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LETTER TO THE EDITOR

# Development of insect-resistant transgenic cotton with chimeric TVip3A* accumulating in chloroplasts

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**Abstract** An optimized *vip3A* gene, designated as *vip3A** was chemically synthesized and a *thi1* gene chloroplast transit peptide coding sequence was attached to its 5′ end to produce the *tvip3A**. *vip3A** and *tvip3A** genes were transformed into *Gossypium hirsutum* cv. Zhongmiansuo35. Of 42 independent transformants, 36 were positive for the *vip3A** or *tvip3A** gene. Four independent transgenic T1 lines with single-copy insertions and unchanged phenotypes (CTV1 and CTV2 for *tvip3A**, and CV1 and CV2 for *vip3A**) were selected by Southern blotting, and subjected to an insect bioassay and field assessment. Four homozygous T2 transgenic lines were then selected and the amount of expressed Vip3A* protein was determined by western blotting and ELISA. The protein concentrations of CTV1 and CTV2 were about three-fold higher than those of CV1 and CV2. As expected, the Vip3A* protein of CTV1 and CTV2 were transported to the chloroplasts, where they accumulated. The Vip3A* protein concentration in the chloroplasts of CTV1 and CTV2 was about 15-fold of that of CV1 and CV2. All four transgenic lines showed 100% mortality against fall armyworm (*Spodoptera frugiperda*) and beet armyworm (*Spodoptera exigua*) by insect bioassay. Moreover, CTV1 and CTV2 exhibited 100% mortality against cotton bollworm (CBW, *Helicoverpa zea*), whereas CV1 and CV2 showed 75.0% and 72.5% mortality against CBW, respectively. The field bioassay indicated that CTV1 and CTV2 were more resistant to CBW than CV1 and CV2. Our results suggest that the two *tvip3A** transgenic lines (CTV1 and CTV2) can be used to develop insect-resistant cultivars and could be used as a resource for raising multi-toxins-expressing transgenic cotton.

**Keywords** *Gossypium hirsutum* L. · *vip3A** and t*vip3A** genes · Insect resistance · Transgenic cotton · Chloroplast transit signal

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## Introduction

The first commercially available transgenic cotton expressing an insecticidal protein (Cry1Ac from *Bacillus thuringiensis* (*Bt*)) was produced in the United Sates in 1995 (Environmental Protection

Agency 1998). The planting area of *Bt* cotton cultivars has steadily increased since then, especially in China and India (http://www.isaaa.org). Although, *Bt* cotton exerts substantial pressure against many lepidopteran pests of cotton, insects have evolved resistance to Bt δ-endotoxins, and some lepidopteran insects are not as sensitive to Cry1A as the cotton bollworm. Many cases of insect tolerance against *Bt* δ-endotoxins have been reported (Cao et al. 2002; Ferre and Van Rie 2002; Shelton et al. 2002; Tabashnik et al. 2003; Zhao et al. 2002). Therefore, the isolation and characterization of new insecticidal toxins is necessary to widen the scope of pest control programs, including delaying the development of resistant insects on transgenic cotton containing Bt δ-endotoxins proteins. To achieve this goal, we have used a new insecticidal protein gene, *vip3A*, to create a new type of transgenic plant.

Vip3A (Vegetative insecticidal protein 3A), a novel insecticidal protein, is secreted during the vegetative phase of *B. thuringiensis* development; in contrast to the crystal δ-endotoxins proteins, which are produced during the reproductive phase of bacterial development (Micinski and Waltman 2005). The Vip3A protein shares no sequence homology with the known crystal δ-endotoxin proteins (Estruch et al. 1996), and exhibits a broader insecticidal spectrum against a wide variety of lepidopteran and coleopteran insects (Doss et al. 2002; Estruch et al. 1996; Mesrati et al. 2005; Selvapandiyan et al. 2001). The mode of Vip3A's action in insects' midgut is also different from that of δ-endotoxin proteins, which minimizes any potential insect cross-resistance to δ-endotoxins (Chabregas et al. 2001; Lee et al. 2003; McCaffery et al. 2006). Initial reports of Vip3A's insecticidal activity by Estruch et al. (1996) indicated 100% mortality of black cutworm (BCW, *Agrotis ipsilon*), beet armyworm (BAW) and fall armyworm (FAW) when added at 140 ng $ml^{-1}$ in the diet. By contrast, the δ-endotoxins Cry1Ac and Cry1Ab exerted relatively lower insecticidal impact on BCW, BAW, and FAW than Vip3A (Estruch et al. 1996; MacIntosh et al. 1990a). However, Vip3A shows similar activities to the Bt δ-endotoxins against various insects. Although Vip3A showed insecticidal activity against the main cotton insects, tobacco budworm (TBW, *Heliothis virescens*) and CBW, 70-fold more Vip3A than Cry1Ac was required to achieve an $LD_{50}$ against TBW, and 25-fold higher to achieve an $LD_{50}$ against CBW. The expression of such large amounts of Vip3A using the CaMV35S promoter in transgenic cotton is theoretically a difficult task to fall armyworm (*Spodoptera frugiperda*) and beet armyworm (*Spodoptera exigua*) achieve, and alternative strategies for increasing transgenic expression of Vip3A are required. Thus, we developed a new approach to achieve higher expression of the Vip3A protein in plants. Based on the results reported by Chabregas et al. (2001) and Chabregas et al. (2003), we chose the coding sequence of the chloroplast transit peptide of THI1 protein to construct a fusion protein gene with *vip3A** gene.

