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Expression of modified snowdrop lectin (*Galanthus nivalis* agglutinin) protein confers insect resistance in *Arabidopsis* and cotton

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Abstract

Background: Cotton is a major fiber crop in the world that can be severely infested by pests in agricultural fields. Identifying new insect-resistance genes and increasing expression of known insect-resistance genes are imperative in cultivated cotton. *Galanthus nivalis* agglutinin, a type of plant lectin that is reportedly toxic towards *Homoptera* sap-sucking pests when administered in their artificial diet or when fed transgenic plant material expressing the gene. The natural GNA gene is mainly expressed in monocotyledons instead of in dicotyledons, thus modification of the gene is necessary to effectively express the GNA gene in transgenic cotton.

Results: We report the artificial synthesis of a modified GNA gene (*ASGNA*), via codon augmentation, and its insertion into *Arabidopsis thaliana* and cotton to test its efficacy as an insect-resistance gene against cotton aphids and *Plutella xylostella*. The level of ASGNA expression in transgenic plants was determined by the enzyme-linked immunosorbent assay (ELISA), and the amount of ASGNA among transgenic plants reached approximately 6.5 μg/g fresh weight. A feeding bioassay showed that survival and reproductive rates of aphids fed transgenic tissues were significantly lower than those fed wild type tissues. Likewise, larvae mortality of *P. xylostella* fed with transgenic plants showed significantly increased levels. Similar results were recorded of aphids feeding on cotton cotyledons with transient expression of ASGNA.

Conclusions: Altogether, results show that *ASGNA* exhibited high insecticidal activity towards sap-sucking insects and is a promising candidate gene to improve insect resistance in cotton and other dicot plants.

Background

Cotton is one of the most important crops for fiber and oil and thus has important economic value in the world. Cotton plants are susceptible to damage by pests and diseases, which decreases yield and quality and results in economic losses [1-3]. For sustainable fiber production and to minimize the damages caused by a diversity of pests, it is essential to develop varieties that can withstand the ravages of various insect pests. Cotton bollworm (*Helicoverpa armigera*), cotton aphid (*Aphis gossypii* Glover), diamondback moth (*Plutella xylostella*) and green mirid bug (*Apolygus lucorum)* are the three major cotton pests that cause severe damage in cotton fields [4]. These insects directly damage cotton plants by eating plants or sucking the sap with their mouthparts [5-7]. Traditionally, chemical pesticides are used to control these pests; however, they cause a number of problems, such as environmental pollution, pesticide residue and increase of production cost [8, 9]. Therefore, cultivating quality cotton with high resistance to herbivory a constant goal of breeders. Transgenic technology is known to offer unique opportunities for controlling pests by using gene-engineering technology to transfer exogenous insecticidal genes into cotton to obtain transgenic insect-resistant cotton [10, 11].

Recently, various insecticidal toxin genes from the bacterium *Bacillus thuringiensis* (Bt) and of trypsin inhibitors (e.g. cowpea trypsin inhibitor, CpTI), plant lectins and ribosome-inactivating proteins have been found to be very effective against cotton pests [12-14]. Expressing toxin protein in crops have shown

significant cost savings in terms of time, labour, money, and reduction in damage to population and environment in the comparison of the chemical insecticides traditionally used [15, 16]. The successful transfer of Bt and other toxin genes into crops, and the resulting higher levels of resistance against pests have been reported in rice, maize and cotton [17-19]. However, use of insecticidal genes is not without its limitations: target insect pests can evolve resistance to a crop's genetically-engineered defenses [20-22]. To minimize the problem of insect adaptation to current insecticidal genes, modifying existing toxin genes or pyramiding various resistance genes against target organisms have found some success. Examples of multi-gene pyramids include fusing multiple Bt toxin genes together, linking Bt toxin with other toxin genes, or linking multiple toxin genes besides Bt [23, 24]. Transgenic upland cotton crops harboring two insecticidal genes, Bt + CpTI or Bt + GNA have been reported to enhance plant resistance to cotton bollworm [25, 26]. To date, no existing publication has reported about transgenic Bt + CpTI or Bt + GNA cotton conferring resistance to both cotton bollworm and cotton aphid.

Cotton aphid, *A. gossypii*, not only affects growth, yield and fiber quality of cotton severely, but also transmits viral diseases and can develop resistance to insecticides [27, 28]. *Galanthus nivalis* agglutinin (GNA), as a plant lectin, was originally isolated from snowdrop bulbs with a specificity towards Manα (1-3) Man-containing oligosaccharides to confer protection against different sap-sucking pests [29]. GNA is a 50 kDa homotetramer which composed of four non-covalently associated monomers. It inhibits the growth and reproduction of pests while being harmless to humans, herbivore and the natural enemies of the aphids [30, 31]. Therefore, GNA is a good candidate gene to research for improving insect resistance in cotton. However, the sequence of GNA gene from monocotyledonous plant *G. nivalis* showing a strong codon usage bias towards towards monocots, thus transgenic dicots (for example cotton, soybean and rape) exhibit low expression levels of GNA. Therefore, modification of the GNA gene sequence is likely necessary to raise expression levels to more efficiently control cotton aphid infestations.

We carried out multiple experiments using two insect pests and two different plant species in this investigation. First, we artificially optimized codons to synthesize a *GNA* gene (*ASGNA*) to more easily express in dicot plants by overlap PCR. The *ASGNA* gene was then transferred into *A. thaliana* by *Agrobacterium*-mediated transformation. The transgenic plants expressed higher levels of the GNA toxin protein in several tissues of the *Arabidopsis* plants. A bioassay of plant resistance to aphids was carried out under artificial conditions, and the result showed that the survival reproductive rates of aphids were significantly reduced in the transgenic group, indicating that transgenic lines have strong resistance to aphid attack. Additionally, a similar bioassay of plant resistance to *P. xylostella* showed that the weights of *P. xylostella* individuals were reduced to a large extent by transgenics. Lastly, we conducted a pest resistance experiment employing transient expression of ASGNA protein in cotton. The results showed that aphid survival and reproductive rates noticeably decreased in aphids exposed to cotton with transient expression of the ASGNA protein. The present study shows that the ASGNA protein is toxic towards aphids and other herbivorous pests, thus demonstrating the potential of using ASGNA for pest control in cotton.

