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Shuangxia Jin
University of Central Florida

Xianlong Zhang
University of Central Florida

Henry Daniell
University of Central Florida

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Pinellia ternata agglutinin expression in chloroplasts confers broad spectrum resistance against aphid, whitefly, *Lepidopteran* insects, bacterial and viral pathogens

Shuangxia Jin, Xianlong Zhang† and Henry Daniell*

Department of Molecular Biology and Microbiology, College of Medicine, University of Central Florida, Orlando, FL, USA

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**Correspondence* (fax +1 407 823 0956; email daniell@mail.ucf.edu)
†Present address: National Key Laboratory of Crop Genetic Improvement, Huazhong Agricultural University, Wuhan, Hubei 430070, China.

Keywords: Broad spectrum biotic stress tolerance, chloroplast transformation, antimicrobial, insect resistance.

Summary

Broad spectrum protection against different insects and pathogens requires multigene engineering. However, such broad spectrum protection against biotic stress is provided by a single protein in some medicinal plants. Therefore, tobacco chloroplasts were transformed with the agglutinin gene from *Pinellia ternata* (*pta*), a widely cultivated Chinese medicinal herb. *Pinellia ternata* agglutinin (PTA) was expressed up to 9.2% of total soluble protein in mature leaves. Purified PTA showed similar hemagglutination activity as snowdrop lectin. Artificial diet with purified PTA from transplastomic plants showed marked and broad insecticidal activity. In planta bioassays conducted with T0 or T1 generation PTA lines showed that the growth of aphid *Myzus persicae* (S*ulzer*) was reduced by 89%–92% when compared with untransformed (UT) plants. Similarly, the larval survival and total population of whitefly (*Bemisia tabaci*) on transplastomic lines were reduced by 91%–93% when compared with UT plants. This is indeed the first report of lectin controlling whitefly infestation. When transplastomic PTA leaves were fed to corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) or the beet armyworm (*spodoptera exigua*), 100% mortality was observed against all these three insects. In planta bioassays revealed *Erwinia* population to be 10 000-fold higher in control than in PTA lines. Similar results were observed with tobacco mosaic virus (TMV) challenge. Therefore, broad spectrum resistance to *homopteran* (sap-sucking), *Lepidopteran* insects as well as anti-bacterial or anti-viral activity observed in PTA lines provides a new option to engineer protection against biotic stress by hyper-expression of an unique protein that is naturally present in a medicinal plant.

Introduction

Agricultural productivity losses caused by herbivorous insects have been estimated to be 10%–20% for major crops grown worldwide (Ferry *et al.*, 2004). Among these herbivorous insects, whitefly and aphids are the most destructive species. Both of them are phloem sap-sucking insects that could obtain nutrients from plant sap, causing significant loss of production. They also transmit various plant viruses through the sap such as the maize mosaic stripe virus (MMSV) (Wang *et al.*, 2005) and tobacco mosaic virus (TMV). Among different types of aphids, peach-potato aphid (*Myzus persicae Sulzer*) is the major destructive pest, which severely affects cotton, tobacco, potato and many vegetable crops (Radcliffe and Ragsdale, 2002). The whitefly *Bemisia tabaci* (*Gennadius*) is the most serious pest in subtropical regions of the world. The B biotype of *Bemisia tabaci* was introduced into the United States in the 1980s (Brown *et al.*, 1995; Ellsworth and Martinez-Carillo, 2001) and causes major losses in cotton (*Gossypium* spp.) and other important crops. Currently, the control of aphid and whitefly populations is mainly dependent upon the use of chemical insecticides, but this has led to the development of insecticide-resistance. In addition, the massive application of pesticides that affect beneficial organisms causes public concern on pesticide safety. Genetic-engineering methods offer solutions for introducing foreign insect-resistance genes into the crop plant genome from native plants. Proteinase inhibitors and Bt endotoxins have been introduced into crop plants and proven to be effective in controlling *Lepidopteran* insects, but these proteins minimally control *Homopteran* or the sap-sucking insects (Yao *et al.*, 2003a; Yu and Wei, 2008).

Plants are also infected by many pathogenic bacteria. *Erwinia carotovora* is one species of the genus *Enterobacteriaceae* containing mostly plant pathogenic bacteria, which causes serious damage in many crops including tomato, tobacco, carrot, potato, leafy greens, squash, green peppers and onion. The plant decay caused by *E. carotovora* is referred to as bacterial soft rot (BSR, Abo-Elyousr *et al.*, 2010).

Plants have evolved by producing insecticidal or antimicrobial proteins including lectins. Plant lectins are classified into 12 families according to their structure and evolution (Jiang *et al.*, 2010). The most commonly studied lectins are chitin-binding lectins, legume lectins, type-2 ribosome-inactivating lectins and mannose-binding lectins. Lectins bind to soluble, brush border membrane receptor proteins in the aphid gut and lead to decrease in nutrient absorption and membrane permeability (Eisemann *et al.*, 1994; Bandyopadhyay *et al.*, 2001). Recent studies show that many plant lectins exhibit insecticidal activity on the sap-sucking *homopetra* insects (Yarasi *et al.*, 2008; Shahidi-Noghabi *et al.*, 2009; Sengupta *et al.*, 2010; Shinjo

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et al., 2011). Mannose-binding lectins are especially important because they confer plant defence against insects (Yao *et al.*, 2003a; Sadeghi *et al.*, 2008; Van Damme, 2008), microbial pathogens (Ye *et al.*, 2001; Freire *et al.*, 2002; Kanrar *et al.*, 2002; Tian *et al.*, 2008; Kheeree *et al.*, 2010; Li *et al.*, 2011) and nematodes (Qi *et al.*, 2008; Bhat *et al.*, 2010).

Several mannose-binding lectin genes have been cloned from plant families including *Iridaceae*, *Alliaceae*, *Orchidaceae*, *Amaryllidaceae*, *Bromeliaceae* and *Liliaceae* (Van Damme, 2008). The *Pinellia ternata* is a traditional Chinese medicinal plant native to China, known as the crow dipper. It is naturally grown in the wild and distributed throughout China and east-Asia (Bensky *et al.*, 2004). Insect bioassay studies showed that lectins or agglutinins of *P. ternata* (PTA) had significant insecticidal activities against cotton or peach-potato aphids when incorporated into their artificial diets (Huang *et al.*, 1997; Pan *et al.*, 1998). Recently, *in vitro* assessment of PTA showed anti-pinewood nematode (Qi *et al.*, 2008) and antifungal activity (Ling *et al.*, 2010). Nuclear transformed tobacco plants also exhibited insecticidal activity against peach-potato aphids (Yao *et al.*, 2003a; Ye *et al.*, 2009), but the level of expression was low.

Similarly, most of the commercial Bt cotton expression level of the truncated CRY protein is around 0.1% of the total soluble protein (TSP) (Perlak *et al.*, 1990, 1991). By contrast, full-length native *cry* genes introduced into the plastid genome; the expression level was very high (McBride *et al.*, 1995; Kota *et al.*, 1999; De Cosa *et al.*, 2001). The highest expression level was up to 45% of the TSP, when the *cry* gene was expressed as an operon with two small open reading frames (De Cosa *et al.*, 2001). Besides the high-level expression of foreign genes, chloroplast genetic engineering exhibits several unique advantages, including transgene containment by maternal inheritance in most crops, multigene engineering in a single step as well as a lack of gene silencing, position and pleiotropic effects (Verma and Daniell, 2007; Daniell *et al.*, 2009; Rigano *et al.*, 2009; Ziegelhoffer *et al.*, 2009; Boyhan and Daniell, 2011; Davoodi-Semiromi *et al.*, 2010; Verma *et al.*, 2010; Clarke and Daniell, 2011).

In this manuscript, we demonstrate that transplastomic tobacco plants expressing the *pta* gene from *Pinellia ternata* exhibit broad spectrum resistance to biotic stress including two sap-sucking *homopetran* insects: peach-potato aphid, whitefly, three *Lepidopteron* insects: beet armyworm, corn earworm and tobacco budworm as well as the bacterial pathogen—*Erwinia carotovora* and the viral pathogen-TMV. Identification and hyper-expression of unique genes like *pta* should open up a new strategy to engineer broad spectrum resistance against biotic stress.

Results

Chloroplast vector design and transformation

To create the tobacco chloroplast expressing *Pinellia ternata* agglutinin (PTA), tobacco leaves were transformed with the chloroplast transformation vector (pLD) containing the *pta* gene by particle bombardment. Site-specific integration of the *pta* gene into the tobacco *trnI/trnA* spacer region of chloroplast genome was achieved by homologous recombination sequences as described previously (Verma and Daniell, 2007; Verma *et al.*, 2008). This *trnI/trnA* site for integration has several distinctive advantages (Daniell *et al.*, 2004). The *psbA* promoter/5′ untranslated region (UTR) increases translation under light and 3′ UTR shown to increase the stability of the transcript (Ruhlman *et al.*, 2010). Therefore, the *pta* gene is regulated by the *psbA* 5′ and 3′ UTR. The *Prrn* of 16S rRNA is a constitutive promoter, which controlled expression of the *aadA* (aminoglycoside 3′ adenylyltransferase) gene and conferred spectinomycin resistance to the plant cell (Figure 1a).

Several transplastomic lines were obtained by gene-gun bombardment as described previously (Verma *et al.*, 2008). Primary shoots emerged 4–6 weeks after selection on the RMOP medium. The shoots from the second round of selection were transferred to the hormone-free half-MSO (with 500 mg/L spectinomycin) medium for rooting and plant regeneration. After the molecular analysis, the PCR- and Southern blot-positive plants were transferred to the green house for further growth and insect or pathogen bioassays. The phenotype of the transplastomic lines was similar to the untransformed control, and all the transplastomic lines were fertile.