*Arabidopsis thaliana* THI1 protein, a thiazole enzyme (encoded by the *thi1* gene), is targeted simultaneously to chloroplasts and mitochondria by a post-translational mechanism (Chabregas et al. 2001; Chabregas et al. 2003). Molecular characterization confirmed that this protein contains a typical chloroplast transit peptide and a mitochondrial presequence-like structure at the N-terminus, enabling dual organelle targeting. In fact, most of the THI1 protein is targeted to the chloroplasts by the transit peptide; only a small amount of protein is targeted to the mitochondria (Chabregas et al. 2001; Chabregas et al. 2003). The use of the THI1 chloroplast transit peptide to direct Vip3A to the chloroplast could increase the intracellular concentration of Vip3A in transgenic cells.

To increase Vip3A expression in transgenic cotton, two strategies were adopted. First, a novel *vip3A* gene (*vip3A**) was designed and synthesized to increase the GC nucleotide content to enhance mRNA stabilization and cotton-preferred codens were used to favor its translation, based on the Vip3A sequence reported by several laboratories (Chen et al. 2002; Estruch et al. 1996; Yu et al. 1997). Second, DNA encoding the chloroplast transit peptide of THI1 was fused to *vip3A** to target the expressed Vip3A* protein to the chloroplasts, allowing accumulation of Vip3A* in the chloroplasts. This gene was referred to as *tvip3A**.

The *vip3A** gene and *tvip3A** were separately introduced into *Gossypium hirsutum* via *Agrobacterium tumefaciens*-mediated transformation. The Vip3A* expression level and the insecticidal activity against FBW, BAW, and CBW were investigated in transgenic *tvip3A** or *vip3A** plants. The results

indicated that the Vip3A* expression level in transgenic *tvip3A** lines was at least three-fold higher than in transgenic *vip3A** lines. Transgenic *tvip3A** plants also showed higher mortality against CBW than transgenic *vip3A** plants. Thus, the optimization of the nucleotide sequence of *vip3A* gene and application of the chloroplast signal were highly effective for increasing Vip3A expression levels and mortality against insects in transgenic cotton plants.

## Materials and methods

### The vip3A* gene and transformation of the recipient cotton variety

The *vip3A** gene was synthesized based on the amino acid sequence of the wild-type *vip3A* genes of *B. thuringiensis* (Fig. 1). The recipient cotton variety was *Gossypium hirsutum* cv. Zhongmiansuo35, a commercially available variety in China, which was provided by Prof. Liu Zhengde. The expression vectors, pBVip3A* and pBTVip3A*, used in this study were constructed in our laboratory and their structures are shown in Fig. S1. *Agrobacterium tumefaciens* LBA4404, harboring pBVip3A* or pBTVip3A*, was used to transform explants of *G. hirsutum* cv. Zhongmiansuo35. Transformation of cotton hypocotyl explants, initiation of callus, and regeneration of transformed plants were performed as described by Wu et al. (2005).

### PCR detection, Southern blot analysis and selection of transgenic plants

Cotton genomic DNA was extracted from young leaves using methods described by Paterson et al. (1993). Polymerase chain reaction (PCR) and Southern blotting were used to ascertain the presence of the *vip3A** gene and the transgene copy number in transgenic plants. PCR analysis was performed using the primer pair VipF (5′-ctcacgtaagggatgacgc-3′) (forward) and VipR (5′-ttgaattgaatacgcatcttc-3′) (reverse), generating a 495 bp amplicon from the *vip3A** gene. The PCR reaction comprised 94°C for 5 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, followed by 72°C for 5 min.

