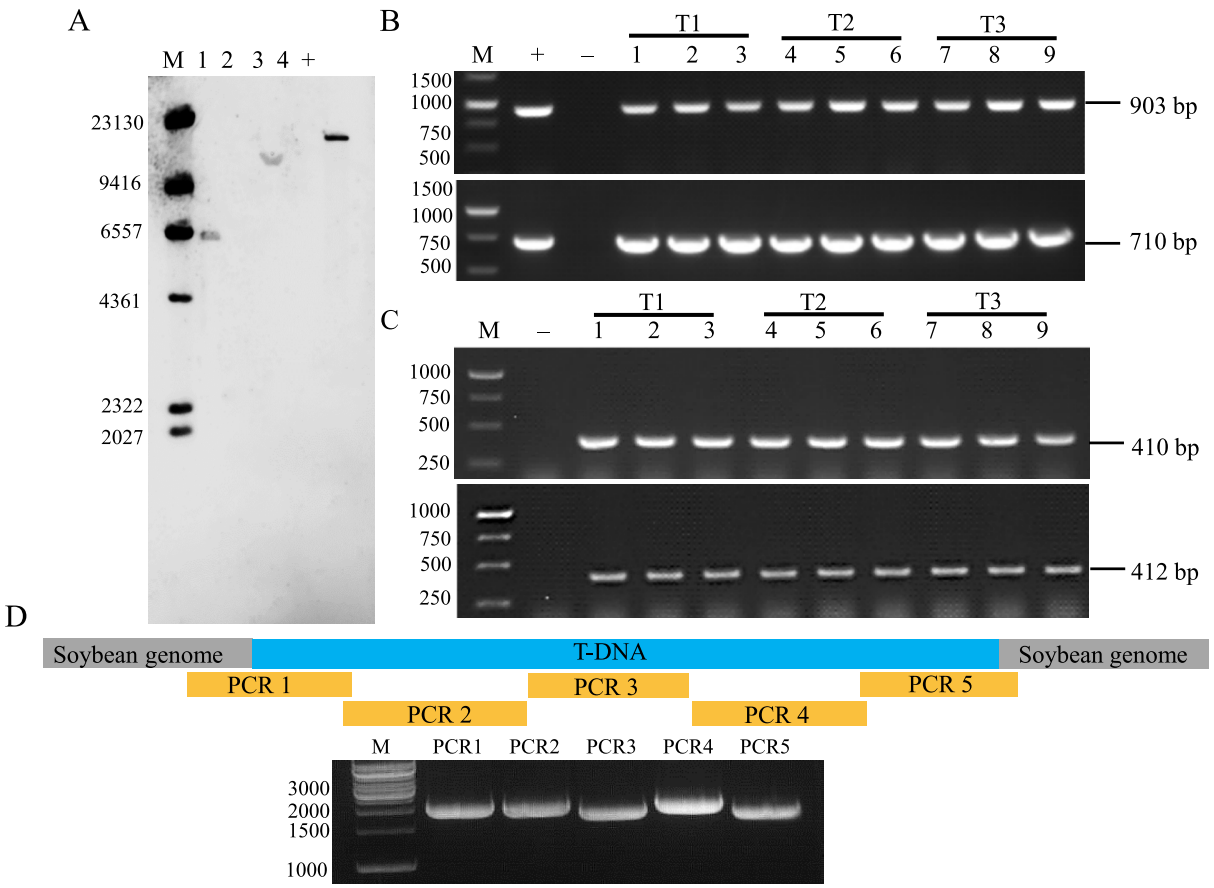

Fig. 2 Southern blot and PCR detection of T-DNA insertion. **A** Southern blot analysis of CAL-16. M, DIG-labeled DNA marker; lane 1, CAL-16 genomic DNA digested with *Eco*R I; lane 2, non-transgenic recipient soybean genomic DNA digested with *Eco*R I; lane 3, CAL-16 genomic DNA digested with *Kpn* I; lane 4, non-transgenic recipient soybean genomic DNA digested with *Kpn* I; lane +, transformation vector digested with *Hin*d III. **B** Gel electrophoresis of PCR products of the inserted genes. Upper, PCR detection of *cry1Ab-vip3*; Lower, PCR detection of *g10-epsps*. M, DNA marker; lane +, transformation vector; lane –, non-transgenic recipient control genomic DNA; lane 1–3, genomic DNA from CAL-16 T1 plants; lane 4–6, genomic DNA from CAL-16 T2 plants; lane 7–9, genomic DNA from CAL-16 T3 plants. **C** PCR detection of the 5'/3' junction between T-DNA and soybean genome. Upper, PCR detection derives from the 5' insertion; Lower, PCR detection derives from the 3' insertion. M, DNA marker; lane +, transformation vector; Lane –, non-transgenic recipient control genomic DNA; lane 1–3, CAL-16 genomic DNA from T1 generation; lane 4–6, CAL-16 genomic DNA from T2 generation; lane 7–9, CAL-16 genomic DNA from T3 generation. **D** Amplification of the inserted T-DNA and its genomic flanking sequences

