

Enhancement of resistance to aphids by introducing the snowdrop lectin gene *gna* into maize plants

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In order to enhance the resistance to pests, transgenic maize (*Zea mays* L.) plants from elite inbred lines containing the gene encoding snowdrop lectin (*Galanthus nivalis* L. agglutinin; GNA) under control of a phloem-specific promoter were generated through the *Agrobacterium tumefaciens*-mediated method. The toxicity of GNA-expressing plants to aphids has also been studied. The independently derived plants were subjected to molecular analyses. Polymerase chain reaction (PCR) and Southern blot analyses confirmed that the *gna* gene was integrated into maize genome and inherited to the following generations. The typical Mendelian patterns of inheritance occurred in most cases. The level of GNA expression at 0.13%–0.28% of total soluble protein was observed in different transgenic plants. The progeny of nine GNA-expressing independent transformants that were derived separately from the elite inbred lines DH4866, DH9942, and 8902, were selected for examination of resistance to aphids. These plants synthesized GNA at levels above 0.22% total soluble protein, and enhanced resistance to aphids was demonstrated by exposing the plants to corn leaf aphid (*Rhopalosiphum maidis* Fitch) under greenhouse conditions. The nymph production was significantly reduced by 46.9% on GNA-expressing plants. Field evaluation of the transgenic plants supported the results from the inoculation trial. After a series of artificial self-crosses, some homozygous transgenic maize lines expressing GNA were obtained. In the present study, we have obtained new insect-resistant maize material for further breeding work.

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1. Introduction

Maize (*Zea mays* L.) is a widely cultivated crop. Yet, its yield and quality are severely affected by pests. One of the major pests causing severe damage every year is aphid, a sap-sucking homopteran insect. Aphids cause mechanical harm and malnutrition to plants by removal of phloem sap. They also transmit various plant viruses such as maize mosaic stripe virus (MMSV) (Zhang and Zhong 1983). There are about 23 types of aphids such as corn

leaf aphid (*Rhopalosiphum maidis* Fitch), peach-potato aphid (*Myzus persicae* Sulzer), cotton-melon aphid (*Aphis gossypii* Glover), birdcherry-oat aphid (*Rhopalosiphum padi* L.), greenbug (*Schizaphis graminum* Rodani), English grain aphid (*Macrosiphum avenae* Fabricius), etc. Among these, corn leaf aphid is the major pest which severely affects maize production (Zhang and Zhong 1983). It has been proved difficult to control aphids by using insecticides. And indiscriminate pesticide usage is harmful to natural predators of aphids, environment and human health

Keywords. *Agrobacterium tumefaciens*; aphid; *Galanthus nivalis* L. agglutinin gene; maize; transgene

Abbreviations used: AP, Alkaline phosphatase; DI, damage index; GNA, *Galanthus nivalis* L. agglutinin; PCR, polymerase chain reaction; RDI, relative damage index; RSs-1, rice sucrose synthase-1.

(Whitten and Oakenshott 1991). Currently emphasis is on the potential of maize transformation technology using conventional breeding methods, and many useful pesticide genes have been found to engineer resistance to insect damage.

Homoptera, including aphids, mainly utilize free amino acids as a nitrogen source present in the phloem. Although proteinases such as cysteine proteinase, trypsin-like proteinase, and cathepsin-like proteinase in the gut of many homoptera show low levels of proteolysis comparing to lepidoptera and other insect groups (Foisac *et al* 2002; Cristofolletti *et al* 2003; Deraison *et al* 2004). Protease inhibitors do not act efficiently on homoptera (Hilder *et al* 1987). Earlier studies showed that the lectin from the snowdrop (*Galanthus nivalis* L. agglutinin; GNA) has a significantly detrimental effect on aphids, brown planthopper (*Nilaparvata lugens* Stl) and other homopteran insects (Habibi *et al* 1993; Powell *et al* 1993; Rahb and Febvay 1993; Down *et al* 1996; Sauvion *et al* 1996; Tang *et al* 1999; Sun *et al* 2001). In those bioassays, the effects of GNA on aphid development included a reduction in growth, delayed onset of nymph reproduction, and, subsequently, lower nymph production per aphid. The possible mechanism of lectin toxicity in insects seems to involve binding of lectin to the gut surface resulting in local lesions on the gut (Eisemann *et al* 1994). Since there is no conclusive evidence of GNA toxicity towards higher animals, care is necessary when we use it. Introducing *gna* under control of a phloem-specific promoter to cereal plants would be a good practice, and potential useful transgenic crops may be produced.

There is still no report on integrating *gna* gene into maize genome, yet. We have generated transgenic maize plants from elite inbred lines containing the gene encoding GNA (Wang *et al* 2005). Since a homopteran is a phloem feeder, protein especially expressing itself in the phloem tissue would be delivered efficiently to the pest. This will minimize any potentially undesirable accumulation of the protein in other parts of the transgenic plant as well as decreasing the risk of potential detrimental effects on human beings. The rice sucrose synthase-1 (RSs-1) promoter can direct phloem-specific expression and it was used by our group to promote *gna*.

Here, we report the production of transgenic maize plants expressing GNA in a phloem tissue-specific manner using *A. tumefaciens*-mediated method and the resistance of transgenic plants to aphids.