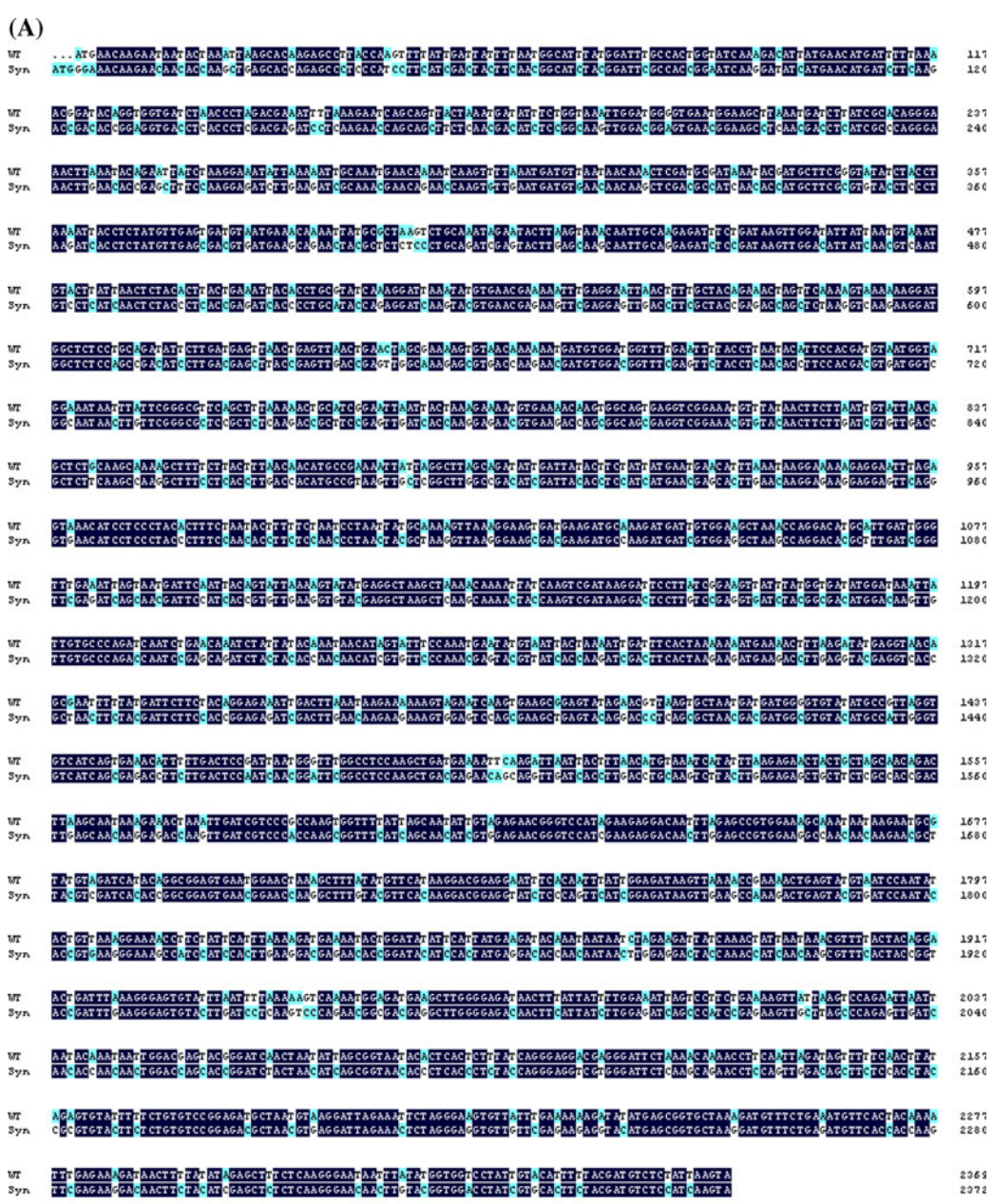
Southern blot analysis was carried out according to the methods suggested by Sambrook and Russell (2001). The vector DNA was used as a positive control. Approximately 20 μg of total genomic DNA was digested with *Hind*III, separated by electrophoresis on a 0.8% agarose gel, and transferred to a nylon membrane (Hybond-N+; Amersham, Buckinghamshire, UK). The amplicon of the *vip3A** gene was used as a probe, which was labeled with α- [$^{32}$P] using a random primer labeling kit (Promega, Madison, WI, USA).

**Fig. 1** Comparison of the chemically synthesized *vip3A** gene with the wild-type *vip3A* gene and the encoded protein sequence. **a** Shaded bases indicate identical sequence between *vip3A** and *vip3A*. Bases modified in *vip3A** were not shaded. **b** The encoded protein sequence of *tvip3A** gene. Italic letters indicate the amino acid residues of chloroplast transit signal of the THI1 protein ▶

In 2008, $T_1$ transgenic cotton plant families derived from self-fertile $T_0$ ($T_0$ stands for the original transgenic plants) transgenic plants were grown (25 plants per family) on the experimental farm of the Institute of Cotton Research, Shanxi Academy of Agricultural Sciences in Yuncheng, China. No insecticide was sprayed during the entire cotton growth period, to allow investigation of the insect resistance of the transgenic plants. Highly insect-resistant lines with normal phenotypes were selected by visual observation and by infestation on detached leaves in the laboratory. The transgene copy number was determined in the resistant lines using Southern blot analysis. Mature seeds were harvested from individual high resistance plants with a single-copy insertion. Homozygous transgenic cotton lines were selected by molecular and genetic analyses of $T_2$ lines in 2009.

