

Transgenic Potato Overexpressing the *Amaranthus caudatus* Agglutinin Gene to Confer Aphid Resistance

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ABSTRACT

Potato (*Solanum tuberosum* L.) was transformed with a pBCACAc vector harboring the *Amaranthus caudatus* agglutinin (ACA) gene mediated by *Agrobacterium tumefaciens* (Smith and Townsend 1907) Conn. One hundred and eighteen of a total 320 regenerated plants were confirmed to possess the ACA gene integrated into the potato genome as detected by polymerase chain reaction (PCR) and Southern blot analysis. Three transgenic events (referred to as PA1, PA2, and PA3) with one copy of the ACA gene insertion and no obvious phenotypic change were selected and further evaluated for expression level of ACA protein and performance in insect bioassays. Based on western blot assays, the content of ACA protein in PA1, PA2, and PA3 events was high at 0.11, 0.32, and 0.29%, respectively, of total soluble leaf protein. The propagation of peach-potato aphids (*Myzus persicae* Sulzer) in the three events was significantly inhibited, with 49, 18.5, and 25 aphids per plant, respectively, compared to 102.5 aphids per plant in nontransformed (NT) controls based on insect bioassays in greenhouse trials. The results of a field trial (in Beijing in 2009) showed that the number of aphids was significantly lower in transgenic plants than in NT. Tuber weight and plant yield of the three transgenic events were significantly higher than those of NT. Our results indicated that PA2 and PA3 can be regarded as potential germplasm to breed new cultivars that may decrease production loss from resistance to aphids and reduced indirect virus damage.

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Abbreviations: ACA, *Amaranthus caudatus* agglutinin; Bt, *Bacillus thuringiensis*; CAS, Chinese Academy of Science; cDNA, complementary DNA; CoYMV, Commelina Yellow Mottle Virus; ELISA, enzyme-linked immunosorbent assay; GNA, *Galanthus nivalis* agglutinin; MS, Murashige and Skoog; NT, nontransformed; OD, optical density; PCR, polymerase chain reaction; PLRV, Potato leaf roll virus; PVY, Potato virus Y; T-DNA, transferred DNA.

POTATO (*Solanum tuberosum* L.) is a staple global food. However, it is highly vulnerable to crop pests and frequently damaged by insect predation and indirect virus infection by homopteran insects. Conventional breeding techniques and traditional insect and virus control measures have limited potential to reduce insect and virus damage as these methods are time consuming, cumbersome, and often unsustainable. Thus, it is useful to devise novel strategies to incorporate effective and durable insect resistance traits in plants (Davies, 1996). Introduction of insect resistance genes into a plant's genome is crucially important and needs to be employed to increase the yield and productivity of potato plants. To attain an effective and durable control against insect attack, it appears promising to introduce genes possessing insecticidal properties (Perlak et al., 1993). Among other genes, Bt [*Bacillus thuringiensis* (Berliner)] genes have been successfully cloned and characterized in numerous transgenic plants to develop protection against lepidopteran pests. The expression of genes encoding the Bt toxin in transgenic potato is now well known to protect against insects such as tuber moth

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(*Phthorimaea operculella* Zeller) and Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Perlak et al., 1993). Aphids are small plant-eating and sucking insects that belong to the super family Aphidoidea (McGavin, 1993). They inflict devastating damage to plants by sucking juice from the phloem tissues. The *Galanthus nivalis* agglutinin (*GNA*) gene has been reported to enhance resistance against aphid attack by reducing fecundity and longevity (Down et al., 1996). Transgenic *GNA* overexpressing potato (Bell et al., 2001), rice (*Oryza sativa* L.) (Nagadhara et al., 2004), and wheat (*Triticum aestivum* L.) (Stoger et al., 1999) have significantly inhibited the growth rate of homopteran pest populations (peach-potato aphids [*Myzus persicae* Sulzer] and plant hoppers [*Nilaparvata lugens* Stal]).