2. Materials and methods

2.1 *Agrobacterium tumefaciens* strain, vector and transformation

Agrobacterium tumefaciens strain AGL0, containing the

standard binary vector pWRG815 (figure 1) constructed by Hu (2000), was used in all experiments. The T-DNA region has a target gene *gna* driven by the phloem-specific promoter RSs-1 and a hygromycin phosphotransferase selective gene (*hpt*) conferring resistance to hygromycin, which is driven by the cauliflower mosaic virus (CaMV) 35S promoter. Outside the T-DNA region, there is another selective gene encoding resistance to spectinomycin (*Spec^R*) for the selection of *A. tumefaciens* by spectinomycin. The *A. tumefaciens* strain and the vector were provided by the Institute of Genetics of Fudan University, Shanghai, China.

Embryogenic type II calli (Li *et al* 2001) derived from the immature embryos of three elite maize inbred lines (DH4866, DH9942, and 8902) were used as materials for *A. tumefaciens*-mediated transformation. In all experiments, bacterial cell densities were adjusted to an optical density (OD₆₀₀) of 0.20 before callus infection. Obtaining type II callus, transformation, selection of transformed calli, and regeneration of plantlets were according to the methods of Quan *et al* (2004). Transformants were selected on selective medium containing 20 mg/l hygromycin; T₀ (plants regenerated from transgenic type II calli),

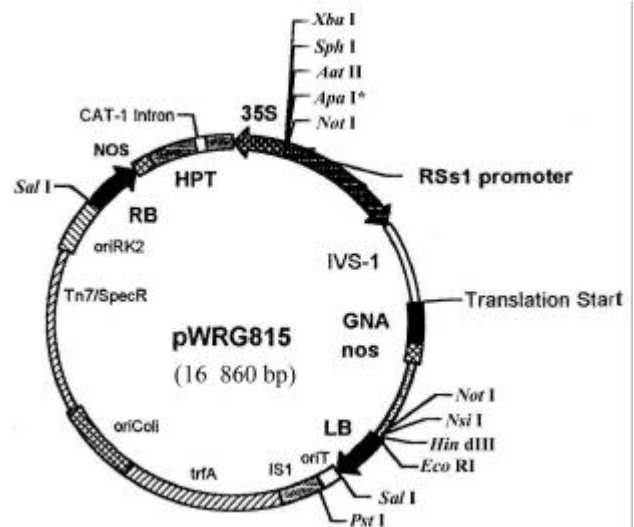


Figure 1. Schematic representation of the plasmid used in maize transformation. The T-DNA region of the vector has a *Galanthus nivalis* L. agglutinin gene (*gna*), driven by the phloem-specific rice sucrose synthase-1 promoter (RSs-1), and a hygromycin phosphotransferase gene (*hpt*) conferring resistance to hygromycin, which is driven by the promoter CaMV35S. Outside the T-DNA region, there is a spectinomycin-resistance gene (*Spec^R*) for the selection of the *Agrobacterium tumefaciens* by spectinomycin. CAT-1 is a modified version of the castor bean catalase gene intron; NOS is a nopaline synthase terminator sequence; LB and RB mean the left and right T-DNA border sequences respectively. **Apa* I should be used at 30°C.

T₁, and T₂ plants were artificially selfed to obtain the next generation.

2.2 Polymerase chain reaction and Southern blot analyses

Polymerase chain reaction (PCR) and Southern blot analyses were used to identify positive transformants. The DNA was prepared from leaf tissue according to the methods of Ausubel *et al* (1995a). For PCR analysis, we used primer pair-specific for the *gna* gene coding sequence as follows: forward primer (GNA1) 5'-ATGGCTAAGGCAGT-CTCCTC-3'; reverse primer (GNA2) 5'-TCATTACTTT-GCCGTCACAAG-3'. The PCR conditions were as described previously (Wu *et al* 2000). The DNA from non-transgenic plants and plasmid pWRG815 was used as a negative and positive control, respectively.

For Southern blot analysis, 15 µg aliquots of DNA were digested with *Xba*I, which recognizes a single site out of the RSs-1-*gna* sequence in the T-DNA region, and fractionated in 0.8% agarose gel. Transfer onto Hybond-N⁺ nylon membrane (Roche Applied Science, Mannheim, Germany) and hybridization were performed according to standard procedures (Ausubel *et al* 1995b). The *gna*-specific probe (generated by PCR) was labelled using a digoxigenin (DIG)-high prime DNA labelling and detection starter kit I (Roche Applied Science, Mannheim, Germany).

2.3 Antibody production and Western blot analysis

Anti-GNA antibodies were raised in rabbits using purified GNA protein (Sigma Chemical, St Louis, MO, USA) through standard protocols (Ausubel *et al* 1995c). The potency test (Ausubel *et al* 1995c) showed that the polyclonal antiserum diluted 1 : 10,000 in Tris-buffered saline (TBS; 50 mmol/l Tris-HCl buffer, pH 7.2, 0.15 mol/l NaCl) containing 0.01% (v/v) Tween-20 could detect 100 pg GNA.