To determine the T-DNA insertion site in the CAL-16 genome, hiTAIL PCR was used to isolate the genomic flanking sequences of the T-DNA insertion. The amplified genomic flanking sequences of the T-DNA match exactly to the genomic sequence of Tianlong 1 at 13,539,527 bp of Chromosome 18. Event-specific PCR was subsequently established by amplifying the joint sequences of the soybean genome and T-DNA, which further confirmed the accuracy of the determined flanking sequences at both ends of the T-DNA.

The whole inserted T-DNA along with its genomic flanking sequences was split into five overlapping segments and amplified by PCR, respectively (Fig. 2D). T hese PCR products were sequenced and the full-length inserted T-DNA sequence was compiled. There was no sequence difference compared to the T-DNA of the transformation construct.

To further confirm whether the T-DNA was stably integrated in the soybean genome, gene-specific PCR of *cry1Ab-vip3A* and marker gene *g10-epsps*, as well as the event-specific PCR of joints of the left and right border with genomic flanking sequences, were conducted for three different generations of CAL-16. All those PCR successfully amplified the expected products (Fig. 2B, C). Those results strongly suggested that the T-DNA is stably integrated into the genome of CAL-16 as a single intact copy.

Expression analysis of Cry1Ab-Vip3A of CAL-16

The spatial and temporal expression of the fusion protein in CAL-16 plants from different generations were analyzed by ELISA. The results showed that insecticidal protein Cry1Ab-Vip3A was expressed constitutively (Fig. 3A), which is expected as the constitutive Cassava Vein Mosaic virus promoter was used to drive the expression. The concentration of Cry1Ab-Vip3A was highest in leaves, followed by seed, the main feeding part of *L. glycinivorella*. It is noteworthy that the fusion protein concentrations in leaves at different development stages were similar (22.3 ~ 24 μg/g fw), indicating CAL-16 possesses high insect resistance throughout the entire growth stage (Fig. 3A). This result also showed that the expression of Cry1Ab-Vip3A was stable between different generations.

Leaf tissue of T10, T11, T12 generations of CAL-16 was analyzed by western blot. With antiserum against