The *Amaranthus caudatus* agglutinin (*ACA*) gene has a function similar to that of *GNA*; however, the underlying mechanism to control aphid damage has not been reported extensively. A specific agglutinin from *Amaranthus caudatus* L. seeds has been reported to enhance the mortality rate of aphids (Rahbe et al., 1995). Additionally, it adversely affects the aphid's growth and developmental stages at a threshold concentration ($68 \mu\text{g mL}^{-1}$), demonstrating that *ACA* has an immense potential to be used against aphid attack (Rahbe et al., 1995). Previously, a full-length complementary DNA (cDNA) of the *ACA* gene was cloned in our laboratory and was integrated into tobacco (*Nicotiana tabacum* L.) through an *Agrobacterium* transformation system that caused enhanced resistance against peach-potato aphids (Guo et al., 2004). We also introduced *ACA* into a cotton (*Gossypium hirsutum* L.) genome that resulted in the development of immunity response against cotton aphid nymphs (*Aphis gossypii* Glover) (Wu et al., 2006). Accumulating evidence suggests that the *ACA* is an anti-aphid gene and a candidate gene to be used to produce transgenic

plants that resist aphid attack and to reduce yield loss due to pests (Wu et al., 2006). Thus, to achieve those goals, we developed the transgenic *ACA* potato.

The present investigation was conducted to produce transgenic potato transformed with the *ACA* gene and to determine if it exhibits the capacity to reduce the growth rate and minimize the survival of aphids feeding on the plants. Our results show that *ACA* transgenic potatoes exhibit relatively more resistance against aphid attack compared to a nontransformed (NT) control. Moreover, the yield losses due to aphid attack were lower in transgenic potatoes than that in NT plants.

MATERIALS AND METHODS

Plants and Insects

Cultured potato plants of the aphid susceptible cultivar 'Ezihuabai' were generously provided by Professor Peng Xuexian, Institute of Microbiology, Chinese Academy of Science (CAS), Beijing, China. Stem sections from shoot cultures were maintained to use as explants. Stem internode pieces were excised from shoots and used to generate transgenic potato plants. Peach-potato aphids were collected from a greenhouse of CAS located in Beijing, China, for aphid bioassays.

Construction of Plant Expression Vector and Transformation of Potato

Full-length cDNA of *ACA* gene (930 bp; GenBank accession no. AY048755) was cloned from *A. caudatus* (Guo et al., 2004), digested with *Bam*H I and *Sal* I, and ligated into a binary vector pBC438 containing *Bam*H I and *Sal* I restriction sites according to a method proposed by Yuan et al. (2001). The *ACA* gene was driven by the phloem-specific promoter from Commelina Yellow Mottle Virus (CoYMV) (Yuan et al., 2001) (Fig. 1). The resulting vector called pBCACAc was transformed into *Escherichia coli* DH5 α that was further verified by performing the same restriction endonuclease digestion assay. After confirmation, pBCACAc was transformed into *Agrobacterium tumefaciens* LBA4404 (An, 1987).

Successful transformation of potato was performed according to a method suggested by Newell et al. (1991) with minor modifications. *Agrobacterium tumefaciens* LBA4404 harboring pBCACAc was allowed to grow for 36 h at 28°C in Luria broth medium (Sambrook and Russell, 2001) supplemented with 50 mg L⁻¹ kanamycin, 50 mg L⁻¹ streptomycin, and 25 mg L⁻¹ rifampicin. The LBA4404 bacteria were resuspended in half-strength liquid Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) at an optical density of 600 nm (OD₆₀₀) = 0.4 before co-cultivation. Stem internode pieces were excised from shoots and were immersed in the resuspended bacterial culture for 5 to 10 min. Stem internode pieces were transferred onto sterile filter paper that was put on the surface of solid MS containing 2.6 g L⁻¹ phytigel (Sigma, St Louis, MO) and were placed at 22°C in the dark for 48 h. Then the stem internode pieces were transferred to solid MS supplemented with 100 mg L⁻¹ kanamycin and 400 mg L⁻¹ cefotaxime and put at 24°C in 16/8 h light/dark. Shoots were derived from stem internode pieces that had been infected with the *Agrobacterium* culture.