Young leaves (1 g) were homogenised in phosphate-buffered saline (PBS) buffer (137 mmol/l NaCl; 2.7 mmol/l KCl; 4.3 mmol/l Na₂HPO₄·7H₂O; 1.4 mmol/l KH₂PO₄), in the presence of 1% (v/v) 2-mercaptoethanol and 10% glycerol. The extract was centrifuged at 2400 g for 5 min at 4°C and the supernatant was collected. The protein concentration of each sample was estimated using a standard Bradford assay (Ausubel *et al* 1995d). After boiling for 2 min, aliquots of 50 µg total protein were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% acrylamide) under reducing conditions (Ausubel *et al* 1995d). The protein was transferred to a polyvinylidene difluoride (PVDF) membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA) using

the BioRad semi-dry transfer system and, thereafter, the membrane was soaked in normal donkey serum blocking solution (2% donkey serum, 1% BSA, 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween 20, 0.05% sodium azide, 0.01 mol/l PBS, pH 7.2) for 2 h at 37°C. The membrane was first probed with polyclonal rabbit anti-GNA antiserum (1 : 10,000 dilution) followed by alkaline phosphatase (AP)-conjugated donkey anti-rabbit secondary antibody (Promega, Madison, WI, USA; 1 : 20,000 dilution). Incubation and washing procedures were carried out according to Ausubel *et al* (1995c). The membrane was incubated in the Western Blue stabilized substrate for AP (Promega) in darkness until bands developed. A semi-quantitative estimation of GNA protein expression levels was obtained by comparing the intensity of bands in the lanes representing tissue samples to standards containing known amounts of purified GNA protein. Blots were subjected to densitometric scan and integrated band areas were used to construct a calibration curve. Data were analysed with Glyko BandsScan software (Glyko, Novato, USA).

2.4 Genetic analysis of transgenic maize plants

PCR-positive T₀ (plants regenerated from transgenic type II calli), T₁ and T₂ plants were artificially selfed to produce the next generation. Twenty-seven transgenic T₀ lines containing a single-copy insertion were selected for genetic analysis. In each generation, 30 plants per line were analysed by PCR as described before and all the plants of a line were derived from an ear. The frequencies of PCR-positive transgenic plants in T₁, T₂ and T₃ generations were examined. According to Mendelian inheritance patterns, for one locus integration, the ratio of *gna*-containing plants to non-*gna*-containing ones should fit the expected 3 : 1 in T₁ generation, 3 : 1 or 1 : 0 (progenies of a homologous line) in T₂ generation, and 3 : 1 or 1 : 0 in T₃ generation.

2.5 Aphid bioassay

Corn leaf aphid nymphs were obtained from a continuous laboratory culture maintained on maize plants at 25°C in an insect culture room, and nymphs produced in the 24 h period were collected for further bioassays. T₂ and T₃ plants from nine independently derived transgenic maize homozygous lines (line Nos A1, A2, A3, B1, B2, B3, D1, D2, D3; A from DH4866, B from DH9942, and D from 8902), together with untransformed maize controls (A0, B0, D0), were challenged by corn leaf aphids and investigated for their effects on aphid survival and the development of aphid population using a protocol essentially the same as described by Hilder *et al* (1995). Plants were

grown inside air-conditioned green house, fertilized and irrigated according to the standard procedure. During the bioassay test, each plant was confined to an insect-proof nylon cage and maintained at 28°C day/20°C night. This system allowed the aphids access to the entire plant, but confined them to a single plant. At the 3rd leaf stage, five neonatal nymphs were introduced with a hair brush to each maize plant on day 0 and the insect survival and population growth were measured at 1-day intervals for a 16-day period. Based on immunoblot staining intensity, the GNA-expression levels in leaf tissue of T₂ plants were approximately at 0.28% (A1), 0.26% (A2), 0.24% (A3), 0.26% (B1), 0.27% (B2), 0.22% (B3), 0.24% (D1), 0.25% (D2), and 0.26% (D3) total soluble protein. In each generation 20 plants were included per line.

The food consumption of aphids has also been studied. After being kept in an empty box for 2-day starvation treatment, 30 corn leaf aphids of 14-day old were inoculated on each plant at the 3rd leaf stage. T₂ plants from the nine transgenic lines mentioned above and untransformed control plants from A0, B0, and D0, were used. After 24 h, the secretory honeydew was quantitatively measured using the ninhydrin test methods of Sun *et al* (2001).

2.6 Field evaluation

T₂ plants from nine GNA-expressing transgenic lines, namely A1, A2, A3, B1, B2, B3, D1, D2, and D3, were evaluated for aphid resistance in the field. Untransformed plants from original inbred-lines A0, B0, and D0, acted as controls. The test field was divided into ten blocks and each block was subdivided into twelve plots that were then randomly assigned to one of the twelve maize lines. Ten plants were planted on each plot. Five plots selected at random for each line were surveyed during the aphid blooming season (in the late July in North China) and all plants within the selected plot were included in the sample. The plants were at the grain-filling stage during this period. On the whole maize plant, aphids preferred feeding on ear husk, ear-site leaf and tassel, especially gathered on the ear-site leaves. After preliminary scoring, we found the number of aphids infesting on the axial surface of the ear-site leaf highly representatives the aphid infestation on the whole plant, so we counted the aphids on the axial surface of the ear-site leaf as representative score for whole plant. A damage score from 0 to 4 depending on the population size of the aphids was given to each plant, where 0 = no aphid; 1 = a few aphids and a little honeydew; 2 = small scattered colonies having a lot of honeydew and no obvious discoloration; 3 = dense colonies and mild leaf discoloration; 4 = dense colonies and severe leaf discoloration. The damage index (DI) and the relative damage index (RDI) were calculated as follows:

(1) $DI = \sum (\text{damage score} \times \text{the number of plants receive-}$

$\text{ing the score}) / (\text{total number of plants} \times \text{the highest score given in the group}).$

(2) $RDI = DI \text{ of the tested transgenic line} / DI \text{ of control}$ RDI was used to determine the aphid resistance of the transformed plants. RDI higher than 0.40 means being sensitive to aphids (represented by S); RDI between 0.25 and 0.40 means middle-resistance to aphids (represented by MR); RDI lower than 0.25 means resistance to aphids (represented by R) (Zhang 1992; Wang and Guo 1999; Xiao *et al* 2001; Teng *et al* 2002). The same evaluation was performed in the T₃ generation.