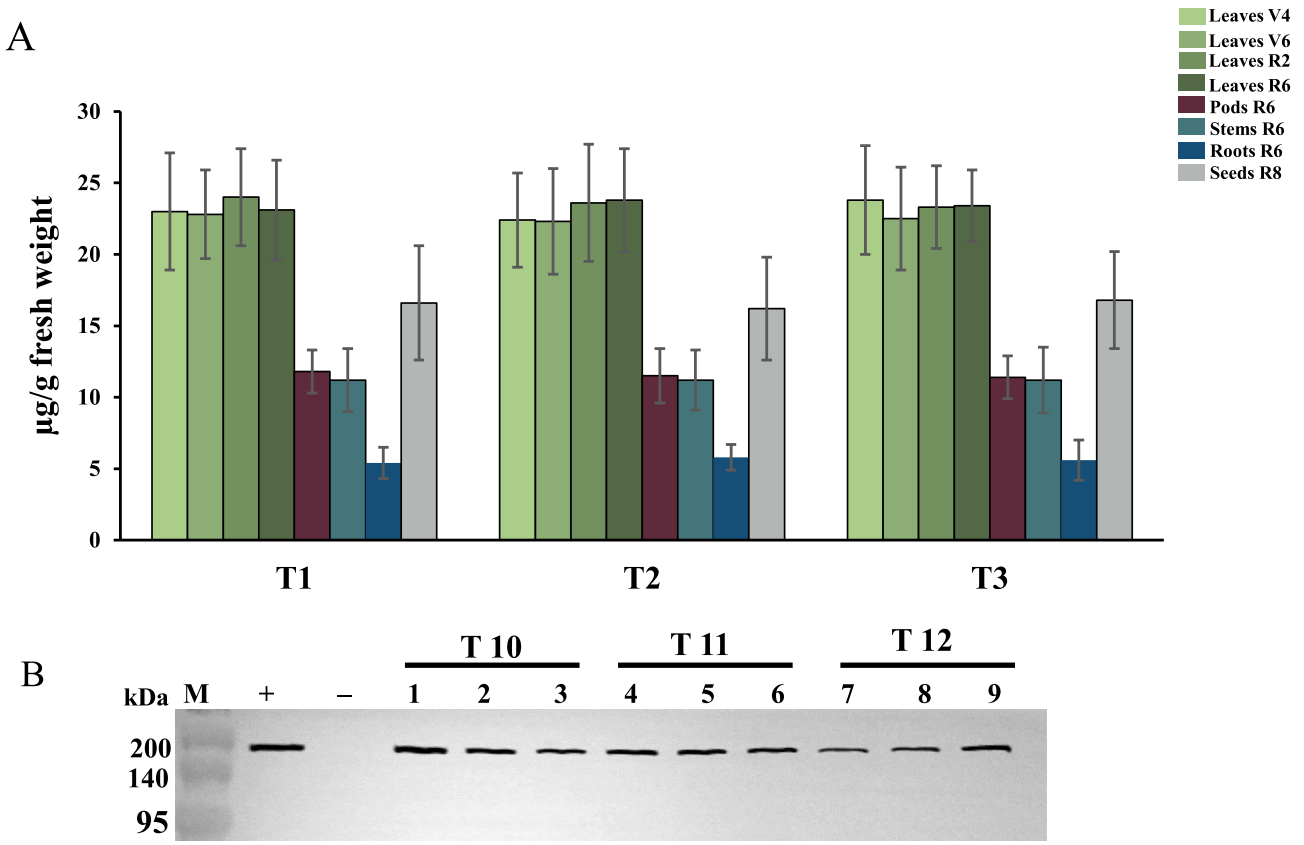

Fig. 3 Analysis of the expression of Cry1Ab-Vip3A in different generations of CAL-16. **A** Quantitative ELISA analysis of Cry1Ab-Vip3A in different tissues of three generations of CAL-16; **B** Western blot analysis of Cry1Ab-Vip3A in generations; M, protein ladder; +, positive control; –, V4 leaf of non-transgenic recipient soybean; lane 1–3, V4 leaf of CAL-16 from T10 generation; lane 4–6, V4 leaf of CAL-16 from T11 generation; lane 7–9, V4 leaf of CAL-16 from T12 generation

Cry1Ab-Vip3A, an expected band of approximately 162 kDa was detected, indicating the Cry1Ab-Vip3A fusion was stably expressed as an intact protein (Fig. 3B).

The above results demonstrated that Cry1Ab-Vip3A was stably expressed in different tissues between generations as an intact fusion protein.

Insect resistance efficacy evaluation of CAL-16

Insect resistance efficacy of CAL-16 against neonates of major soybean lepidopteran pests, *S. litura*, *S. exigua*, *H. armigera*, *A. Ipsilon*, and *S. frugiperda*, was evaluated by leaf disc insect bioassay. The mortality of all five insect species feeding on CAL-16 leaves reached 100% 3 days post-infestation, which was significantly higher than the mortality obseved on non-transgenic soybean leaves. In addition, almost no tissue consumption was observed in the CAL-16 leaf-disc, whereas the non-transgenic line leaf-disc suffered serious tissue consumption among all the five tested pests. (Fig. 4), demonstrating that CAL-16 was highly resistant to *S. litura*, *S. exigua*, *H. armigera*, *A. Ipsilon*, and *S. frugiperda*.

