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Transgenic Tobacco Expressing *Zephyranthes grandiflora* Agglutinin Confers Enhanced Resistance to Aphids

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Abstract Plant lectins have been reported as transgenic resistance factors against a variety of insect pests. Herein, homologous analysis demonstrated that *Zephyranthes grandiflora* agglutinin (ZGA) exhibited high similarity with other monocot mannose-binding lectins (MBLs). Phylogenetic analysis revealed that it had taxonomical relationships with insecticidal MBLs. Subsequently, a plasmid expression vector pBI121 containing *zga* gene (pBIZGA) was constructed using the *zga* sequence, under the control of CaMV35S promoter and nos terminator. pBIZGA was then integrated into the genome of *Nicotiana tabacum* L. Polymerase chain reaction and Southern blot analysis demonstrated that this *zga* gene was integrated into the plant genome. Western blotting and agglutinating activity analysis also showed that transgenic tobacco plants expressed different levels of ZGA. Carbohydrate inhibition analysis indicated that recombinant ZGA and the native shared the same carbohydrate-binding specificity. Moreover, genetic analysis confirmed Mendelian segregation (3:1) of the transgenic in T1 progenies. In planta bioassays on T0 plants and their progenies indicated that expressed ZGA had an effect on reducing the survivability and fecundity of tobacco aphids (*Myzus nicotianae*). These findings demonstrate that the novel *zga* gene of ZGA can be expressed in crop plants susceptible to various sap-sucking insects.

Keywords *Zephyranthes grandiflora* agglutinin · Tobacco aphid (*Myzus nicotianae*) · Bioassay · Insect resistance · Transgenic tobacco

Abbreviations

pBIZGA	expression vector pBI121 containing *zga* gene
ZGA	*Zephyranthes grandiflora* agglutinin
CaMV35S	35S cauliflower mosaic virus promoter
zga	*Zephyranthes grandiflora* agglutinin gene
MBLs	mannose-binding lectins
GNA	*Galanthus nivalis* agglutinin

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ZCA	*Zephyranthes candida* agglutinin
gna	*Galanthus nivalis* agglutinin gene
ASAL	*Allium sativum* leaf agglutinin
WGA	wheat germ agglutinin
6-BA	6-benzylaminopurine
ConA	concanavalin A
T0	the first generation of transgenic plants
T1	the first progenies of T0

Introduction

Lectins are carbohydrate-binding proteins that are widely distributed in animals, plants, and microorganisms [1]. They bind carbohydrates reversibly and possess the ability to agglutinate cells or precipitate polysaccharides and glycoconjugates [2]. Lectins have attracted great interest because they can be involved in protein–carbohydrate interactions [3], seed storage [4], growth regulation [5], plant development [6], antitumor [7, 8], and defense against pests and pathogens [9–11]. Numerous investigations on the insecticide activity of plant lectins have been carried out because of the mechanism for plant defense.

Compared with other insect pests, it is more difficult to prevent the sap-sucking insects from attacking crop plants [12, 13]. Most of these insects belong to the Homoptera, such as aphids, brown plant hopper, and green leafhopper. The sap-sucking pests, different from Lepidopteran and Coleopteran group of chewing insects, were feed on the plant phloem sap to satisfy their nutritional need [14]. Although the application of the pesticide provides temporary relief, the liberal dispersion of the chemicals is costly for the farmers and hazardous for the environment [15]. Moreover, none of the toxins of *Bacillus thuringiensis* has been found to be toxic to sap-sucking insects [16, 17], and protease inhibitor approaches have shown a little influence on these pests [18]. Therefore, much research has been concentrated on finding the effective insecticidal agents against insects of the Homoptera.

Recent studies have demonstrated that some mannose-binding lectins (MBLs) are capable of offering a defense mechanism against Hemipteran groups of sap-sucking pests [19]. *Galanthus nivalis* agglutinin (GNA), one member of MBLs, has received much attention for its remarkable toxicity. Meanwhile, some studies with transgenic tobacco, wheat, tomato, potatoes, and rice plants expressing GNA have been shown to be deleterious on aphids, leafhoppers, and plant hoppers [20–22]. Due to the promising results, it is valuable to isolate and utilize novel lectin genes toxic to sap-sucking insects. Interestingly, hitherto, some mannose-binding lectin genes have been cloned from *Pinellia ternate*, *Allium sativum*, *Allium cepa*, *Zephyranthes candida*, etc. Furthermore, when the lectins were added in artificial diet or expressed transgenically in plant systems, they were shown to resist the attack of the sap-sucking insects including aphids, brown plant hopper, and green leafhopper [23–26]. These lectins could adversely affect the survivability or the fecundity of the insects and/or their ability to transmit pathogens [27, 28]. It was demonstrated that lectins bind to the soluble and brush border membrane enzymes in the midgut of insects, and these enzymes as receptors may decrease the absorption of nutrients and/or the permeability of the membrane [29, 30]. Also, it was found to bind to receptors which play the key roles in aphid-mediated virus transmission [31].

Zephyranthes grandiflora is a traditional Chinese medicinal herb and ornamental plant species, belonging to the Amaryllidaceae. In our previous study, the full-length cDNA of *zga* was cloned using the rapid amplification of cDNA ends–polymerase chain reaction

(RACE-PCR) protocol from bulbocodium of *Z. grandiflora*. The deduced amino acid sequence indicates that a preprotein with 164 amino acid residues is firstly translated and then processed to a mature protein with 106 amino acids [32]. Homology analysis of the deduced amino acid sequence of *Z. grandiflora* agglutinin (ZGA) with GNA and other insecticidal MBLs revealed that they shared a high degree of sequence similarity (81% with GNA, 81.8% with ZCA) [23, 32]. Accordingly, it is speculated that ZGA may also have a similar insecticidal activity with other insecticidal MBLs and it could be utilized as a potential candidate agent to control sap-sucking insects through genetic engineering.