Agricultural characteristics such as plant height, plant shape and ear site of these T₂ and T₃ plants were also recorded individually and compared with untransformed plants from the three original lines.

3. Results

3.1 Maize transformation

More than 200 regenerated transgenic plants were obtained from three maize inbred-lines DH4866, DH9942, and 8902. Under the same transforming manipulation, the efficiencies of transgenic plantlet production were at 5.9% (DH9942), 6.7% (8902), and 12.3% (DH4866) respectively. The calculation was the number of PCR-positive regenerated plants divided by the number of initial infected calli. Only one plant from a survival callus after selection was taken for count. Genotype showed an effect on the transformation efficiency.

3.2 Molecular analysis of transgenic plants

Transgenic plants from the three maize inbred-lines DH4866, DH9942, and 8902 were identified. In PCR assays, the expected 500-bp band was obtained from T₀ to T₃ plants (data not shown). Southern blot analysis of 36 PCR-positive primary transformants confirmed the presence of the *gna* gene in the plant genome. Most of the transformants showed a single hybridization band with the *gna*-specific probe. But the size of the band was different from each other, indicating their independent transgenic status. Only four of the 36 independent transformants showed two bands, and one showed three bands. Representative results of Southern blot in cases of single copy integration are shown in figure 2. In order to avoid possible effects such as gene silencing caused by multicopy insertion, only transformants with a single-copy insertion were used in the GNA expression analysis and insect resistance tests.

Twenty-four T₀ transgenic plants at the 11th leaf stage were subjected to immunoblot analyses and leaf tissue of their 8th leaf was used. The transgenic plants expressed a

12 kDa polypeptide corresponding to the GNA protein standard, and the expression level varied from 0.13% to 0.28% total soluble protein, which was based on immunoblot staining intensity. Nine T_0 plants with the highest expression levels of GNA, namely A1, A2, A3, B1, B2, B3, D1, D2 and D3, were selected and self-fertilized (A from DH4866, B from DH9942, and D from 8902). Isogenic transgenic lines for *gna* were identified in T_2 and T_3 generations by PCR and Southern blot. The 8th leaves of their progenies (T_1 , T_2 , and T_3 plants) at the 11th leaf stage were assayed for GNA expression levels by immunoblot analyses. Compared with the parent, there was no significant difference in progeny plants in GNA expression levels. Figure 3a shows representative results of Western blot analysis of T_1 , T_2 and T_3 plants.

We selected 10 GNA-expressing plants at the 13th leaf stage and extracted total soluble protein from each leaf separately. Semi-quantitative estimations of the samples were performed by Western blot. The results showed that GNA expression level in the 6th leaf (the first to the fifth leaf had perished) was lowest, which nearly could not be detected by Western blot. The GNA expression levels in the 10th and 11th leaf were highest, which were about 0.25% total soluble protein (figure 3b,c).

3.3 Genetic analysis

Genetic analysis confirmed Mendelian segregation of the *gna* gene in most of the transgenic lines by χ^2 -square. However, three out of the 27 lines tested did not obey the

Mendelian segregation (A8, B5, and B7). For A8, it obeyed the Mendelian segregation in T_1 generation; however, only six out of 30 plants tested were PCR-positive in its T_2 generation, and only two out of 30 plants were PCR-positive in T_3 generation. Lines B5 and B7 did not obey the Mendelian segregation even in T_1 generation and the transgene *gna* was lost in T_2 generation. The mechanism of the abnormal genetic phenomena is still unclear and the representative genetic analysis data is shown in table 1.

3.4 Aphid bioassay

The aphid bioassays were performed on T_2 and T_3 young plants of nine independent transgenic lines. The surviving adult aphids and progeny nymphs produced were recorded. The results from T_2 generation are summarized in figure 4. The survival frequency of inoculated insects on the plants over the assay period (16 days) was ca. 64%, and there was not obvious difference between the GNA-expressing plants and the non-transgenic plants (figure 4a,b). In contrast, the fecundity of the aphids that developed on the plants showed a strong dependence on GNA expression, as shown in figure 4c. Control plants showed a mean of ca. 22 nymphal aphids per plant. However, aphid number on GNA-expressing plants was drastically reduced, with reductions ranging from ca. 40.9% to ca. 63.6% in different lines. When data for GNA – (control plants) and GNA + (GNA-expressing plants) were pooled (using GNA expression as a grouping variable), the reduction in nymphs per plant was shown to be statistically