L. glycinivorella, a severe pest infesting soybean seeds, prefers moderate temperature and high humidity environments. Sanya (18°14′35.02" N 109°30′18.00" E), Hainan Province, China, characterized by its tropical marine monsoon climate and all year-round humidity, presents an ideal location for testing the resistance of CAL-16 to *L. glycinivorella* under natural conditions. In March 2019, CAL-16 and no-transginic soybeans were planted at the agricultural experiment station of Zhejiang University in Sanya. No insecticide was applied during the whole growing season. At maturation, serious damage by *L. glycinivorella* was observed in the non-transgenic soybean, whereas no damage was observed on CAL-16 (Fig. 5A). Statistical analysis of the damaged pod proportion per plant revealed that average 92.89% pods of the non-transgenic soybeans were infested by *L. glycinivorella*, meanwhile no bored pods were observed in CAL-16 (Fig. 5B). Approximately 2.77 adult soybean pod borers were found per plant of non-transgenic soybeans, whereas, no soybean pod borers were found on CAL-16 (Fig. 5C). Those results indicate that CAL-16 exhibits very high resistance to *L. glycinivorella*, even in the region with severe soybean pod borer infestations.

Agronomic traits of CAL-16

Agronomic traits of CAL-16 were measured in the growing season of 2019, at Changxing County (30°53′9" N 119°37′57" E), Huzhou City, Zhejiang Province, China, with non-transgenic recipient Tianlong 1 planted as control. Major agronomic traits, including germination rate, plant height, height of the first pod, number of branches, number of nodes on the main stem, pod count per plant, and yield were measured. As shown in Table 1, all the measured agronomic traits of CAL-16 was virtually identical to those of non-transgenic recipient soybean. These results demonstrated that the genetic modification which confers insect resistance, did not exert negative impact on agronomic traits.

Discussion

Pest insect resistance development is a major concern of IR transgenic crops (Zhao et al. 2011; Tabashnik and Carriere 2017). Pyramiding different insecticidal proteins with

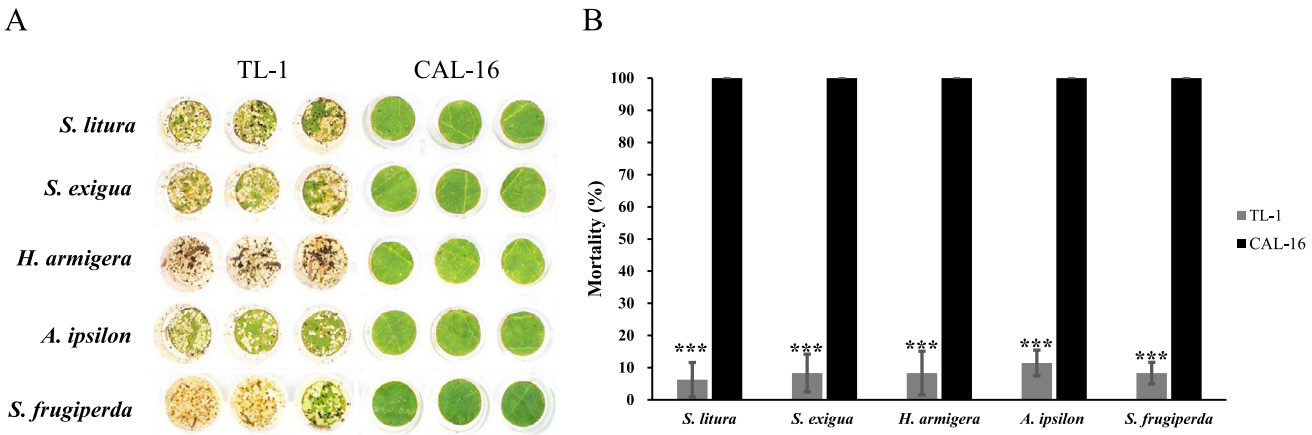

Fig. 4 Leaf disc insect bioassay of five majoy soybean lepidopteran pests. **A** Leaf discs of CAL-16 inoculated with five major soybean lepidopteran pests for 3 days. The second uppermost trifoliate leaf of V4 stage was used for this assay. Each well was inoculated with two neonates. Non-transgenic recipient soybean leaves at the same stage were used as a control. **B** Leaf disc insect bioassay mortality statistic. The mortality was recorded at 3 days after inoculation. Error bars show SE ($n=96$). P values were obtained by Student's two-tailed t test. *** $P<0.001$

