A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Establishment of test systems for evaluating ingestion RNA interference-induced lethality against *Tetranychus urticae,* a representative sucking pest

흡즙성 해충인 점박이응애에 대한 섭식 **RNA** 간섭에 의한 치사력 검증 체계 구축

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UNDER THE DIRECTION OF ADVISER SI HYEOCK LEE
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF
SEOUL NATIONAL UNIVERSITY

By
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Major in Entomology
Department of Agricultural Biotechnology
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FOR THE DEGREE OF MASTER OF SCIENCE
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Establishment of test systems for evaluating ingestion RNA interference-induced lethality against *Tetranychus urticae*, a representative sucking pest

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Ung Gyu Lee

Abstract

Double-stranded RNA-mediated interference (RNAi) has been universally applied as a specific, potent and successful technology for gene manipulation and emerged as a potential pest control strategy when combined with the plant transgenesis. To establish the RNAi-based control strategy against the two spotted-spider mite (*Tetranychus urticae* Koch), a worldwide notorious sucking pests in facility cultivation, test systems for evaluating ingestion RNA interference-induced lethality were established. As a prescreening tool prior to the development of transgenic crops, protocols for the agroinfiltration in the soybean and kidney bean were established and optimized. The maximum efficiency of hairpin RNA delivery in soybean was determined to be obtained by sea sand and syringe in the soybean and kidney bean, respectively. Transient expression of target hairpin RNA reached the maximum level at 24-h post-agroinfiltration in

both soybean and kidney bean. The agroinfiltrated soybean plants expressing the COPA and aquaporin 9 (AQ9) hairpin RNA induced 64.1% and 39.5% mortalities of *T. urticae* at 144-h post-infestation. The target gene knocksown was observed in the infested mites, confirming that the observed mortality was likely resulted from RNAi. In summary, agroinfiltration was determined as an effective pre-screening tool for evaluating the RNAi-induced lethality of plants expressing target hairpin RNA prior to the generation of transgenic plants.

To confirm the proof of concept that the constitutively expressed hairpin RNA in plant can also cause RNAi-induced lethality against *T. urticae*, a representative sucking pest, an Arabidopsis plant lines constitutively expressing the COPA hairpin RNA were generated via floral-dip method. When infested with *T. urticae*, the transgenic Arabidopsis resulted in *T. urticae* mortality and transcript downregulation. These findings strongly demonstrated that sap-feeding *T. urticae* can be actually killed by in planta expression of target lethal gene, such as COPA, and RNAi-based transgenic plants can be exploited as a novel alterative mean to control sucking pests *T. urticae*, as well.

Key words: *Tetranychus urticae*, RNA interference, Feeding RNAi system, Agroinfiltration

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LITERATURE REVIEW

1. *Tetranychus urticae*

The two spotted-spider mite, *Tetranychus urticae* Koch (Acari, Tetranychidae) is one of the most serious pests known as extremely polyphagous such as ornamental plants and vegetable crops grown in greenhouses worldwide. It is known to feed on over 900 host species due to various host range. The TSSM is the most economically important phytophagous mite in the world (Van Leeuwen, Vontas et al. 2010).

Spider mite females can lay more than 200 eggs. They are lay 3 to 14 eggs per day. It completes development from egg to adult stage within 7 – 8 days at 27.5 ºC - 32.5 ºC (Helle and Sabelis 1985). Development of *T. uritcae* is influenced by host plants, plant nutrition, leaf age, moisture stress and temperature. TSSM feed excessively and reproduce quickly in spring and fall (Helle and Sabelis 1985).

To control of *T. urticae*, insecticides and acaricides have been used steadily. However, TSSM has a ability to rapidly develop resistance to almost all types of acaricides in many countries (Knowles 1997), due to its short life cycle, high biotic potential and arrhenotokous parthenohenesis reproduction (Saito, Tabata et al. 1983).

2. RNA interference

RNA interference (RNAi) also called post transcriptional gene silencing (PTGS) is a biological process in which inhibit gene expression that was caused by the destruction of specific mRNA molecules (Fire, Xu et al. 1998, Elbashir, Lendeckel et al. 2001, Hannon 2002) .

By the late 1990s, RNAi was discovered and described by Andrew Fire and Craig C. Mello those work on RNA interference in the nematode worm *C. elegans* (Montgomery 2004). When exogenous double-stranded RNA (dsRNA) are injected into *C. elegans*, it was confirmed to induce that dsRNA is bound with complementary intracellular mRNA and suppresses the expression of a gene (Fire, Xu et al. 1998).

RNAi is a defense mechanism against foreign dsRNA such as virus. In RNAi response, RNAs of 21 – 23 nucleotides in length, called small interfering RNAs (siRNAs), are snipped from longer dsRNA by an RNase 3 enzyme called Dicer. The antisense strand of the siRNA is used by an RNA-induced silencing complex (RISC) to guide messenger RNA (mRNA) cleavage, so promoting mRNA degradation (Elbashir, Lendeckel et al. 2001, Hannon 2002). RNAi has an important role in regulating expression of protein coding genes and mediate resistance to both exogenous parasitic and exogenous pathogenic nucleic acid (Hannon 2002).

RNAi technology is an important tool for gene function studies by *in vitro* and *in vivo* conditions by using loss-of-function strategy (Fire, Xu et al. 1998, Turner, Davy et al. 2006). RNAi and this technology could be used as a tool for control of insect pests (Price and Gatehouse 2008, Huvenne and Smagghe 2010). The target genes and their application methods for RNAi experiments in insect pest species has been tested from 2005 (Zhang, Li et al. 2013).

3. Agroinfiltration

Agroinfiltration is a method used in plant biology and especially lately in plant biotechnology to induce transient expression of desire gene or protein in a plant. The transfer DNA (abbreviated T-DNA) is the transferred DNA of the tumor-inducing (Ti) plasmid from bacteria such as *Agrobacterium tumefaciens*. The T-DNA is transferred from bacterium into the host plant's DNA genome. The T-DNA is bordered by 25-base-pair repeats on each end and initiation of transfer is at the right border and terminated at the left border and requires the vir genes of the Ti plasmid.

In the method a suspension of *Agrobacterium tumefaciens* is introduced into a plant leaf by direct injection conventionally using syringe or by vacuum infiltration, or brought into association with plant cells immobilised on a porous support (plant cell packs), whereafter the bacteria transfer the desired gene into

the plant cells via transfer of T-DNA.

"Agrobacterium" suspension into the leaves through the stomata into the mesophyll tissue. This can result in nearly all of the cells in any given leaf being in contact with the bacteria.

Once inside the leaf the Agrobacterium remains in the intercellular space and transfers the gene of interest as part of the Ti plasmid-derived T-DNA in high copy numbers into the plant cells. The gene is then transiently expressed through RNA synthesis from appropriate promoter sequences in all transfected cells (no selection for stable integration is performed).

CHAPTER 1.

Establishment of agroinfiltration system for in planta temporary expression of *Tetranychus urticae* hairpin RNA

Establishment of agroinfiltration system for in planta temporary expression of *Tetranychus urticae* hairpin RNA

Abstract

Agroinfiltration-based transient expression of coatomer subunit alpha (COPA) and aquaporin 9 (AQ9) genes from the two-spotted spider mites, *Tetranychus urticae*, in soybean induced RNA interference (RNAi)-based lethality to *T. urticae* (Dubey, Lee et al. 2017). To establish an optimized agroinfiltration protocol for the evaluation of hairpin RNA-induced mortality of *T. urticae*, the efficiency of different Agrobacterium delivery methods (sea sand, carborundum and syringe) was evaluated using soybean and kidney bean as host plants. In addition, transient and spacial expression patterns of COPA hairpin RNA in host plants were investigated to determine the extent of translocation of expressed hairpin RNA in host plants. The sea sand and syringe methods showed the highest expression level in soybean and kidney bean, respectively. Considering the resulting tissue damage, however, syringe appeared the best choice for agroinfiltration in both soybean and kidney bean. In the case of spacial expression pattern, the partially agroinfiltrated apical region of a leaf showed more relative expression levels in both soybean and kidney bean compared to the basal region. The untreated leaves adjacent to agroinfiltrated leaves were determined to express hairpin RNA though

the expression level was low. These findings suggest that hairpin RNA can be dispersed within a leaf and translocated to adjacent leaves. Finally, the transient expression level of hairpin RNA was highest at 48-h post-agroinfiltration in both soybean and kidney bean, suggesting that *T. urticae* bioassay needs to be initiated before 48 h following agroinfiltration. In conclusion, when using soybean or kidney bean for agroinfiltration, Agrobacterium delivery by the syringe method and the use of whole agroinfiltrated leaf for *T. urticae* bioassay at 24-h post-agroinfiltration are recommended.

