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Expression of the *Galanthus nivalis* agglutinin (*GNA*) gene in transgenic potato plants confers resistance to aphids

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ABSTRACT

Aphids, the largest group of sap-sucking pests, cause significant yield losses in agricultural crops worldwide every year. The massive use of pesticides to combat this pest causes severe damage to the environment, putting in risk the human health. In this study, transgenic potato plants expressing *Galanthus nivalis* agglutinin (*GNA*) gene were developed using CaMV 35S and ST-LS1 promoters generating six transgenic lines (35S1-35S3 and ST1-ST3 corresponding to the first and second promoter, respectively). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis indicated that the *GNA* gene was expressed in leaves, stems and roots of transgenic plants under the control of the CaMV 35S promoter, while it was only expressed in leaves and stems under the control of the ST-LS1 promoter. The levels of aphid mortality after 5 days of the inoculation in the assessed transgenic lines ranged from 20 to 53.3%. The range of the aphid population in transgenic plants 15 days after inoculation was between 17.0 ± 1.43 (ST2) and 36.6 ± 0.99 (35S3) aphids per plant, which corresponds to 24.9–53.5% of the aphid population in non-transformed plants. The results of our study suggest that GNA expressed in transgenic potato plants confers a potential tolerance to aphid attack, which appears to be an alternative against the use of pesticides in the future.

1. Introduction

Aphids are considered as the largest group of sap-sucking pests that cause significant yield losses in agricultural crops worldwide [1–3]. They induce damage to their host plants, modifying plant metabolism, ingesting plant nutrients from the phloem, and vectoring plant-pathogenic viruses [4,5]. *Macrosiphum euphorbiae* (Thomas), and *Myzus persicae* (Sulzer) aphid species constitute one of the major potato pests affecting the production of this crop [6], which is considered the fourth most important worldwide [7].

Despite the improvement of biopesticides like toxins derived from entomopathogenic fungus [8] and bacteria [9], and spiders venom [10] that have specificity for target pest species [11] and do not affect mammals [12], these substances remain in the epidermis, being effective only for crewing insects. Notwithstanding the fact they combat aphids, which are able to penetrate their long acupuncture mouthparts into plant phloem, it is necessary to search for effective substances that must be able to reach these active sites with a long-time permanence and low digestion ratio

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once it has been ingested by the insect. One candidate substance that complies with the aforementioned requirement is the snowdrop lectin *Galanthus nivalis* agglutinin (GNA), which is able to cross the midgut epithelium [13] remaining stable and active within the insect gut after having been ingested. It has been reported that GNA confers resistance to crewing and sap-sucking insects in rice, tobacco, cotton, rape, and wolfberry [14–16], without toxicity to higher animals [17]. The use of genetic engineering that allows the synthesis of GNA to improve the resistance of important crops like potato to aphids is yet a pending issue that needs to be implemented.

Transgenic technology has allowed the expression of a broad spectrum of promoters, which potentially are involved in the synthesis of lectins, one of them, the CaMV 35S promoter, is most widely used in transgenic plants, since it not only affects the associated transgene, but also exerts influence in thousands of base pairs up- or downstream of the insertion site on a given chromosome [18,19]. However, the foreign gene, driven by CaMV 35S promoter, is expressed in all tissues during plant growth and development [20]. It causes consumption of excessive matter and energy within the cells for the expression of the target gene, affecting its temporal and spatial effectiveness [21]. Furthermore, there are other promoters that are only expressed in photosynthetic tissues; this is the case of ST-LS1, a light-inducible promoter whose expression has been detected in leaves and photosynthetic stems of potato [22–24]. In the present study, the transgenic potato plants expressing *GNA* gene driven by CaMV 35S and ST-LS1 promoters respectively were obtained to find the difference of tissue-specific expression and to estimate the resistance of the transgenic potato plants to aphids. Our aims were to obtain the transgenic potato plants expressing the *GNA* gene driven by CaMV 35S and ST-LS1 promoters, respectively, to find the difference of the tissue-specific expression of the *GNA* gene and further to improve the resistance of potato to aphids.