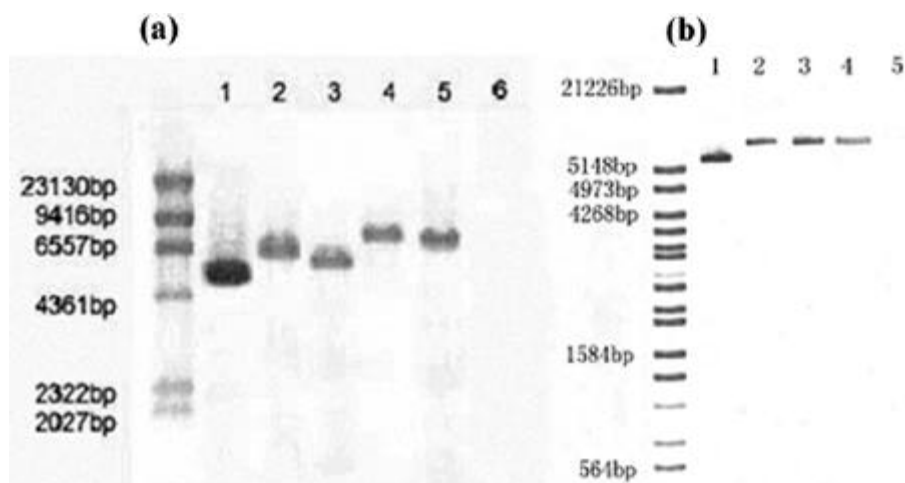


Figure 2. Representative Southern blot results of transgenic genomic DNA digested with *Xba*I and probed with the coding sequence of the *Galanthus nivalis* L. agglutinin gene (*gna*). Lambda *Eco*RI/*Hind*III marker was used. (a) Lane 1, positive control (RSs-1-*gna* fragment from plasmid pWRG815); lanes 2–5, T_1 plants coming from four individual transgenic events; lane 6, negative control (untransformed plant). (b) Lane 1, positive control (RSs-1-*gna* fragment from plasmid pWRG815); lanes 2–4, T_1 , T_2 , and T_3 plants coming from a T_0 plant; lane 5, negative control (untransformed plant).

significant at $P < 0.01$ (by student's *t*-test), with a mean reduction of 46.9% (figure 4d). The development of aphids was also retarded by GNA. Aphids inoculated on the GNA-expressing plants took ca. 7.4 days to reach maturity and start to produce nymphs, which was 2.2 days longer than that of the negative control group (ca. 5.2 days). In the food consumption assay, compared with non-transformed controls, the quantity of secretory honeydew in T₂ lines A1, A2, A3, B1, B2, B3, D1, D2, and D3, was reduced by 64%, 57%, 42%, 55%, 62%, 32%, 38%, 45%, and 52%, respectively. GNA exhibited an antifeedant effect with aphids. Results from the aphid toxicity bioassay in T₃ generation (data not shown) were similar to the results of T₂ generation.

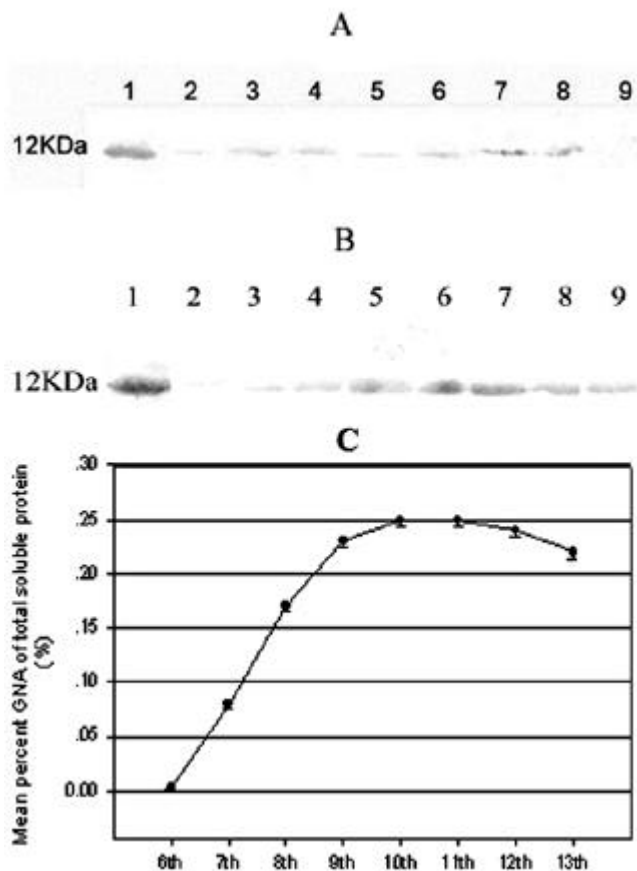


Figure 3. (a) Representative results of Western blot analysis of leaf extracts from T₂ and T₃ plants. Equal amounts of total soluble protein (50 µg) were loaded into each lane. Lane 1, purified GNA protein (500 ng); lanes 2–8, transgenic plants (lanes 2–4, T₁ plants; lanes 5–6, T₂ plants; lanes 7–8, T₃ plants); lane 9, untransformed plant. (b) The Western blot results of different leaves on one GNA-expressing plant. Lane 1, Purified GNA protein (500 ng); lanes 2–9, protein extracted from the 6th to 13th leaf (from left to right in turn) of the same transgenic plant. (c) Chart of expression of GNA in different leaves from 10 plants at 13-leaf stage (mean ± SE).

The data of the field investigation are given in tables 2 and 3. Compared with the untransformed control, the percentage of plants getting a score of 5 in the transgenic lines dropped by 87.8% (T₂ generation) and 86.3% (T₃ generation). Figure 5 shows a typical aphid-resistant transgenic plant and a control one. All these results indicate that the GNA-expressing plants can reduce the population density efficiently by retarding the development and reducing the fecundity of aphids.

Compared with original elite inbred-lines, the transgenic plants (from T₂ to T₃ generations) had no obvious change in agricultural traits such as plant height, plant shape and ear position. We did not find any obvious alteration in the combining ability of these progenies from transformed plants also.