Transgene integration into the chloroplast genomes

PCR analysis with the primer pair 3P-3M (Figure 1a) was used to test site-specific integration of the transgene cassette into the chloroplast genome. Site-specific transgene integration resulted in a PCR product of 1.65 kb with the 3P-3M primer pair (Figure 1c). Spectinomycin-resistant mutant plants as well as the untransformed control did not show any PCR products, indicating that they are negative for transgene integration. The primer pair 5P-2M was used to confirm integration of the *pta* gene, which produced 2.44-kb PCR product. PCR result revealed the expected-size products in the transplastomic lines (Figure 1d, lanes 1–6), conforming integration of the gene of interest.

The *Sma I*-digested tobacco chloroplast DNA blots were hybridized with ^{32}P d-CTP labelled *trnI*- and *trnA*-digested flanking sequence probe (Figure 1b). This probe revealed a single 4.0-kb hybridizing fragment in the untransformed control tobacco as expected. In the PTA transplastomic lines, only a 6.5-kb fragment was observed (Figure 1e). The absence of the 4.0-kb fragment in transplastomic lines confirmed that all chloroplast genomes were transformed (subject to the detection limit of Southern blot), and therefore, they were considered to be homoplasmic.

High-level expression of PTA in transplastomic lines

Crude protein extracts from untransformed control and PTA transplastomic lines were resolved on 14% SDS–PAGE gels. Western blot analysis of mature leaf extracts from all four selected transplastomic lines (3-month-old plants) showed the presence of a single polypeptide around 12 kDa, when probed with anti-his-tag antibody (Figure 2a, lanes 1–4).

To quantify expression levels of the PTA in transplastomic lines, enzyme-linked immunosorbent assay (ELISA) was performed in all four transplastomic tobacco lines. Because the PTA was fused with 6 × His-tag, ELISA was performed using the his-tag antibodies to quantify the PTA-his-tag fusion proteins. His-tag PTA accumulated up to 5.16%–9.27% of the TSP (Figure 2b). The line C.2.1 and D2.1 showed the lowest (5.16%) and highest (9.27%) expression levels, respectively. Such variation of expression levels among these PTA lines could be due to leaf samples harvested from the greenhouse at different intensity of illumination or their developmental stages because the *psbA* promoter/UTR is regulated by light or during development.

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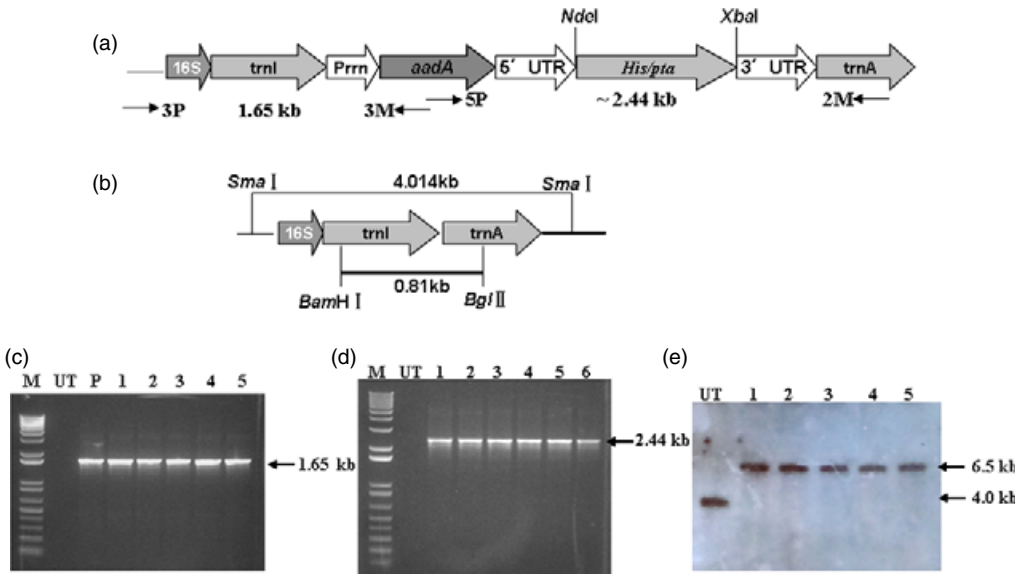

Figure 1 pLD-UTR-his- pta chloroplast transformation vector and transgene integration. The *pta* gene contained 810 bp encoding 269 amino acids. (a) primer annealing sites (3P/3M and 5P/2M) and expected products of wild-type and transplastomic lines (b) Schematic representation of the chloroplast flanking sequences used for homologous recombination, probe DNA sequence (0.81 kb), when digested with SmaI (c) and (d) PCR analysis using primer pairs 3P/3M or 5P/2M for evaluation of site-specific integration of the transgene cassette into the chloroplast genome. Lane 1–5 in (c) and lane 1–6 in (d) are transplastomic lines; UT, untransformed control; P, positive control; M, plus DNA ladder. (e) Southern blot hybridized with the flanking sequence probe. The size of left franking sequence is 1.2 kb, and the right is 1.0 kb. Untransformed chloroplast genome shows a 4.0 kb while *Pinellia ternata* agglutinin (PTA) lines show 6.5-kb hybridizing fragment.

Purification of the PTA from transplastomic lines

Purification of His-Tag PTA protein was performed using His affinity chromatography nickel-chelate-charged columns, and the purified PTA was separated by SDS–PAGE or non-reduced PAGE gels. The results showed that only one polypeptide with molecular weight around 12 kDa was observed in SDS–PAGE gels, which was similar to the previous reports (Figure 3 lane 2–7) (Van Damme *et al.*, 1995; Qi *et al.*, 2008). In the non-reduced PAGE, the PTA showed larger molecular weight (around 50 kDa) (Figure 3, Lane 8–10), confirming that the PTA formed tetramers within chloroplasts and this is essential for its biological activity. The computer analysis revealed that PTA has two putative conserved domains and three mannose-binding boxes like lectins from other *Araceae* and *Amaryllidaceae* species (Yao *et al.*, 2003b).

Agglutination activity of recombinant PTA

The agglutination activity of recombinant *Galanthus nivalis* agglutinin (GNA) purchased from Sigma Chemical Co. (St. Louis, MO) used as a positive control and PTA was carried out using serial dilutions of purified proteins. These results showed that PTA could conjugate the rabbit blood cell even at very low concentration-2 μg/mL (Figure 4). There was more conjugation with increased concentration of PTA and GNA. Most of the blood cells (>75%) were conjugated by the PTA when its concentration was 16 μg/mL or higher in the blood cell suspension, which was similar to the GNA's conjugation activity (Figure 4, Table 1). Inhibition of agglutination by sugars showed that recombinant PTA exhibited similar specificity for mannose to GNA (Longstaff *et al.*, 1998). The agglutination activity of PTA (20 μg/mL) and GNA (20 μg/mL) was inhibited by mannose at the concentration of 50–75 mM and 75 mM, respectively, but no inhibition by glucose, sucrose or maltose was observed (Table 2).

Biological activity of PTA purified from transplastomic leaves on the aphid and whitefly in artificial diet assays

Biological activity of PTA against aphids is shown in Figure 5a. These results showed that survival of nymphs were significantly lower than that of control after 24 h ($P < 0.01$). Average aphid number declined from 100 to 39.7 over a 4-day period on the artificial diet with 400 μg/mL PTA. However, on an average 80 aphids survived in the control group (approximately two-fold higher than the artificial diet, with 400 μg/mL PTA). These experiments also revealed that higher concentration of PTA showed significantly higher toxicity to the aphid. Four PTA concentrations showed significant difference in toxicity to the aphid ($P < 0.01$). Even when 50 mg/L PTA was used, the PTA still exhibited its anti-aphid activity. Furthermore, with the extension of the exposure time to the PTA, more aphids were killed on the artificial diet with PTA (Figure 5a).

For the whitefly bioassay with the same artificial diet as used for aphid, the results showed that groups with four concentrations of PTA had significantly less ($P < 0.01$) whiteflies when compared with the control group after 24 h (Figure 5b). After 96 h, the average number of whiteflies on the control artificial diet was 80.6. However, there was only an average of 47.7 on the PTA treatment when 400 μg/mL PTA was added, which was approximately 59% of the control group. Among four PTA (50,100, 200 and 400 μg/mL) groups, all showed significant toxicity to the whitefly. Higher dose showed significantly higher toxicity to the whitefly ($P < 0.01$) (Figure 5b).

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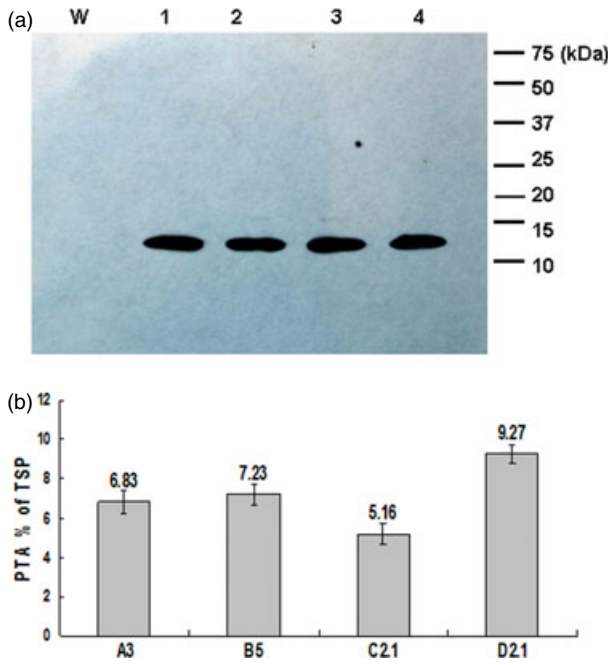

Figure 2 Western blot and quantification of *Pinellia ternata* agglutinin (PTA) by ELISA in transplastomic lines. (a) Western blot analysis of transplastomic PTA lines. Leaf total lysates from wild-type and transplastomic lines were resolved by SDS–PAGE and transferred to nitrocellulose membrane. W, wild-type; 1–4, transplastomic lines: A3, B5, C2.1 and D2.1. (b) ELISA quantification of PTA in four transplastomic lines. The recombinant his-tag protein (Gen Script Inc, Piscataway, NJ) was used as standard for the ELISA. The ELISA for the same plants from four transplastomic lines was repeated three times, and the data and bars indicate mean ± SD (standard deviation).