2. Materials and methods

2.1. Plants and insects

The potato cv. 'Atlantic' was propagated *in vitro* by subculturing single-node cuttings on Murashige and Skoog medium [25] supplemented with 3% sucrose and 0.45% agar. Plantlets were grown in 150-mL flasks under white fluorescent light during 16 h and in the dark during 8 h, at a temperature of 24 ± 2 °C. Microtubers were induced in the dark at 24 ± 2 °C in an MS medium supplemented with 8% sucrose and 0.45% agar [26]. Green peach aphids (*M. persicae*, Sulzer) were collected from Yuzhong County in Gansu Province, China. Aphids were reared in a light incubator at 25 ± 2 °C under a 14 h light/10 h dark photoperiod, and fed with plantlets of potato cv. 'Atlantic'.

2.2. Construction of the plant expression vector

The fragment of *GNA* gene with nucleotide sequence (GenBank accession No. M55556.1) [27] was digested with *Bam*H I and *Sal* I from the clone vector and ligated into a binary vector pBI121 [28] and pBI121-ST-LS1 [24] resulting in the recombinant vectors pBI121-CaMV35S-GNA and pBI121-ST-LS1-GNA. These vectors were transformed into *Escherichia coli* DH5α respectively, and further verified by the same restriction endonuclease digestion. After that, pBI121-CaMV35S-GNA and pBI121-ST-LS1-GNA were transformed into *Agrobacterium tumefaciens* LBA4404, respectively, using the freeze-thaw method [29].

2.3. Transformation of potato

Potato transformation was performed according to the protocol of Si et al. [30]. Microtuber slices of potato cv. 'Atlantic' were co-cultured (media-MS + 1 mg/L IAA + 0.2 mg/L GA_3 + 0.5 mg/L 6-BA + 2 mg/L ZT) for 2 days with *A. tumefaciens* LBA4404 containing the plasmid pBI121-CaMV35S-GNA and pBI121-ST-LS1-GNA, respectively, then transferred into a selection media supplemented with 50 mg/L kanamycin. When green buds sprouted from the surface of the slices and reached a length of 1 cm, they were transferred to a selective rooting medium containing 100 mg/L of kanamycin and 200 mg/L of carbenicillin. Plantlets with well-developed roots were propagated for further molecular analysis.

High-quality DNA was isolated from the leaves of putatively transformed and non-transformed (NT) control potato plants for PCR according to the method proposed by Edwards et al. [31]. The part of the coding sequence of the *GNA* gene was amplified using a PCR Screening Kit (GenStar, Beijing, China) with forward primer (5′-GCGGATCCATGGCTAAGGCAAGTCTCC-3′) and reverse primer (5′-GTACGAGCTCTTACTTTGCCGTCACAAGCT-3′). Amplification was performed in a thermal cycler (T100™, BIO-RAD) programmed for one cycle of 3 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 30 s at 66 °C, and 1 min at 72 °C. A final extension step was performed for 5 min at 72 °C. The amplification products with 500 bp in length were separated by electrophoresis on 1.0% agarose gels treated with GoldView II staining.

2.4. Gene expression analysis by qRT-PCR

Total RNAs were isolated from the transgenic lines and NT control using RNAsimple Total RNA Kit (lot#N2822, TIANGEN, Beijing, China) following the manufacturer's instructions. Reverse transcription was performed in 20 μL reaction mixture with the RevertAid First Strand cDNA Synthesis Kit (Cat No: 3K1622, Thermo Scientific) and qRT-PCR amplification was performed in 20 μL of the reaction mixture with the SuperReal PreMix Plus (SYBR Green) (lot#N3113, TIANGEN, Beijing, China), 10 μM of each primer (*ef1a* as an internal control gene and forward and reverse primers: 5′-CAAGGATGACCCAGCCAAG-3′ and 5′-TTCCTT ACCTGAACGCCTGT-3′, and the gene-specific forward and reverse primers: 5′-CTCACCACTTACGCACAAGC-3′ and 5′-CGGCAATATCCTCTTTCTCG-3′). Reactions were conducted with an ABI3000 device (Applied Biosystems 3000 Real-Time PCR System) using the default cycling conditions (30 s at 95 °C and 40 cycles of 95 °C for 5 s, 60 °C for 34 s, 15 s at 95 °C, 1 min at 60 °C and 95 °C for 15 s). Each experiment was repeated three times independently. After