In this study, we report for the first time that *zga* can be successfully integrated into the genome of tobacco, and the transgenic plant synthesized and correctly processed a fully active ZGA protein. Further in planta bioassays demonstrate that the transgenic plant can adversely affect survivability and fecundity of aphids when expressed transgenically in tobacco. As mentioned above, these results would offer us enlightenment for comprehending the significant role for this lectin in further anti-insect investigations.

Materials and Methods

Bioinformatics Analysis of the Sequence of ZGA

The multiple sequence alignment was performed with the CLUSTAL W program [33]. Phylogenetic tree was constructed by the Molecular Evolutionary Genetics Analysis (MEGA 4) software [34, 35].

Plasmid Construction

Total RNA was extracted from *Z. grandiflora* bulbocodium using an RNA Extraction Kit (Takara Biotechnology, Dalian, China) and reversely transcribed with a cDNA synthesis primer AP (5′-GGCCACGCGTCGACTAGTAC$(T)_{21}$-3′) using a Reverse Transcription Kit (TaKaRa, Dalian, China). The *zga* gene was amplified using a PCR reaction using the gene-specific primer2 (5′-AGAGGATCCACCCAAACAAGCAAGTAAAC-3′) and primer3 (5′-TTCGAGCTCTAGCCTCACTCGTCTCATAAT-3′), designed according to the published sequence **(**GenBank accession no. AY288522**)** with a *Bam*HI and a *Sac*I site, respectively. PCR products were recovered by using a PCR Fragment Recovery Kit (TaKaRa, Dalian, China) and were inserted into the pMD18-T vector (TaKaRa, Dalian, China).The recombinant plasmid was completely digested with *Bam*HI and *Sac*I to release a fragment containing the coding sequence of the *zga* and the latter was inserted into *Agrobacterium* binary vector pBI121 predigested with *Bam*HI and *Sac*I to generate pBIZGA. The recombinant plasmid, pBIZGA, contained the selectable marker neomycin phosphotransferase gene (nptII) conferring kanamycin resistance and the *zga*, both driven by CaMV35S. The schematic T-DNA structure is represented in Fig. 3. The constructed plasmid was then transformed into DH5α. The resulting clone that contained the gene encoding for ZGA where the DNA sequence was determined was verified.

Transformation of *Agrobacterium tumefaciens*

The plasmid was transferred into *A. tumefaciens* strain EHA105 by use of direct transformation of $CaCl_2$ competent cells. Transformed bacteria were selected on YEB plates containing kanamycin (50 mg/L) and rifampycin (50 mg/L) and random colonies

were chosen for plasmid minipreparation. The presence of the introduced plasmid was confirmed by restriction enzyme analysis.

Transformation and Regeneration of Transgenic Tobacco Plants

The tobacco (*Nicotiana tabacum* L.) cultivar "Bairihong" was used for transformation. Leaves from subculture tobacco (40 days old) were used as a source for transformation explants. The transformation was performed essentially as described previously by Horsch et al. [36]. *A. tumefaciens* strain EHA105 containing pBIZGA was grown for 2 days at 28 °C in YEB supplemented with 50 mg/L kanamycin and 50 mg/L rifampycin. The bacteria (OD_{600}=0.8) were collected and suspended in hormone-free MS liquid medium before use [37]. The leaf discs of approximately 0.5–1.0 cm^2 were immersed in the bacterial suspension for 5 min, transferred onto MS solid medium supplemented with 1.0 mg/L 6-benzylaminopurine (6-BA), and incubated at 26 °C in the dark for 2 days. After the cocultivation, the discs were placed on selection medium (MS medium supplemented with 1.0 mg/L 6-BA, 100 mg/L kanamycin, 500 mg/L ampicillin, and 250 mg/L timentin) and cultured for 2 weeks at 26 °C under a 16-h light/8-h dark photoperiod (fluorescent light of 40 μmol/m^2s, Philips cool white fluorescent light) until callus developed. The callus were then transferred onto the same fresh selection medium and cultured for 2 weeks in the same condition until shoots developed. The regenerated green healthy shoots were separated from the explants and transferred to hormone-free MS medium containing 100 mg/L kanamycin, 500 mg/L ampicillin, and 250 mg/L timentin for rooting. The well-rooted plants were eventually transferred to soil in pots in greenhouse.

PCR and Southern Blotting Analyses

Putative transformants and the control (wild type) plants were used for PCR analysis. The total DNA was extracted from young leaves using the cetyl trimethylammonium bromide method of Graham [38]. PCR analysis for the detection of the *zga* gene was performed using primer2 and primer3. For PCR analysis, DNA was denatured at 94 °C for 3 min followed by 30 amplification cycles (94 °C for 50 s, 56 °C for 50 s, 72 °C for 50 s) and finally 10 min at 72 °C. The expected product size was about 550 bp.