Results

Cloning of the *ASGNA* gene

In order to optimize the codons and achieve high levels of *GNA* expression in cotton, we analyzed the sequence of *GNA* and found that eight codons were infrequently used in dicotyledons (Fig. 1a). We replaced these eight codons with frequently used dicotyledonous codons based on the codon usage bias without changing the amino acids. Then six pairs of primers were designed and *GNA* was synthesized by gene splicing by overlap extension (SOE-PCR). We obtained a 512-bp *GNA* fragment after conducting six PCRs (Fig. 1b and c, Fig. S1), and the sequencing result confirmed that the codons were successfully replaced (data not shown).

Expression of *ASGNA* in transgenic *A. thaliana*

The full-length cDNA fragment of *ASGNA* was cloned into a plant expression vector under the control of a constitutive modified CaMV 35S promoter (Fig. 2a). The resultant construct was then transformed into *Arabidopsis* plants. After selection of T_0 seeds with kanamycin, the *GNA* transformation was successfully achieved in several independent clones. The independently generated transgenic lines were analyzed by PCR amplification to confirm transformation. The expected DNA fragment was only identified in transformants, indicating that the *ASGNA* gene had successfully integrated into *Arabidopsis* genomic DNA (Fig. 2b, Fig. S2). The homozygous transgenic plants were selected and their progeny were used for subsequent experiments. We performed an ELISA assay to detect protein expression of ASGNA in three transgenic lines. The results showed that the three transgenic lines had similar expression levels of ASGNA in various tissues, of which GNA protein contents were approximately 6 μg/g fresh weight (Fig. 2c). In addition, the leaves of transgenic plants exhibited higher GNA protein levels when compared with protein levels of the other tissues. Thus leaves were used for further experiments.

Effects of ASGNA toxin on aphids

To test whether the expression of ASGNA in transgenic *Arabidopsis* plants contributed to greater defense against aphids, the bioassay with *A. gossypii* was carried out. Second-instar aphids were transferred to fresh detached *Arabidopsis* leaves (Fig. 3a and b), and after 18 hours of feeding, we observed the number of aphids on transgenic *Arabidopsis* plants was significantly less than the numbers on wild-type plants, indicating that aphids were likely avoiding plants of the three transgenic lines expressing ASGNA. Pairwise multiple comparisons revealed non-significant differences among the three transformed ASGNA lines (Fig. 3c). In the survival analysis, significant differences of survival curves were found between transgenic lines and the untransformed control (Fig. 3d). Moreover, we noticed that reproductive rate and body width of surviving aphids in the ASGNA plants were affected in comparison to those of the control group (Fig. 3e and f). In wild-type plants, the body width of surviving aphids was approximately 1.1-millimetre, while in intransgenic plants, the body breadth of aphids was reduced to 0.8 millimetre (Fig. 3e). Statistical analysis of the fertility rate showed that aphids on control plants was nearly 100 percent,

whereas on transgenic lines, the rate was reduced to about 35% (Fig. 3f). These results indicate that transgenic plants transformed with an *ASGNA* gene have strong resistance to aphids.

Effects of ASGNA toxin on *P. xylostella*

Plutella xylostella is also an important cotton pest that is difficult to control. In order to test the efficacy of GNA proteins expressed in plants against *P. xylostella*, a bioassay with *Plutella xylostella* was performed with the transgenic *Arabidopsis*. Three homozygous lines from independently transformed plants were assayed for *Plutella xylostella* resistance. *Plutella xylostella* larvae were placed on the transgenic plants and wild-type plants, respectively. When compared to non-GNA-transformed controls, *Plutella xylostella* that fed on transgenic plants showed significantly increased levels of larva mortality (Fig. 4a). Larva weights were also reduced to a large extent after seven days from the start of the experiment. Larva on transgenic plants weighed approximately 6 mg, while larva on control plants weighed about 14 mg (Fig. 4b). Furthermore, we noticed that larva from the three transgenic lines (lines 1-5, 3-1 and 5-2) had lower cocoon proportion. Of the larva fed on wild type plants, the cocoon proportion was nearly 60%, and the cocoon proportion was significantly reduced in line 1-5, 3-1 and 5-2 (Fig. 4c). Pest mortality of ASGNA transgenic lines were high, ranging from 45.1% to 55.3% while mortality of the controls were lower, ranging from 9.8% to 22.6%. The average mortality rates from the transgenic and control groups were 50.5% and 16.8%, respectively. The highest larva mortality was observed from individuals placed on transgenic line 5-2 which was 38.5% higher than that placed on the wild type (Fig. 4d). These results indicate that the resistance levels of transgenic lines containing ASGNA were significantly improved compared to the levels of non-transformed controls.

Transient expression of ASGNA in cotton cotyledons resulted in enhanced resistance to aphids

Transient gene expression is an attractive alternative method that allows transgenes to be assayed more rapidly and easily, so we conducted a transient transformation in cotton cotyledons to test for insect resistance (Fig. 5a). In order to observe ASGNA expression, a GFP (Green Fluorescent Protein) gene was introduced and fused with ASGNA to construct a recombinant fusion protein. The *Agrobacterium* cultures containing GFP or the ASGNA-GFP expression vector were infiltrated into cotton cotyledons by injection. We detected strong GFP fluorescence after three days and confirmed ASGNA expression at the RNA level by real-time PCR (data not shown). The strong fluorescence indicated that ASGNA was highly expressed in cotton cotyledons (Fig. 5b).