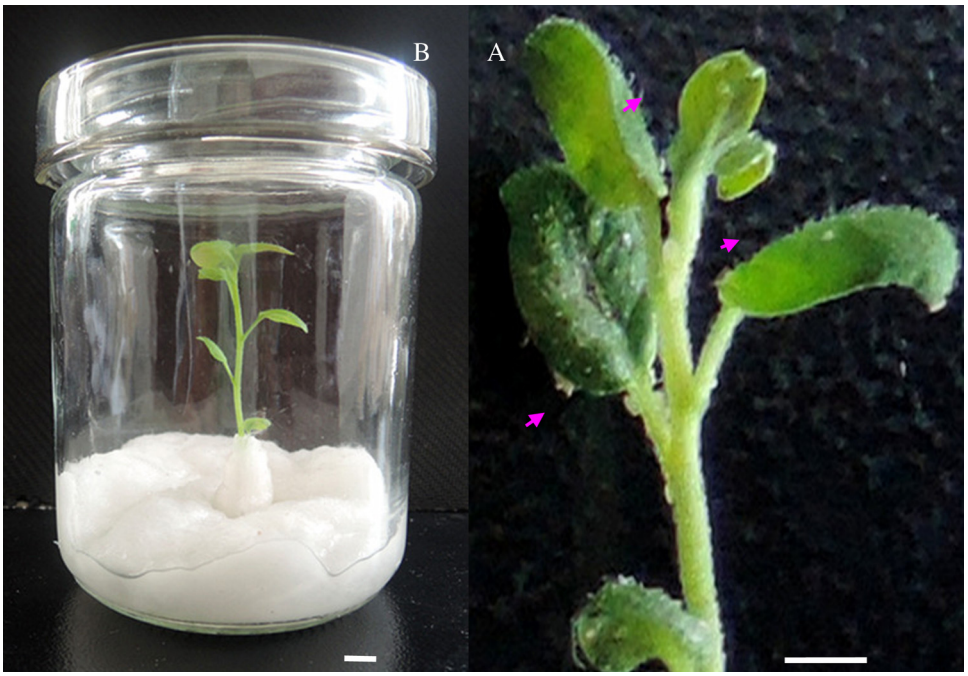

Fig. 1. Insect bioassay setup was used to identify the resistance to aphids of the transgenic potato plants and NT. Left: insect bioassay setup. Right: enlarged view of the being tested potato plants. Inset in A shows aphid culture on potato plants. The bar represents 5 mm.

each reaction, dissociation curve analysis was carried out to verify the specificity of the amplification and $2^{-\Delta\Delta Ct}$ was used to calculate the relative expression levels.

2.5. Evaluation of resistance of the transgenic potato plants to aphids

Insect resistance bioassays were performed according to the method described by Cooper et al. [32] and Du et al. [33]. Thus, a 75-mm-long sample from the top of the stem was detached from 25-day-grown transgenic and NT plants. Stem wounds were wrapped in a wet tampon and each plant was placed into a glass cage (65 mm in diameter, 90 mm in height) (Fig. 1A). The plants were inoculated with two aphid larvae in the first-instar larva state. Five plants were used per transgenic individual and control plant. Three replications were performed for each individual line. All the cages were placed in the illumination incubator (LRH-300-G) at 24 ± 1 °C. The survival of insects within the cages was monitored at an interval 24 h for 15 days (Fig. 1B). The mean survival and mortality per plant was calculated as the numbers of surviving and dead (in the four days following inoculation) aphids per day. ANOVA followed by Duncan's multiple-range tests were conducted to compare differences in mortality and survival rate among transgenic and NT control plants for the insect bioassay experiment.