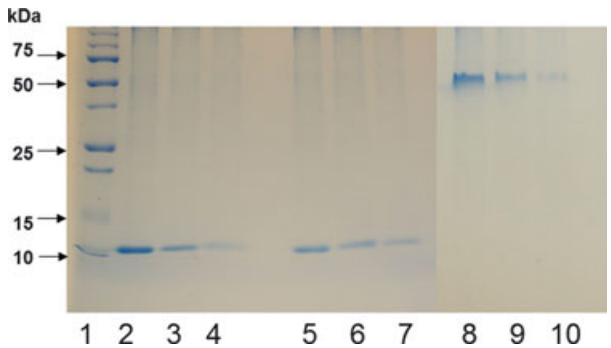

Figure 3 SDS–PAGE and non-reducing PAGE of purified *Pinellia ternata* agglutinin (PTA) protein. Lane 1: Protein standards; Lanes 2–4, the first three washes (or fractions) of the purified PTA from line C2.1; Lane 5–7, the first three washes (or fractions) of the purified PTA from line D2.1.Lane 8–10, three fractions of purified PTA on the non-reducing PAGE gel.

Effect of PTA purified from transplastomic leaves on the development of corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) and beet armyworm (*Spodoptera exigua*)

Pinellia ternata agglutinin at the concentration of 200 μg/mL impregnated into the artificial diet and with the control was investigated for their effects on larval survival and development. Larval survival ratio of corn earworm was 56.7% when reared on diet impregnated with PTA, while 86.7% on the control diet 10 days after initiating the test. For tobacco budworm, 53.3% of the larvae survived on the PTA diet when compared with 93.3% of the larvae on the control diet. Similarly, only 56.7% larvae of beet armyworm survived on the PTA diet, as compared to 93.3% in the control diet (Table 3). All the larval survival ratio of these three insects on PTA diet was significantly lower than that of control groups ($P < 0.01$).The average weight of the larvae from corn earworm, tobacco budworm and beet armyworm 10 days after initiating the experiment was 38.3, 48.8 and 114.5 mg, respectively, on PTA diet, which were significant lower than that of control diet with 86.3, 107.8 and 211.1 mg, respectively ($P < 0.01$). There was a significant reduction ($P < 0.01$) of pupa weight on PTA diet (192.5, 212.8 and 104.1 mg for corn earworm, tobacco budworm and beet armyworm, respectively) when compared with on the control diet (313.9, 286.4 and 192.5, respectively, Table 3). The larval development period of corn earworm, tobacco budworm and beet armyworm was prolonged by 2.6, 1.7 and 1.1 days, respectively, when larvae were reared on PTA impregnated diet. For the pupa development, there was 1.9, 1.7 and 2.0 days delay in larvae reared on PTA impregnated diet when compared with the control group (Table 3).

Decrease of fecundity of aphid and whitefly in two successive generations of transplastomic PTA lines

In planta bioassay was used to evaluate the biological activity of PTA in transplastomic lines. The aphid population was monitored over a 21-day period after release of 30 third instar aphids in the nylon cage (Figure 6a,b) as described previously (Jin *et al.*, 2011), and the aphid population was measured at 3-day intervals. These results showed that both transplastomic lines strongly inhibited aphid growth and fecundity. On the leaf surface of UT plants, there was heavy colonization of aphids (Figure 6c), but the PTA transplastomic plants showed negligible colonization (Figure 6d). Figure 7a shows the aphid population change during the 21-day test period of two transplastomic lines from T0 generation when compared with UT plants. At least 20 plants were evaluated from each line. The aphid population on UT plants increased steadily and soared up to a maximum on day 21 with >400 aphids per plant. On transplastomic line C2.1, the aphid population increased in the first 9 days and then decreased gradually to approximately 40 aphids per plant on day 21, which was 10.3% of the UT aphid population. The aphid population on the other transplastomic line D2.1 increased and reached its maximum on day 12 and then declined gradually to approximately 30 aphids per plant on day 21, which was only 8% of the UT aphid population.

Transplastomic T1 plants derived from the C2.1 and D2.1 were exposed to aphids using the same method to evaluate inheritance of the resistance trait. In planta bioassay revealed that transgenic T1 plants from C2.1 and D2.1 lines showed aphid survival and growth pattern similar to their T0 parents (Figure 7b).

The in planta bioassay showed that transplastomic lines (T0 generation) were resistant to whitefly (Figure 6f), while the untransformed control (UT) plants showed a heavy colonization of whitefly on the surface of the leaves (Figure 6e). The total population (including immature and adults) of whitefly on untransformed control was 1391/plant, which was 13.5

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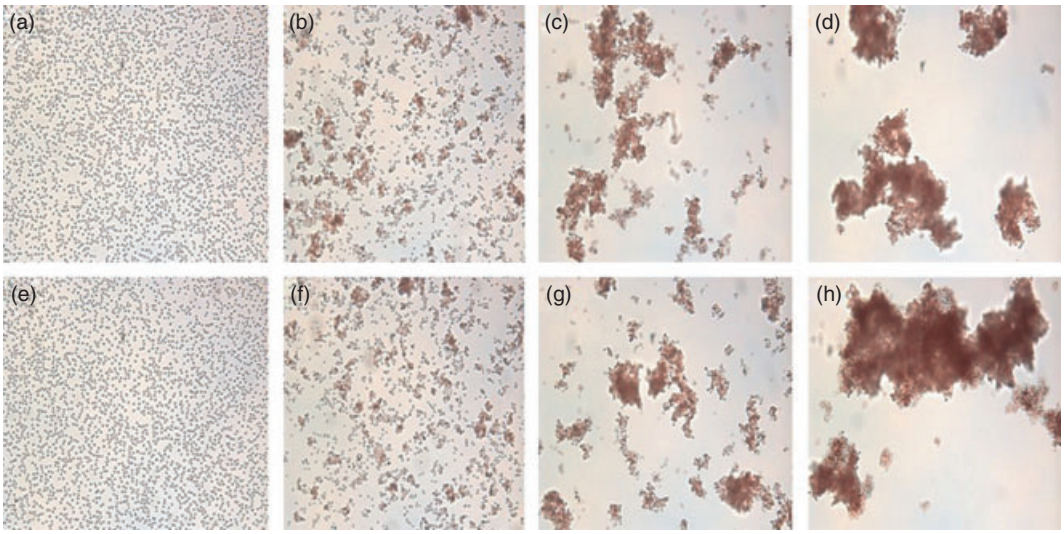

Figure 4 Hemagglutination activity of *Pinellia ternata* agglutinin (PTA) in the rabbit blood cells. (a) and (e): PBS buffer without lectin as the negative control (indicated as ck) was added into the blood cell suspension and incubated for 1 h at room temperature. All blood cells were scattered and no conjugation happened. (b-d): PBS buffer with PTA added into the blood cell suspension at a final concentration of 2, 8, 16 μg/mL, respectively. (f–h): PBS buffer with *Galanthus nivalis* agglutinin (GNA) was added into the blood cell suspension at a final concentration of 2, 8 and 16 μg/mL, respectively. Partial of blood cells was conjugated by the GNA at the concentration of 2 and 8 μg/mL. All blood cells were conjugated when 16 μg/mL GNA was added. The PTA showed similar hemagglutination activity to the GNA.

Table 1 Hemagglutination assay of *Pinellia ternata* agglutinin (PTA)

Agglutination concentration (μg/mL)	Rabbit red blood cells conjugation	
	Galanthus nivalis agglutinin	PTA
64	++++	++++
32	++++	++++
24	++++	++++
16	++++	++++
8	+++	+++
4	+++	++
2	++	++
1	+	+
0.5	+	+

Note: ++++ denotes more than 75% of cells were conjugated by PTA; +++ denotes 50%–75% of cell were conjugated; ++ denotes 25%–50% of cell were conjugated; + denotes <25% of cell were conjugated.

Table 2 Minimum concentrations of sugars required to cause inhibition of hemagglutination by *Galanthus nivalis* agglutinin (GNA) (20 μg/mL) and recombinant *Pinellia ternata* agglutinin (PTA) (20 μg/mL)

Sugar	Minimum concentration for inhibition	
	GNA (mM)	PTA (mM)
Glucose	>200*	>200
Sucrose	>200*	>200
Maltose	>200*	>200
Mannose	75	50–75†

*No inhibition at a final sugar concentration of 250 mM, the highest concentration used in this experiment.

†Data range is provided for different preparations of purified PTA.

(102/plant) and 11.7 (118/plant) times more than those in the transplastomic line C2.1 and D2.1 of T0 generation, respectively (Figure 8a). Similarly, the total population of whitefly on untransformed control was 1258/plant, which was 10.6 (119/plant) and 12.5 (100/plant) times more than those in the transplastomic line C2.1 and D2.1 of T1 generation (Figure 8b). At least 20 plants were evaluated from each line.