The bioassay using second-instar aphids to feed on cotton cotyledons for 24 hours showed that survival rates of aphids on transiently-expressed-ASGNA cotyledons was significantly less than that on the transiently-expressed-GFP group (Fig. 5c). The numbers of aphids were also counted three days after their transfer to cotyledons. We found that aphid aphids exposed to transiently-expressed-GFP cotyledons was no difference in populations when compared with that of the control group (Fig. 5d). In contrast, aphid populations on transiently-expressed-ASGNA cotyledons were reduced by 50% when compared with that of the control group, showing that transient expression of ASGNA in cotyledons had inhibitory effects on the growth and reproduction of aphids.

Discussion

Compared with pesticide residue and ecological destruction caused by the long-term use of chemical pesticides, transgenic plants conferring pest resistance would be an alternative tool for pest managing. Because some pests have evolved resistance to the toxin proteins that confer resistance to pests in transgenic cotton [32], new and more effective insect-resistance genes need to be developed.

Plant lectins are non-immune origin glycoproteins that selectively bind to carbohydrate, agglutinate cells or precipitate polysaccharides and glycoconjugates [33-35]. Recent advances in our understanding of the biochemical and molecular mechanisms of plant lectins were provided by research focused on GNA-related lectins. Snowdrop (*G. nivalis*), a menmber of (GNA)-related ectinfamily, which havehas been exclusively isolated from a subgroup of monocotyledonous plants in 1987 [29]. Considering that GNA is relatively safe to mammals because they lack the appropriate lectin receptors, the encoded gene of GNA has been cloned and modified to confer pest resistance in plants via genetic engineering since snowdrop lectin was firstly isolated. Yuan *et al.* (2001) modified the gene coding sequence of GNA by site-directed mutagenesis based on codon usage bias to improve gene expression of lectin in *Nicotiana tabacum* [36]. Western blot analysis showed that the expression level of GNA in GNA34m (modified GNA) transgenic tobacco plants was 0.25% of the total soluble proteins and was much higher than that in GNA34 (transgenic plants. Moreover, the GNA34m lines exhibited greater aphid resistance when compared to that of the GNA34 transgenic lines. In our study, the full-length coding sequence of the ASGNA gene was artificially synthesized and modified with respect to the dicot-biased codon usage. The ASGNA gene was transformed into *Arabidopsis* plants and GNA protein levels in transgenic plants were approximately 6 μg/g fresh weight. In addition, there were no significant differences in ASGNA protein contents among the three transgenic lines. Previous studies report that expression of GNA protein constituted 0.4% of total soluble protein in transgenic lettuce and 0.3% to 0.4% of the total soluble protein in transgenic potato [37, 38]. In this study, the GNA content accounted for 0.41–0.47% of total soluble protein, which is higher than that of. We suspect that our synthetic ASGNA gene, which was based on the preferred codons of dicotyledons, was more suitable for expression in *Arabidopsis*.

The toxicity level of GNA lectin may vary according to the type and age of insect, as well as the type of animal. For instance, GNA has a moderate level of toxicity to insect pests with chewing mouthparts (e.g., Lepidoptera, Diptera) and a significant level of toxicity to insect pests with piercing-sucking mouthparts (e.g., aphid, brown planthopper and nematode), while it is nearly non-toxic to humans and herbivore [39]. Thus, GNA is valuable for improving insect resistance in plants while reducing potential harm to humans and other non-target animals. In potato, GNA expressed under the CaMV 35S promoter confers some resistance to aphid attack; the aphid population exposed to transgenic plants ranged from 24.9–53.5% of the aphid population exposed to non-transformed plants. Yuan et al. (2001) constructed a plant expression vector with cloned GNA and a double CaMV 35S enhancer and CoYMV promoter with tissue-specific expression in the phloem. Then they *Agrobacterium*-transformed tobacco plants with the construct. The transformed tobacco plants exhibited strong anti-aphid activity that reduced the aphid population by 45%–60% on average [37]. Li and Romeis (2009) fed lacewing flies with an artificial diet

containing different concentrations of GNA and found that GNA not only delayed development of lacewing larvae, but also reduced longevity, daily fecundity and total fecundity of adults [40]. In our bioassay of transgenic *Arabidopsis*, the aphid population on transgenic plants was less than fifty percent of the population on control plants, indicating that the transgenic *Arabidopsis* had greater resistance to aphids. Furthermore, the average aphid population of transiently-expressed-ASGNA cotyledons reduced to about half that of wild-type rates, indicating that ASGNA is toxic to piercing-sucking insect pests. Therefore, ASGNA is a viable candidate gene for conferring pest resistance to cotton aphids in cotton breeding.

Nontoxicity to non-target insects is very important for agricultural applications of insect-resistance genes. Phytolectin is a mannose-binding lectin found in monocotyledons and has strong similarities to GNA in amino acid sequences, binding specificity and molecular structure. Peumans et al. (1997) fed leek flowers that highly expressed phytolectin to the non-target insect bees and found that the phytolectin lost its activity during the conversion of nectar into honey [41]. Down et al. (2000) found that transgenic plants expressing GNA had no significant effects on development and survival of *Adalia bipunctata* larvae that preyed on the aphids fed with these transgenic plants [42]. These results suggest that GNA may be nontoxic to small predators of aphids and other pests. However, whether or not the synthesized ASGNA gene has deleterious effects on beneficial insects remains to be further studied.

Conclusions

This work shows that the artificially synthesized *ASGNA* was more suitable for expression in dicotyledons and have a higher level of ASGNA expression in transgenic Arabidopsis. The *ASGNA* transgenic plants showed high insecticidal activity towards sap-sucking insects, transient expression of ASGNA in cotton cotyledons also show enhanced resistance to aphids. All this results indicated that expressing the ASGNA protein in transgenic plants can be a useful approach for controlling pests in the future.