3. Results

3.1. Potato transformation and GNA *gene expression analysis by qRT-PCR*

After three weeks of infection with *A. tumefaciens* LBA4404 containing the plasmid pBI121-CaMV35S-GNA

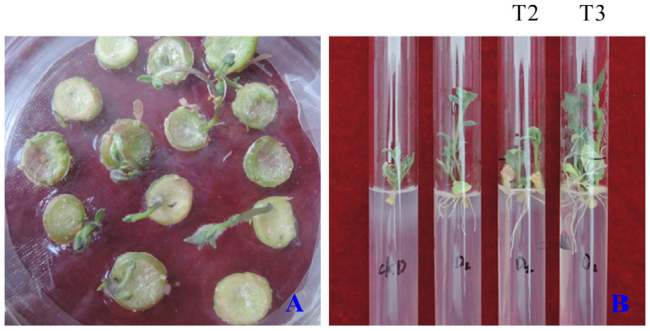

Fig. 2. Potato transformation. A. Shoot formation directly from transgenic microtuber discs of potato cv. 'Atlantic' after three weeks of culture in a selective medium (MS + 1 mg/L IAA + 0.2 mg/L GA3 + 0.5 mg/L 6-BA + 2 mg/L ZT + 50 mg/L kanamycin + 200 mg/L carbenicillin) and incubated under a photoperiod with 16 h light/8 h dark cycles at 24 °C. B. Roots were formed in about 10 days when green shoots were transferred to the selective rooting medium (MS + 100 mg/L kanamycin + 200 mg/L carbenicillin). NT: non-transformed potato plant as a negative control; T1-T3: three transgenic potato plants.

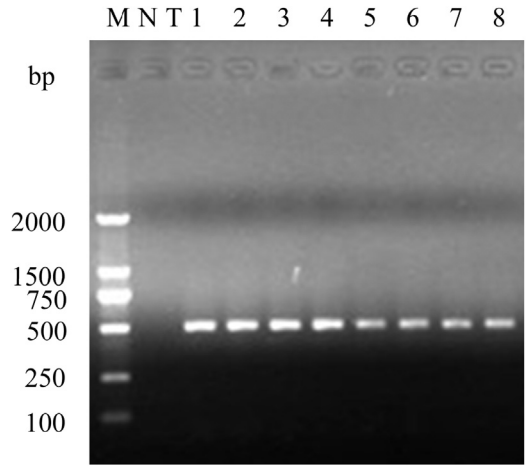

Fig. 3. Transgenic potato plants verification by PCR assay. M: DL2000 marker (TaKaRa); NT: non-transformed potato plant as negative control; 1: plasmid pBI121-CaMV35S-GNA as positive control; 2–8: seven transgenic potato plants.

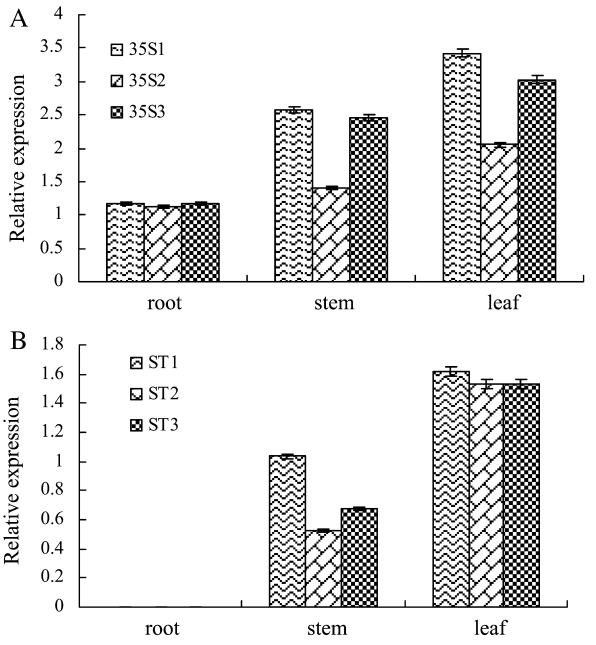

Fig. 4. Tissue-specific expression assay of *GNA* gene in the transgenic potato plants by qRT-PCR. A. Transgenic potato plants transformed with pBI121-CaMV35S-GNA. 35S1–35S3 are three different transgenic plant lines. B. Transgenic potato plants transformed with pBI121-ST-LS1-GNA. ST1-ST3 corresponds to different transgenic plant lines. Error bars indicate standard deviations obtained from three independent replicates.