1. Introduction

RNA interference (RNAi) is a gene silencing mechanism in many organisms (Tabara, Sarkissian et al. 1999, Aravin, Naumova et al. 2001, Wesley, Helliwell et al. 2001). As a novel strategy of pest control, RNAi has recently been applied for the generation of transgenic plants expressing hairpin RNAs that target species-specific genes (Baum, Bogaert et al. 2007, Mao, Cai et al. 2007, Zha, Peng et al. 2011). Once ingesting the *in planta*-expressed hairpin RNA in transgenic plants, insects exhibited significantly increased mortality due to knockdown of target genes, offering the possibility of RNAi-based transgenic plants for managing devastating agricultural pests (Price and Gatehouse 2008, Huvenne and Smagghe 2010, Zha, Peng et al. 2011).

The two spotted-spider mite, *Tetranychus urticae Koch*, is a notorious and polyphagous sucking pest that causes serious damage to a wide variety of crop species worldwide. Although synthetic acaricides have been used extensively to manage *T. urticae*, emergence of acaricide-resistant populations has dramatically reduced the efficacy of conventional acaricides (Kim, Park et al. 2006). Based on RNAi, a protocol for ingestion RNAi via kidney bean leaf disc-mediated systematic delivery of double stranded RNA (dsRNA) has been recently established against *T. urticae* (Kwon, Park et al. 2013, Kwon, Park et al. 2016). Feeding dsRNA targeting the coatomer subunits epsilon (COPE) and beta 2 (COPB2) resulted in high mortality (Kwon, Park et al. 2013, Kwon, Park et al. 2016). In addition, dsRNA targeting the coatomer subunit alpha (COPA) and the aquaporin 9 (AQ9) genes induced high lethality when ingested by *T. urticae* (Spring, Robichaux et al. 2009, Lee, Dubey et al. 2016).

Agroinfiltration has been used as the versatile technique in plant biotechnology. Universal usage of agroinfiltration inducing transient expression of gene and protein has been studied for the investigation of gene-for-gene or protein-for-protein interactions between plant resistance and pathogen avirulence genes such as PTGS (Post-Transcriptional Gene Silencing) part of a defense mechanism against virus infection (Kapila, DeRycke et al. 1997, Bendahmane, Querci et al. 2000, Stephan, Slabber et al. 2011). And transient expression of hairpin RNA

homologous to a rapidly replicating plant tobamovirus forced virus to be interfered with multiplication in a sequence-dependent manner (Tenllado, Barajas et al. 2003). Also, recent study report that use of Agrobacterium-mediated transient hairpin RNAs induced RNAi and lethality to *T. urticae* (Dubey, Lee et al. 2017). For this technique, abrasion or gentle pressure needs to let Agrobacterium mixture with the transformed gene constructs to penetrate through the stomata in plants leave and to enters the intercellular space of the leaf, the light green color getting more deepen, reflecting a successful infiltration (Sparkes, Runions et al. 2006, Chen, Lai et al. 2013). Therefore, understanding of physical structure and anatomy to the leaf are essential for successful agroinfiltration (Wroblewski, Tomczak et al. 2005, Manavella and Chan 2009, Andrieu, Breitler et al. 2012). As tool of Agrobacterium delivery, syringe infiltration has been used as a conventional way of agroinfiltration and for several plant species with proving substantial benefits (Santi, Batchelor et al. 2008) and carborundum has been used in several case of agroinfiltration as well (Jin, Wang et al. 2015) However, sea sand has been usually employed for grinding plants tissue (Mohri, Yamamoto et al. 1996).

In this study, I investigated the efficiency of agroinfiltration to the soybean and kidney bean depending on Agrobacterium delivery methods (sea sand, carborundum and syringe) by comparing the relative expression level of COPA

hairpin RNA. Following agroinfiltration with carborundum, the transient and spacial expression patterns of COPA hairpin RNA in host plants and COPA gene knockdown in *T. urticae* were evaluated.

2. Materials and methods

2.1 Growth conditions of host plants

To grow soybean (Glycine max) and kidney bean (Phaseolus vulgaris), nursery box soil was made up of Baroker (Seoul Bio, Seoul, South Korea) and PER-LITE (Green Fire Chemicals, Chungnam, South Korea) in a 3:1 ratio. And two kinds of the seeds were surface-sterilized for 10 min in a 10% (v/v) bleach solution and then washed twice with sterile water. Seedlings were raised in shallow dark plastic pots (10 cm diameter and 8 cm deep) in a plant growing room under 25±1 °C, 55±5% and RH 16:8 (L:D) photoperiod.

2.2 *T. urticae* strain and rearing

The PyriF strain, which is susceptible to acaricide and has the most similar genetic background to other green-type *T. urticae* (Kwon, Kim et al. 2011), has been reared on kidney bean plants in the insectary under 25±1 °C, 55±5% RH and a 16:8 (L: D) photoperiod since 2007. And PyriF strain from kidney bean plants was adapted to soybean plants for three month and has been reared at the same

condition since 2016 (Dubey, Lee et al. 2017).

2.3 Construction of hairpin RNA vector

In order to prepare the hairpin corresponding to part of COPA gene, partial sense and antisense COPA (accession no. NW015449938) fragments inserted into an RNAi vector (pPZP: PIN). The RNAi vector was constructed by inserting the entire hairpin-forming cassette from the T-vector insert into pPZP: PIN. Partial COPA cDNA sequences was amplified in sense and antisense orientation by reverse transcriptase using random hexamer and then cloned into the pLPS-T TA Topo Vector (Elpis Biotech, Inc., Daejeon, South Korea). The clones in this vector were confirmed by restriction enzyme digestion analysis, after that, the confirmed COPA gene sequences and GUS intron were then cloned into the RNAi vector using XhoI and BamHI restriction sites.

2.4 *Agrobacterium tumefaciens* transformation

A. tumefaciens competent cells transformation was electroporated by Gene Pulser II system (Bio-Rad Laboratories, Inc., Hercules, CA). 50–100 ng of purified binary vector containing a cloned gene was added to the top of 50 μl frozen competent cells. The mixture of competent cells and vector was placed on ice for 2 min and then transferred into a chilled 0.2 cm electrode gap cuvette (Bio-

Rad Laboratories, Inc., Hercules, CA). The electroporation parameters were performed following 25 μF and 200 Ω, using a 2.5 kV pulse for duration of 5 ms. After adding YEP media (1 ml) to the electroporation mixture, the mixture was transferred to a sterile tube and incubated in a dark rotary shaker (28 °C, 200 rpm, 2–4 h). 200 μl of cultures were spread on YEP medium containing spectinomycin, rifampicin and chloramphenicol (50, 30 and 25 μg/ml, respectively) and grown at 28 °C for 48 h.