Methods

Plant materials

Arabidopsis thaliana ecotype Columbia-0 (wild type) was ordered from ABRC (Ohio State University) and the seeds were planted on agar plates containing Murashige and Skoog salts, 1% Suc (w/v), and 0.7% (w/v) agar, and adjusted to a pH of 5.7. After vernalization at 4°C for at least 2 d, seedlings were grown in an illuminated growth chamber at 23°C. Germinated seedlings were used for *Agrobacterium*-mediated transformation and the putative transgenic plants were then moved to pots containing soil of equal proportions of clay, sand, and peat moss (1:1:1). Finally, the plants were moved to a greenhouse and subjected to various molecular analyses and an insect bioassay.

Gossypium hirsutum (Xuzhou 142) seeds used in this study were acquired from the Institute of Cotton Research of the Chinese Academy of Agricultural Sciences (Anyang, China). The seeds were planted in

containers of sand (one seedling per container) and grown under a 16-h light and 8-h dark cycle at 30°C in a climate-controlled greenhouse located at Shaanxi Normal University.

Synthesized ASGNA and transgenic plants

The original *GNA* sequence was obtained from National Center for Biotechnology Information (GenBank: M55556.1). The *ASGNA* gene was designed according to the dicot-bias of codon usage of the GNA gene. The 12 long fragments of *ASGNA* gene were synthesized by the Sangon Biotech company and ASGNA was obtained using the overlapping PCR method (Table S1) [43]. Primers for ASGNA are listed in Table S1 in Supporting Information. Then the gene was cloned into the plant expression vector pART27. The constructs were introduced into *Arabidopsis* by the *Agrobacterium tumefaciens* floral-dip procedure [44], and transgenic plants were screened using 50 mg/mL kanamycin.

DNA isolation and PCR

The DNA was isolated using improved CTAB methods. *Arabidopsis* plant leaves (0.1 g) were ground into powder in liquid nitrogen. Then, 0.6 ml CTAB extraction buffer was added and the lysate was incubated at 65°C for 30 min. The DNA was purified by adding an equal volume of a mixture of chloroform: isoamyl alcohol (24:1) followed by centrifugation at 8000 *× g* for 10 min at 4°C. The supernatant was mixed with 0.6 volume of isopropanol and then subjected to centrifugation at 8000 *× g*. The precipitate was washed twice with 75% ethanol and then dissolved in 300 µl sterile water. Then we added NaAc (1/10 volume of 3 M, pH 5.2) and two volumes of ethanol to the dissolved precipitate and incubated each sample for 10 min at -20°C. The tube was centrifuged at 8000 *× g* for 5 min and the pellet was then washed twice with 75% ethanol and re-dissolved in sterile water.

The primer sequences used for this study are listed in Supplementary Table 1. The PCR thermal cycler program was as follows: 95°C for 3 min, 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C elongation for 1 min. The PCR amplification products were electrophoresed on a 1% agarose gel and purified with the E.Z.N.ATM Gel Extraction Kit (OMEGA Bio-Tek, USA).

Determination of ASGNA expression levels in transgenic plants

Total protein was extracted from different fresh transgenic tissues (leaf, stem petal, root and silique). Ground leaves were put in 1.5 ml micro tubes with 400 µl of protein extraction buffer (150 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.1% NP40, 50 mM, Tris-HCl (pH 7.5), and EDTA-free complete protease inhibitor cocktail). The samples were vortexed to homogenize each one and incubated at 4°C for 2 hours and then centrifuged at 13,000 *× g* for 10 min. The supernatant was eluted and stored in new 1.5 ml tubes and Bradford reagent was used to quantify proteins. The expression of GNA protein was detected by an ELISA kit (Meuxuan biotechnology, Shanghai, China). In brief, 25 µl of either plant extract or purified GNA toxin standards (to obtain final concentrations from 5 to 60 ng of GNA sample in extract buffer) in ELISA plates coated with an anti-GNA antibody were incubated at 37°C for 2 h in an airtight container with 25 µl of alkaline phosphatase enzyme conjugate. Unbound protein was removed by washing with phosphate-

buffered saline and 0.01% Tween 20 (v/v). Wells were again washed and then the assay was developed by the addition of 50 µl pnitrophenyl phosphate substrate solution and absorbances were read at 400 nm in a microtitre plate reader (Epoch, BioTek, Vermont). Levels of GNA were determined from the GNA calibration curve. Each sample was assayed in triplicate.

Insect bioassay

Aphid larvae and *P. xylostella* were collected from the Shaanxi Normal University field station (E, 108°93', N, 34°17', Shaanxi Province, Northwest China) in July and grown under laboratory conditions for a feeding bioassay. To investigate whether transgenic plants expressing **ASGNA** could confer enhanced **resistance** to *P. xylostella*, insect larvae either fed on control or transgenic plant tissue placed in petri-dishes, one larvae of each species per dish. To investigate aphid resistance of **ASGNA plants**, each whole plant was confined in an insect-proof fine-mesh nylon cage and 10 late-instar aphid nymphs were introduced with a hair brush to plant leaves of each plant on Day 0. Survival and growth rates of the insect populations were determined at 2-day intervals for a 14-day period. Cocoon proportion was calculated as follows: (number of larva with cocoon/number of total larva) × 100%. All the experiment was repeated three times (each independently derived transgenic line was micropropagated into three cloned plants).

Transient GNA expression in cotton

Cotyledon disks excised from 10–12-day-old cotton seedlings were used for the GNA transient expression analysis. *Agrobacterium* cultures were grown overnight at 28°C in LB medium containing the antibiotics 50 µg/ml kanamycin and 25 µg/ml gentamicin, as well as 10 mm MES and 20 µm acetosyringone. The cells were pelleted by centrifugation at 1500 *g* at room temperature for 5 min and re-suspended in infiltration culture containing 10 mm $MgCl_2$, 10 mm MES and 200 µm acetosyringone. Cell suspensions were incubated at room temperature for at least 3 h. *Agrobacterium* cultures containing the GNA expression vector were infiltrated into two fully expanded cotyledons of 2-week-old plants using a needle-less syringe. To facilitate the infiltration, small holes were punched with a needle on the underside of the cotyledon. These experiments were repeated at least three times with more than six plants for each construct per repeat.