### RNA preparation and transcriptional analysis

To determine the expression level of *vip3A** in transgenic cotton plants, Reverse transcription polymerase chain reaction (RT–PCR) analysis was conducted. Total RNA was extracted from young leaves of the transgenic and non-transformed (NT) control plants using Trizol reagent according to the manufacturer's instructions (Invitrogen, Life Technologies, Carlsbad, CA), and the sample was then treated with RNase free DNase I (Promega, Madison, WI). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the SuperScript™ First-Strand


(A)
(B)

Synthesis system for RT–PCR (Invitrogen Life Technologies, Carlsbad, CA). One microliter of cDNA from the reverse transcription reaction was amplified using the same primer pair used to amplify the *vip3A** probe. A fragment of the cotton *GhUBI1*gene (GenBank, accession number EU604080) was amplified from the same cDNA sample as an internal control using primers UBI-F: 5′-ctgaatc ttcgctttcacgttatc-3′ and UBI-R: 5′-gggatgcaaatcttcgt taagac-3′.

### Detection of Vip3A* protein in transgenic plants

Total protein was extracted from fully expanded young leaves of transgenic homozygous lines according to Sambrook and Russell (2001). The protein concentration in the samples was determined by the Bradford method (Bradford 1976). To identify Vip3A* expression, western blot analyses were performed. The Vip3A* protein content in transgenic plants was further quantified by enzyme-linked immunosorbent assay (ELISA) according to Sambrook and Russell (2001). Approximately 20 μg of soluble protein was loaded in each well for western blot analysis, and about 1 μg of soluble protein was applied in each well for the ELISA assay. Rabbit antiserum against Vip3A* (1:3,000 v/v) prepared in our laboratory and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Ig)G (Promega, Madison, WI, USA; 1:5,000 v/v) were used as primary and secondary antibodies, respectively, in both protein assays.

Chloroplast isolation was carried out according to procedure of the Chloroplast Isolation Kit from Sigma–Aldrich (P 4937) with minor modification, mainly, the fully expanded young leaves of cotton plants were kept in the dark overnight before isolating the chloroplasts, and the leaf homogenate was centrifuged for 3 min at 250*g*. Protein extraction from chloroplasts and leaf remnants (leaf homogenate subtracted chloroplasts) was the same with the method used to extract proteins from leaves and were assayed by ELISA analysis as described above. The Vip3A* content in chloroplast and leaf remnant were expressed as ng/μg chloroplast total soluble protein and ng/μg leaf remnant total soluble protein, respectively.

### Evaluation of insect-resistance of transgenic plants in laboratory

The egg masses of FAW and CBW were donated by the Plant Protection Institute of the Chinese Academy of Agricultural Sciences, egg masses of BAW were collected from the cotton plants grown in the field, and all were allowed to hatch in the laboratory.

The insect bioassay on transgenic plants was performed according to Guo et al. (2003) with slight modifications. Fully expanded young leaves were detached from transgenic plants and cut into discs of uniform diameter. Each leaf disc was placed in a plastic box containing a piece of wet filter paper of 30 mm diameter. They were inoculated with four first-instars larvae of FAW, BAW, and CBW, respectively. Four days later, surviving larvae were counted and the insect mortality was recorded. The insect mortality was used to verify the level of insect-resistance of a transgenic plant. Four replicate experiments were performed per transgenic plant, five boxes per replicate.

### Evaluation of insect resistance and agronomic performance of transgenic cotton in the field

Evaluation of the insect resistance of transgenic plants in the field was carried out by artificial infestation and natural infestation of CBW at the experimental farm of the Institute of Cotton Research, Shanxi Academy of Agricultural Sciences in Yuncheng, China. The materials tested included four homozygous *vip3A** and *tvip3A** transgenic lines (CV1, CV2, CTV1, and CTV2), *cry1Ac* transgenic line BR-98-2 (previously produced in our laboratory), which was used as a positive control, and the NT cultivar Zhongmiansuo35 as a susceptible control. The seeds of these lines were sown in a completely randomized block design with three replicates. Each plot consisted of 80 plants, which were grown in four rows. Within a row, the seeds were sown 30 cm apart, with 60 cm between the rows. No insecticide was applied for lepidopteran pest control during the whole growing season. Each cotton plant was infested with five first-instar larvae of CBW at the flowering stage. The number of squares and bolls damaged by CBW were counted in the field.