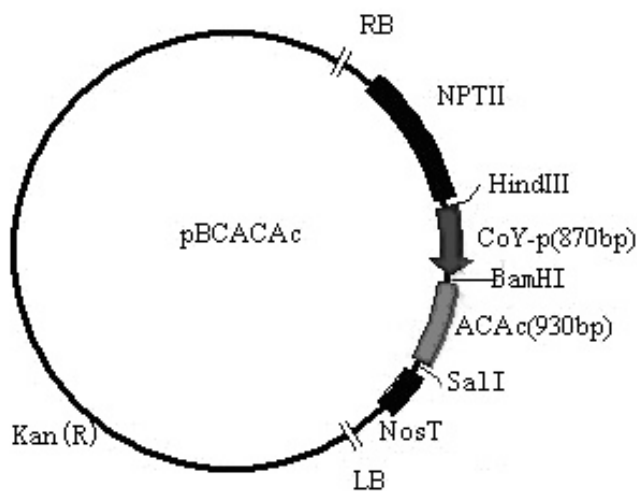


Figure 1. Schematic diagram of the transferred DNA (T-DNA) structure of the plant expression vector pBCACAc NPT II, neomycin phosphotransferase gene; CoY-p, Commelina Yellow Mottle Virus promoter; Nos T, transcriptional termination sequence of nopaline synthase gene; LB, left border of T-DNA; RB, right border of T-DNA.

Then the putatively transformed shoots were rooted on the solid medium containing MS salts, 100 mg L⁻¹ kanamycin, and 400 mg L⁻¹ cefotaxime. Plants were regenerated from the selected shoots and grown to maturity in the greenhouse.

Polymerase Chain Reaction Detection and Southern Blot Analysis

High quality DNA was isolated from the leaves of putatively transformed and control potato plants for performing polymerase chain reaction (PCR) and Southern blot assays. The coding sequence of the *ACA* gene was amplified using the PCR Screening Kit (TaKaRa, Dalian, China) with forward (5'-CCATGGCAGGATTAC-CAGTG-3') and reverse (5'-TAGTTGTTGGATCCCAATTC-3') primer pair, and the size of amplicon was determined to be 912 bp. Analysis of the amplicons was done by electrophoresis with 1.0% agarose gels treated with ethidium bromide staining. For Southern blot hybridization, genomic DNA of potato was digested with *Hin*-*d*III, which recognized a single site in the CoY-p-*ACA* sequence in the transferred DNA (T-DNA) region (shown in Fig. 1), and electrophoresed on 1.0% argrose. The DNA was transferred onto nylon membrane Hybond-N⁺ (Amersham, Buckinghamshire, UK) and the blot was performed according to a method proposed by Sambrook and Russell (2001). The fragment of the *ACA* gene was amplified by PCR as above as the probe in hybridization. The probe labeling and membrane hybridization was done according to a method reported by Guo et al. (2004).

Western Blot Analysis and Enzyme-Linked Immunosorbent Assay

Total proteins were isolated from the fresh young leaves of the three transgenic events, and 50 µg total soluble proteins or 50 ng *ACA* protein induced in *E. coli* per lane were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to a method described by Sambrook and Russell (2001). After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride membrane. The filter was labeled with polyclonal rabbit anti-*ACA* serum (1:3000 v/v) produced by our laboratory as a primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega, Madison, WI; 1:5000) as a secondary antibody. The immunoblot reaction pattern was scanned by an Acer 320P scanner (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and analyzed with IMAGEMASTER software (Amersham Pharmacia Biotech Inc.). The *ACA* protein content in the transgenic events was further confirmed by enzyme-linked immunosorbent assay (ELISA) according to Sambrook and Russell (2001) using the same primary and secondary antibodies as the western blot assay.