Abbreviations

PBS: phosphate-buffered saline; **PNP**: pnitrophenyl phosphate; **GNA**: Galanthus nivalis agglutinin; ELISA: enzyme-linked immuno sorbent assay; GFP: green fluorescent protein;

Declarations

Ethics approval and consent to participate

This research did not involve any human subjects (including human material or human data), or protected animals or protected plant species as materials.

Availability of data and materials

All of the data and materials supporting our research findings are contained in the methods section of the manuscript. Details are provided in the attached supplementary data.

Competing Interests

The authors declare that they have no competing interests.

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Author contributions

J.Y. designed the research. P.H., S.W. L.T., X.H. and S.C. performed the research. Y.J. and Z.W. analyzed the data. P.H. and J.Y. wrote the paper. All authors have read and approved the final manuscript.

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Figures

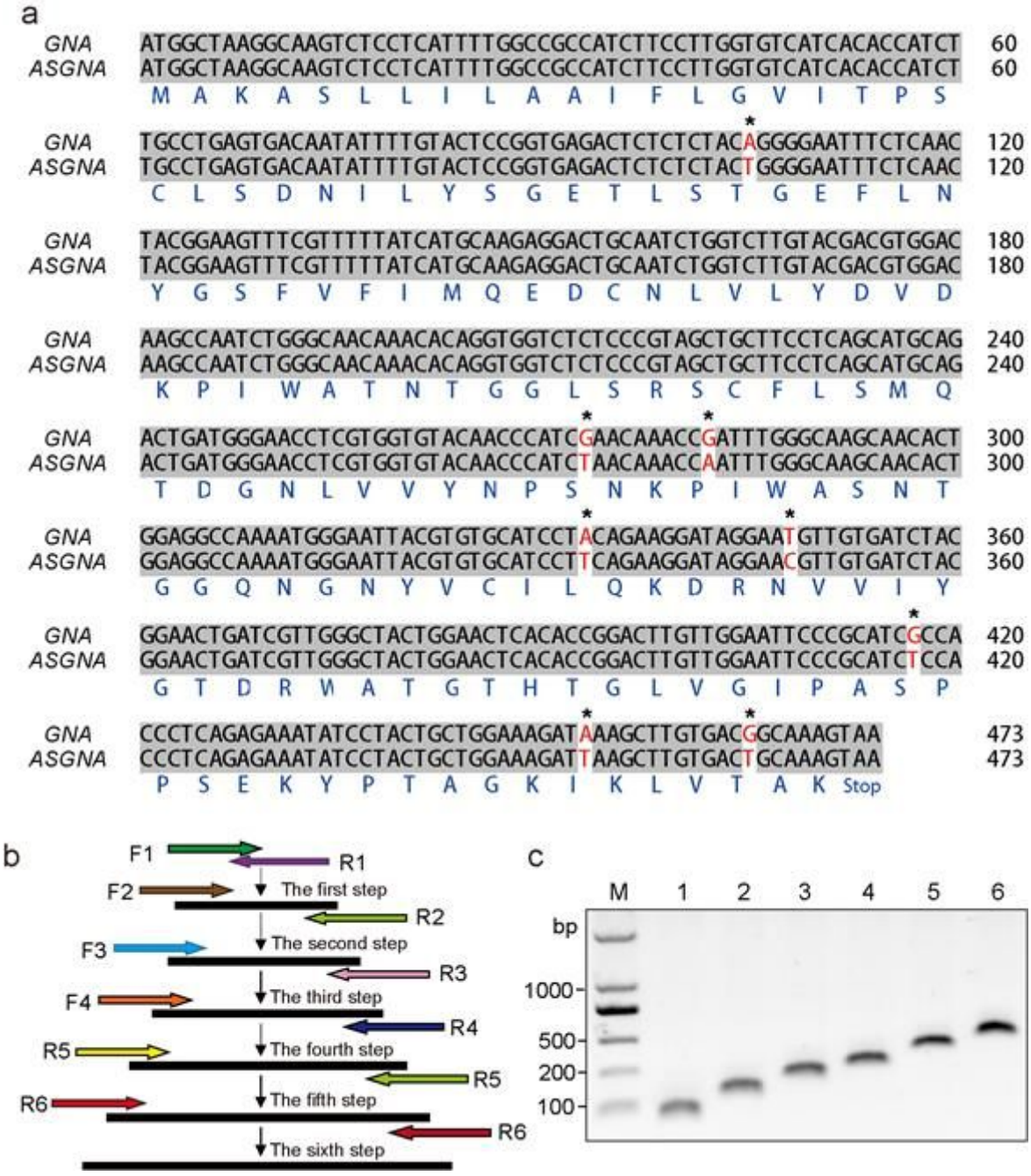

Figure 1

Cloning of ASGNA genes. (a) Sequence alignment of Galanthus nivalis agglutinin (GNA) with selected codons artificially replaced to modify GNA (ASGNA). Numbers flanking the sequences are nucleotide positions. The replaced nucleotides are indicated by asterisks (*). (b) Schematic diagram of the synthesis of the ASGNA gene by overlap PCR. Primer pairs used in PCR amplifications are F1/R1 (step 1), F2/R2 (step 2), F3/R3 (step 3), F4/R4 (step 4), F5/R5 (step 5) and F6/R6 (step 6). (c) Thed amplification of the ASGNA gene by six overlapping PCRs. M: Marker. 1-6 are PCR products from step 1 to step 6, respectively.