and pBI121-ST-LS1-GNA respectively, green buds sprouted from the surface of the microtuber slices (Fig. 2A). Roots were formed in about 10 days (Fig. 2B) when the buds were transferred to a selective rooting media. PCR assay demonstrated that transformed plants showed a 500 bp amplification product, whereas no product was found in NT plants (Fig. 3). Here, we selected three transgenic lines 35S1-35S3 from the CaMV 35S promoter and three transgenic lines from the ST-LS1 promoter for further analysis. qRT-PCR analysis showed that the *GNA* gene was expressed in roots, stems and leaves of the transgenic potato plants transformed with pBI121-CaMV35S-GNA containing the constitutive promoter CaMV 35S (Fig. 4). However, in the transformation carried out by pBI121-ST-LS1-GNA, the *GNA* gene was expressed in all the organs except roots. *GNA* gene expression was higher in leaves than in stems of the transgenic plants (Fig. 4).

3.2. Bioassay of resistance of the transgenic potato plants to aphids

3.2.1. Aphid mortality analysis

The aphid mortality levels after 5 days of inoculation in the transgenic lines ranged between 20 (35S1) and 53.3% (ST1) (Fig. 5). Differences (at $P < 0.05$) in the mortality level against the NT control were found, except for 35S1 and 35S2 lines.

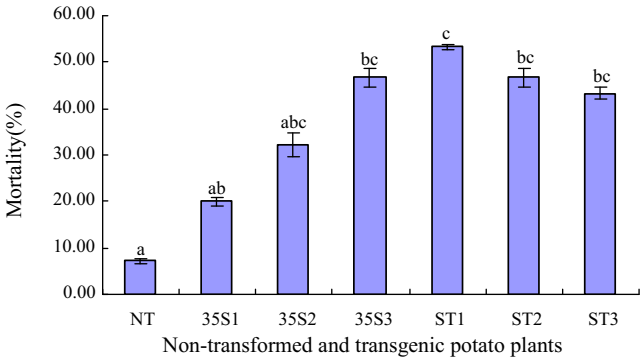

Fig. 5. Mortality analysis of first-instar larvae in the first five days following the inoculation in non-transformed and transgenic potato plants. NT: non-transformed potato plant; 35S1–35S3: transgenic plants driven by the CaMV 35S promoter; ST1–ST3: transgenic plants driven by the ST-LS1 promoter. Different small letters indicated a significant difference at $P < 0.05$ by Duncan's multiple-range test. Error bars indicate the standard deviations obtained from three independent replicates.

3.2.2. Aphid survival analysis

The aphid population on NT plants increased at a steady rate and soared up to a maximum of 68.3 ± 0.95 individuals per plant (Fig. 6). On transgenic lines, the aphid population increased more slowly than NT and rose to a maximum ranged from 17.0 ± 1.43 (ST2) to 36.6 ± 0.99 (35S3) individuals per plant, which corresponded from 24.9 to 53.5% of NT aphid population (Fig. 6).

4. Discussion

Potato is the world's fourth-largest food crop, following maize, wheat, and rice. Because it supplies dietary fibers, carbohydrates, high quantities of proteins, vitamins and minerals, potato is usually regarded as a starchy food or a vegetable. China is one of the largest potato-producers worldwide. Aphids significantly impact agricultural and horticultural crops, either by causing direct damage to plants through feeding on the phloem, or indirectly by acting as vectors for plant-pathogenic viruses [34]. There are many measures, such as genetic engineering, which have been applied to reduce the damage caused by aphids to crops [10,35–37]. Aphids hinder worldwide potato production; therefore, to effectively control the damage of the aphid has become an important issue in potato production.