Genomic DNA was extracted for Southern blot analysis from PCR-positive transformants and the control plants. Twenty micrograms tobacco genome DNA digested with *Bam*HI was electrophoresed in the 0.8% agarose gel along with undigested pBIZGA plasmid and transferred onto nylon membrane (Hybond-N+, Schleicher & Schuell). The membrane was hybridized overnight at 42 °C with digoxigenin (DIG)-labeled *zga* cDNA probes and washed 2×5 min in the ample 2× SSC, 0.1% SDS at 15–25 °C under constant agitation, then washed 2×15 min in 0.5×SSC, 0.1% SDS at 65–68 °C, finally exposed to X-ray film (Fuji film) for 16 h.

According to the results from PCR and Southern blotting analyses, 21 T0 generations of transgenic lines harboring the *zga* gene were selected for further analysis. Two- to 3-week-old lines of transgenic tobacco plants rooted in the culture tubes were transferred to a soil mixture for biofunctional analysis.

Western Blot Analysis of Transgenic Tobacco Plants

Protein expression in transgenic tobacco plants was evaluated by Western immunoblotting. The total proteins were extracted from 0.5 g fresh leaves by grinding in 500 μL phosphate-

buffered saline buffer (PBS, pH 7.2). Insoluble materials were removed by centrifugation at 10,000×*g* for 30 min at 4 °C. Proteins were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting was performed as described previously [39]. Native ZGA protein was used as the positive control.

Extraction of Proteins

Putative transformants (plant nos. 2, 6, 7, 8, 10, 15, and 18, three cloned replicate plants per line) and wild type plants as control were cultured in greenhouse maintained at 16-h light/8-h dark photoperiod, 75% relative humidity, and 26 °C temperature. Protein was extracted from young leaves in two volumes (*v*/*w*) of Tris–HCl (50 mM, pH 6.8, containing 0.2 M NaCl). After standing overnight at 4 °C, the homogenate was filtered through cheese cloth and centrifuged at 3,000×*g* for 15 min. The supernatant was filtered through filter paper (Whatman 3MM) and the cleared extract was applied to hemagglutination assays and electrophoresis analysis. The concentration of total proteins was determined by the folin phenol method [40] with bovine serum albumin as standard.

Recombinant ZGA Protein Analysis

Assays of hemagglutinating activity and carbohydrate inhibition of hemagglutinating activity were used to check for the biological activity of the recombinant ZGA protein. The assay was carried out by the serial dilution method [41] in 96-well microtiter U plates using untreated rabbit erythrocytes (2% suspension in PBS) against standards of purified native ZGA. Hemagglutination was assessed visually after 1 h at room temperature. Hemagglutinating activity of extracts was expressed as concentration (micrograms per milliliter) of total soluble plant protein.

Inhibition of hemagglutination activity was studied using a series of carbohydrates and glycoproteins. Purified native ZGA, total soluble proteins of control and no. 15 tobacco plants (at a concentration of recombinant ZGA equal to purified native ZGA) were used. Different concentrations of the carbohydrate/glycoprotein were mixed with these lectins. After 30 min incubation, the erythrocyte suspension was added and hemagglutination evaluated. Carbohydrate/glycoprotein used for this assay were Man-α(1,3:1,6)-mannotriose, Man-α(1,3)-Man, Man-α(1,2)-Man, Man-α(1,6)-Man, D-mannose, D-glucose, D-lactose, maltose, D-galactose, sucrose, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, thyroglobulin, ovomucoid, and fetuin.

Aphid Bioassay Test on Transgenic Tobacco T0 Lines

Six independently derived transgenic tobacco T0 lines (plant nos. 2, 6, 7, 10, 15, and 18, three cloned replicate plants per line), together with untransformed tobacco controls, were challenged by tobacco aphids (*M. nicotianae*) and investigated for their effects on aphid survival and the development of aphid population using a protocol essentially the same as described by Rahbe et al. [18]. All bioassays were carried out at 26 °C under an approximate 16-h light/8-h dark photoperiod regime. Insects were reared on untransformed tobacco plants in growth chambers maintained at the abovementioned conditions. Each plant (20–30 cm tall) was confined to an insect-proof fine-mesh nylon cage, ten third instar aphid nymphs were introduced with a hair brush to the leaves of each plant on day 0. The insect survival was measured at 2-day intervals for a 20-day period. The effect of ZGA on aphid fecundity was assessed by the total number of nymphs produced per individual

transgenic plants at the end of the entire bioassay period. The mean data was expressed as the percentage of total aphids surviving on the respective days. The experiment was repeated three times (each independently derived transgenic plant was micropropagated into three).

Genetic Analysis of Segregation of *zga* Gene in T1

Four independent primary transgenic T0 plants containing a single-copy insertion (plant nos. 6, 7, 15, and 18) were selected for genetic analysis. T1 (the first progenies of T0) seeds obtained from these self-pollinated T0 plants were surface sterilized by soaking them in 10% bleach and 0.1% Triton X-100 for 30 min. Subsequently, they were washed twice with sterile water and dried. The seeds were sown onto MS basal medium and DNA was isolated from the germinating seedlings. Afterwards, PCR analyses for *zga* gene have been done using *zga* gene-specific primers (primer2 and primer3). The reaction mixtures were analyzed in 1% agarose gel. After separation of the amplified product of the ZGA sequence, segregation pattern of *zga* gene in progeny plants were calculated. According to Mendelian inheritance patterns, for one locus integration, the ratio of *zga*-containing plants to non-*zga*-containing ones should fit the expected 3:1 in T1 generation. The surviving green plantlets were transferred to soil and grown under greenhouse condition for further development.