2.5 Agroinfiltration methods

To prepare agrobacterium, a single colony from *A. tumefaciens* EHA 105 possessing binary vectors with the COPA gene was selected and cultured in 10 ml YEP media containing the three antibiotics and grown overnight in a dark rotary shaker (28 °C, 200 rpm). 100 μl of overnight culture was transferred to 100 ml YEP media in airationed flask and grown until the optical density at 600 nm (OD600) reached 0.6–0.8. The cells were centrifuged (4 °C, 9000 rpm, 5 min) and then pellet were resuspended in 10 ml of agroinfiltration media (10 mM of MgCl2 and MES, pH 5.2) containing 100 μl of acetosyringone (200 μM).

As different Agrobacterium delivery methods, Sea sand (Junsei Chemical, Tokyo, Japan), carborundum (Sicheng Abrasives, Zhengzhou, China) and 5 ml syringe (Korea vaccine, Ansan, South Korea) were used to penetration of

agrobacterium. To cause abrasion, 0.5 g of Sea sand and carborundum were added to resuspended agrobacterium and plant leaves were gently pressed by hand with the latex free gloves (Taeshin Bioscience, Namyangju, South Korea) until the surface of whole leaf turn to be dark green overall. In case of syringe infiltration, the back side of the leaf was gently injected by needleless syringe filled with resuspended agrobacterium with creating a small dark green zone. Subsidiarily, 48 h post-agroinfiltration leaves were collected for comparing the physical damage from the three materials visually.

2.6 Detection of hairpin RNA in agroinfiltrated host plants

For detecting efficiency of time-dependent agroinfiltration, carborundum agroinfiltration was conducted to host plants and treated leaves were collected at 0, 12, 24, 48, 72 and 96 h post-infestation, respectively. Whole agroinfiltration of one leaf was prepared by removing unnecessary leaves except the top of two leaves going to be agroinfiltrated. Then one leaf was agroinfiltrated, the other adjacent leaf was kept in normal condition for detection of transferred hairpin RNA. In case of partial agroinfiltration, at the middle of leaves surface were horizontally marked to divide apical and basal region. Subsequently, apical or basal region were agroinfiltrated and the other region left without agroinfiltration. And all agroinfiltrated host plants were grown in incubator under 28±1 °C, 55±5%

RH and a 16:8 (L: D) photoperiod for 48 h.

2.7 Feeding RNAi via agroinfiltrated host plants

Previously, the mites (PyriF) adapted to soybean and kidney bean were synchronized. Fifty to hundred female mites were inoculated onto each host plant which was agroinfiltrated before 24 h in incubator under 28±1 °C, 55±5% RH and a 16:8 (L: D) photoperiod. After inoculation of mites, the insectary containing host plant placed on the incubator for 96 h. And mites were collected at 0, 6, 12, 24, 48, 72 and 96 h post-infestation, respectively.

2.8 Total RNA extraction and cDNA synthesis

Total RNA was extracted from soybean, kidney bean and female mites using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's instructions. First, 50 female mites were homogenized in 100 µl of TRI reagent using a 1.5 ml tube with sterilized plastic pestle and then centrifuged (4 °C, 12000 g, 15 min). In case of plants, one soybean leaf and half of kidney bean leaf were homogenized in 1 ml of TRI reagent using sterilized mortar pestle and then centrifuged (4 °C, 12000 g, 15 min) respectively. The transparent supernatants were then transferred to isopropanol half amount of TRI reagent for precipitation. The precipitates were washed twice with 75% ethanol and then

dissolved in nuclease-free water. To prevent undesirable genomic DNA contamination, DNase I (Takara Korea Biomedical Inc., Seoul, South Korea) was treated to 10 μg total RNA at 37 °C for 30 min. The total RNA was then precipitated with 3 M sodium acetate (pH 5.2) and chilled 100% ethanol. cDNA was synthesized from 1 μg of purified RNA with random hexamer using the SuperScript Ⅵ synthesis kit (Invitrogen, Carlsbad, CA). The synthesized cDNA was stored at -20 °C until use.

2.9 Quantitative real-time PCR (qPCR) analysis

To compare the relative expression of target genes, 25 ng of diluted cDNA was used as the template and both actin and cyclophilin A primers were designed as an internal reference gene for soybean, kidney bean and *T. urticae*, respectively. Also, hairpin COPA RNA in host plants and COPA transcript in *T. urticae* were detected by using COPA gene-specific primers (Table 1). qPCR was performed by LightCycler 96 System (Roche Diagnostics, Indianapolis, IN, USA) and conducted by using the SYBR premix Ex Taq II (Takara Korea Biomedical Inc., Seoul, South Korea) in 20 μl reaction mixtures containing 5 pM of gene-specific forward and reverse primers respectively. The following qPCR cycles were used: 95 °C for 30 s, followed by 45 cycles of 95 °C for 10 s, 57 °C for 10 s and 72 °C

10 s. The Ct values were obtained from the threshold line (fluorescence = 0.2) that was automatically established by the instrument (Light Cycler 96, Roche Diagnostics, Indianapolis, IN, USA). For determination relative transcript amount of COPA in soybean, kidney bean and *T. urticae*, $2^{-\Delta\Delta Ct}$ method was used and melting curve was confirmed for exact single target amplification (Livak and Schmittgen 2001).

Table 1. The primers used in this study

Primer name	Oligonucleotide sequence	Remarks
5_COPA_PCR	5´ GATGATTCCATGAAAGGTCCT 3´	Amplification of COPA cDNA for cloning
3_COPA_PCR	5´ CTGGTCTCTCTTTACTGGTTT 3´	
5_COPA_qPCR	5´ GTCAGTCAGATGCAGTTGTC 3´	Quantification of COPA transcript
3_COPA_qPCR	5´ GAGGTGATGTTGGATGGAA 3´	
5_CyclophilinA_qPCR	5´ TTTCAATGGCCAATGCCGGA 3´	Internal reference of *T. urticae* in qPCR
3_CyclophilinA_qPCR	5´ CGACGTGTTTACCATCAAGCC 3´	
5_Actin_qPCR	5´ATCTTGACTGAGCGTGGTTATTCC 3´	Internal reference of soybean in qPCR
3_Actin_qPCR	5´ GCTGGTCCTGGCTGTCTCC 3´	

2.10 Statistical analysis

All experimental groups were designed to one independent experiment containing more than three replicates. Statistical significance of differences was calculated using non parametric one-way analysis of variance (ANOVA) ($p <$

0.05). All statistical analysis was performed with Prism 3 (GraphPad Software, Inc).

3. Results and discussion

3.1 Development of RNAi vector for *in plant* expression of hairpin RNA

The ability of RNAi vector using CaMV 35S promoter was confirmed to express enough hairpin RNA inducing RNAi in agroinfiltrated soybeans and lethality to *T. urticae* (Dubey, Lee et al. 2017). Based on this study, kidney bean, which grown worldwide and is susceptible to *T. urticae*, was utilized as another model plant to verify the applicability of agroinfiltration. Because pPZP vector was designed to express COPA gene-specific hairpin RNA in agroinfiltrated host plants, the hairpin construct was composed of sense and antisense fragment from COPA cDNA (276 bp), which are palindrome containing inserted GUS fragment (246 bp) (Fig. 1). Thus, transiently transcribed COPA gene cassette in host plants was determined to produce hairpin RNA. Although the amounts of COPA hairpin RNA is hard to be quantified precisely, clear silencing patterns were observed in COPA transcript in *T. urticae* (Dubey, Lee et al. 2017). As a common form of inducing gene silencing in plants, siRNA has that function and is translocated in plant cells by RNA-dependent RNA polymerase (RdRp) (Dunoyer and Voinnet 2005). In our study, even transiently expressed hairpin RNA was induced by

agroinfiltration system, it couldn't be inferred what is major form which ingested into *T. urticae* and response to *T. urticae* cells transcript level. However, long dsRNAs produced in transgenic plants were processed into siRNA and dsRNA to suppress gene expression in the Hemipteran Insect (Zha, Peng et al. 2011).