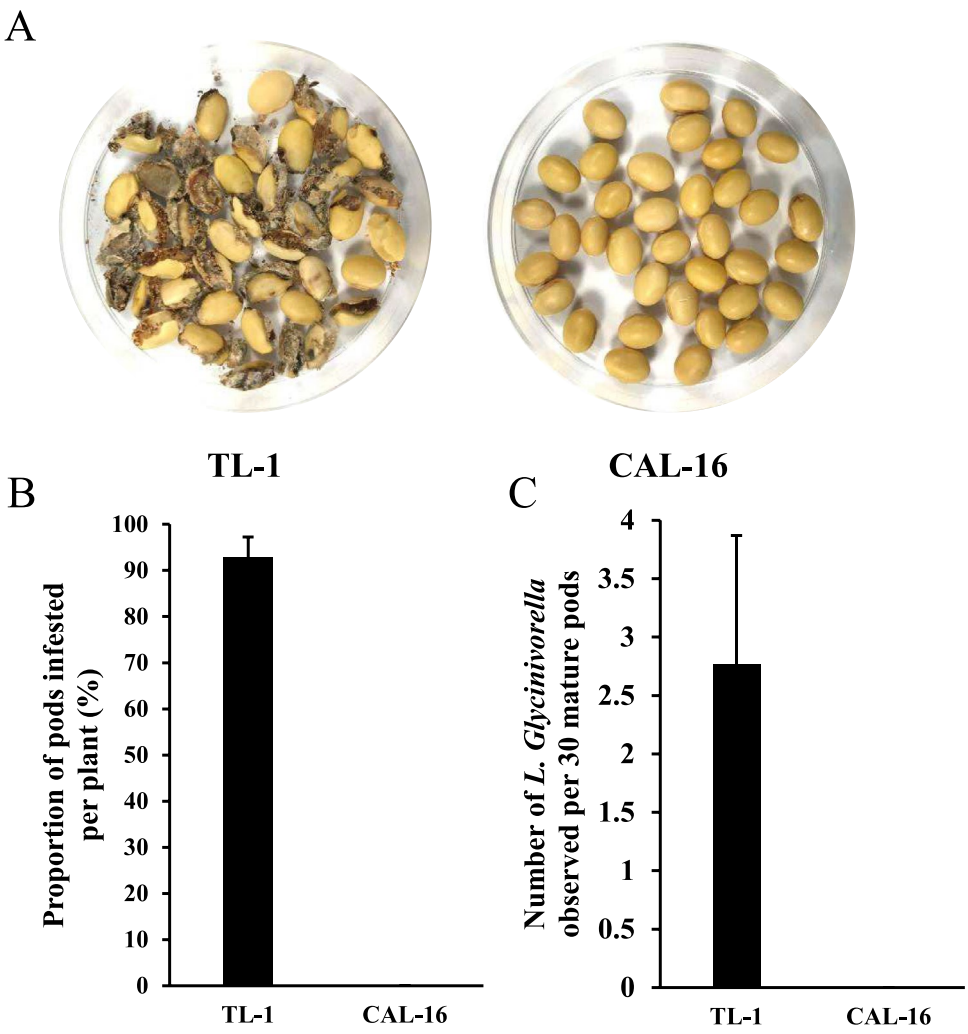

Fig. 5 Field insecticidal activity evaluation of CAL-16 against *L. Glycinivorella*. **A** Seed damage of CAL-16 by *L. Glycinivorella*. The seeds in each dish came from a single plant picked at random. **B** The proportion of pods infested by *L. Glycinivorella* per plant. **C** Number of *L. Glycinivorella* observed per 30 mature pods. CAL-16 and non-transgenic control soybeans were cultivated in Sanya City, Hainan Province, China without the application of insecticides. Error bars show SE ($n = 90$)

Table 1 Agronomic traits comparison of CAL-16 and non-transgenic recipient control

Agronomic trait	TL-1	CAL-16	*P* value
Gemination rate (%)	99.25 ± 0.96	98.75 ± 0.96	0.18
Plant height (cm)	39.60 ± 5.82	38.07 ± 7.21	0.46
First pod height (cm)	10.26 ± 2.08	10.15 ± 1.87	0.85
Number of nodes of the main stem	13.05 ± 0.69	13.20 ± 0.52	0.44
Branch numbers	4.20 ± 0.95	4.35 ± 1.18	0.66
Number of pods per plant	55.15 ± 7.24	56.35 ± 6.15	0.58
Hundred seeds weight (g)	18.5 ± 0.18	18.5 ± 0.16	0.87
Days to maturity (day)	92.0 ± 0.00	92.0 ± 0.00	1.00
Yield (kg ha^{-1})	1879.63 ± 133.88	1920 ± 114.14	0.48