Aphid Bioassay Test on Transgenic Tobacco T1 Lines

PCR-positive transgenic T1 plants derived from two T0 plant lines (plant nos. 6 and 15) showing high ZGA expression and enhanced resistance to tobacco aphids were challenged again by the aphids and investigated for their effects on aphid survival and fecundity using the method mentioned earlier.

Statistical Analysis of the Data

All the data were confirmed in at least three independent experiments. Standard deviation of the mean was estimated to measure the precision of the estimate of the mean. To detect significant differences between treatments and control, statistical unpaired two-tailed *t* tests were employed. $P<0.05$ was considered to be significant. All these data was analyzed by SPSS 10.0 (SPSS, Chicago, IL, USA).

Results

Bioinformatics Analysis of the Sequence of ZGA

The CLUSTAL W program was used to compare the amino acid sequence of ZGA with that of other monocot MBLs. Homologous analysis showed that the identity between ZGA and GNA, *Lycoris radiata* agglutinin, *Z. candida* agglutinin (ZCA), *Amaryllis vittata* agglutinin, *Narcissus pseudonarcissus* agglutinin, *A. sativum* agglutinin II (ASAII), *A. sativum* leaf lectin, and *A. cepa* lectin was 81%, 64%, 81.8%, 75.1%, 59.5%, 43.7%, 37.3%, and 37.1%, respectively (Fig. 1). These results revealed that ZGA belongs to the family of monocot MBLs. Furthermore, ZGA has three mannose-binding sites of four conserved amino acid residues (QDNY), which is similar with these MBLs above. The mannose-binding sites are relevant to biological activity.

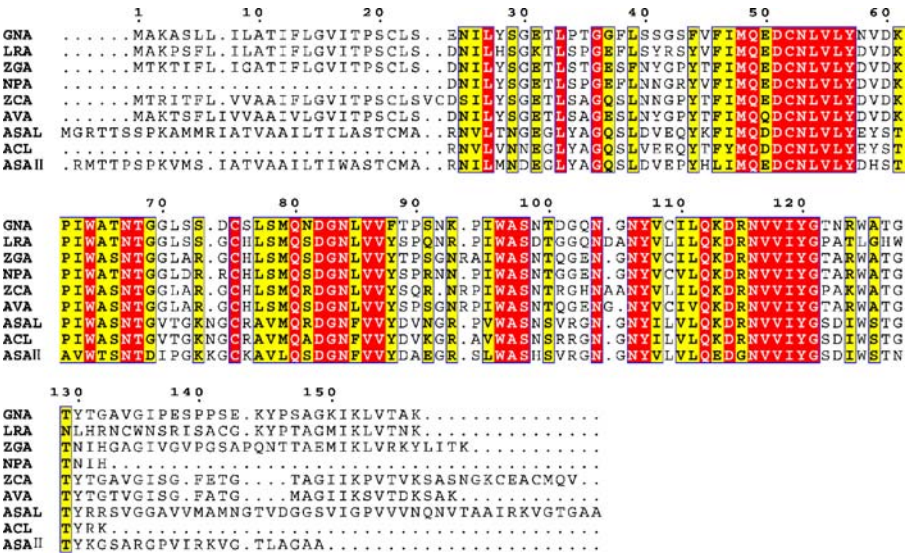

Fig. 1 Multi-alignment of the predicted ZGA amino acid sequence with other monocot MBLs from family Amaryllidaceae and Liliaceae. Identical amino acid residues are indicated with *dark red* background and *white* foreground. The conservative amino acids are indicated with *yellow* background and *black* foreground. *GNA G. nivalis* agglutinin (AAL07474), *LRA Lycoris radiate* agglutinin (AAP20877), *ZGA Z. grandiflora* agglutinin (AAP37975), *ZCA Z. candida* agglutinin (AAM27447), *AVA A. vittata* agglutinin (AAP57409), *ASAL A. sativum* leaf agglutinin (P83886), *ACL A. cepa* lectin (AAR23522), *NPA N. pseudonarcissus* agglutinin (1NPL_A), *ASAII A. sativum* agglutinin II (AAA32645). GNA, ZCA, ASAL, ACL, NPA, and ASAII all posses insecticidal activity toward sap-sucking insects

To trace the evolutionary relationships of ZGA and insecticidal MBLs, a phylogenetic tree was constructed by the Molecular Evolutionary Genetics Analysis (MEGA 4) software, using most available sequences of insecticidal MBLs (Fig. 2). A big cluster contained ZGA, ZCA, GNA, *Chimonanthus praecox* lectin, ASAII, *A. sativum* leaf lectin, and *A. cepa* lectin. So, we speculate that ZGA may have a similar insecticidal activity with other four lectins.

Generation and Selection of ZGA Transgenic Tobacco Plants

A plasmid pBIZGA was constructed according to the method of Schardl [42], and the transgenic tobacco plants expressing ZGA were produced by infecting leaf discs with engineered *A. tumefaciens* containing this pBIZGA construct (Fig. 3). Thirty-one lines of the first generation of transgenic plants (T0) showing resistance to kanamycin were generated. PCR analysis revealed that 27 out of 31 putative independent transgenic T0 plants were positive for the *zga* gene (550 bp), while no PCR products were obtained from the nontransformed tobacco plants (representative samples shown in Fig. 4).