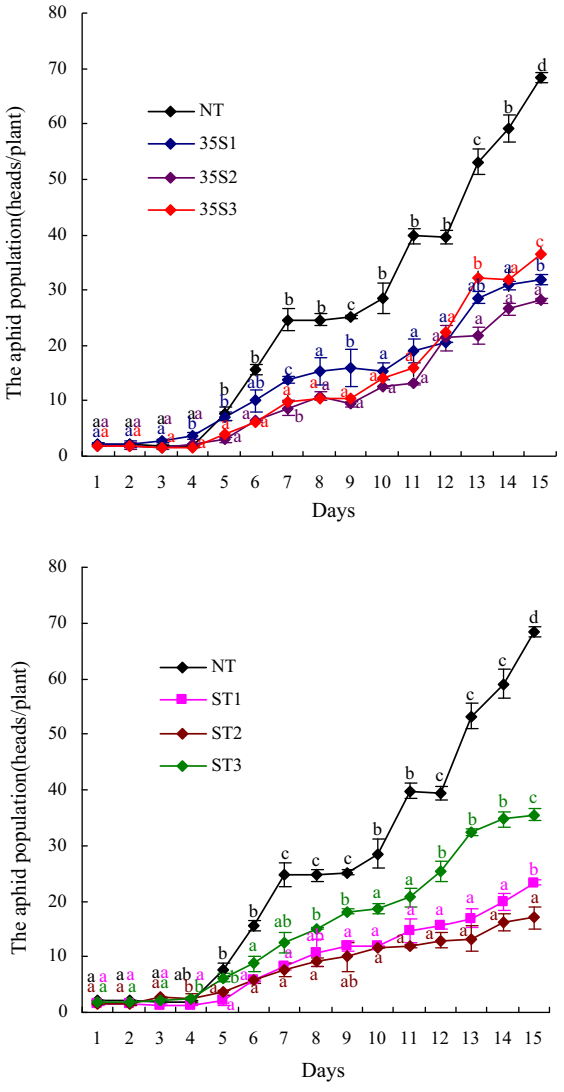

Fig. 6. Survival analysis of the aphid population during the first 15 days after inoculation with first-instar aphid larvae. NT: non-transformed potato plant; 35S1–35S3: transgenic plants driven by CaMV 35S promoter; ST1–ST3: transgenic plants driven by the ST-LS1 promoter. Different small letters indicate a significant difference at $P < 0.05$ by Duncan's multiple-range test. Error bars indicate the standard deviations obtained from three independent replicates.

Since the snowdrop lectin (GNA) was separated in 1987 [38], its encoded gene was cloned and applied to plant genetic engineering [27]. Because of lacking receptors in mammals, GNA is relatively safe to the human body. Compared with pesticide residue and ecological destruction caused by the long-term use of chemical pesticides, GNA is regarded as the safer and more efficient approach to reduce the damage for crops against insects. In our experiment, the result showed that *GNA* could express in the transgenic potato plants and represent high mortality to aphid. It was found that the quantity of aphids on the transgenic potato plants was far less than on non-transgenic plants.

Similar to other phloem-specific promoters (RSs, ubi1) with insecticidal effects in sap-sucking homopteran [14], our study detected insecticidal effects toward the green peach aphid *M. persicae* (Sulzer) in transgenic potato plants with CaMV 35S and ST-LS1 promoters. However, the result from qRT-PCR assay showed that the expression of the *GNA* gene driven by the ST-LS1 promoter in the transgenic plants was higher in leaves than in stems, and no expression in roots (Fig. 4), and even the highest mortality (ST1) and lowest survival (ST2) of aphids were found in lines generated with this promoter.

Because tuber is the edible portion of potato, in accordance with our results, we hypothesize a no or less expression of *GNA* driven by ST-LS1 promoter in this organ of transgenic potato plants, guaranteeing a more safety to its use as food. However, this hypothesis will have to be tested in the future. Moreover, our study has been done *in vitro* conditions in plantlets, so it is needed to further grow plants in the field to assay their resistance to aphids. In addition, as potato is not the only plant-hosting aphids in China, the protection of other aphid hosts must be taken into consideration in order to prevent and control the damage caused by aphids for potato.

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