Insecticidal effects of PTA expressed in chloroplasts on corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) and beet armyworm (*Spodoptera exigua*)

The fourth-instar larvae of corn earworm, tobacco budworm and beet armyworm consumed almost the whole leaf disc form untransformed control within 48 h and continued to grow well (Figure 9a-1, b-1 and c-1). While feeding on PTA leaf form T0 generation plants of transplastomic line C2.1, insects consumed only small amount of leaf material, and all insects died within 4 days (Figure 9a-2, b-2 and c-2). T1 plants derived from the transplastomic C2.1 line were also challenged with all three insect species. To test the PTA insecticidal effect in the young plant, leaf samples from 5-week-old transplastomic plants (T1 generation) were used for the insect bioassays. Similarly, insects on the untransformed control leaves consumed almost the entire young leaf and grew healthy (Figure 9a-3, b-3 and c-3). For the transplastomic leaves, all insects consumed more samples than mature leaves but after 48 h, stopped feeding and died within 4 days (Figure 9a-4, b-4 and c-4).

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Resistance of transplastomic PTA lines to *Erwinia carotovora*

The anti-bacterial ability of PTA was studied by challenging with *Erwinia* soft rot pathogen (*Erwinia carotovora*) either by using syringe or sand paper inoculation method as described previously (Lee *et al.*, 2011). On the first day of inoculation with *Erwinia*, signs of damage were evident on leaves of untransformed control plants in the region of the inoculation. On the third day after inoculation, all inoculated untransformed tobacco leaves underwent severe necrosis, whereas in transplastomic PTA leaves, no or mild damage was observed. Inoculation of tobacco plants with *E. carotovora* using the sand paper method resulted in areas of necrosis surrounding the point of inoculation in untransformed control in all four cell densities –10^8, 10^6, 10^4 and 10^2 cfu/mL (Figure 10a), whereas transplastomic PTA-inoculated leaves showed no or mild necrosis (Figure 10b). Even inoculation of 10^8 cfu/mL *E. carotovora* cells resulted in no or mild necrosis in PTA transplastomic leaves. In contrast, untransformed control plants displayed clear necrosis even when inoculated with 10^2 cells cfu/mL only. Similar results were observed in bacterial inoculation using the syringe method. Transplastomic PTA leaves infected with *E. carotovora* showed a slight discoloration within the area of inoculation of 10^8 cfu/mL (Figure 10c), and untransformed control plants displayed obvious necrosis even with low concentration of bacteria-10^2 cfu/mL (Figure 10d).

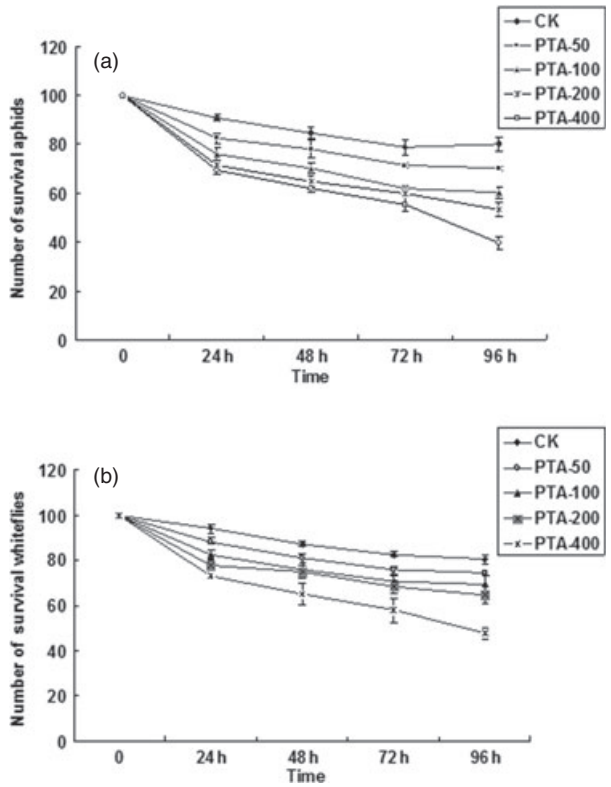

Figure 5 Evaluation of biological activity of *Pinellia ternata* agglutinin (PTA) on aphid and whitefly. (a) Anti-aphid efficacy of PTA; (b) Anti-whitefly efficacy of PTA. Total 100 nymphs of the third instar of aphid or 100 newly emerging adult whitefly were inoculated onto Petri dishes filled with the artificial diet as a replicate. Three replicates were set up for each treatment and control. Difference between PTA and control is significant at $P < 0.01$ throughout each assay. CK: artificial diet without PTA; PTA-50, 100, 200 and 400 show artificial diet with 50, 100, 200 and 400 μg/mL purified PTA, respectively.

To estimate the colonization of *E. carotovora* in the host PTA and UT leaves, the bacterial population in the inoculation area was evaluated. Bacterial suspensions (1.0×10^5 cfu/mL) of *E. carotovora* were injected into the leaf samples of transplastomic and UT through a syringe. Following inoculation, *E. carotovora* population in UT and PTA tobacco leaves was less than 1×10^5 cfu/cm^2 on 0 day post-inoculation (dpi). However, the bacterial populations soared up to 2.0×10^8 cfu/cm^2 when the UT was inoculated with of *E. carotovora* under the same conditions on 3 dpi. By contrast, *E. carotovora* populations in two transplastomic PTA lines-C2.1 and D 2.1 were 7×10^3 and 1×10^4 cfu/cm^2 on 3 dpi (Figure 11). The *E. carotovora* population in UT was 10 000 times larger than in the PTA samples on 3 dpi. In addition, no apparent symptoms of necrosis were observed in any of the PTA transplastomic plants. The data shown here are an average of six different experiments and demonstrate that the PTA transplastomic plants are resistant to *E. carotovora* pathogen infection.

Resistance of transplastomic PTA lines to tobacco mosaic virus

To evaluate the anti-viral activity of the PTA in conferring resistance to TMV, 1-month-old transplastomic plants from lines C2.1 and D2.1 T1 generation were challenged with TMV for 30 days. In susceptible untransformed control plants, TMV

Table 3 Biological activity of *Pinellia ternata* agglutinin (PTA) against *Helicoverpa zea*, *Heliothis virescens* and *Spodoptera exigua*

Insect species	Treatment	Survival rate (%) (10DAI)	Larval weight (mg/larval) (10DAI)	Pupal weight (mg/pupa)	Larval period (Days)	Pupar period (Days)
Helicoverpa zea	Control	86.7 ± 5.8	86.3 ± 9.5	313.9 ± 22.8	15.5 ± 1.1	14.2 ± 1.0
	PTA	56.7 ± 11.5	38.3.±13.4	192.5 ± 17.8	18.1 ± 1.3	16.1 ± 1.0
Heliothis virescens	Control	93.3 ± 5.8	107.8 ± 9.6	286.4 ± 17.0	16.4 ± 1.0	13.4 ± 0.7
	PTA	53.3 ± 5.7	48.8 ± 5.6	212.8 ± 18.7	18.1 ± 0.8	15.1 ± 1.2
Spodoptera exigua	Control	93.3 ± 5.8	211.1 ± 13.2	192.5 ± 21	12.1 ± 0.8	6.8 ± 0.7
	PTA	56.7 ± 5.8	114.5 ± 5.0	104.1 ± 15	13.2 ± 0.7	8.8 ± 0.7

Purified PTA was added into the artificial diet at 200 μg/mL to test its activity. The control treatment did not include the PTA. Five ml artificial diet with or without 200 μg/mL PTA was dispensed into each Petri dish and allowed to dry for 2 h. Three *Lepidopteran* insects, corn earworm, tobacco budworm and the beet armyworm, were challenged with the PTA. Ten neonate larvae of each species of insect were released in each replicate. Three replicates were set up for each treatment. Laval mortality and weight were recorded on the tenth day after releasing larvae. After obtaining the data, the live larvae were placed back on the artificial diet, and the date of pupation and adult emergence was recorded. DAI: Days after initiating the experiment.

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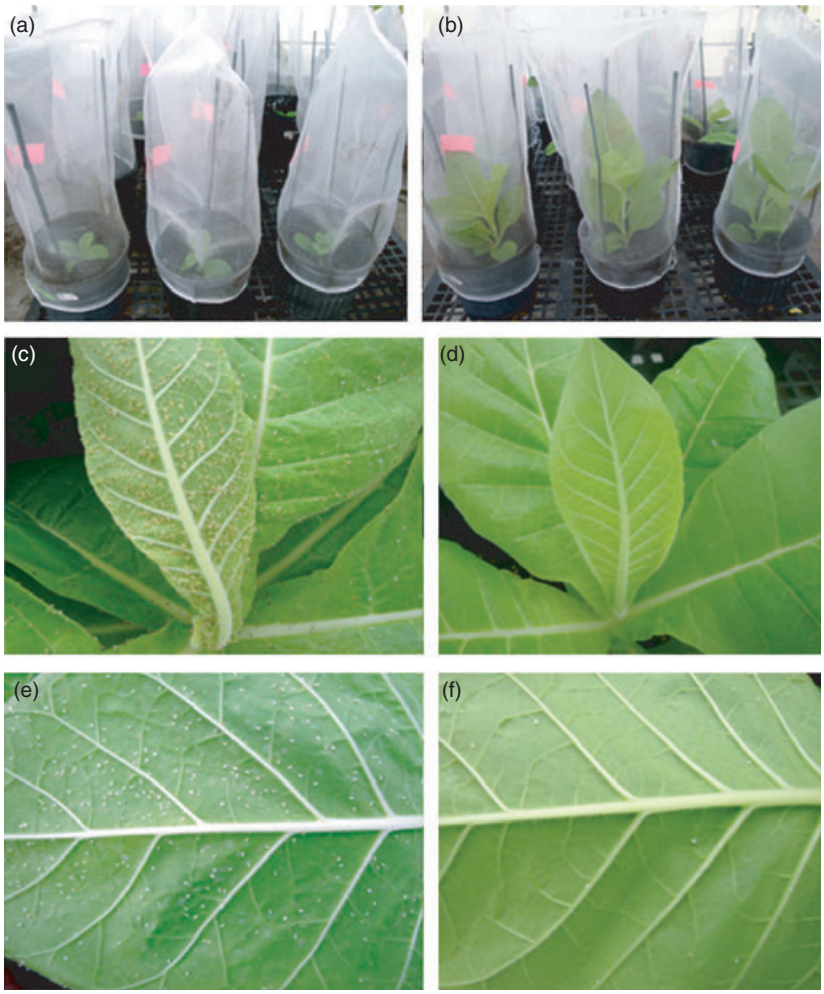