4. Discussion

Developing a genotype-independent transformation system is a major goal of transgenic work. However, all the methods that lead to successful modification of plant genome today are still affected by the genotype of the acceptor plant. Although high transgenic efficiencies (5%–50%) were reported when using model plant materials such as maize inbred line A188 (Schlappi and Hohn 1992; Escudero *et al* 1996; Ishida *et al* 1996) and hybrid line Hi-II (Hi-II parent A × Hi-II parent B, each of these parents is derived from tissue culture selection of an A188 × B73 line; Zhao *et al* 2000; Frame *et al* 2002) in *A. tumefaciens*-mediated transformation, the transgenic efficiency was low or even zero in many other inbred-lines, especially in the elite inbred-lines of cultivated maize (Shen *et al* 1993; Escudero *et al* 1996; Ishida *et al* 1996). In this study, using *A. tumefaciens*-mediated method, snowdrop lectin gene *gna* driven by a phloem specific promoter R_{Ss}-1 was successfully introduced into embryogenic calli derived from immature embryos of three elite maize inbred-lines DH4866, DH9942 and 8902. The transgenic efficiencies were less than 12.3%, and genotype showed an obvious effect on the transformation efficiency. Fertile transgenic plants were regenerated after antibiotic selection. The genetic transformation system was very efficient for maize crop improvement because the elite inbred-lines were selected as acceptor genotypes.

Southern blot analyses revealed that, out of the 36 transformants, four had two-copy insertion of the transgene, and one had three-copy insertion. It is not uncommon for *Agrobacterium*-mediated transformation to result in the integration of multiple copies of the transgene (Kumar and Fladung 2000). A possible explanation is illegitimate recombination acts as the major mechanism by which multi-copy transgene integration is brought about in *Agrobacterium*-mediated transformation (Iyer *et al*

2000) or/and the multiple position insertion of transgene in the plant genome. After insertion of transgene, further rearrangements or eliminations may occur, for example by duplication or deletion during meiosis, which seems to frequently happen during plant development (Matzke *et al* 1999). However, a single mechanism is unlikely to account for all the different repeat structures seen resulting from *Agrobacterium*-mediated transformation.

Most transgenic lines showed normal Mendelian segregation. However, three out of 27 test lines did not show Mendelian segregation in T₁ and/or T₂ generation. This may be explained by self-DNA protection system. Self-DNA protection systems occur in all higher organisms, and transgene might be recognized as invader and either modified to make it non-functional or eliminated from the genome. The ability to excise foreign DNA that has integrated into the genome is a very effective defense against this potentially mutagenic event. Mechanisms for detection of integrated foreign DNA include the recognition of aberrant structural features, sequence repeats and anomalous sequence composition, location within the genome ("position effect"), and the disruption of normal genome functions. However, relatively little is known

about the processes by which transgene DNA is integrated into the plant genome (Iyer *et al* 2000).

Earlier studies on artificial diet bioassays showed that GNA had significant detrimental effect on the development of several species of aphids, such as the peach-potato aphid (Sauvion *et al* 1996) and the glasshouse potato aphid (*Aulacorthum solani* Kaltentbach; Down *et al* 1996). It was reported that potatoes expressing GNA under the constitutive promoter CaMV 35S significantly reduced the fecundity of aphids (Down *et al* 1996; Gatehouse *et al* 1996). Transgenic plants with RSs-1-*gna*, such as tobacco (Shi *et al* 1994), tomato (Wu *et al* 2000), rice (Rao *et al* 1998; Sudhakar *et al* 1998) and wheat (Stoger *et al* 1999), have also shown a specific expression in phloem and gained an effective protection against homopteran pests including aphid, brown planthopper and others. Evidences showed that GNA expression at a level above 0.04% of total soluble protein in transgenic wheat plants had a significant effect ($P < 0.02$, by Student's *t*-test) on the fecundity of grain aphid, which could be linked to expression levels of the foreign protein in different transgenic lines of wheat (Stoger *et al* 1999). In other artificial diet bioassays, the effect of GNA on rice

Table 1. Genetic analysis of transgene *gna* in transgenic plants and their offsprings.

Line ^a	Progeny ^b	Total number of plants tested	Number of PCR-positive plants	Expected segregation	X ²	P
A5	T ₁	30	19	3 : 1	0.242	> 0.05
	T ₂	30	18	3 : 1	0.400	> 0.05
	T ₃	30	19	3 : 1	0.242	> 0.05
A8* ^c	T ₁	30	18	3 : 1	0.400	> 0.05
	T ₂	30	4	3 : 1	6.760	< 0.01
	T ₃	30	2	3 : 1	8.301	< 0.01
B1	T ₁	30	21	3 : 1	0.044	> 0.05
	T ₂	30	30	1 : 0	0.00	> 0.995
	T ₃	30	30	1 : 0	0.00	> 0.995
B5*	T ₁	30	2	3 : 1	8.301	< 0.01
	T ₂	30	0	—	—	—
B7*	T ₁	30	4	3 : 1	6.760	< 0.01
	T ₂	30	0	—	—	—
D3	T ₁	30	21	3 : 1	0.044	> 0.05
	T ₂	30	30	1 : 0	0.00	> 0.995
	T ₃	30	30	1 : 0	0.00	> 0.995

^aThe correspondence between the codes and the original inbred lines is as follows: A represents DH4866, B represents DH9942, and D represents 8902.

^bT₀ plants (plants regenerated from transgenic type II calli) selfed to produce T₁ plants; T₁ and T₂ plants selfed to produce the next generation. Plants of a line were derived from an ear. According to Mendelian inheritance patterns, for one locus integration, the ratio of *gna*-containing plants to non-*gna*-containing ones in each line should fit the expected 3 : 1 in T₁ generation, 3 : 1 or 1 : 0 (progenies of a homologous line) in T₂ generation, and 3 : 1 or 1 : 0 in T₃ generation.