Regenerated plants were verified for the presence of *zga* by Southern blot analysis. All of the 27 independent *zga* PCR-positive T0 plants were further analyzed by Southern blot hybridization. Genomic DNA digested with *Bam*HI from these transgenic plants showed that 21 out of 27 T0 plants gave one or more hybridization signal DIG-labeled *zga* cDNA as a probe (representative samples nos. 2, 4, 6, and 8 shown in Fig. 5). The gene copy number for *zga* was 2, 2, 1, and 1 in nos. 2, 4, 6, and 8, respectively. Thus, it could be

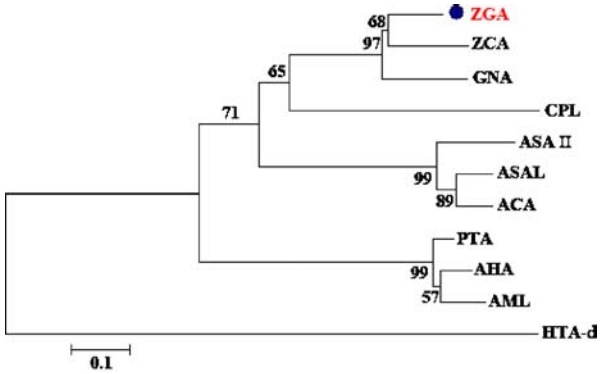

Fig. 2 Dendrogram of ZGA with other insecticidal MBLs. The DNA sequences were aligned by the Clustal W program and the tree was generated by neighbor-joining methods within MEGA 4. The insecticidal activity of these lectins was displayed as follows: *ZCA Z. candida* agglutinin (AF503622), *Rhopalosiphum padi*; *GNA G. nivalis* agglutinin (M55556), *Myzus persicae*, *Nilaparvata lugens*, *Sogatella furcifera*, *L. oleracea*, *Aulacorthum solani*; *CPL C. praecox* lectin(DQ352145), *L. erysimi*; *ASA II A. sativum* agglutinin II (M85171), *Myzus persicae*, *M. nicotianae*, *L. erysimi*, *Spodoptera littoralis*, *Nephotettix virescens*, *Nilaparvata lugens*, *Dysdercus cingulatus*; *ASAL A. sativum* leaf agglutinin (ASU58947), *Myzus persicae*, *M. nicotianae*, *L. erysimi*, *Spodoptera littoralis*, *Nephotettix virescens*, *Nilaparvata lugens*, *Dysdercus cingulatus*; *ACA A. cepa* agglutinin (L12171), *L. erysimi*, *Brassica juncea*; *PTA*, *Pinellia ternata* agglutinin (DQ092435), *Myzus persicae*; *AHA Arisaema heterophyllum* agglutinin (AY289926), *L. erysimi*; *AML Alocasia macrorrhizos* lectin (DQ340864), *L. erysimi*, *Nephotettix virescens*, *Nilaparvata lugens*; *HTA-d Helianthus tuberosus* agglutinin-d (AF477034), *Myzus persicae*

deduced that *zga* was integrated into the genome of transgenic T0 tobacco plants. No signal was detected in the untransformed control tobacco plant (Fig. 5, lane N).

Recombinant ZGA Protein Analysis

Crude protein extracts from transgenic plants were tested to hemagglutinate rabbit erythrocytes. The seven selected T0 lines (plant nos. 2, 6, 7, 8, 10, 15, and 18, three cloned replicate plants per line) possessed variable hemagglutinating activities. The no. 7 independent line showed strong activity at 3.13 μg/mL of total soluble protein. However, the activity of one dependent line (no. 8) was especially weak, even if at 200 μg/mL (Table 1). Hemagglutinating activities depended on the concentration of recombinant ZGA in the total soluble protein; hence, we concluded that the no. 7 line had the highest expression level among the lines. In addition, the control plants tested were devoid of any

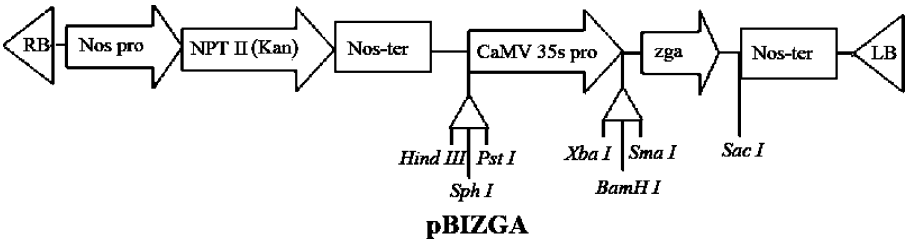

Fig. 3 Schematic representation of transformation plasmid (*pBIZGA*). *CaMV35S* cauliflower mosaic virus 35S promoter, *NPTII* neomycin phosphotransferase gene, *NOS-ter* 3′-termination region from *Agrobacterium* nopaline synthase gene, *zga Z. grandiflora* agglutinin gene

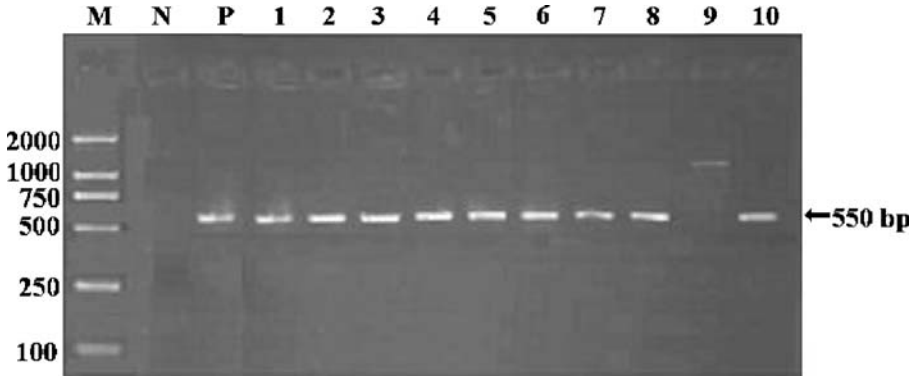

Fig. 4 Representative PCR analysis for the presence of the *zga* gene in transgenic tobacco plants. *M* DNA marker DL2000 (bp), *N* untransformed plant (negative control), *P* positive control (*zga* gene fragment), *nos. 1–10* independent transgenic plants. The *arrow* indicates the expected PCR product (550 bp)

hemagglutinating activity at all. These results indicated that the recombinant ZGA protein possessed the same biological activity as the native.