Figure 6 In planta bioassay of *Pinellia ternata* agglutinin (PTA) transplastomic and UT plants with aphid or whitefly. (a) Mesh-bag cage placed on each pot (40-day-old, 6–7 leaf stage) on day 0 for insect bioassays. (b) Release of plants from cages after insect bioassays. (c) UT plant heavily colonized with mature and immature aphids (d) PTA transplastomic plants with negligible colonization of aphids. (e) UT plant heavily colonized with mature and immature whiteflies. (f) PTA transplastomic plants with negligible whiteflies.

multiplied and spread throughout the leaves, causing typical, necrosis, mosaic and wrinkle symptoms on 30 dpi (Figure 12a-left and 12b left). However, the PTA leaves from two different lines did not show obvious signs of TMV infection and the plants grew well (Figure 12a-right; 12b-right). Ten plants from each PTA line and control were evaluated. These results suggest that the *pta* gene confers resistance to TMV infection.

Discussion

The hemagglutination activity is the key determinant of the role of lectins in different organisms. Previous studies showed that GNA-related lectin has two-domains, containing one to three mannose-binding sites. These sites are the carbohydrate-binding site, which specify the carbohydrate recognition function (Chandra *et al.*, 1999; Shridhar *et al.*, 2009). The rabbit blood cell conjugation experiment is the classic method used to determine the lectin's hemagglutination activity. In the present study, the chloroplast-derived PTA showed very similar hemagglutination activity to the commercial GNA (purified from *G. nivalis*), indicating that the procedures of expression and folding of the PTA in chloroplast were similar.

Pinellia ternate belongs to *Araceae*, and the lectins from *Araceae* family have been shown to be tetrameric proteins composed of lectin subunits of 12–14 kDa (Van Damme *et al.*, 1995; Qi *et al.*, 2008). In this study, the SDS–PAGE showed a single polypeptide band around 12 kDa in purified PTA samples and crude extraction from transplastomic plant leaves. However, non-reducing gel yielded a larger-size band with an apparent molecular mass of around 50 kDa, which confirms that chloroplast-derived PTA is a tetramer composed of four 12-kDa subunits similar to other lectins from *Araceae* specie (Van Damme *et al.*, 1995).

Previous reports showed that lectins had insecticidal activity (Rahbe *et al.*, 1995). The lectin gene-GNA from snowdrop was demonstrated to confer enhanced resistance to aphids (Hilder *et al.*, 1995; Gatehouse *et al.*, 1996; Yao *et al.*, 2003a; Dutta *et al.*, 2005; Ye *et al.*, 2009) and planthoppers (Rao *et al.*, 1998; Foissac *et al.*, 2000; Nagadhara *et al.*, 2004). Transgenic tobacco expression of leaf (ASAL) and bulb (ASAII) agglutinins from *Allium sativum L.* (garlic) exhibited resistance to the cotton leafworm (*Lepidoptera: Noctu*idae) (Sadeghi *et al.*, 2008). Recent insect bioassay and transgenic studies showed that PTA had significant insecticidal activity against peach potato

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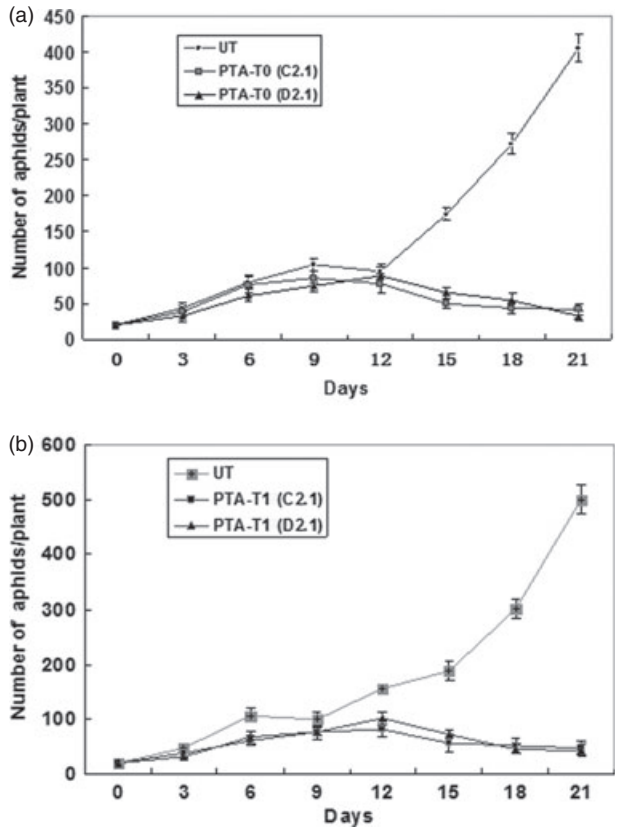

Figure 7 Aphid bioassay on transplastomic PTA tobacco T0 and T1 generations in C2.1 and D2.1 lines. In these bioassays, plants were maintained at 25 ± 2 °C, and at least 20 different individual plants were used from two different transplastomic lines. (a) Aphid population on UT and transplastomic PTA T0 generation plants. (b) Aphid population on UT and PTA T1 generation lines. Points and bars indicate mean ± SD (standard deviation). Thirty third instar aphid nymphs were introduced on each plant on day 0, and insect survival was measured at 3-day intervals for a 21-day period.

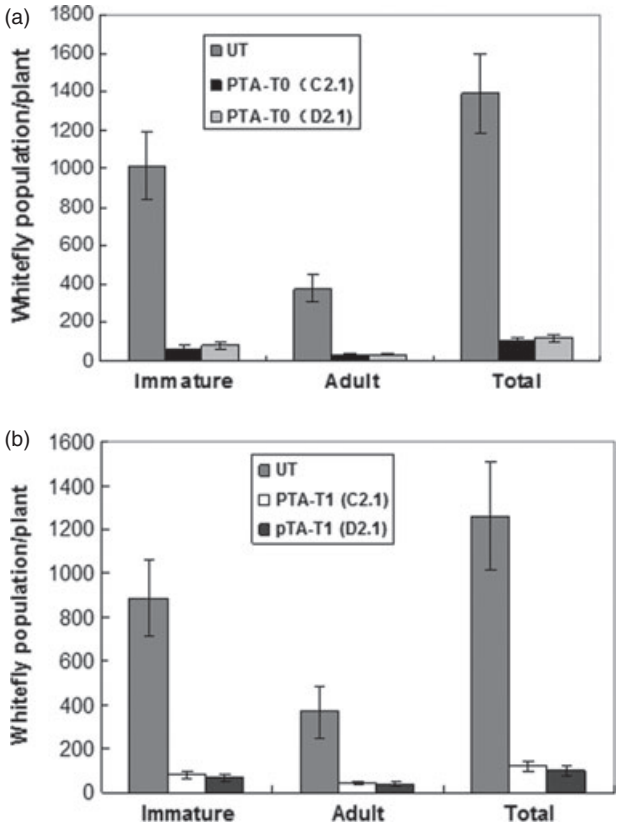

Figure 8 Whitefly population on UT and two *Pinellia ternata* agglutinin (PTA) lines. In these bioassays, plants were maintained at 25 ± 2 °C, and at least 20 different individual plants were used from two different transplastomic lines. (a) Whitefly population on UT and PTA T0 generation plants. (b) Whitefly population on UT and transgenic PTA T1 generation lines. For each transplastomic line and the control, data are shown as mean ± SD (standard deviation).

(*Myzus persicae Sulzer*) when expressing the PTA in tobacco plants *via* nuclear transformation (Yao *et al.*, 2003a). PTA also exhibited anti-pinewood nematode activity (Qi *et al.*, 2008) and antifungal activity (Ling *et al.*, 2010). However, there was no report on insecticidal effects of lectins to the most destructive sap-sucking insect—whitefly that belonged to the order of *Hemiptera*. The ability of the whitefly to spread virus had the most negative impact on global food production. In 1986, a new whitefly strain, *B. tabaci*, 'biotype—B', caused substantial loss to poinsettias in Florida and then Texas and Arizona, and by 1991, this had spread throughout the United States, where it caused more than $500 million in damage to cotton, tomato and many vegetable crops (Brown *et al.*, 1995). Most whitefly species are important in the transmission of plant diseases. For example, *Bemisia tabaci* and *B. argentifolii* transmit bean golden mosaic, tomato yellow leaf-curl, African cassava mosaic, bean dwarf mosaic, bean calico mosaic, tomato mottle and other Begomoviruses. In 1997 Tomato yellow leaf-curl begomovirus, TYLCV was discovered in Florida, USA. This viral disease was transmitted by the whitefly, *Bemisia argentifolii.* In the present study, both the artificial diet assay with PTA purified from chloroplasts and in planta bioassays showed that PTA significantly affected whitefly. Therefore, PTA is suitable for using in genetic transformation of crops, vegetables and ornamental plants for minimizing the crop loss because of whiteflies.