^c*Represents line which did not show Mendelian segregation of *gna*.

brown planthopper survival showed a clear dose response curve (Powell *et al* 1995), and artificial diet bioassays with other aphids have also shown that the insecticidal effects of GNA increase with concentration, although very low levels of the protein can cause stimulation of growth (Sauvion *et al* 1996). Results obtained from maize lines, containing the RSs-1-*gna* construct, cannot be extrapolated to establish any correlation between expression levels with insecticidal effects, since expression levels determined for these lines are from the whole leaf

tissue, and GNA in these lines is only expressed in phloem cells. Since aphids are phloem feeders, the expression level determined for this line must greatly underestimate the level of GNA delivered to the aphid via the phloem sap. In late July and August in the North China, which is the infestation season for corn aphids, maize plants enter their grain filling stage. On a whole plant, aphids were found to prefer to feed on positions such as ear husk, leaves near to ear, and tassel. The pattern of the GNA mean expression levels from the 6th to

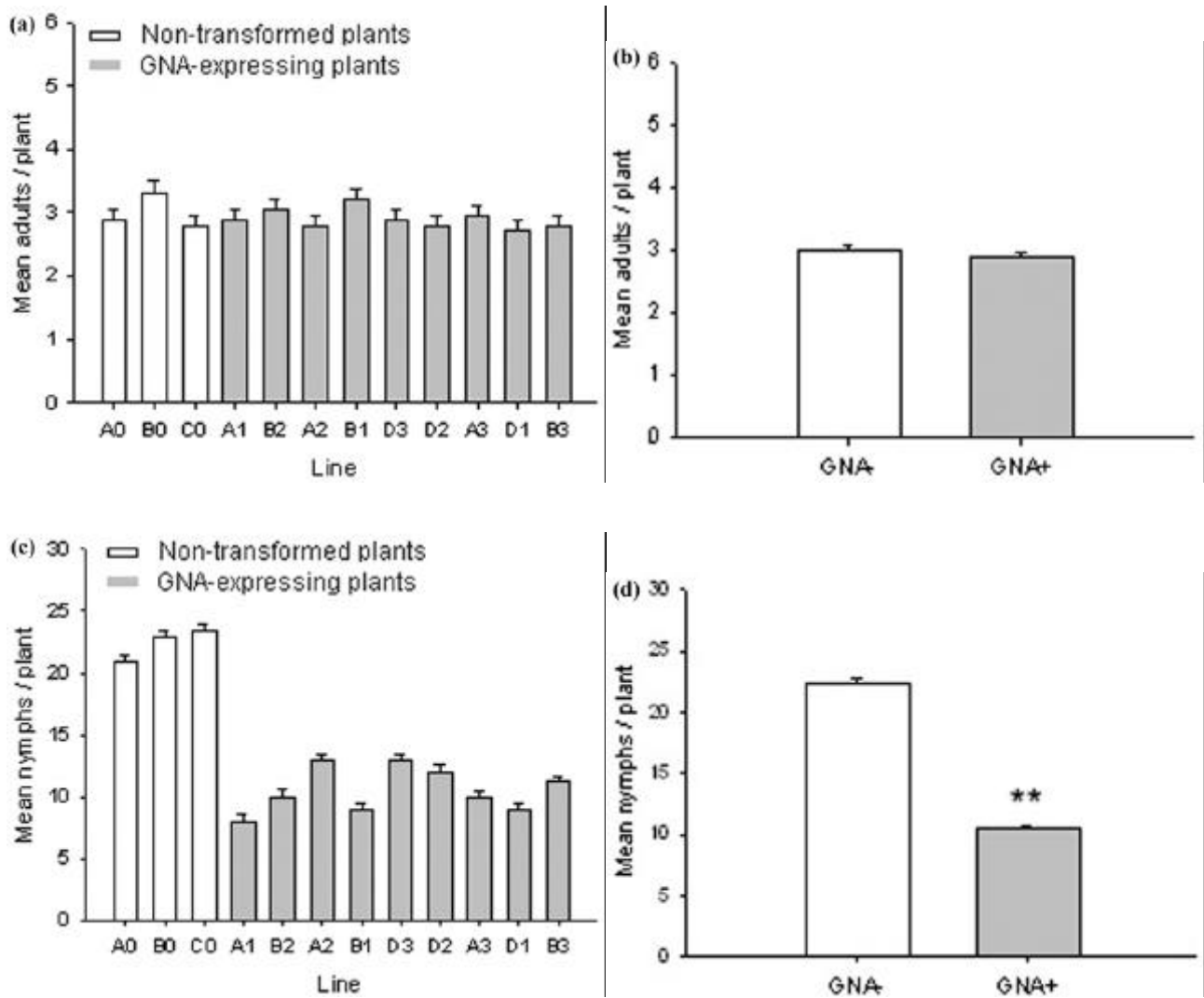


Figure 4. Survival and fecundity of aphids reared on GNA-expressing maize plants. Five neonatal aphids were transferred to each plant, and survival of these aphids to adulthood and their production of nymphs were determined 16 days later (allowing to distinguish aphids between the first and second generation clearly). Each line consisted of 20 plants. In (a) and (c), the GNA-expressing plants on the x-axis are arranged in order of decreasing expression levels. (a) Survival of adult aphids per plant. (b) Pooled analyses for total adults survival per plant, where data from all control plants and data from transgenic plants expressing GNA above 0.20% total soluble protein are pooled as GNA- ($n = 60$) and GNA+ ($n = 180$) respectively. (c) Total nymph production per plant. (d) Pooled analyses for total nymph production per plant, where data from all control plants and data from transgenic plants expressing GNA above 0.20% total soluble protein are pooled as GNA- ($n = 60$) and GNA+ ($n = 180$) respectively. **Highly significant difference at $P < 0.01$ (by Student's *t*-test).