**Table 1** Agronomic traits and insect mortality of transgenic cotton lines and NT control

| Code of lines | Plant height (cm) | Branches per plant[a,b] | Bolls per plant | Boll weight (g) | Lint percent (%) | Damage rate of square and boll by CBW (%) | Insect mortality (%) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | FBW | BAW | CBW |
| CTV1 | 102.5 ± 3.5 | 12.8 ± 1.2 | 21.5 ± 2.1 | 5.4 ± 0.3 | 37.2 ± 1.5* | 1.1 ± 0.7** | 99.5 | 100 | 99.0 |
| CTV2 | 108.3 ± 4.2 | 12.1 ± 1.4* | 22.3 ± 2.3* | 5.2 ± 0.2 | 38.5 ± 1.2 | 0.6 ± 0.5** | 99.0 | 98.5 | 98.5 |
| CV1 | 99.8 ± 3.8 | 13.0 ± 0.9 | 20.1 ± 1.9 | 5.5 ± 0.4 | 38.4 ± 1.3 | 17.5 ± 4.5** | 100 | 99.0 | 77.5** |
| CV2 | 116.4 ± 4.1* | 13.2 ± 1.5 | 21.4 ± 2.5 | 5.3 ± 0.3 | 39.1 ± 1.6 | 19.5 ± 5.3** | 99.5 | 99.5 | 72.5** |
| NT | 106.7 ± 3.1 | 13.4 ± 0.8 | 20.7 ± 1.6 | 5.4 ± 0.3 | 38.7 ± 0.9 | 82.5 ± 11.5 | 0 | 0 | 0 |

* $t$ value significance at 0.05 level

** $t$ value significance at 0.01 level

[a] Eighty plants of each line in a replicate were investigated and the experiment was done in three replicates.

[b] Values are given as means ± standard deviation

Investigation of the agronomic performance of transgenic homozygous cotton lines was conducted in the field. Plant height, number of branches per plant, and number of bolls per plant were measured at maturity, and all the plants from each plot were harvested for measurements of boll weight and determination of lint percentage. Data on plant height, branches, bolls, boll weight, lint percentage, plant damage, and insect mortality were analyzed by a paired comparison Student's t-test. Statistically significant results at $P < 0.01$ or $P < 0.05$ are indicated in the Table 1.

## Results

### Modification of the vip3A gene, construction of the tvip3A* gene, and construction of plant expression vectors

The sequences of wild-type *vip3A* genes were compared using DNAman Software. The genes were highly similar and five variable sites were found, causing variations in the amino acid sequence: Q284 K, T291P, E406G, K742G, and P770S. Moreover, wild-type *vip3A* gene was very A+T-rich, which could cause lower expression of *vip3A* genes in the plant expression system. Thus, to optimize the expression of *vip3A* in cotton plants, the sequence of the wild-type *vip3A* gene was modified in three ways: (1) reduction of the amount of A+T nucleotides, (2) deletion of unstable factors in the mRNA transcript, and (3) optimization of the codon usage bias. The modified gene was chemically synthesized and designated as *vip3A**. DNA encoding the chloroplast transit signal sequence of the *thi1* gene was added to the 5′ end of the modified *vip3A** gene using PCR, to generate a chimeric gene designated as *tvip3A**. The DNA sequences of wild type and modified *vip3A** genes and their coding protein sequence were shown in Fig. 1.

*vip3A** and *tvip3A** were introduced into the plant expression vector pBin438 (Li et al. 1994) using *Bam*H I and *Sal* I sites to construct pBVip3A* and pBTVip3A*, respectively. The *vip3A** and *tvip3A** genes in the T-DNA region were placed under the control of a strong constitutive CaMV35S promoter(Fig. S1).

### Identification of transgenic cotton lines

Cotton hypocotyl explants were transformed with pBVip3A* and pBTVip3A* according to the method described by Wu et al. (2005). It was rather a difficult task to obtain transgenic cotton regenerated plantlets, and it took up to 16 months to raise 20 independent transgenic plants from each construct, which were identified by selection on kanamycin. The difficulties to produce transgenic cotton plants mainly come from the long time period and low rate of somatic embryogenesis and plant regeneration. In many cases, it takes 12–18 months to develop a transgenic cotton plant (Firoozabady et al. 1987; Bayley et al. 1992; Trolinder and Goodin 1987; Wu et al. 2005).

PCR was used to detect the presence of the *vip3A** and *tvip3A** genes in the genomes of the transgenic plants and the results were shown in Fig. S2 A. A total of 36 PCR positive transgenic plantlets were

selected, transferred into pots and cultured in greenhouse conditions for further experiments.

Southern blotting assay of PCR positive transgenic plants showed the presence of one or two bands per lane (Fig. S2 B), indicating that the representative transformants contained one or two copies of *vip3A** or *tvip3A**. Most of the transgenic cotton plants developed flowers and seeds. Based on their unchanged phenotypes, single copy insertion of the target gene and high insect-resistance (as determined by a preliminary bioassay), four independent transgenic lines were selected. These lines, designated as CTV1 and CTV2 (*tvip3A**) and CV1 and CV2 *(vip3A**), were used for further analysis of Vip3A* expression, insecticidal activity, and their agronomic performance.

### *Expression of vip3A** and *tvip3A** genes *in transgenic plants*

The transcriptional expression of *Vip3A** was detected by RT–PCR (Fig. 2a), where strong bands were observed in the four transgenic lines, while no band corresponding to Vip3A* mRNA were detected in NT plants.