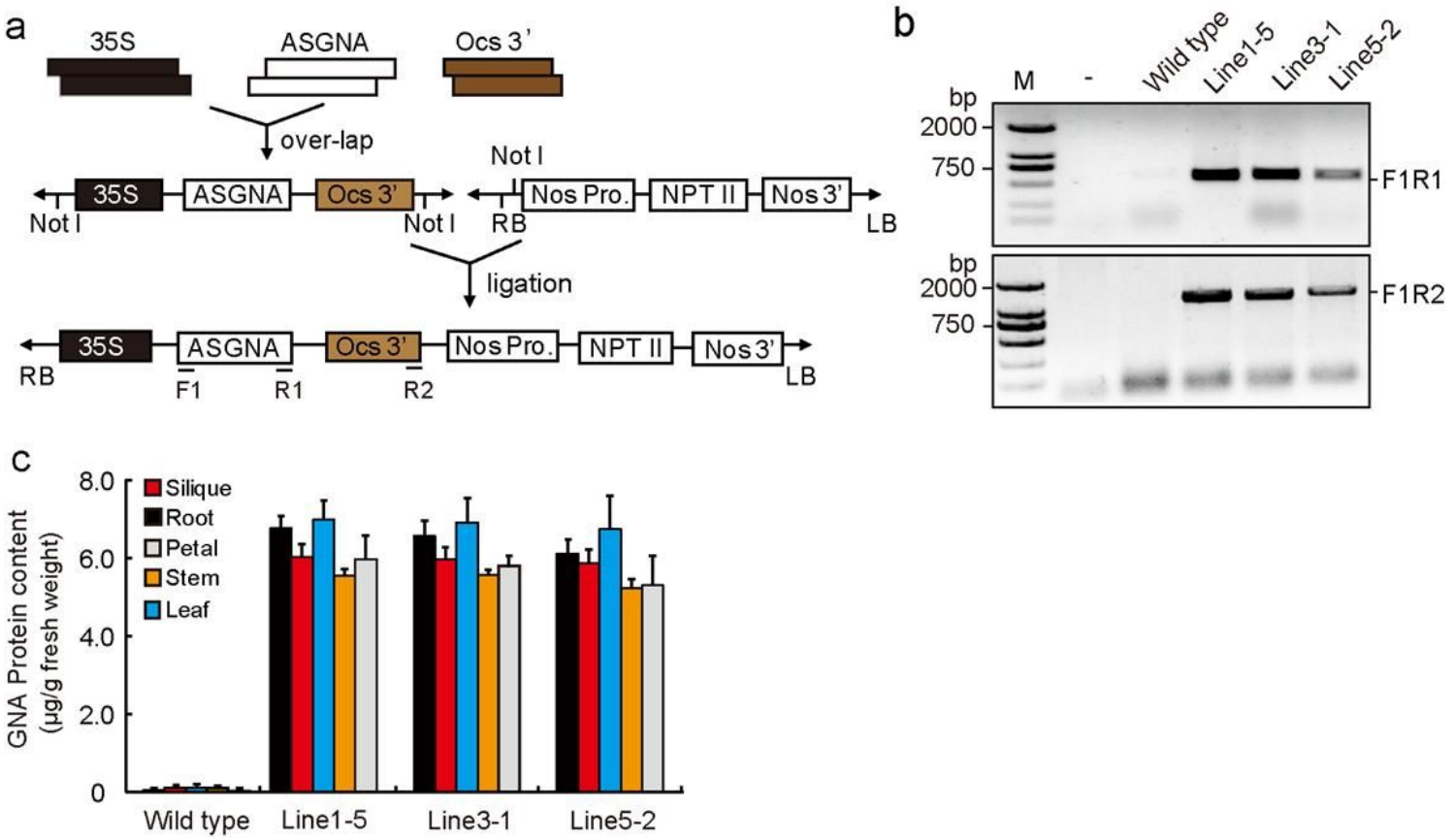

Figure 2

Generation and confirmation of transgenic ASGNA plants. (a) Schematic representation of the CaMV35S-ASGNA expression construct in a plant transformation vector. 35S, CaMV35S promoter. NPT II, neomycin phosphotransferase II gene. RB, right border. LB, left border. (b) Detection of transgenic plants by amplification of ASGNA using the genomic DNA template. Lane M, DNA marker DL2,000. Lane "-," no-template control. The primers used in PCR amplification are shown in A. (c) Protein expression of ASGNA in transgenic lines. ELISA was performed to measure the total protein extracted from different organs of transgenic lines. Three replicate assays were set up using a single crude extract from plant tissues.