Table 2. Field evaluation of aphid resistance of T₂ plants.

Line ^a	Mean per centage GNA of total soluble protein ^b	Damage index	Relative damage index ^c
A1	0.28	0.21	R
A2	0.26	0.21	R
A3	0.24	0.24	MR
A0	0	0.86	–
B1	0.26	0.25	MR
B2	0.27	0.29	MR
B3	0.22	0.29	MR
B0	0	0.82	–
D1	0.24	0.24	MR
D2	0.25	0.23	MR
D3	0.26	0.20	R
D0	0.00	0.92	–

^aFifty plants per line from nine isogenic transgenic lines were examined. Non-transgenic plants from their original inbred lines A0, B0, and D0, were used as negative controls. Data were collected in the late July in North China, which is the aphid blooming season and all the plants in the evaluation were at the grain-filling stage.

^bThe 8th leaf from five plants at 11-leaf stage per line were selected to estimate the GNA-expressing level.

^cS means the relative damage index (RDI) > 0.40, being sensitive to aphids; MR means 0.25–RDI 0.40, middle-resistance to aphids; R means RDI < 0.25, being resistant to aphids.

Table 3. Field evaluation of aphid resistance of T₃ plants.

Line ^a	Mean percentage GNA of total soluble protein ^b	Damage index	Relative damage index ^c
A1	0.28	0.21	R
A2	0.27	0.21	R
A3	0.24	0.23	MR
A0	0	0.87	–
B1	0.26	0.26	MR
B2	0.27	0.24	MR
B3	0.22	0.25	MR
B0	0	0.84	–
D1	0.22	0.24	MR
D2	0.26	0.22	MR
D3	0.24	0.20	R
D0	0.00	0.88	–

^{a,b,c}See footnote of table 2.

the 13th leaves showed that the younger mature leaves (9th–12th) have the highest GNA concentration (above 0.23% total soluble protein). Ears will produce from the axil of the 10th, the 11th, or the 12th leaf that aphids usually infest. These leaves have higher GNA expression levels within the whole plant and will provide a better resistance to aphids as well as reducing the yield lost.

In the present study, the GNA expression levels in leaf tissue varied from 0.13% to 0.28% total soluble protein.

The progeny plants from these parental transformants showed enhanced resistance to the inoculated corn leaf aphids, significant at $P < 0.01$. Although the survival of aphids reared on the GNA-expressing plants was not affected, their fecundity showed a strong dependence on GNA expression. Aphid reproduction was markedly reduced on the GNA-expressing plants. The onset of nymph production of aphid reared on the GNA-expressing plants was about 2.2 days delayed, and this might partly account

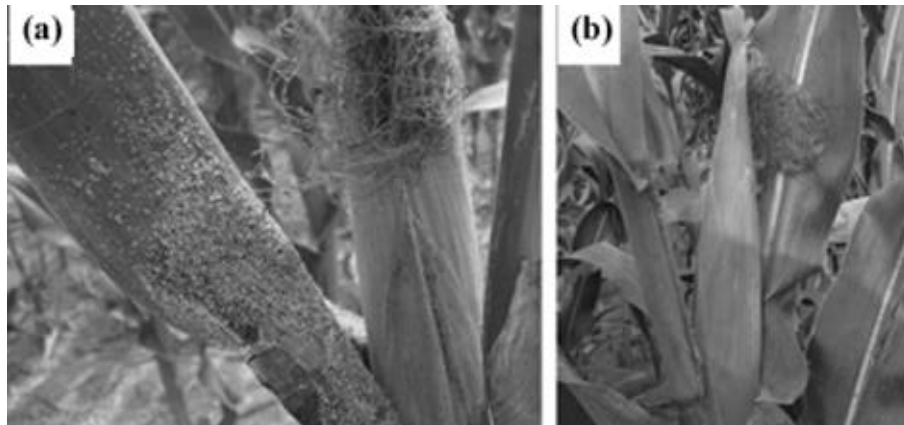


Figure 5. Influence of the snowdrop lectin transgene *gna* in T_2 maize plant in the field. Aphid population on the ear-site leaf was examined. (a) Nontransgenic plant (negative control plant). (b) A typical aphid-resistant GNA-expressing plant.

for the lower nymph number per plant in the 16-day period bioassay. These results were comparable with earlier data of the effects of GNA on aphids and other homopterans using *in vitro* assays (Down *et al* 1996; Sauvion *et al* 1996; Stoger *et al* 1999). The resistance of transgenic maize plants to aphids was significantly improved. Many investigations showed that GNA exhibited a multimechanistic mode of action having both antifeedant (Powell *et al* 1995) and systemic toxic effects (Powell *et al* 1998) against brown plant hopper. In this study, GNA also showed an antifeedant effect on aphids. Since aphid is responsible for the transmission of a number of plant viruses, the antifeedant property of GNA enhanced the resistance of plants to some virus diseases by reducing the feeding frequency of aphids (unpublished data).

After a series of artificial self-crosses, some homozygous transgenic lines highly expressing GNA (above 0.20% of total soluble protein) were obtained. These lines have shown significant resistance to aphids. The agricultural traits and combining ability of their original inbred-lines have been stably inherited in most cases. Thus, we have obtained new maize self-lines with resistance to aphids for further breeding work.

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