The inhibition assays showed that Man-α(1,3:1,6)-mannotriose, Man-α(1,6)-Man, Man-α(1,3)-Man Man-α(1,2)-Man, and D-mannose exerted strong inhibitory influence on the hemagglutination of both native and recombinant ZGA. However, there was no inhibition by D-glucose, D-lactose, maltose, D-galactose, sucrose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine up to 200 mM. Among tested glycoproteins, their hemagglutination could only be inhibited by thyroglobulin, whereas ovomucoid and fetuin were not inhibitory at all. These results showed that recombinant ZGA displayed the same carbohydrate-binding specificity as the native. The total soluble proteins of control plant tested were devoid of any hemagglutinating activity at all, despite the addition of sugars (data not shown).

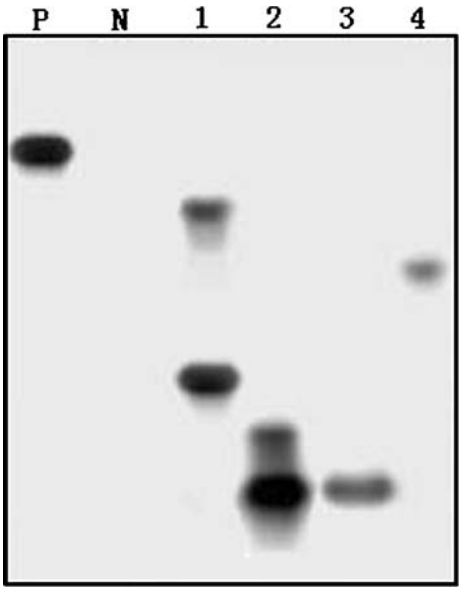

Fig. 5 Representative Southern blot analysis for the presence of the *zga* gene in transgenic tobacco plants. Genomic DNA of transgenic and control plants was digested with *Bam*HI and separated by 0.8% agarose gel electrophoresis along with undigested pBIZGA plasmid, then hybridized with the DIG-labeled *zga* probe. *P* undigested pBIZGA (positive control), *N* untransformed plant (negative control), *lanes 1–4* independent transgenic plants nos. 2, 4, 6, and 8

Table 1 Agglutinating activity of transgenic plants toward rabbit erythrocytes.

	Concentration of total soluble protein (μg/mL)										
	200	100	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.20
Control	−	−	−	−	−	−	−	−	−	−	−
2	+	+	+	+	+	−	−	−	−	−	−
6	+	+	+	+	−	−	−	−	−	−	−
7	+	+	+	+	+	+	−	−	−	−	−
8	+	−	−	−	−	−	−	−	−	−	−
10	+	+	+	−	−	−	−	−	−	−	−
15	+	+	+	+	+	+	+	−	−	−	−
18	+	+	+	+	+	+	−	−	−	−	−

+ agglutination, − nonagglutination, *Control* nontransgenic tobacco plant, *2–18* transgenic tobacco plants

Expression of ZGA in Transgenic Tobacco Plants

Western blot analyses of leaf extracts from eight presumptive T0 transformants showed the presence of a polypeptide of about 12 kDa (Fig. 6, lanes 3–10), corresponding to the purified native ZGA protein (Fig. 6, lane P), when probed with anti-ZGA primary antibodies. No such band was observed in untransformed control plant (Fig. 6, lane N), which was in coincidence with the Southern blot analysis. It was noteworthy that all of the ZGA transgenic tobacco lines had normal growth phenotypes both in the culture tubes and soil mixture.

Effect on Survivability of Aphids when Feeding on Transgenic Plants

Aphid bioassay test was used to study the insecticidal activity of ZGA expressed in transgenic plants. The survival and growth of aphids were monitored with late instar aphid nymphs at an interval of 2 days for 20 days on T0 lines (plant nos. 2, 6, 7, 10, 15, and 18) as well as on T1 progenies of ZGA-expressing transgenic plants (nos. 6 and 15). Untransformed plants were used as negative control.

It was obvious to observe the differences in insects' survival between T0 plants and control plants after 4 days. Relatively higher mortality levels were observed on the expressive transgenic plant lines nos. 6, 7, 15, and 18, while all four lines expressed ZGA at a high level. The growth of aphid population on the four lines was significantly declined to an average of 37.5±3.60 (no. 6), 52.5±4.13 (no. 7), 49.75±4.32 (no. 15), and 61±1.33 (no. 18) insects per