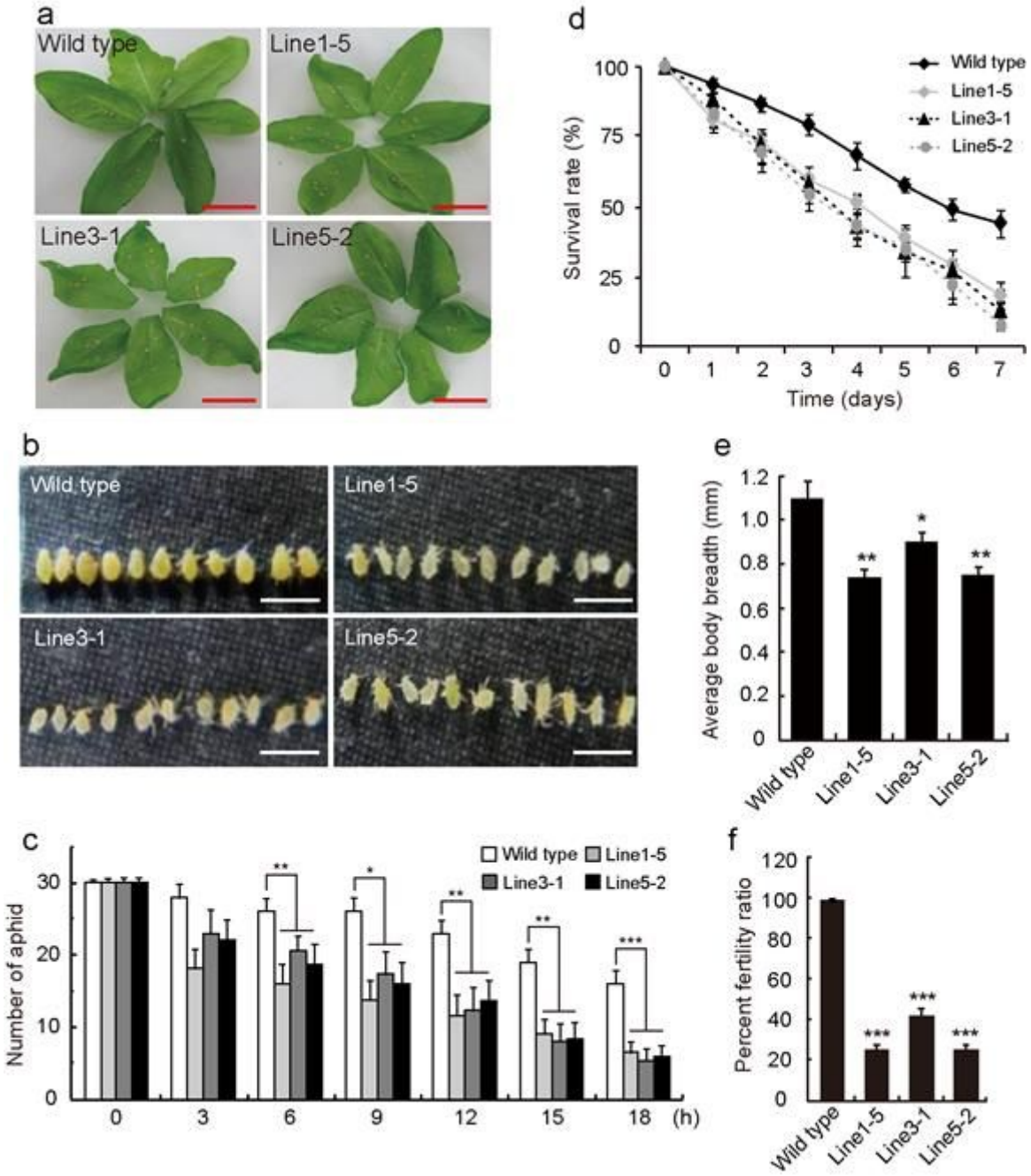

Figure 3

Aphid feeding bioassay of ASGNA transgenic lines. (a) Arabidopsis leaves were fed to aphids. Bars = 1.0 cm. (b) Morphological characteristics of aphids fed wild type or ASGNA transgenic leaves. Bars = 0.5 cm. (c) The numbers of aphids feeding on transgenic plants expressing ASGNA significantly reduced over time. The significance differences between transgenic plants and control plants were determined from the independent samples; the error bars represent SE of the means. *, P<0.05. **, P<0.01. ***, P<0.001. (d-f) Survival rate, body breadth and percent fertility analysis of aphids fed on different plant lines. Each experiment was performed with three biological replicates, and the error bars represent SE of the means. *, P<0.05. **, P<0.01. ***, P<0.001.