For most nuclear transformation with lectin genes, the expression level was low (Fitches *et al.*, 1997), in the range of 0.07%–1.3% TSP (Ripoll *et al.*, 2003; Saha *et al.*, 2006). However, in the present study, the highest expression of PTA observed was up to 9.27% TSP, up to 130-fold higher than nuclear transgenic plants. Previous reports revealed that the insect resistance level of transgenic lectin plants was directly proportional to the level of expression of foreign lectin (Yao *et al.*, 2003a). The higher expression achieved in this research contributes the potential to reduce the resistance of the sap-insect to the lectin by the high dose strategy. The aphid nymph survival and the growth of aphid population were significantly (>90%) reduced in transplastomic lines with high PTA expression. The whitefly bioassays showed similar insecticidal effects (>90%) as observed in the aphid bioassay. The higher insect resistance level recorded in transgenic PTA plants may be due to its higher expression level or the unique insecticidal characteristics of PTA (Yao *et al.*, 2003a). To our knowledge, this is the first report that transgenic plant with lectin gene conferring resistance to sap-sucking insect-whitefly,

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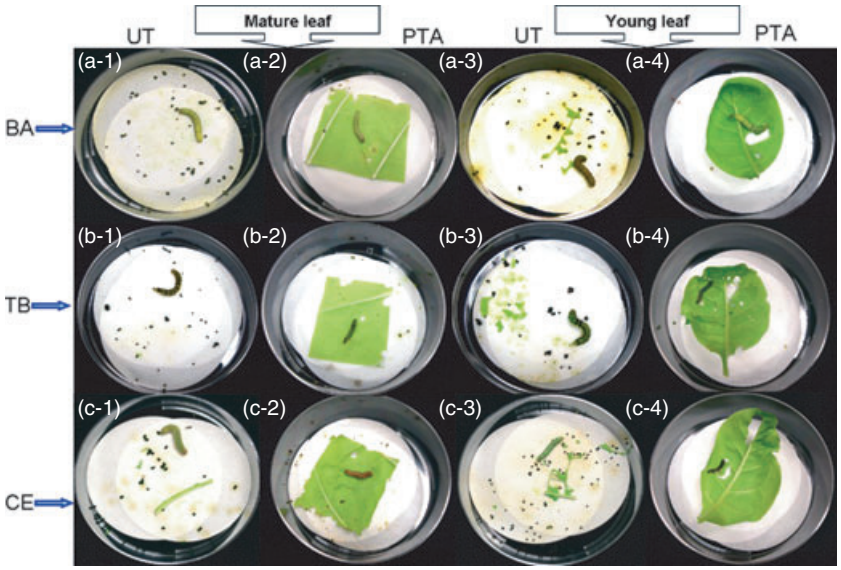

Figure 9 Leaf bioassay of wild-type and *Pinellia ternata* agglutinin (PTA) chloroplast transplastomic leaves against corn earworm (CE) tobacco budworm (TB) and beet armyworm (BA). (a-1, b-1, c-1) UT leaf bioassays with BA, TB, CE; (a-2, b-2, c-2) PTA T0 plant leaf bioassays with BA, TB, CE; (a-3, b-3, c-3:) UT leaf bioassays with BA, TB, CE; (a-4, b-4, c-4) PTA T1 plant young leaf bioassays with BA, TB, CE. Photos were taken 2 days after bioassay.

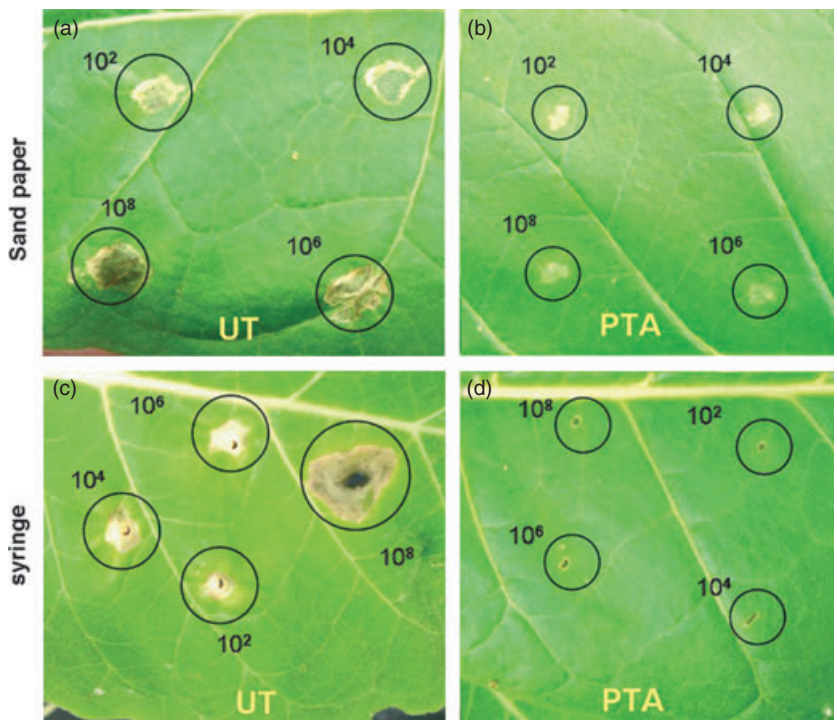

Figure 10 In planta antibacterial bioassays of *Pinellia ternata* agglutinin (PTA) transplastomic T1 generation tobacco. Five- to 7-mm areas of untransformed and T1 transplastomic PTA tobacco leaves (a, b) were scraped with fine-grain sandpaper. Twenty microliter of the 10^8, 10^6, 10^4 and 10^2 cells from an overnight culture of *Erwinia carotovora* was inoculated in each prepared area. In the parallel study, twenty microliter of the 10^8, 10^6, 10^4 and 10^2 cells from an overnight culture of *E. carotovora* was injected into leaves of UT (c) PTA transplastomic (d) plants. Photos were taken 5 dpi (day post-inoculation).

the most destructive insect on the subtropical region of the world.

The *Pinellia ternata* is an edible herb native to China. The raw or semi-cooked roots and leaves have been consumed for more than 2000 years in ancient China as a medicinal plant for relieving cough and inflammation (Wei and Peng, 2003; Bensky *et al.*, 2004). However, because of higher levels of expression of PTA in transplastomic plants, preclinical and clinical studies should be carried out to evaluate its impact on humans or other non-target systems. However, it is important

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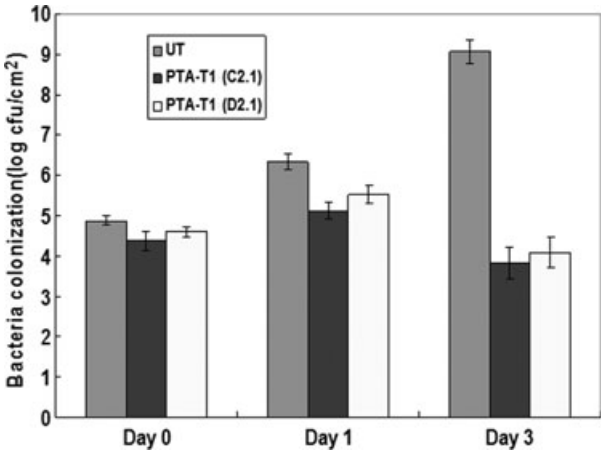

Figure 11 Bacterial populations in *Pinellia ternata* agglutinin (PTA) and UT leaves. Bacterial pathogen (*Erwinia carotovora*) inoculated in two PTA T1 transplastomic lines and untransformed (UT) tobacco plants. Bacterial suspensions (1.0×10^5 cfu/mL) of *E. carotovora* were infiltrated into the leaf of transplastomic and untransformed tobacco (UT), respectively. The infiltrated area of individual plants was punched-out from the inoculated areas of an individual plant. The bacterial populations in the inoculated plant were detected on 0, 1 and 3 dpi (day post-inoculation). All values represent means of six replicates with standard deviations shown as error bars.