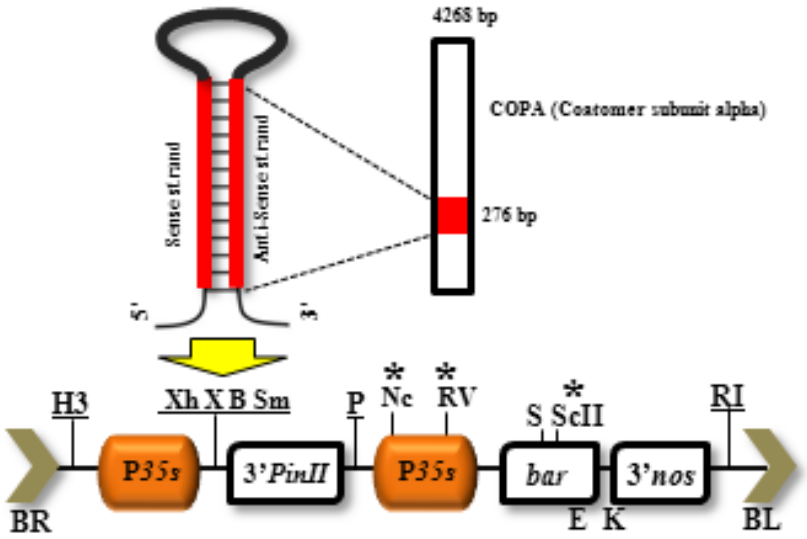

Fig. 1. Detailed schematic representation of the RNAi expression vector construct and the cDNA fragment of *T. urticae* COPA gene inserted in this vector.

3.2 Identification of efficient Agrobacterium delivery methods

To determine the most effective way of Agrobacteriun suspension into plant tissue without causing significant damages to host plants, the efficiencies of three different delivery methods were evaluated and compared. Because leaves damage

is related with density of *T. urticae* population (Tehri, Gulati et al. 2014), these factors can disrupt mites feeding behaviors and ingestion causing RNAi to *T. urticae*. First of all, qPCR analysis was used for comparing relative expression level of Agrobacterium delivery methods. Relative expression level of COPA hairpin RNA corresponding to *T. urticae* gene was significantly highest when using sea sand agroinfiltration to soybean compared with carborundum and syringe ($P < 0.05$) (Fig. 2). On the other hand, when using syringe agroinfiltration

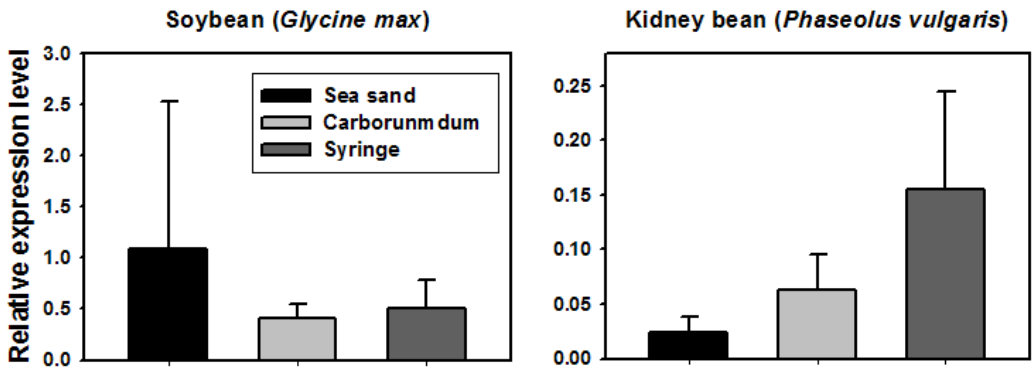

Fig. 2. Relative transient expression level of COPA hairpin RNA in 48 h post-agroinfiltration soybean (A) and kidney bean leaves (B) by different Agrobacterium delivery methods

showed a significantly highest relative expression level of COPA hairpin RNA in kidney bean compared with sea sand and carborundum ($P < 0.05$). As compared to efficiency of carborundum agroinfiltration used in previous research (Dubey,

Lee et al. 2017), COPA hairpin RNA expression when using sea sand was 2.67-fold higher in soybean and it was 2.79-fold higher in kidney bean when using syringe (Fig. 2). Tissue damage of leaves at 48 h post-agroinfiltration was mini-

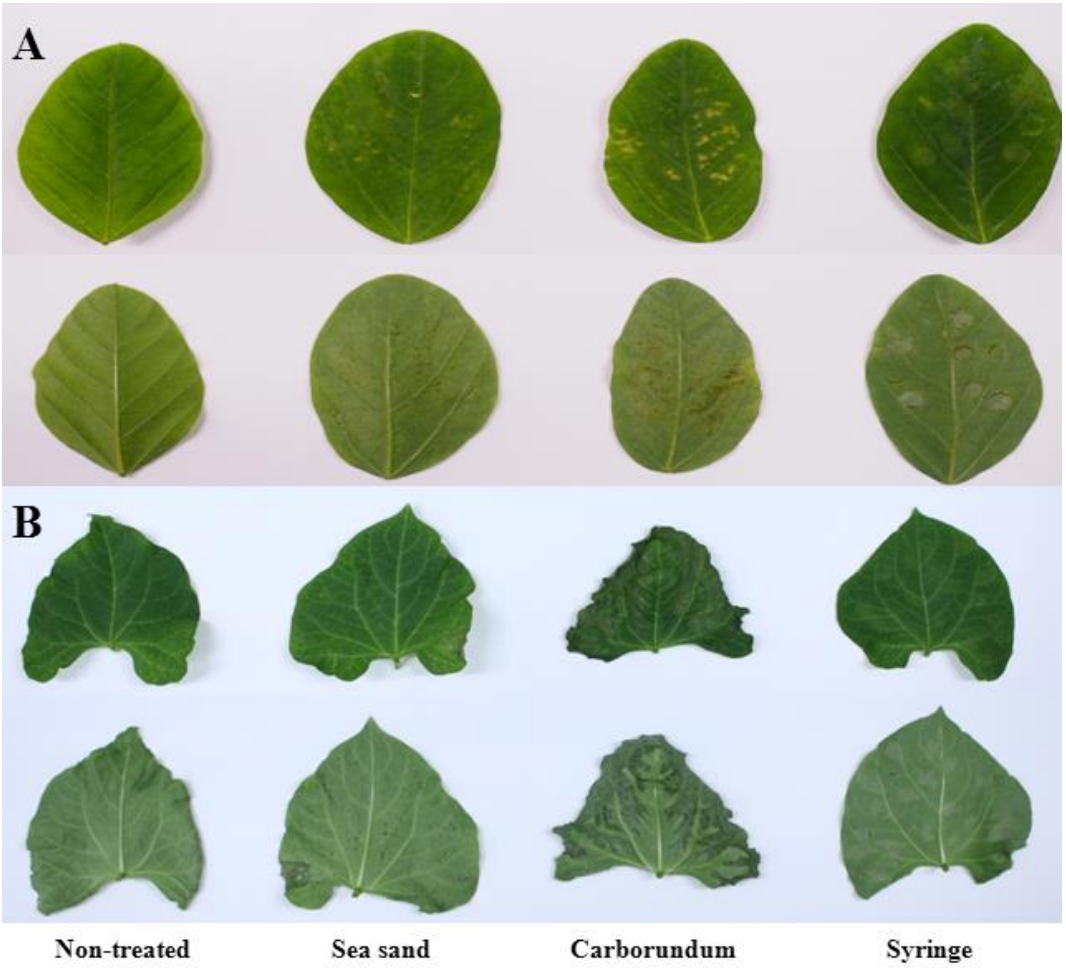

Fig. 3. Comparison of leaves appearance caused by different Agrobacterium delivery methods to soybean and kidney bean agroinfiltrated with *T. urticae* COPA gene and the control leaves without infiltration

mum in soybean with syringe and appropriate for bioassay (Fig. 3). In conclusion, if considering both aspects of efficiency and leaf damage, sea sand and syringe can be suitable as Agrobacterium delivery methods to soybean and kidney bean, respectively.