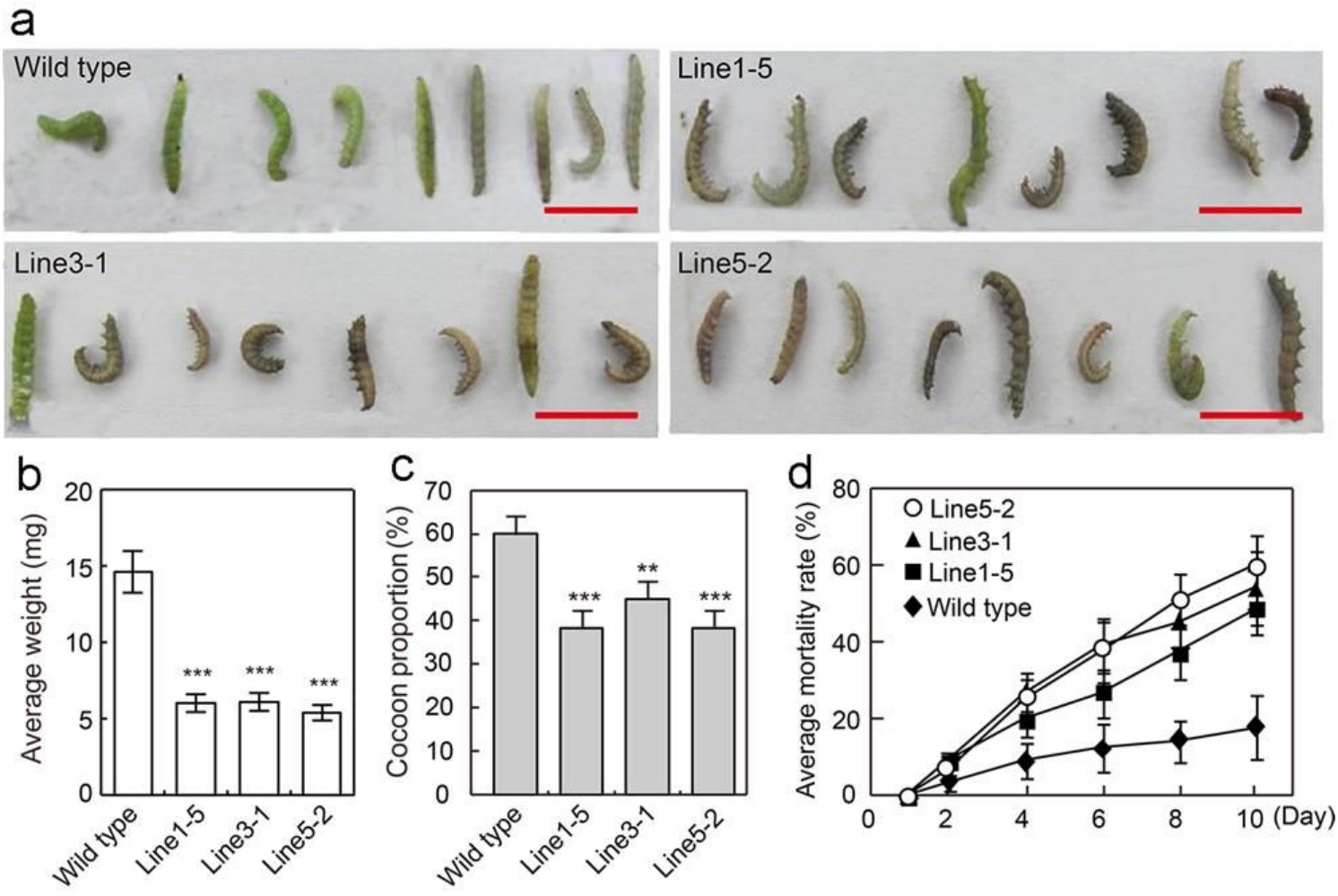

Figure 4

Effect of transgenic Arabidopsis lines expressing ASGNA on Plutella xylostella larvae. (a) The body characteristics of larvae fed on different plant lines, including a non-transformed wild type control. (b) The weight of larvae fed with transgenic plants expressing ASGNA significantly decreased. Error bars indicate standard errors of means of three biological replicates. ***, $P < 0.001$. (c) Analysis of cocoon proportion of larvae fed on transgenic lines. ***, $P < 0.001$, **, $P < 0.006$. (d) Larva mortality rates of individuals fed on ASGNA transgenic leaves significantly increased compared to that of the control.

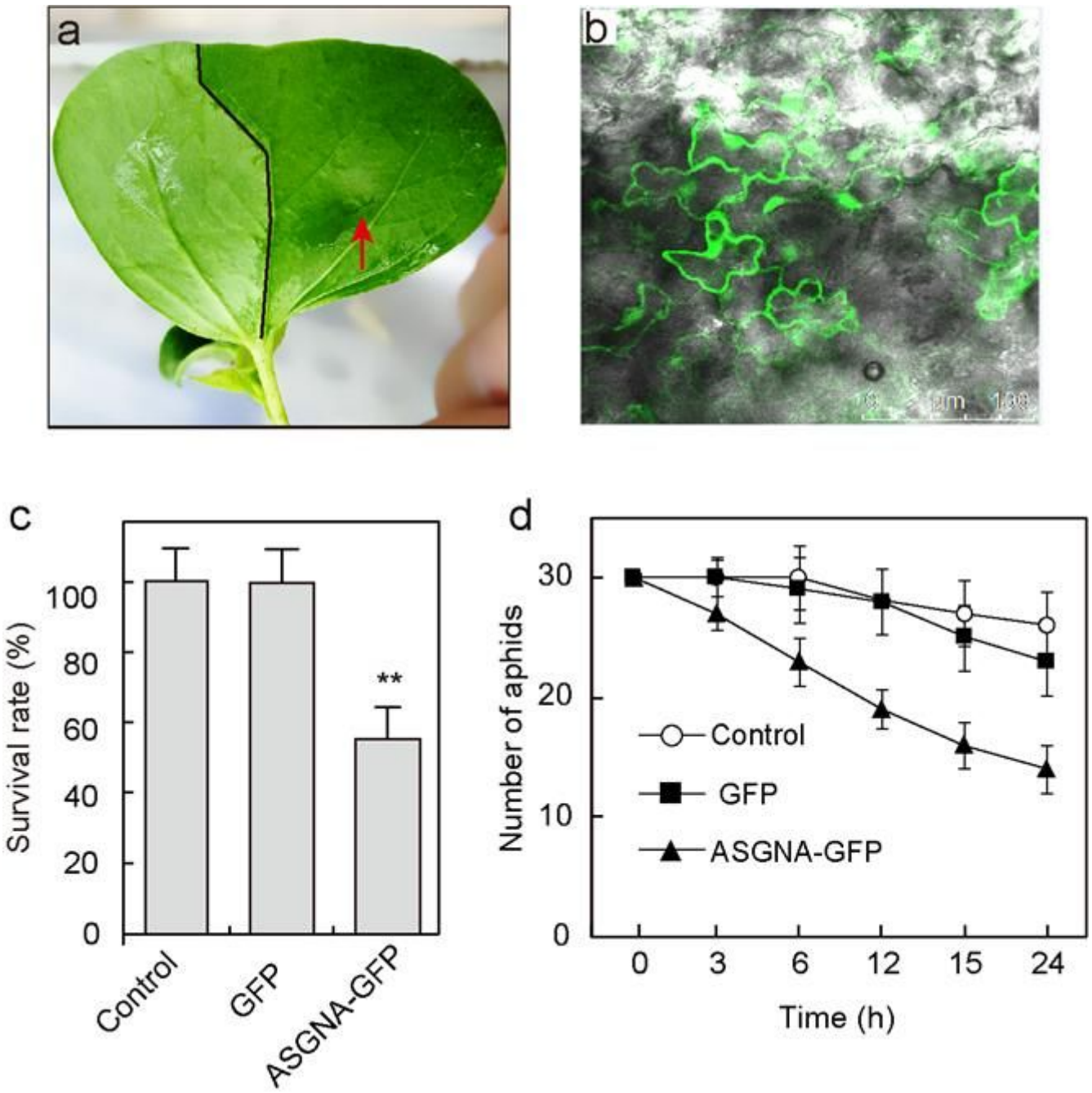

Figure 5

Analysis of insect pest resistance and the transient expression of ASGNA in cotton cotyledons. (a) Half of each cotton cotyledon was injected for transient expression of ASGNA. The red arrow depicts the injection point and the black line represents the boundary between the injected and non-injected halves of the cotyledon. (b) Protein expression was detected by GFP fluorescence. Each experiment was performed in three biological replicates, and the error bars represent SEs of the means. **, $P < 0.01$. (c) Survival rates of aphids significantly decreased after feeding on transiently-expressed ASGNA cotton cotyledons. The error bars represent SEs of the means. **, $P < 0.01$. (d) The number of aphids was significantly reduced after feeding on cotyledons expressing ASGNA.

Supplementary Files

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- Supplementalfigures.pdf
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