Evaluation of Insect Survival and Population Development on Transgenic Events in Greenhouse

The insect survival and population bioassays were done after allowing aphid feeding on the potato plants. The tubers of transgenic potato events and NT were planted in 16 cm pots filled with soil containing two parts clay and one part vermiculite in the greenhouse in Beijing in 2009. Fifteen-day-old transgenic and NT potato plants after tuber germination, about 10 cm tall, were used to assess anti-aphid metabolic effects of *ACA*. Three independent transgenic

potato events (8 plants per event) were infested with aphids that were collected from a greenhouse population that was maintained on potato plants, and the leaves infested with aphids were shielded with a 30 mesh screen cage. For insect survival and population development assays, five aphids were released on each plant in the evening, and observations were recorded every 3 d up to 21 d after infestation. The same trial was replicated three times in a greenhouse. Data were obtained to determine the inhibition rate of aphid population development, according to the calculation described by Yuan et al. (2001). The data were subjected to ANOVA (SAS Institute, 2004) to test for differences between transgenic and control plants.

Evaluation of Insect-Resistance and Agronomic Performance of Transgenic Events in the Field

Further evaluation of the inhibition of transgenic plants to aphid population growth was performed through artificial infestation and natural infestation of peach-potato aphids in the field in Beijing in 2009. The materials tested included three *ACA* transgenic events (PA1, PA2, and PA3) and the NT cultivar 'Ezihuabai' as a susceptible control. The tubers of these events were sown in a randomized complete block designed plot with three replicates. Each plot consisted of 100 plants grown in four rows. The distance between plants in a row was 25 cm and the distance between rows was 50 cm. No insecticide was applied for aphid control during the entire growing season. Each potato plant was infested with five peach-potato aphids at 30 d after germination. The number of aphids was counted at 25 d after infestation. The plant height, number of branches per plant, number of tubers per plant, mean weight per tuber, and yield per plant were measured at maturity. Data on plant height, branches, tubers, tuber weight, plant yield, and inhibition of aphid propagation of the three transgenic events and NT control were analyzed by ANOVA (SAS Institute, 2004) for randomized complete block designs. Means were separated with a Duncan's multiple comparison test at $p < 0.05$.

RESULTS

Construction of the Plant Expression Vector and Potato Transformation and Regeneration

Potato stem internode pieces inoculated with *A. tumefaciens* LBA 4404 harboring pBCACAc, produced regenerating shoots with a frequency of 65% in the absence of selection and 12% on medium containing 100 mg L⁻¹ kanamycin monosulfate. Plants regenerated on the kanamycin-containing medium were further assayed for kanamycin resistance by excising new shoots and inserting into medium containing an increased level of kanamycin. Of a total 320 regenerated plants, 118 (37%) were kanamycin resistant. Thus the transformation efficiency of the explants (stem internode pieces) was about 4.4%.

Polymerase Chain Reaction and Southern Blot Analyses

Polymerase chain reaction analysis revealed that the kanamycin-resistant regenerated plants were all positive for the

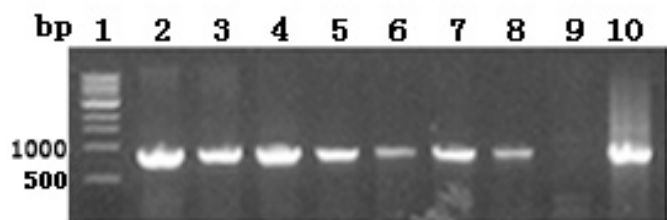


Figure 2. Polymerase chain reaction (PCR) analysis of transgenic potato plants. Lane 1: DNA ladder marker; lane 2 to 8: independent transgenic *Amaranthus caudatus* agglutinin (ACA) gene potato plants; lane 9: nontransformed (NT) plant; lane 10: pBCACAc vector.