3.3 Transient and spacial expression pattern of COPA hairpin RNA in agroinfiltrated host plants

The relative expression of COPA hairpin RNA increase rapidly from initiation state and reached the highest level at 24 h post-agroinfiltration, and then reduced steadily in both agroinfiltrated soybean and kidney bean leaves (Fig. 4).

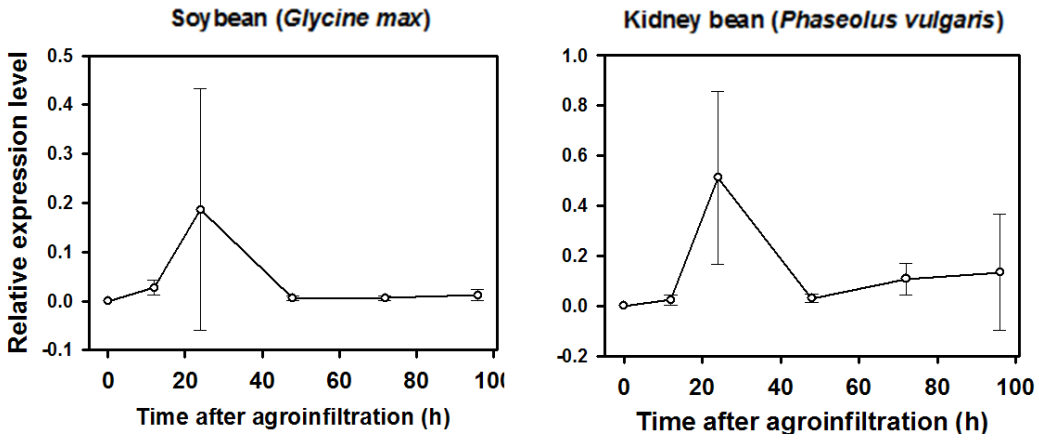

Fig. 4. Transient and spacial expression pattern of COPA hairpin RNA in agroinfiltrated host plants

Based on these results, the 48 h post-agroinfiltration time was determined as the critical point when comparing the relative expression level of hairpin RNAs. In spacial expression patterns of COPA hairpin RNA, the transcript level of COPA hairpin RNA in adjacent treated leaves was 12.6- and 79.8 fold higher than in adjacent non-treated leaves (Fig. 5).

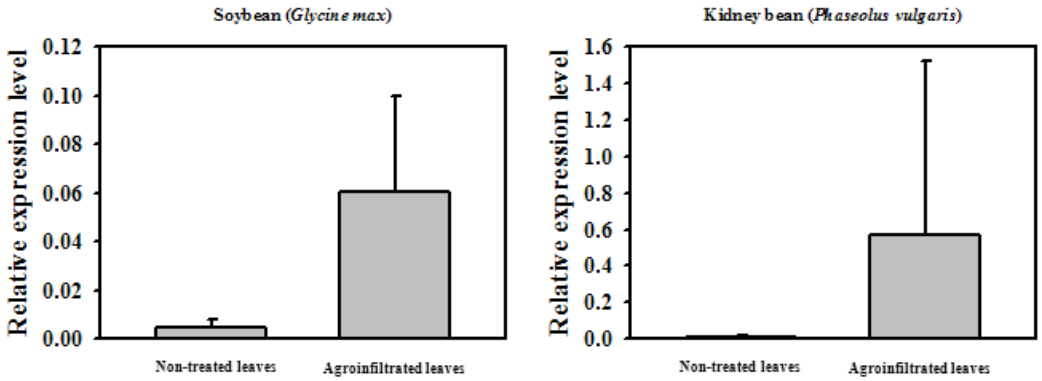

Fig. 5. Differential expression of COPA hairpin RNA between agroinfiltrated and non-treated leaves

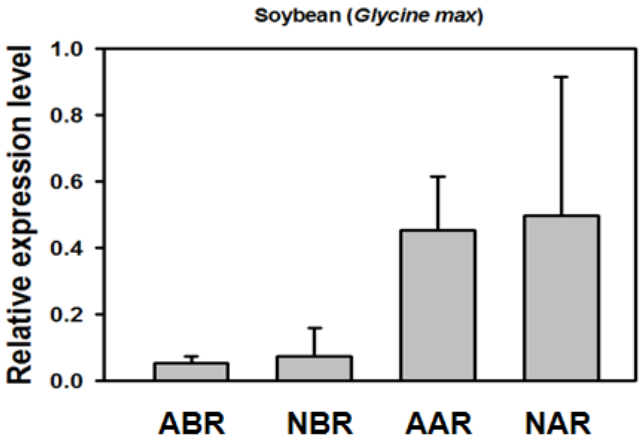

Fig. 6. Differential expression of COPA hairpin RNA between agroinfiltrated and non-treated leaves

When a single leaf was agroinfiltrated in the center, the relative expression in the apical region (AAR) within the leaf showed 5.99-fold higher levels than non-treated basal region (NBR) in soybean (Fig. 6). Similarly, non-treated apical region (NAR) showed 9.15-fold higher expression levels than agroinfiltrated basal region (ABR) in soybean. Therefore, these results suggest that hairpin RNA can be translocated within a treated leaf, especially to the apical region, but to non-treated leaves only at a negligible level.

3.4 Confirmation of COPA knockdown in *T. urticae* induced by host plants expressing COPA hairpin RNA

The COPA transcript level in *T. urticae* was also investigated by qPCR over time after infestation. Following infestation of *T. urticae* to agroinfiltrated host plants, COPA transcript level in *T. urticae* was sharply decreased at 6 h and stayed without considerable fluctuation until 96 h post-infestation (Fig. 7). As the COPA hairpin RNA level decreased after 48 h post-agroinfiltration, the constant knockdown of target gene in *T. urticae* may suggest that the constant presence of target hairpin RNA in host plant is not essential for maintaining the silenced status of the targeted gene. Since agroinfiltration appears to be effective in inducing gene silencing, it can be employed as a dsRNA (or hairpin RNA) delivery tool to identify genes responding to dsRNA ingested by *T. urticae* (Kwon, Park et al.

2017). Unlike this case, dsRNA expressed transiently in host plant was insufficient to induce RNAi in a pathogen, *Phytophthora nicotianae*, (Zhang, Wang et al. 2011).