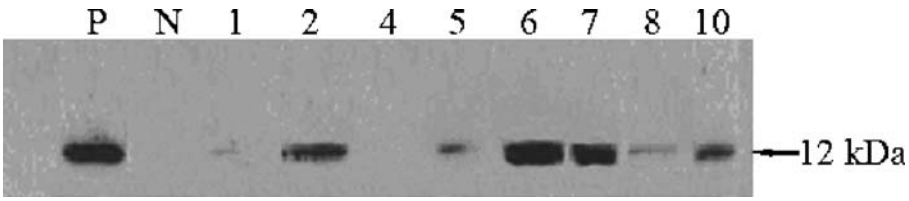

Fig. 6 Representative Western blotting analysis for the expression of *zga* gene in transgenic tobacco plants. Protein was extracted from young leaves and 30 μg of crude protein was loaded in each lane. *P* 100 ng purified native ZGA protein (positive control, 12 kDa), *N* untransformed plant (negative control), *1*, *2*, *4*, *5*, *6*, *7*, *8*, *10* crude protein extracts from eight independent transgenic T0 plants nos. 1, 2, 4, 5, 6, 7, 8, 10, respectively

plant, respectively, compared to the control plants (197.5±6.06 per plant) at the end of the period (Fig. 7). There was significant difference of the aphid survival between the control and each of nos. 6, 10, 15, and 18 plants ($P<0.05$) constantly from day 6 to day 20 throughout the bioassay (data not shown).

Effect on Fecundity of Aphids when Feeding on Transgenic Plants

The nymphs started developing into nonfeeding alate form after 4 days of the bioassay period. It was expected that the nymphs would develop into adults and the adults would give rise to a cycle of fresh generation of nymphs after 12 days. However, it was observed that the increase of the nymphs' population was adversely affected when these insects were fed on the transgenic plants (Fig. 7). The number of nymphs produced per plant was decreased by 81% (no. 6), 73.7% (no. 7), 74.8% (no. 15), and 69.1% (no. 18), compared with controls. The inhibition ratio of several plants could reach up to 97.2%. Nevertheless, aphid population on two transgenic T0 line (nos. 2 and 10) were lower than that on the control during most of the assay period, but since day 10, they showed no significant difference, reflecting the low expression levels of the *zga* gene in the transgenic plants.

In transgenic plants of T1 generation, the progeny of aphid nymphs declined to an average of 11.8±2.73 (no. 6, $n=25$) and 18.5±3.28 (no. 15, $n=25$) on day 20, respectively, compared to 41.3±2.65 on controls. Aphid fecundity inhibitions were 71.4% (no. 6) and 55.2% (no. 15) ($P<0.05$). The fecundity of aphids was also notably retarded.

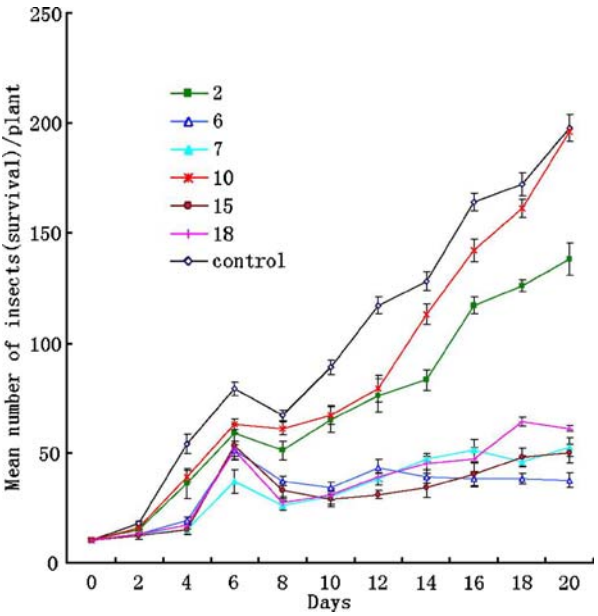

Fig. 7 Aphid bioassay tests on transgenic tobacco line nos. 2, 6, 7, 10, 15, and 18. Ten late instar aphid nymphs were introduced into each plant on day 0 and the insect survival and growth of aphid population were measured at 2-day intervals for a 20-day period. *Points* and *bars* show the mean±SE

Test for Inheritance of the Transferred Lectin Gene in T1 Generation

T1 progenies of the four independently derived primary transgenic T0 plants (plant nos. 6, 7, 15, and 18) were analyzed for the presence of the *zga* gene by PCR. Results point toward the fact that the *zga* gene in all four lines was inherited at a segregation ratio of 3:1, indicating integration of the *zga* transgene into a single genetic locus (Table 2).

Discussion

Recently, much interest has been focused on the potential application of MBLs in integrated insect management via transgenic technology [20]. ZGA, which is a member of the monocot MBLs, has received much attention for its remarkable biological activities [32]. The homologous analysis showed that ZGA has three mannose-binding sites of four conserved amino acid residues (QDNY), similar with other MBLs (Fig. 1). Phylogenetic analysis suggested that ZGA, GNA, ZCA, *A. sativum* leaf lectin, *C. praecox* lectin, ASAII, and *A. cepa* agglutinin have taxonomical relationships (Fig. 2). In the present study, a genetic engineering approach was undertaken to express this novel insecticidal lectin ZGA in tobacco to investigate the effect on the survivability and fecundity of the sap-sucking insects *M. nicotianae*.

The findings of hemagglutination and carbohydrate-binding assays revealed the same biological activity with the native (Tables 1 and 2). The inhibition assays indicated the presence of the active mannose-binding groove in the recombinant lectin (Table 2). The test demonstrated that the preprolectins [43] are correctly cotranslationally and posttranslationally processed in the tobacco cells into fully active mature lectin polypeptides. A very similar phenomenon was found in transgenic tobacco that expressed *A. sativum* leaf agglutinin (ASAL) and ASAII [44]. It was noteworthy that the hemagglutinating activities of the seven selected T0 lines rely on the expression levels of ZGA. Similar results were reported for the expression of pea lectin in potato plants and GNA in papaya, and indirect calculations of recombinant lectin content were made based on the assay results [45, 46].