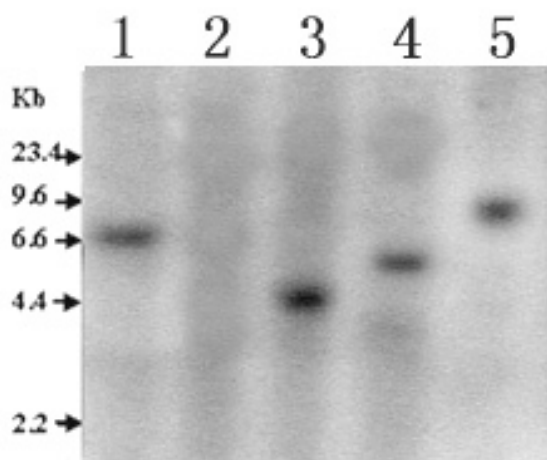


Figure 3. Southern blot assay of the three transgenic potato events used in this study. The pBCACAc plasmid and transformed potato DNA were digested with Hind III (only one Hind III restriction site is present within the transferred DNA [T-DNA] region). The *Amaranthus caudatus* agglutinin (ACA)-specific probe was polymerase chain reaction (PCR) amplified from pBCACAc vector. Lane 1: pBCACAc vector; lane 2: nontransformed (NT) plant; lane 3 to 5: independent transgenic ACA potato plants, PA1, PA2, and PA3, respectively; DNA markers are shown on the left side.

ACA gene (Fig. 2). The result of Southern hybridization analysis suggested that the ACA gene had been incorporated into potato genomic DNA. Three transgenic potato events with single copy ACA gene insertion (Fig. 3) and no obvious phenotypic changes were selected as the representative events for further studies and are referred to as PA1, PA2, and PA3. The different hybridization patterns (Fig. 3) also indicated that the three events resulted from different independent transformation events.

Western Blot Analysis

Immunoblot analysis of the three transgenic events exhibiting the desired agronomic and morphological traits showed that ACA protein was invariably present in their leaf proteins (Fig. 4). The content of ACA protein was estimated to be about 0.11, 0.32, and 0.29%, respectively, of total soluble protein in the leaves of these transgenic plants. To further confirm the ACA protein level in transgenic events, ELISA was

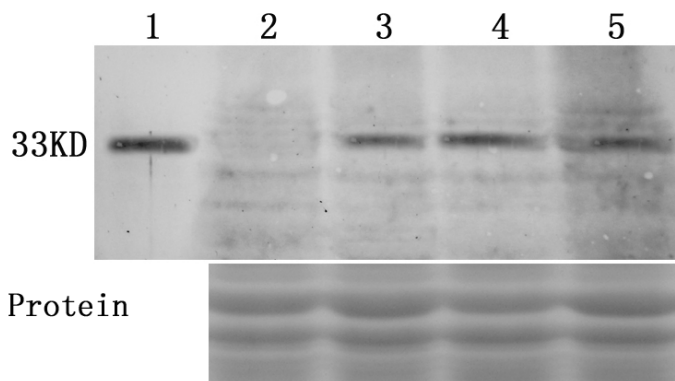


Figure 4. Western blot analysis of total protein isolated from leaves of transgenic potato events. Upper portion: The total protein of leaves from potato plants was loaded at 50 μ g per lane; 50 ng *Amaranthus caudatus* agglutinin (ACA) protein expressed in *Escherichia coli* was used as a positive control. Lane 1: ACA protein; lane 2: nontransformed (NT) plant; lane 3 to 5: independent transgenic ACA potato plants, PA1, PA2, and PA3, respectively; protein size is shown on the left side. Lower portion: the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel of potato total proteins stained with Coomassie brilliant blue as a control of amount of protein loading.

performed as a supplementary trial. The amount of ACA was calculated by subtracting the OD value of NT control leaf extract from the three transgenic events (Supplemental Fig. S1). The result of ELISA showed that the content of ACA protein in transgenic events was consistent with that estimated from western blot analysis. These results confirmed that the ACA gene was not only inserted into genomes of the transgenic events but also translated into ACA protein. Because the CoYMV promoter is phloem specific, the expression level of ACA protein in phloem tissue should be theoretically higher than that detected in whole leaf tissues.