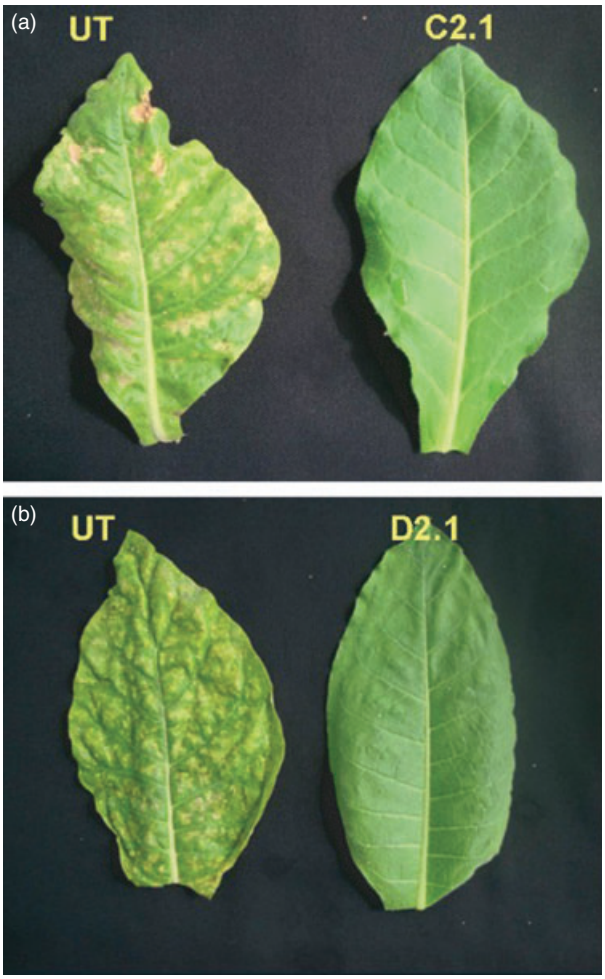

Figure 12 Response of plant expressing *Pinellia ternata* agglutinin (PTA) and untransformed control (UT) to tobacco mosaic virus (TMV) infection. (a) TMV-inoculated leaf from UT plant and PTA line-C2.1 T1 generation plant (b) TMV-inoculated leaf from UT plant and transplastomic PTA line –D 2.1 T1 generation plant. Pictures were taken on 30 dpi (day post-inoculation).

to develop strategies for introducing traits to confer broad spectrum protection against biotic stress. More researchers focus on the multigene transfer (MGT) as an approach to generate plants with multiple traits for protection against biotic stress. However, there are several technical hurdles limiting the wide range application of MGT in plant biotechnology. For the conventional stacking strategy of MGT, transgenes are stacked by crossing homozygous transgenic parent, which is labour-intensive and time-consuming because multiple breeding generations are required. As for the cotransformation MGT, it needs special vector (BIBAC, TAC) (Hamilton *et al.*, 1996) or is technically challenging (artificial plant chromosomes, Houben *et al.*, 2008) or only suitable for a limited number of plant species (operon system for plastid transformation, De Cosa *et al.*, 2001; Quesada-Vargas *et al.*, 2005). Therefore, developing a single gene with multiple functions is highly desirable.

In the last decade, several reports have already described the effect of PTA on aphids and armyworm, but the knowledge about the mechanism of action of PTA (mannose-binding) is still limited. Biochemical and molecular characterization of plant lectins demonstrated that they may interact with glycolipids, glycoproteins and oligosaccharides. It is generally accepted now that most plant lectins interact with foreign glycans. Therefore, they may play an important role in defence response against different insects, pathogens (fungi, bacteria and virus) and phytophagous invertebrates (Peumans and Van Damme, 1995; Van Damme *et al.*, 1998; Ma *et al.*, 2010). Regarding insecticidal activity of the GNA-related lectins, most researchers agree with their preferential binding to high mannose-type glycan chains, which are typical constituents of insect glycoproteins (Van Damme 2008). Previous reports showed that the mannose-binding plant lectins may interact with glycosylated receptors in the insect mid-gut. One of the major receptors for GNA was identified as a subunit of ferritin, suggesting that GNA may interfere with the insects iron metabolism (Du *et al.*, 2000).

Although antiviral activity of lectins in plants is novel and surprising, this has been previously observed against human viral infections. Plant lectins were shown to inhibit HIV replication in lymphocyte cell cultures by inhibition of virus-cell fusion (Hansen *et al.*, 1989; Matsui *et al.*, 1990; Balzarini *et al.*, 1991). Muller *et al.* (1988) reported that *Gerardia savaglia* lectin could inhibit virus replication by preventing virus adsorption. However, Balzarini *et al.* (1991, 1992) showed that plant lectin could prevent fusion of HIV particles with their target cells. Keyaerts *et al.* (2007) demonstrated that the plant lectin interacts both at virus entry and release. In addition to the antiviral effect of mannose-specific lectin on HIV, these plant lectins also showed antiviral effects on respiratory syncytial virus, cytomegalovirus and influenza A virus (Balzarini *et al.*, 1991, 1992, 2004).

In conclusion, the high-level expression of PTA in transplastomic tobacco plants provides significant levels of protection against three insects species form the order of *Lepidoptera*—tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and the beet armyworm (*spodoptera exigua*);

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two species from *Homopetra*: aphid and whitefly; bacterial pathogen: *E. carotovora* and viral pathogen: TMV. A single protein conferring multiple functions for pathogen resistance makes the *pta* gene a promising candidate to be used in the genetic engineering for enhanced insect, bacterial and viral resistance and may make a substantial contribution for crop protection. This may provide additional global food security for crop production and storage.

Experimental procedures

Construction of chloroplast transformation vector

The *Pinellia ternate* plants were obtained from Plant Germplasm Center of College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China. The coding sequence (CDS)—810 bp of *Pinellia ternate agglutinin* (*pta*) gene (NCBI: AY191305)—was PCR amplified from *Pinellia ternate* genomic DNA using forward (5′-AGACATATGGCCTCCAAGCTCCTCCT CT-3′) and reverse (5′-AGCATGCAAGCTTTCTAGATTAATGA TGATGATGATGATGATTCACCTTCTCCGTCACC-3′) primers, respectively. Sequence for restriction enzymes (*Nde*I and *Xba*I 6xHis-Tag) was added in the forward and reverse primers, respectively, to facilitate its cloning into the pLD-utr vector (Verma *et al.*, 2008). Full-length PCR-amplified *pta* gene was ligated into the PCR Blunt II Topo vector (Invitrogen, Carlsbad, CA). The *pta-his* gene was then released from Topo vector by digesting with *Nde*I and *Xba*I enzymes and cloned into the pLD-utr vector (Verma *et al.*, 2008) to create pLD-utr-*pta-his* vector. All cloning steps were carried out according to Sambrook and Russell (2001).

Bombardment and selection of transplastomic plants

Sterile tobacco (*Nicotiana tobacum* var. Petit Havana) young leaves (4–6 weeks old) from *in vitro* grown seedlings were bombarded with gold particles coated with plasmid DNA pLD-utr-*pta-his*, and transplastomic plants were regenerated as described previously (Daniell *et al.*, 2004; Verma *et al.*, 2008).

PCR analysis to confirm transgene integration

Total plant genomic DNA was isolated from untransformed (UT) and putative transplastomic tobacco leaves using the Qiagen (Valencia, CA) DNeasy plant mini kit. PCR analysis was performed using two sets of primers. The 3P (5′-AAAACCCGTCCT-CAGTTCGGATTGC-3′)/3M (5′-CCGCGTTGTTT CATCAAGCC TTACG-3′) primer pair anneals with the *aad*A gene in the vector, and the native chloroplast DNA, to check site-specific integration of the selectable marker genes into the chloroplast genome. The 5P primer (CTGTAGAAGTCACCATTGTTGTGC) anneals with the *aadA* gene, and the 2M primer (TGACTGCCC ACCTGAGAGCGGACA) anneals with the *trnA* gene in the vector, which were used to confirm integration of the transgene expression cassette (Lee *et al.*, 2011).

The PCR analysis was performed as described previously (Verma *et al.*, 2008). Leaves from the PCR-positive shoots were cut into small pieces and regenerated on selection medium for additional rounds of selection to achieve homoplasmy (Verma *et al.*, 2008).

Confirmation of homoplasmy and maternal inheritance

Confirmation of site-specific transgene integration and homoplasmy was performed by Southern blot analysis according to laboratory protocol (Kumar *et al.*, 2004). In brief, total tobacco genomic DNA (2 μg) from UT and transplastomic plants from third round of selection was digested with *Sma*I and then separated on a 0.8% agarose gel. After following the laboratory procedure, the gel was transferred onto a nylon membrane. The chloroplast flanking sequence probe was obtained by digesting pUC-Ct plasmid (Verma *et al.*, 2008) DNA with *Bam*HI and *Bgl*II, which generated a 0.81-kbp fragment. The membrane was hybridized with flanking probe labelled with [α-^{32}P] dCTP using Stratagene QuickHyb hybridization solution followed by manufacturer's protocol. Homoplasmic plants were transferred to the greenhouse, and seeds were collected. Seeds from untransformed and T0 homoplasmic plants were germinated *in vitro* on spectinomycin-containing medium to evaluate maternal inheritance.

Western blot analysis of PTA transplastomic tobacco plants

Leaves from UT and PTA transplastomic plants (100 mg) were ground in liquid nitrogen into fine powder and resuspended in 200 ìl of plant extraction buffer [(100 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 200 mM Tris–HCl pH8, 0.05% Tween-20, 0.1% sodium dodecylsulphate (SDS), 14 mM â-mercaptoethanol, 200 mM dithiothreitol, 200 mM sucrose, Roche complete mini EDTA-free protease inhibitor cocktail)] using mechanical pestle. Protein extracts from UT and PTA transplastomic plants were separated in 14% SDS–PAGE and transferred to nitrocellulose membranes for immunoblotting as described previously (Verma *et al.*, 2008). After transfer, membrane was blocked in PTM (1 × PBS, 0.1% Tween-20, 3% milk) for 2 h at room temperature and then incubated overnight at 4 °C with 1: 2500 dilution of anti-His primary antibody (GenScript Inc, Piscataway, NJ), followed by incubation in 1: 3000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Southern biotech, Birmingham, AL) for 1.5 h at room temperature. A SuperSignal West Pico chemiluminescence substrate Kit (Pierce, Rockford, IL) was used for autoradiographic detection.