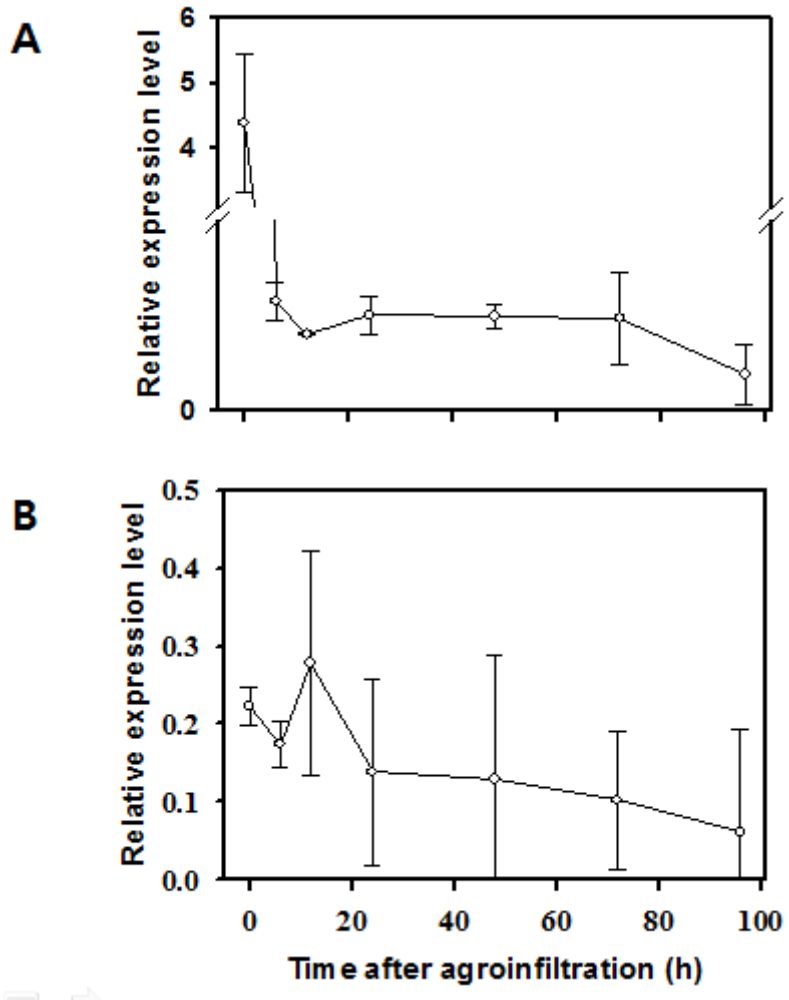

Fig. 7. Extent of COPA gene knockdown in TSSM fed agroinfiltrated soybean and kidney bean plants transiently expressing COPA hairpin RNA

CHAPTER 2.

Agroinfiltration-based expression of hairpin RNA in soybean plants for RNA interference against *Tetranychus urticae*

Agroinfiltration-based expression of hairpin RNA in soybean plants for RNA interference against *Tetranychus urticae*

Abstract

The coatomer subunit alpha (COPA) and aquaporin 9 (AQ9) genes from the two-spotted spider mite, *Tetranychus urticae*, were previously determined to exhibit RNA interference (RNAi)-based lethality when their double-stranded RNAs were systemically delivered via multi-unit chambers (Kwon et al., 2016). In current study, the hairpin RNAs of the COPA and AQ9 were transiently expressed in soybean plants by agroinfiltration. When *T. urticae* was fed with the soybean plants agroinfiltrated with the COPA and AQ9 hairpin RNA cassettes, the cumulative mortality increased significantly at 6 days post-infestation. Quantitative PCR analysis revealed that the transcript level of both COPA and AQ9 was significantly reduced in *T. urticae* after 2 days post-infestation, thereby confirming that the significant increases in mortality resulted from the knockdown of COPA and AQ9 transcripts. Our findings demonstrate the utility of COPA and AQ9 as potential genes for plant host-mediated RNAi control of *T. urticae*. In addition, we proved the usefulness of agroinfiltration as a rapid validation tool for confirming the RNAi-based lethality of target genes against arthropod pests before producing transgenic plants as agroinfiltration requires less time and skill

to validate transgene function. Furthermore, these findings prove the concept that hairpin RNA expressed in plant hosts can also induce RNAi and eventually kill *T. urticae*, a sap-sucking pest.

1. Introduction

RNA interference (RNAi) is a common gene silencing mechanism in both plants and animals (Tabara, Sarkissian et al. 1999, Aravin, Naumova et al. 2001, Wesley, Helliwell et al. 2001). The application of RNAi for pest control has recently been attempted for Coleopteran and Lepidopteran insects, in which double-stranded RNA (dsRNA) targeting a single gene was delivered via feeding (Baum, Bogaert et al. 2007). When insects ingested the tissue of transgenic plants expressing target hairpin RNA, they exhibited significantly increased mortality, confirming the possibility of *in planta* RNAi as a new pest control strategy (Price and Gatehouse 2008, Huvenne and Smagghe 2010).

The two spotted-spider mite, *Tetranychus urticae Koch,* is one of the most notorious herbivorous pests that cause serious damage to a wide variety of crop species. Various synthetic acaricides have been used extensively to control *T. urticae*. However, the development of acaricide resistance has dramatically reduced the efficacy of conventional acaricides. As an alternative control strategy based on RNAi that can mitigate the acaricide resistance problem, a protocol for

feeding RNAi against *T. urticae* via the leaf disc-mediated systematic delivery of dsRNA has been recently established (Kwon, Park et al. 2013, Kwon, Park et al. 2016). Feeding dsRNA targeting coatomer I (COPI) genes, such as coatomer subunits epsilon (COPE) and beta 2 (COPB2), resulted in high mortality (Kwon, Park et al. 2013, Kwon, Park et al. 2016). Further screening of *T. urticae* genes for RNAi revealed that dsRNA targeting coatomer subunit alpha (COPA), another COPI subunit gene, induced high lethality when ingested by *T. urticae* (Lee, Dubey et al. 2016). The transcript level of the COPI gene was significantly reduced (up to 30%) following dsRNA treatment, indicating that the toxicity resulted from RNAi against the target gene. Therefore, the COPI subunit genes appear to be good candidates for *in planta* RNAi against *T. urticae*. In addition, the aquaporin 9 (AQ9) gene, of which gene product is involved in aquaporin formation, exhibited high lethality when knocked down, suggesting its potential utility as a lethal gene for RNAi. AQ9 is primarily known as a membrane transporter protein that is involved in regulating the flow of water and several small molecules across cellular membranes (Spring, Robichaux et al. 2009).

A substantial time commitment is required to generate and screen transgenic plants, particularly crop plants expressing hairpin RNA against such candidate genes; therefore, it is desirable to evaluate RNAi efficacy via transient expression methods prior to generating transgenic plants expressing target hairpin RNA.

Agroinfiltration has proven to be a powerful tool for transient gene expression in plants. It has been used for the rapid analysis of gene function and post-transcriptional gene silencing as well as the investigation of gene-for-gene interactions between plant resistance and pathogen avirulence genes (Kapila, DeRycke et al. 1997, Bendahmane, Querci et al. 2000). In conventional agroinfiltration, gentle pressure is used to force a cell suspension of *Agrobacterium* carrying the desired gene constructs to penetrate leaf air space through the stomata (Sparkes, Runions et al. 2006). Consequently, leaf architecture and anatomy is a crucial factor in successful agroinfiltration (Wroblewski, Tomczak et al. 2005, Manavella and Chan 2009, Andrieu, Breitler et al. 2012). Successful agroinfiltration resulting in T-DNA transfer and the subsequent expression of a foreign gene is a multistep process involving *Agrobacterium* delivery, bi-directional signaling between organisms, and bacterial recognition and binding. Although agroinfiltration has been effectively used for many plants, efforts to use this method for soybean have been largely unsuccessful (Van der Hoorn, Laurent et al. 2000).

Here, we employed agroinfiltration to transiently express hairpin RNA targeting *T. urticae* COPA and AQ9 genes in soybean plants. Following transient expression, we evaluated the RNAi lethality, target gene knockdown efficiency, and degree of protection against *T. urticae*.

2. Materials and methods

2.1 Plant materials and growth conditions

The potting media was prepared by mixing sand, soil and farmyard manure in a 1:1:1 ratio. Earthen pots and the soil mixture were sterilized by autoclaving for 1 h at 121 C and 15 psi, and the soybean seeds were surface-sterilized for 10 min in a 10% (v/v) bleach solution and then rinsed twice with sterile water. Seedlings were raised in shallow pots (10-cm diameter and 8-cm deep) in a plant growth chamber [16 h, 25 C 16:8 (L: D) photoperiod]

2.2 *T. urticae* rearing

The PyriF strain, which has the most similar genetic background to green-type *T. urticae*, had been reared on kidney bean plants (*Phaseolus vulgaris*) in an insectary (25 ± 1°C, 55 ± 5% RH, 16:8 (L: D) photoperiod) since 2007 and was then adapted to soybean plants for three months.