Evaluation of Insect Resistance and Agronomic Performance

To assess the aphid resistance of transgenic events, five peach-potato aphids were infested on the surface of a single leaf of each 15-d-old plant planted in a greenhouse. The observation of aphid growth and count of aphid population were performed at 3-d intervals up to 21 d. Host resistance was determined 21 d after infestation and the inhibition rate of aphid population growth was calculated. We observed that the aphids that fed on the three transgenic events displayed a retarded growth and reduced fecundity and longevity, though data were recorded only for aphid number. The mean numbers of aphids collected from each plant throughout the assay are presented in Fig. 5. Aphid numbers were lower in three selected transformed events than those of the control ($p < 0.05$). On the leaves of the control, aphid number increased from 5 to 102.5 (20.5 times greater) per plant over a 21-d period whereas it increased relatively slowly from the five aphids per plant to 49, 18.5, and 25 aphids, respectively, on

plants of transgenic events, PA1, PA2, and PA3. Based on the data illustrated in Fig. 5, the inhibition rates of aphid population growth of three transgenic events compared with NT control were 52.0, 81.9, and 75.5%, respectively.

To further assess the inhibiting activity of transgenic potato events expressing ACA protein to peach-potato aphids in the field, a field trial of the three transgenic potato events (PA1, PA2, and PA3) was conducted to investigate their aphid resistance and agronomic performance. The numbers of aphids per plant in PA1, PA2, and PA3 were 52, 6, and 13 aphids at 25 d post infestation while that in the NT control was 162 aphids, illustrating higher inhibition of transgenic events to aphid population growth compared with the NT control (7th column in Table 1). Therefore, the inhibition rates of aphid population growth of three transgenic events were 78.0, 96.3, and 92.0%, respectively. The result of aphid resistance in the field trial was similar to that in the greenhouse trial. Under field conditions, without application of pesticide, tuber weight and yield per plant in three transgenic events were significantly higher ($p < 0.05$) than those in the NT control. However, based on the data shown in Table 1, all three transgenic potato events displayed insignificant differences in agronomic performance for plant height, branch number, and tuber number in comparison with the NT control, 'Ezihuabai'.

DISCUSSION

Based on the statistics of FAO (FAO, 2008), among all the food crops in the world, the potato product ranks the fourth crop, behind corn (*Zea mays* L.), rice, and wheat. However, major reduction in the quantity and quality of potato is caused by insect damage, especially aphid damage due to sucking juice of plants and spreading virus. Improving insect resistance has been an important plant breeding goal for a long time. Several potentially useful insecticidal proteins have been identified and protocols are being optimized to confer sustainable control over insects and pests when expressed in transgenic potato (Bell et al., 2001; Davies, 1996; Down et al., 1996; Newell et al., 1991; Perlak et al., 1993). One of the best examples is snowdrop lectin (GNA). Bell et al. (2001) reported that GNA-expressing potato significantly reduced the levels of pest damage. Though they showed that transgenic GNA potato plants augment the control against Bright-line Brown-eye moth (*Lacanobia oleracea* Linnaeus), no data available showed that GNA could resist peach-potato aphids. Here we report

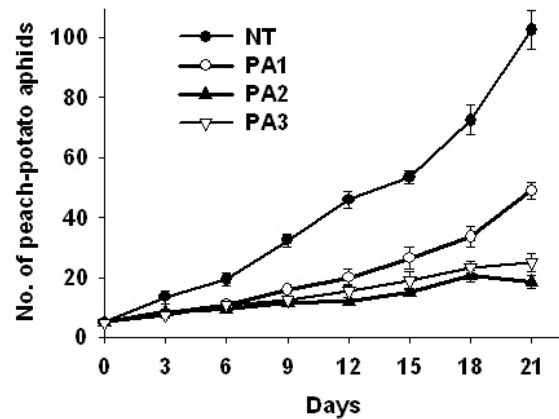


Figure 5. The number of surviving aphids in transgenic and control potato events in the greenhouse trial.

for the first time that transgenic potato plants expressing the ACA gene impart a potentially effective check on potato aphid population growth. This finding paves the way toward further research regarding progress and development to incorporate ACA-mediated insect resistance into the desired plants.