Purification of *Pinellia ternata* agglutinin

Purification of His-Tag PTA protein was performed using His affinity chromatography nickel-chelate-charged columns (Clontech, Mountain View, CA) following manufacturer's protocol. Briefly, transplastomic leaves were ground in plant extraction buffer [100 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl pH8, 0.05% Tween-20, 0.1%, 200 mM dithiothreitol, 200 mM sucrose, Roche complete mini protease inhibitor cocktail] using mechanical pestle. Then, samples were sonicated for 5 min on ice and centrifuged for 20 min at 4 °C. The clear supernatant was loaded on His60 Ni column equilibrated with equilibration buffer (50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole; pH7.8) and shaken for 1 h at 4 °C. The column was placed in vertical position and kept for 30 min to settle the resin at the bottom. The resin was washed with 20 mL of wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, 40 mM imidazole, pH7.8) followed by another wash with the same buffer of pH 7.2. Then, the target protein was eluted three times with 2 mL elution buffer each (50 mM sodium phosphate, 300 mM sodium chloride; pH 7.4) with different imidazole concentrations (250, 400 and 800 mM). The eluted fractions were collected, and the presence of target protein and purity was checked by 14% SDS–PAGE. To detect the

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polymer of PTA, 8% native PAGE gel was prepared without reducing agent, SDS and two-mercaptotoethanol in the sample buffer and without SDS in the electrophoresis buffer. After electrophoresis, the protein was detected by Coomassie Brilliant Blue (G-250) staining (Bio-Rad Laboratories, Hercules, CA).

Quantification of PTA by ELISA

The recombinant his-tag standard protein (GenScript Inc) and crude extracts of PTA leaves from four transplastomic lines (Line A3, B5, C2.1 and D2.1) were diluted using ELISA coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). The 96-well microtiter EIA plate was coated with 100 ìl of recombinant his-tag standard serially diluted from 50 to 1.56 ng/ml and different dilution of PTA crude extracts (1 : 500–1 : 16 000). Plates were incubated overnight at 4 °C. After incubation, each plate was washed three times with 1 × PBS-T (1 × PBS +0.1%, Tween-20) followed by two times washing with water. The coated wells were blocked with 200 ìl of PTM (PBST with 3% dry milk) and incubated for 1 h at 37 °C. After washing, the plate was incubated with 1 : 2500 dilution of rabbit anti-his-tag primary antibody (GenScript, Piscataway, NJ) for 1 h at 37 °C followed incubation with 1 : 3000 dilution of HRP-conjugated goat anti-rabbit IgG antibody (Southern biotech) for 1 h at 37 °C. Plates were washed and incubated with 100 μL TMB (3, 3′, 5, 5′-Tetramethylbenzidine) substrate for 5–10 min. Then, the reaction was stopped by adding 0.2N H_2SO_4. The absorbance was read with a microplate reader (BioRad, model 680) at 450 nm. The ELISA for the same samples from four transplastomic lines was repeated three times.

Hemagglutination assay of PTA

The purified PTA and *G. nivalis* agglutinin (GNA as positive control; Sigma Chemical Co.) were used for hemagglutination assays. Ten per cent washed rabbit blood cells was purchased from lampire® biological laboratories (PA) and diluted by phosphate-buffered saline (10 mM Na_2HPO_4/NaH_2PO_4, pH 7.2, 130 mM NaCl; PBS) into 1%. The 90-μL blood cell suspension was mixed with 10 μL of PTA solution to a final concentration of 0.5, 1, 2, 4, 8, 16, 24, 32 and 64 μg/mL, respectively and inoculated at room temperature for 1 h. The PBS buffer without PTA was added into blood cell suspension as the negative control. After incubation, samples were observed under the microscope and photographed. The inhibition of hemagglutination by PTA and GNA was carried out with serial dilutions of appropriate carbohydrates.

Artificial diet feeding trial of PTA to whitefly (*Bemisia tabaci*) and aphid (*Myzus persicae Sulzer*)

Purified PTA (50, 100, 200 and 400 μg/mL) was added into the artificial diet (Southland Products Incorporated, Lake Village, AR) to test its activity to whiteflies and aphids. The artificial diet without PTA was used as the control in this experiment. A total of 100 nymphs from third instars of aphids were inoculated onto two petri dishes (9 cm diameter, 50 insects in each dish) filled with the artificial diet. Similarly, 100 new emerging adult whiteflies were also inoculated. The feeding dishes were inoculated in a SANYO growth cabinet, illuminated with a 16/8 h light/dark regime at 25 °C. The survival of aphid nymphs and whitefly was recorded daily. Three replicates were set up for each treatment and controls. Differences between treatments were determined by one-way analysis of variance (ANOVA).

Artificial diet feeding trial of PTA to corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) and the beet armyworm (*spodoptera exigua*)

Purified PTA (200 μg/mL) was added into the artificial diet (Southland Products Incorporated) to test its insecticidal activity. The artificial diet without PTA was used as the control. Five ml artificial diet with or without PTA was dispensed into Petri dishes (3.5 cm diameter) and allowed to dry for 2 h on the Laminar air flow cabinet. Three *Lepidopteran* insects, corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) and the beet armyworm (*Spodoptera exigua*), were challenged with the PTA. All the eggs were obtained from Benzon Research laboratory (http://www.benzonresearch.com/insect-list.htm) and were hatched at 25 °C. Ten neonate larvae of each species were released in each Petri dishes containing the artificial diet. All dishes were kept at 25 °C and 12 h photoperiod. Larval mortality and weights were recorded on the tenth day after releasing the larvae. All the larvae were weighed using electronic balance. After obtaining data, the live larvae were placed back onto the artificial diet, and the dates of pupation and adult emergence were recorded. Three replicates were set up for each treatment. Differences between treatments were determined by one-way analysis of variance (ANOVA).

In planta aphid and whitefly bioassays

The aphid and whitefly bioassays were performed using T0 and T1 young PTA transplastomic and untransformed (UT) plants as described previously (Jin *et al.*, 2011). For aphid bioassay, untransformed and transplastomic PTA plants (45-day-old, 6–7 leaf -stage) were covered with insect-proof nylon mesh bag, and thirty-third instar nymphs were introduced on each individual plant. The total number of adults and emergence of immature insects were recorded every 3 days for a total period of 21 days. In case of whitefly *in planta* bioassay, 30 new emerging adults were introduced on each individual plant, and colonization of whitefly population was evaluated as described by Jindal and Dhaliwal (2009). After 25 days, the mesh bags were removed from the plants, and the total number of adults and immature whitefly were counted by naked eyes or using a hand magnifying glass. In both assays, cultures were maintained at 25 ± 2 °C, and at least 20 different individual plants were used from two different transplastomic lines.

Lepidopteran insect bioassays with detached transplastomic leaves

Insect bioassays with untransformed and transplastomic detached leaves were also conducted against three *Lepidopteran* insects: corn earworm (*Helicoverpa zea*), tobacco budworm (*Heliothis virescens*) and the beet armyworm (*Spodoptera exigua*). Leaf bioassays were conducted with excised leaf material (3 × 3-cm^2) from T0 mature transplastomic and UT plants and placed on 9-mm plastic Petri dishes laid with Whatman® filter paper soaked with distilled water. One insect larva (fourth-instar) was introduced per sample, and leaf consumption and mortality were evaluated after 5 days. Experiments were repeated three times. To test the young leaf insecticidal activity, the whole leaf from 5-week-old transplastomic T1 and UT plants was fed to fourth instar insect larva following the same method described previously.

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In planta assay for *Erwinia* soft rot

To verify the bacterial pathogen resistance of PTA, untransformed (UT) and PTA transplastomic leaves from T1 generation were inoculated with *Erwinia* suspension culture. *Erwinia carotovora* strain was grown overnight at 25 °C in 5 mL of nutrient broth (NB) medium (Difco, Franklin Lakes, NJ), and serial dilutions of bacterial cells were prepared immediately. Five- to 6-mm^2 areas of greenhouse grown UT and PTA transplastomic tobacco leaves were scraped with sandpaper, and 15 μL of 10^8, 10^6, 10^4 and 10^2 of *Erwinia* cells was inoculated to each prepared area. In a parallel study, 15 μL of the same dilutions of *Erwinia* cells was injected into leaves of UT and PTA leaves by a syringe. Experiments were repeated at least six times. Photos were taken after 5 dpi.

To investigate colonization of *Erwinia* in plant samples, leaves of UT and transplastomic T1 generation tobacco were infiltrated with 20 ìl of bacterial suspension (1.0×10^5 cfu/mL) through syringe as described by Lee *et al.* (2011). Leaf discs (0.8 cm^2) were punched off from the inoculated areas after 0, 1 and 3 dpi. The bacterial population inside the leaf was calculated as follows: leaf disc was ground in 100 ìl of sterilized water in a 1.5-mL microcentrifuge tube. The suspension was serially diluted with sterilized water and was then plated on nutrient broth agar medium (Difco). *Erwinia* colonies were counted after 1 day of incubation at 25 °C. Experiments were repeated at least for six times.

Tobacco mosaic virus bioassay and phenotypic analysis

For TMV bioassay, full-length infectious TMV RNA transcripts were generated by *in vitro* transcription of *Kpn*I-linearized Klenow-filled pTMV004 vector using T7 RNA polymerase (Promega, Madison, WI), as described previously (Dinesh-Kumar and Baker, 2000). The TMV bioassay was performed using a protocol essentially the same as described by Lee *et al.* (2011). Briefly, *in vitro*-generated TMV transcripts were rub-inoculated onto TMV-sensitive tobacco plants for virus regeneration, and then, these infected leaves were harvested 14 dpi and re-inoculated onto wild-type tobacco plants for virus multiplication. The inoculum for plant infection was prepared by grinding TMV-infected tobacco leaf tissue in 10 mM sodium phosphate buffer, pH 7.0. The leaf sap with TMV was then injected into the main veins of 1-month-old PTA transplastomic and UT tobacco plant leaves using a syringe. Plants were observed for development of resistance or susceptibility to TMV 30 dpi. Ten plants from two PTA lines-C2.1 and D2.1 separately were used in this bioassay. Photos were taken 30 dpi.

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