2.3 Hairpin RNAi vector construction

The hairpin RNA-expressing RNAi construct was prepared by inserting partial sense and antisense COPA (Accession no. NW015449938) and AQ9 (Accession no. XM015937828) gene fragments into an RNAi vector (pPZP: PIN). The RNAi vector was prepared by inserting the entire hairpin-forming cassette from the T-

vector insert into pPZP: PIN. Partial COPA and AQ9 cDNA sequences were amplified in sense and antisense orientation by reverse transcriptase using gene-specific primers (Table 1) and then cloned into the pGEM-T easy vector (Promega, Madison, WI). The clones in the intermediate vector were confirmed by sequencing and restriction digestion, and the confirmed COPA and AQ9 gene sequences and GUS intron were then cloned into the RNAi vector using *Xho*I and *BamH*I restriction sites. Likewise, a partial fragment (211 bp) of pQE30 plasmid was cloned into the same RNAi vector (pPZP: PIN) and used as a negative control for agroinfiltration (Fig. 1 A).

2.4 *A. tumefaciens* transformation

A. tumefaciens competent cells were transformed using an electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA). Purified binary vector (50-100 ng) containing a cloned gene was added to the top of 50 µl frozen competent cells. The mixture of competent cells and vector was incubated on ice for 2 min and then transferred into an ice-cold 0.2 cm electroporation cuvette. The electroporation parameters were as follows: 25 µF and 200Ω, using a 2.5 KV pulse for duration of 5 ms. YEP media (1 ml) was added to the electroporation mixture; the resulting mixture was then transferred to a sterile tube and incubated with shaking (28°C, 200 rpm, 2-4 h). Cultures (200 µl) were plated on YEP

medium containing spectinomycin, rifampicin and chloramphenicol (50, 30 and 25 μg/ml, respectively) and grown at 28°C for 2 days.

2.5 Agrobacterium colony restriction analysis

To obtain positive clones harboring the gene inserts, plasmid DNA was isolated from the *Agrobacterium* and verified by restriction digestion with suitable enzymes as described below. Colonies were grown in YEP media for 48 h at 28°C and then used for plasmid isolation. The isolated plasmids were then subjected to double restriction digestion with the desired enzymes and the digested products were separated on a 0.8% agarose gel in TAE buffer at 5 V/cm current.

2.6 Agroinfiltration

A single isolated colony of *A. tumefaciens* EHA 105 harboring binary vectors carrying each gene of interest was inoculated in YEP media containing appropriate antibiotics and grown overnight in a rotary shaker (200 rpm at 28°C). An aliquot of the overnight culture (500 μl) was diluted to 100 ml in YEP media and grown until the optical density at 600 nm (OD_{600}) reached 0.6-0.8. The cells were then precipitated by centrifugation (5,000 rpm, 10 min, 4°C) and resuspended in agroinfiltration media containing 100 μM acetosyringone to an

OD_{600} of 0.6-0.8. Soybean leaves were agroinfiltrated by mechanical abrasion using carborundum (Tenllado and Diaz-Ruiz 2001).

2.7 Quantitative real-time PCR (qPCR) detection of dsRNA transcripts in agroinfiltrated soybean leaves

qPCR was performed to assess the production of dsRNA in agroinfiltrated soybean plants. Total RNA was isolated using the TRIzol method (Invitrogen, Carlsbad, CA) from agroinfiltrated leaves expressing COPA, AQ9 and negative control. cDNA was synthesized from total RNA (1 µg) using random hexamer primers (Table 1) and the SuperScript cDNA synthesis kit (Invitrogen). qPCR was conducted by using the DNA Green Master mix (Roche Diagnostics, Indianapolis, IN, USA) in 20-µl reaction mixtures containing 5 pM of each gene-specific primer set (Table 1) and 25 ng of cDNA template. The following thermal program was used: 95°C for 10 m, followed by 45 cycles of 95°C for 10 s, 55°C for 10 s and 72°C 10 s. The actin and cyclophilin A genes were used as internal reference genes for the soybean and *T. urticae*, respectively. The Ct values were obtained from the threshold line (fluorescence = 0.2) that was automatically established by the instrument (Light Cycler 96, Roche Diagnostics, Indianapolis, IN, USA). $2^{-\Delta\Delta Ct}$ method was used to quantify the relative change in transcription level of COPA and AQ9 in *T. urticae* (Livak and Schmittgen 2001). Melting curve analysis

following qPCR indicated that all reactions produced single target amplicons.

Table 1. The primers used in this study

Primer name	Oligonucleotide sequence	Remarks
5_COPA_PCR	5′ GATGATTCCATGAAAGGTCCT 3′	Amplification of COPA cDNA for cloning
3_COPA_PCR	5′ CTGGTCTCTCTTTACTGGTTT 3′	
5_AQ9_PCR	5′ ACAAACTGAGGGTGCAAATGC 3′	Amplification of AQ9 cDNA for cloning
3_AQ9_PCR	5′ TTTTGAGCCGGCCAATGAAG 3′	
5_COPA_qPCR	5′ GTCAGTCAGATGCAGTTGTC 3′	Quantification of COPA transcript
3_COPA_qPCR	5′ GAGGTGATGTTGGATGGAA 3′	
5_AQ9_qPCR	5′ GTCAGCGATACCAATTGC 3′	Quantification of AQ9 transcript
3_AQ9_qPCR	5′ TCTCACCCTCAACAGGTCAT 3′	
5_CyclophilinA_qPCR	5′ TTTCAATGGCCAATGCCGGA 3′	Internal reference of *T. urticae* in qPCR
3_CyclophilinA_qPCR	5′ CGACGTGTTTACCATCAAGCC 3′	
5_Actin_qPCR	5′ATCTTGACTGAGCGTGGTTATTCC 3′	Internal reference of soybean in qPCR
3_Actin_qPCR	5′ GCTGGTCCTGGCTGTCTCC3′	

2.8 Total RNA extraction from *T. urticae*

Total RNA was extracted from female mites using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Briefly, 50 female mites were homogenized in 100 µl of TRI reagent using a 0.5 ml glass-glass micro homogenizer (Kontes glass company, Vineland, NJ) and then centrifuged at 12,000 g. The supernatants were then collected and precipitated with isopropanol. The resulting pellets were washed twice with 75%

ethanol and then dissolved in nuclease-free water. Any contaminating genomic DNA was removed by treating the total RNA (20 μg) with DNase I (1.25 units; Takara Korea Biomedical Inc., Seoul, South Korea) for 20 min at 37°C. The total RNA was then precipitated with 3 M sodium acetate (pH 5.2) and pre-chilled absolute ethanol. The purified RNA (1 μg) was then used for random hexamer-primed cDNA synthesis. The diluted cDNA (25 ng/μl) was used as the template for qPCR using cyclophilin A as an internal reference gene.

2.9 Bioassay and mortality evaluation

Mites (PyriF) were adapted to soybean plants and maintained. The mite populations were synchronized prior to infesting on agroinfiltrated soybean plants. Fifty female mites were inoculated onto individual agroinfiltrated soybean leaves, and their mortality was evaluated at 2, 4 and 6 days post-infestation.

2.10 Data analysis

T. urticae mortality data were obtained from two to five independent experiments (a total of 5-18 replicates). Relative expression level of hairpin RNAs in soybean leaf was measured from three to four independent experiments (a total of 6-11 replicates). For the evaluation of knockdown extent of target genes in *T. urticae*, two to four independent experiments (a total of 4-5 replicates) were

conducted. The data were represented by the mean and standard deviation from all of the experiments. Non parametric one-way analysis of variance (ANOVA) was used to analyze the significance of differences between the control and agroinfiltrated soybean plants ($P< 0.05$).

3. Results

3.1 Development of RNAi constructs for agroinfiltration

The pPZP gene constructs were designed to express COPA and AQ9 gene-specific hairpin RNAs in agroinfiltrated soybean plants. Sense fragments of COPA and AQ9 cDNA (276 bp and 329 bp, respectively) were joined to the corresponding antisense fragments via a 246 bp GUS fragment (Fig 1A and 1B). When transcribed in soybean plants, these COPA and AQ9 gene cassettes should form hairpin RNA.