We obtained 26 transgenic potato events with one foreign gene insertion and acceptable agronomic traits and selected three events as representatives to evaluate the aphid resistance of the ACA protein. The content of ACA protein in PA2 and PA3 was higher than that in PA1 by western blot assay and ELISA; therefore, the number of aphids surviving in PA2 and PA3 plants was significantly lower than that in PA1 plants. The yield per plant in PA2 and PA3 events was significantly higher than that in the PA1 event likely due to reducing number of the aphids.

The effect of virus infection has always been a serious problem for potato growers and breeders. Besides feeding damage, aphid vectors are also mainly responsible for spread of common viruses, namely Potato virus S (PVS), Potato virus Y (PVY), Potato leaf roll virus (PLRV), and so on. Potato virus S and PVY are both contact and nonpersistently aphid-transmitted types, while PLRV is persistently aphid transmitted (Paul, 2004). The effect of viruses is as crucially important as direct insect damage, because losses due to the viruses and allied pathogens are not confined to the year of infection (Paul, 2004). In our study, the usefulness of transgenic potato events in the control of potato aphids through field trials verified that the ACA gene could

Table 1. The agronomic traits and aphid resistance of transgenic potato events and nontransformed (NT) control in a field trial conducted in Beijing in 2009. One hundred plants of each event in a replicate were investigated and the experiment was conducted in three replicates.

Lines [†]	Plant height (cm) [‡]	No. of branches per plant [‡]	No. of tubers per plant [‡]	Mean weight per tuber (g) [‡]	Yield per plant (g) [‡]	Mean no. of aphids per plant [‡]
PA1	59.5 ± 4.2a	3.7 ± 0.3a	5.9 ± 0.7a	105 ± 13.5b	620 ± 38.5b	52 ± 22.6b
PA2	60.2 ± 4.7a	3.2 ± 0.2a	6.8 ± 0.7a	121 ± 17.2c	823 ± 48.5d	6.6 ± 4.8c
PA3	58.6 ± 3.6a	3.5 ± 0.4a	6.4 ± 0.6a	116 ± 15.8bc	743 ± 42.6c	13 ± 6.5c
Ezihuabai (NT)	58.9 ± 4.4a	3.4 ± 0.3a	6.2 ± 0.5a	88 ± 16.6a	546 ± 31.5a	162 ± 51.5a

[†]Values are given as means ± standard deviation.

[‡]Means within a column followed by different letters are significantly different at $p < 0.05$.

be an effective candidate gene for genetic modification of plants against aphid attack. Producing ACA transgenic plants shows promise to reduce the population of aphids; this, in turn, would prevent the virus spread to some extent.

Aphid population growth on transgenic potato plants in the field trial was less than that in the greenhouse trial. This suggests that aphids can move from resistant to the susceptible plants in the field, while they cannot freely migrate among the plants for the plant shielded by screens in the greenhouse. Although the three transgenic events showed greater inhibition of aphid population growth compared with NT in the field, the number of surviving aphids among the transgenic plants from the same events showed huge differences, which may be due to lack of uniformity of aphid infestation. Distribution of aphids on plants in the field is not uniform according to several reports (Gibson, 1972; Hodgson, 1978; Kidd, 1976). Thus, five aphids per plant were supplemented by artificial infestation to ensure all plants were infested by aphids. Because aphids can move among leaves and plants, it is difficult to assess some life parameters of aphids inoculated in plants, such as survival of infested aphids, mortality, mean daily offspring, and so on. However, it is easy to count the total number of aphids on plants, and thus we can evaluate the aphid resistance of the transgenic potato events only by this parameter.

Because the aphids suck the juice of plant by inserting their stylet into the phloem, we used a phloem-specific promoter, CoYMV promoter, to drive the ACA gene expression. Thus the content of ACA protein in phloem is theoretically higher than those in other tissues, which can better control aphid damage compared with a constitutive expression promoter. Subject to food safety and environmental safety assessments, the two transgenic events, PA2 and PA3, with high content of ACA protein, good agronomic performance, and high aphid resistance can be used as cultivars or as germplasm to breed new cultivars to control aphid damage.

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