Value represent means ± SE

P values were obtained by Student's two-tailed *t* test

different insecticidal spectra and mechanisms of action has been utilized as an effective strategy for insect resistance management. (Tabashnik et al. 2013; Xiao and Wu 2019). Cry insecticidal protein and Vip3 insecticidal proteins have different insecticidal modes of action and show strong complementary insecticidal profiles (Gupta et al. 2021). In this study, we introduced the fusion protein Cry1Ab-Vip3A into soybean by *Agrobacterium* transformation system. Compared to conventional event staking or molecular stacking of multiple gene expression cassettes into a single T-DNA

(Singh et al. 2016; Zafar et al. 2022), the fusion strategy employed in the present study not only minimizes the construct size, but also ensures the equal expression of the two genes. Cry1Ab-Vip3A was designed by fusing Cry1Ab and Vip3A via an 8- amino acid peptide linker (GGAGGAGG). Previous research has demonstrated that Cry1Ab-Vip3A will generate active trypsin-resistant Cry1Ab core and trypsin-resistant Vip3A core upon activation by trypsin, or insect mid gut juice (Xu et al. 2018), suggesting that the fusion protein had equivalent activity of combined individual Cry1Ab and Vip3A toxins in insect midgut. In this study, we showed Cry1Ab-Vip3A was expressed as an intact fusion in transgenic soybean. It is evident that the fusion protein was correctly processed into individual toxins in target insect midgut, since high insecticidal efficacy was observed among the transgenic soybean events.

The insertion site and sequence of an exogenous gene can potentially lead to unstable expression of the exogenous gene (Van Blokland et al. 1994; Matzke et al. 1994). For a commercially viable transgenic event, it is essential that transgenes were expressed stably across different generations for maintaining the corresponding traits. For CAL-16, the insecticidal activity assays indicated that its insect resistance efficacy has not changed significantly up to T12 generation. This was further validated by no detection of significant changes in the fusion protein expression level among plants of different generations by ELISA. This study also found that the fusion protein was constitutively expressed in different tissues and at different growth stages, which is consistent with our observation that CAL-16 provides high protection for the whole plants and throughout the growth stage.

Over-expression of *Bt* insecticidal protein may lead to changes in agronomic traits, such as stunting (Chakraborty et al. 2016). To study the effect of the fusion protein on the agronomic traits of soybean, field trial was conducted by planting CAL-16 and its recipient soybean. No statistically significant difference was found in key agronomic traits between CAL-16 and WT lines, including gemination rate, plant height, number of pods per plant and yield. The field trial demonstrated that the genetic modification did not negatively impact the agronomic traits, which is prerequisite for the commercial cultivation of CAL-16.

Currently, the commercial cultivation of *Bt* transgenic soybeans mainly involves the expression of Cry proteins. The Cry proteins currently utilized are highly effective against certain primary pests, but not all lepidopteran insects. The non-target pests which are insensitive to these Cry proteins could emerge as major pests (Wan et al. 2008). Vip3A proteins show different insecticidal mode of action and are complementary to Cry proteins in insecticidal activity profiles. Particularly, Vip3 displays high activity toward the important soybean pest genera of *Agrotis* and *Spodoptera*, which are poorly controlled by Cry proteins and cause serious damage to soybean (Donovan et al. 2001; Zhu et al. 2019). By expressing two types of insecticidal proteins with different modes of action and spectrums, CAL-16 confers resistance to much more broad spectra of lepidopteran pests and may also help to delay the resistance development of target insects. CAL-16 has the potential to become a leading event of new generation of IR soybeans for commercially planting due to its broad-spectrum resistance. To further validate the high efficacy of CAL-16, soybean pests of other major soybean planting countries and pests with resistance to Cry proteins may be used for subsequent tests.

Conclusion

The transgenic soybean event CAL-16 expressing a fusion of two *Bt* insecticidal proteins with different modes of action is highly resistant to major soybean lepidopteran pests, including *H. armigera*, *S. litura*, *A. ipsilon*, *S. exigua*, *S. frugiperda*, and *L. glycinivorella*. Vip3A has not been utilized among the transgenic soybean events commercially released so far, thus CAL-16 is quite compatible with the current products for management of pest insect resistance development.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information file.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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