In the study, Western blot analysis demonstrated that the recombinant ZGA had a molecular weight similar to the purified native protein, confirming that processing, i.e., removal of leader sequence and C-terminal extension, was correct. Moreover, it also showed that most of the transgenic plants expressed ZGA at different levels (Fig. 6). A similar phenomenon was also found when other lectin genes such as *gna* were expressed [47] due to various reasons such as epigenetic silencing and cosuppression, position effects, transgene structure [48]. More importantly, these results are in agreement with the aforementioned hemagglutinating activity assays that the transgenic plants with a high

Table 2 Segregation analysis of *zga* gene in T1 progenies of transgenic tobacco lines.

T1 line no.	Total assayed	Expected ratio	PCR for *zga* gene			
			zga+	*zga*$^-$	χ^2	*P* value
6	30	3:1	20	10	1.11	0.29
7	30	3:1	22	8	0.04	0.83
15	30	3:1	24	6	0.40	0.53
18	30	3:1	23	7	0.04	0.83

Four-week-old T1 progeny plants were analyzed by PCR for the presence of the *zga* gene

level of ZGA expression (Fig. 6, lanes 2, 6, and 7) could exhibit a high hemagglutinating activity (Table 1).

Since it was important to express an alien gene after successful integration and inheritance in a transgenic approach, we investigated the insecticidal effect of ZGA in T0 and T1 plants. Herein, the aphid survival was significantly decreased to 37.5±3.60 (mean±SD) on the no. 6 T0 line compared with 197.5±6.06 in the case of untransformed plant. The nos. 6 and 15 T1 generation caused a reduction in aphid nymphs number to 11.8±2.73 and 18.5±3.28, respectively, while the number on control was up to 41.3±2.65. Comparable results were described in an earlier study by Hilder et al. who report that the mortality rate of aphids reared on transgenic GNA tobacco leaves was observed to be 8.3±2.5 compared with 38.8±8.6 on the control [21]. The survival of white-backed plant hopper was reduced by more than 90% and the fecundity was also greatly affected when fed on GNA rice plants [49]. Foissac et al. reported that when fed on GNA-expressing transgenic rice, green leafhopper and brown plant hopper showed 29% and 53% reduction of survival [47]. Likewise, the mean survival percentage of mustard aphids on 35SASAL2 and RSsASAL4 T0 mustard plants were approximately 18.66±0.86% and 23.33±0.66% after 9 days of bioassay period, respectively, compared with control mustard plants [50]. As to the ASAL-expressing transgenic tobacco plants, the survival of peach potato aphid significantly decreased to 16±1.37% on the AS11 line, compared with control [51]. Similar observation was recorded by Yao et al. in the case of PTA tobacco plants [25].

In addition to the decrease in aphid survival, the fecundity of aphids feeding on ZGA-expressing tobacco plants was also reduced, compared with those feeding on control. Various degrees of reduction in fecundity have been reported by different groups using different combinations of promoter, target insects, and source of insecticidal proteins. Previous studies already demonstrated that insect fecundity was decreased by 30% and from 30% to 65%, respectively, when studied on transgenic rice and wheat plants expressing GNA [16, 52]. Kanrar et al. demonstrated a 35% reduction in fecundity when mustard aphids were feed with an artificial diet containing 0.1% wheat germ agglutinin (WGA) [29]. Similar results were reported that the fecundity of mustard aphids feeding on transgenic plants has decreased by 60–64% and 35–39% in the case of 35SASAL and RSsASAL plants, respectively [51]. The survival percentage of *M. nicotianae*, as evident from the present study, had been reduced in the case of ZGA transgenic samples in the range of 55.2–81%, which is significantly lower than WGA plants. In general, aphid propagates parthenogenetically under favorable condition, which sparks a huge population in a very short time. Therefore, reduction of fecundity by plants expressing ZGA would be of considerable significance in controlling the rapidly growing aphid population.

However, at present, the mechanisms underlying the reduction in larval biomass of insects fed on lectins are not clear. Previous studies demonstrated that GNA and ConA bind to the soluble and brush border membrane enzymes in the midgut of *Lacanobia oleracea* [29]. It was suggested that binding of lectins to these receptors may decrease the permeability of the membrane. Different from the mechanism that results in mortality, the effect on fecundity seemed to depend on the influence of the lectin to the physiology of the insects. A depression in feeding was found when *Lipaphis erysimi* was allowed to feed on ALAL mustard plants [50]. Starvation might be caused by the deterrent effect, and accordingly affect the survival, development of *M. persicae* [21], and fecundity of *L. oleracea* [53]. In the present study, we also found such a feeding depression, some of the aphids moved around the leaf surface when treated on transgenic plants, while such a phenomenon was not found on the untransformed tobacco plants. More thoughtful analyses

would better explain and ensure the mechanisms' effect on the survivability and fecundity of the insects in our further study.

In conclusion, the present study demonstrate that, in small-scale trials carried out under controlled environment conditions, expression of *zga* transgenic tobacco plants afford significant levels of protection against aphids, one of the most serious insect pests causing significant yield losses of crops. These results suggest that the *zga* gene can be fruitfully employed in genetic engineering for enhancing insect resistance as well as for the projected demands for production by increasing crop yield.

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