Total protein from the *vip3A** and *tvip3A** transgenic lines was isolated from the leaves and subjected to western blotting, along with an NT control sample. Figure 2b shows that a band of ~89 kDa corresponding to Vip3A*in size and specifically reacted with the Vip3A* antiserum was appeared in all transgenic plants, but not in the NT plant, indicating that Vip3A* was expressed efficiently in all four transgenic lines. At the same time, Fig. 2b also shows that there are no extra reactive protein species shown in the whole western blot image indicating that in our experimental condition we did not find unprocessed, aggregated or degraded Vip3A* products. The sizes of the proteins expressed from the *vip3A** and *tvip3A** transgenic lines were the same, indicating that the chloroplast transit signal peptide had probably been cleaved from the precursor TVip3*A to produce the mature Vip3A*protein.

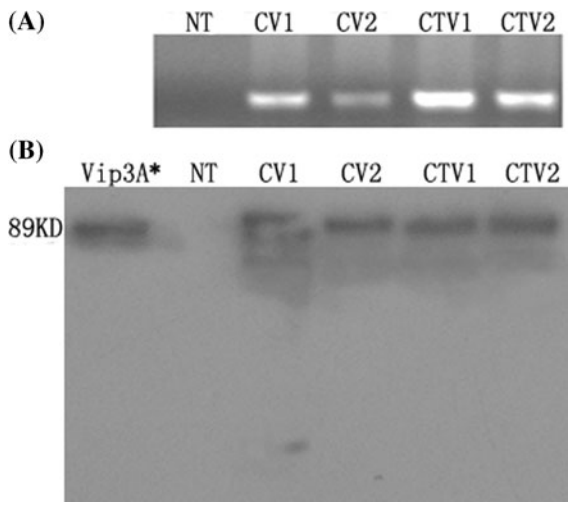



**Fig. 2** RT–PCR and western blot analysis of four transgenic lines. **a** RT-PCR analysis of total mRNA from transgenic cotton lines. **b** Western blot analysis of total soluble protein isolated from leaves of transgenic cotton lines. Fifty ng of Vip3A* protein expressed in *E. coli* was used as a positive control. Approximately 20 μg of soluble protein extracted from cotton young leaves was loaded in each well

ELISA was then used to quantify Vip3A* expression in the four homozygous transgenic lines. The amount of Vip3A* was calculated by subtracting the OD value of NT leaf extract from the four transgenic lines (Fig. 3). The transgenic cotton lines with *tvip3A** gene (CTV1 and CTV2) showed about a three-fold higher level of Vip3A* expression ($11.54 \pm 1.0$ 5 and $10.85 \pm 0.92$ μg $g^{-1}$ of leaf fresh weight (mean ± SE), or $6.12 \pm 0.25$ and $5.95 \pm 0.18$ ng μ$g^{-}$1 of total soluble protein, respectively) than lines with vip3A* gene (CV1 and CV2) ($2.94 \pm 0.20$ and $2.55 \pm 0.12$ μg $g^{-1}$ leaf fresh weight, or $1.78 \pm 0.11$ and $1.71 \pm 0.12$ ng.μ$g^{-1}$ of total soluble protein, respectively). The higher expression of Vip3A* in *tvip3A** transgenic lines is likely to be the result of targeting and accumulating Vip3A* in the chloroplasts.

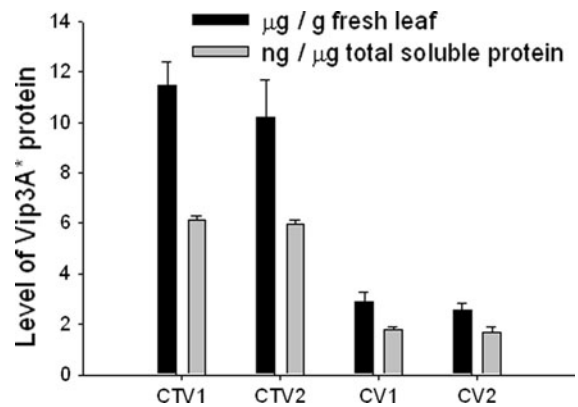



**Fig. 3** ELISA analysis of Vip3A* protein in fresh leaves from transgenic lines

### Accumulation of Vip3A* protein in chloroplasts

To check whether the chloroplast transit signal had efficiently targeted the Vip3A* protein to the chloroplasts, chloroplasts were isolated from leaves of transgenic plants and the NT control. Total proteins were extracted from the chloroplasts and the remnant faction of the leaves (leaf subtracted chloroplasts). The Vip3A* contents of the chloroplast and the remnant faction of leaf were determined by ELISA analysis. The ELISA results (Fig. 4) showed that the average amount of Vip3A* in chloroplasts from the two transgenic *tvip3A** cotton lines was about 15-fold of that from transgenic *vip3A** cotton lines. The Vip3A* protein levels of chloroplasts in the transgenic *tvip3A** cotton lines reached $23.17 \pm 1.05$ ng $\mu g^{-1}$ and $21.68 \pm 0.98$ ng $\mu g^{-1}$ chloroplast total soluble protein, respectively, while that of the transgenic vip3A* lines were only $1.47 \pm 0.12$ and $1.53 \pm 0.10$ ng $\mu g^{-1}$ chloroplast total soluble protein. In addition, the Vip3A* contents in the leaf remnant of transgenic *tvip3A** cotton lines were obviously less than those of transgenic *vip3A** lines. The above results suggested that Vip3A* protein in CTV1 and CTV2 lines was mainly accumulated in chloroplasts.

### Evaluation of insect resistance and agronomic performance

To investigate the insecticidal activity of Vip3A* protein expressing transgenic lines against insects that normally infest cotton, leaves sampled from the transgenic cotton lines and NT control were used to feed the larvae of FBW, BAW, and CBW in the greenhouse. This insect bioassay showed almost 100% mortality of FBW, BAW, and CBW on detached leaves of transgenic lines CTV1 and CTV2 after 96 h of infestation. By contrast, nearly 100% survival of these insects was recorded on the leaves of NT control plants. Transgenic lines CV1 and CV2 also showed 100% mortality to FBW and BAW, but only 75.0 and 72.5% mortality to CBW (Fig. S3) were shown after 96 h infestation.

Field trials of the four transgenic cotton lines (CTV1, CTV2, CV1 and CV2) were conducted to investigate their insect resistance and agronomic performance. The mortalities of three cotton pests on the detached leaves of transgenic lines planted in field shown in Table 1 Column 8, were consistent with the insect bioassay of transgenic lines planted in greenhouse. Under field conditions without application of pesticide, about 82.5% of the square and bolls of the NT control were damaged, whereas little or no damage was observed on the transgenic *tvip3A** plants (CTV1 and CTV2; 1.1 and 0.6%, respectively). However, slightly more damage was observed on the squares and bolls of the transgenic *vip3A** lines (CV1 and CV2; 17.5 and 19.5%, respectively). Based on the data (Table 1) from the field experiments, except for insect resistance, all four transgenic cotton lines displayed insignificant differences in agronomic performance, such as boll-number, plant-height, branch-number, boll-weight, and lint percentage compared with the NT control, Zhongmiansuo 35.

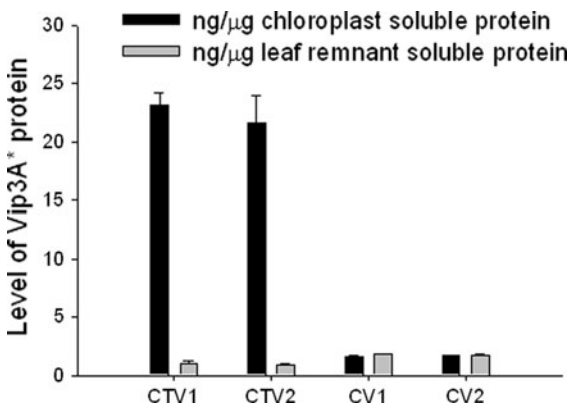



**Fig. 4** ELISA analysis of Vip3A* protein in chloroplasts and remnants from leaves of transgenic lines

## Discussion

In the present study, a number of transgenic cotton lines with high insect-resistance to the tested insects were developed via an *Agrobacterium*-mediated transformation system. Transgenic plants carrying the *vip3A** or *tvip3A** genes exhibited approximately 100% mortality against FBW and BAW. About 25% of the tested CBW larvae survived on the detached leaves of transgenic *vip3A** plants; however, the transgenic *tvip3A** lines showed nearly 100% mortality against CBW. In field studies, infestation with CBW on the terminal leaves of transgenic cotton caused more damage to squares and bolls of transgenic *vip3A** lines than to those of transgenic *tvip3A**

lines. The laboratory and field assessments showed that there was higher mortality of CBW on transgenic *tvip3A** plants than on transgenic *vip3A** plants. Some reports have proposed transgenic cotton lines with multiple resistance genes might show better insect resistance than transgenic cotton lines with single *vip3A* gene insertion (Anilkumar et al. 2008; Bommireddy and Leonard 2008; Kurtz et al. 2007; Micinski and Waltman 2005; Sena et al. 2009; Singh et al. 2008). These reports also indicated that transgenic cotton lines with a single *vip3A* are resistant to some cotton insects, such as *H. zea* and *Heliothis virescens*; however, the survival of both insects on cotton plants is higher than on transgenic cotton lines expressing *B. thuringiensis* endotoxin proteins or a combination of these toxin proteins. We hypothesized that the Vip3A level in transgenic cotton is not enough to kill these insects. The $LC_{50}$ values of *B. thuringiensis* toxin protein for CBW were reported to be 17 ng/cm$^2$ Cry 1Ab, 4–20 ng/cm$^2$ Cry 1Ac, and 420 ng/cm$^2$ Vip3A, respectively (Estruch et al. 1996; MacIntosh et al. 1990a; MacIntosh et al. 1990b). Thus, the toxicity of Vip3A is about 20-fold lower than Cry 1Ab and Cry 1Ac to CBW. Therefore, we modified the *vip3A** gene by codon-optimization and the addition of a chloroplast transit peptide to increase the expression and stability of the *vip3A** gene in transgenic cotton lines. The concentration of Vip3A* in the transgenic *tvip3A** cotton lines was three-fold higher than that in the transgenic *vip3A** cotton lines, which resulted in a higher mortality against CBW compared with transgenic *vip3A** cotton. Our results suggest that the Vip3A* protein mainly accumulated in the chloroplasts of *tvip3A** cotton lines at a higher concentration and is possibly more stable than Vip3A* located in the cytoplasm. De Cosa et al. (2001) reported that foreign proteins could be more stable and accumulated in the chloroplasts, which might be because of lower protease activity in comparison with the cytoplasm. The fact that the Vip3A*content in chloroplasts of CV1 and CV2 lines was higher than expected might reflect in our experimental conditions. Some Vip3A* protein probably could be absorbed onto the surface of chloroplasts or could diffuse into the chloroplasts. However, the absolute amount of Vip3A* in the cytoplasm should be much higher than that in chloroplast in CV1 and CV2 lines. The exact reason by which the Vip3A* protein ends up in the chloroplast fraction in CV1 and CV2 lines needs to be studied.

The toxicity of Vip3A toward CBW was lower in transgenic plants than that of Cry1Ab and Cry1Ac (Estruch et al. 1996; MacIntosh et al. 1990a; MacIntosh et al. 1990b). In the current study, we evaluated toxicity of transgenic Cry1Ac cotton lines (BR-98-2, previously developed in our laboratory) and the four transgenic cotton lines (CV1, CV2, CTV1 and CTV2) under field conditions. The results showed that the BR-98-2 and the transgenic *tvip3A** cotton lines (CTV1 and CTV2) were completely resistant, whereas the transgenic *vip3A** cotton lines (CV1 and CV2) were moderately resistant to CBW. The concentration of Cry1Ac protein in BR-98-2 assayed using the Agdia Bt-Cry1Ab/1Ac ELISA Kit was 2.05 μg/g leaf fresh weight, which was similar to that of CV1 and CV2. On the other hand, CTV1 and CTV2 expressed Vip3A* up to three-fold higher than CV1 and CV2. Our findings indicate that resistance to CBW of cotton expressing Cry1Ac is undeniably superior to Vip3A, although the *vip3A* cotton appears to be very effective against FAW and BAW. Thus, it is necessary to increase the stability and expression level of Vip3A in transgenic cotton to reach a level conferring full resistance. Nevertheless, overexpression of a foreign protein might negatively influence the growth and development of transgenic plants (Maqbool et al. 1998). In this study, the modifications we made to the coding sequence of Vip3A, in particular the addition of the chloroplast transit peptide, resulted in accumulation of Vip3A* in the chloroplast in an amount sufficient to confer 100% mortality against CBW, without altering the agronomic traits. In this study, two transgenic *tvip3A** cotton lines, CTV1 and CTV2, showed the higher expression level (about 11 μg/g leaf fresh weight) of Bt toxins than previously reported transgenic Bt crops. So far, Bt protein levels expressed in commercial Bt cultivars (cotton, maize, and potato) generally range from 1 to 11 μg/g leaf fresh weight, which is sufficient to meet the requirements of the "high dose/refuge" strategy (Cohen et al. 2000).

There is a growing concern about the potential threat of insects developing resistance to Bt endotoxins (Cry1Ab, Cry1Ac, Cry1F, and their combinations), especially with the widespread adoption of Bt crops over recent years (Kurtz et al. 2007; Sena et al. 2009; Singh et al. 2008). To date, many resistant

insect strains have emerged in the laboratory, greenhouse, and/or field conditions (Cao et al. 2002; Ferre and Van Rie 2002; Shelton et al. 2002; Tabashnik et al. 2003; Zhao et al. 2002). To meet the insect-damage challenge in plants, various management techniques have been implemented, such as high dose/refuge strategy, gene stacking, and special expression methodologies (Jackson et al. 2007; Kurtz et al. 2007; Sena et al. 2009; Singh et al. 2008). However, the most important strategy should be to search for novel insecticidal genes, especially those encoding proteins with different insecticidal mechanisms compared with Bt endotoxin proteins. Vip3A is a newly discovered insecticidal toxin protein that shares no amino acid sequence similarity with that of known B. *thuringiensis* endotoxins. Evidence suggests that Vip3A protein can bind to the brush border membrane vesicles (Lee et al. 2003), with receptor binding and ion channel properties (Lee et al. 2006; Lee et al. 2003). Therefore, Vip3A toxin, along with B. *thuringiensis* endotoxins, might be considered to broaden the base for attaining better insect control in transgenic crops. Although the *vip3A* gene is at an early stage of utilization, we foresee a huge potential for the Vip3A toxin in combination with *B. thuringiensis* endotoxins to raise transgenic crops with a broader insecticidal spectrum and also to avoid cross-resistance, to delay the development of Bt-resistant insects.

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