3.2 Transient expression of COPA- and AQ9-specific hairpin RNAs in agroinfiltrated soybean plants

Expression of hairpin RNAs corresponding to COPA and AQ9 transcripts was confirmed by qPCR using COPA and AQ9 gene-specific primers. Further validation was obtained by PCR using forward primers for COPA or AQ9 and a reverse primer for the GUS intron region. This qPCR analysis revealed that the

hairpin RNA transcript levels of COPA and AQ9 in soybean leaves were 23.7- and 32.9-fold higher compared with that of internal reference gene (actin) at 2 days post-agroinfiltration (Fig. 2). No hairpin RNA of COPA or AQ9 was detected in the soybean leaves agroinfiltrated with pQE30 (negative control).

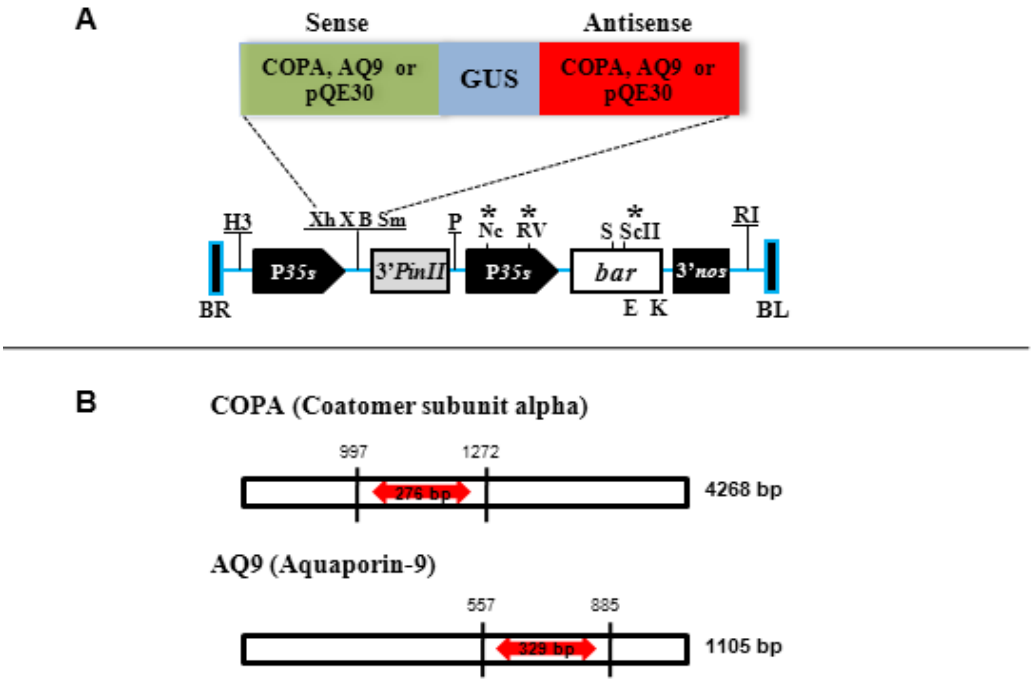

Fig. 1. Detailed schematic representation of the RNAi expression vector construct (A) and the cDNA fragment of genes (COPA and AQ9) used in this study (B).

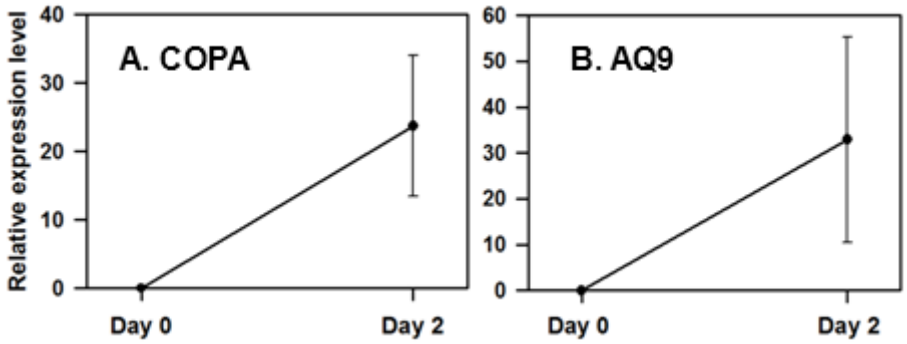

Fig. 2. Relative transient expression levels of *T. urticae* COPA (A) and AQ9 (B) hairpin RNAs in agroinfiltrated soybean plants after 48 h.

3.3 Lethality of target hairpin RNA-expressing soybean plants against *T. urticae*

Two days after the infestation on COPA-agroinfiltrated soybean leaves, a low level of *T. urticae* mortality (5.6 ± 4.9%) was observed. The mortality increased to 37.2 ± 11.9% and 64.1 ± 14.2% at 4 and 6 days post-infestation, respectively (Fig. 3A). The mortality also increased for AQ9 gene-agroinfiltrated soybean plants, from 7.1 ± 7.7%, after two days to 25.3 ± 21.7% and 39.5 ± 20.5% after 4 and 6 days, respectively (Fig. 3B). *T. urticae* mortalities for COPA and AQ9 gene-agroinfiltrated soybean leaves significantly increased after 6 ($P < 0.01$) and 4 ($P < 0.05$) days compared with that of pQE30 (control)-agroinfiltrated soybean leaves ($P > 0.05$) (Fig. 3C). These results demonstrated that agroinfiltrated soybean plants expressing COPA and AQ9 hairpin RNAs were lethal to *T. urticae*. Furthermore, both COPA- and AQ9-agroinfiltrated soybean leaves exhibited

considerably reduced damages by *T. urticae* compared with control leaves (Fig. 4).

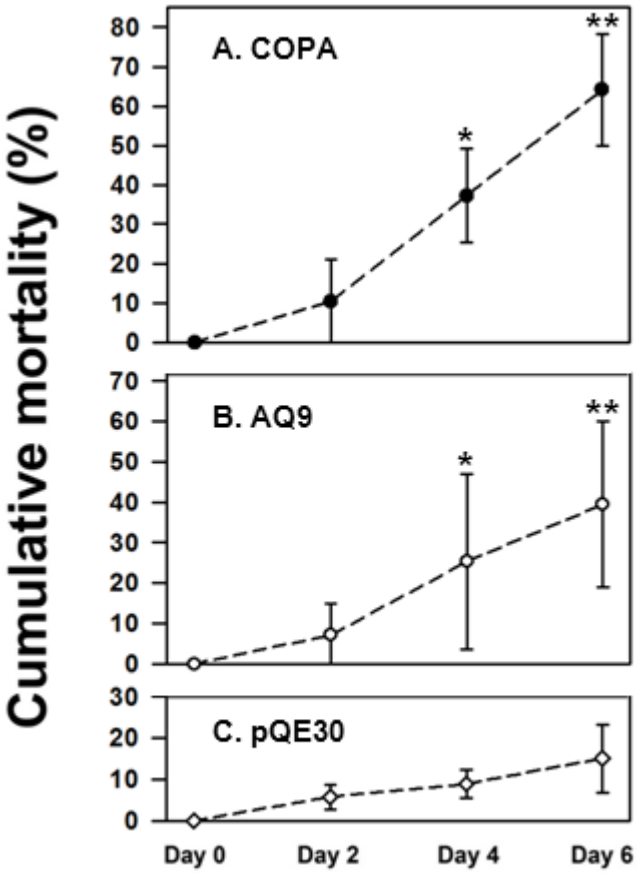

Fig. 3. Lethality of soybean plants agroinfiltrated with *T. urticae* COPA (A), AQ9 (B) or pQE30 (C) gene cassettes against *T. urticae*. The data represent the average percent mortality ± SD from two to five independent experiments (a total of 5-18 replicates). The asterisks (*) represent significant difference in *T. urticae* mortality in agroinfiltrated soybean plants over pQE30 control ($P < 0.05$)

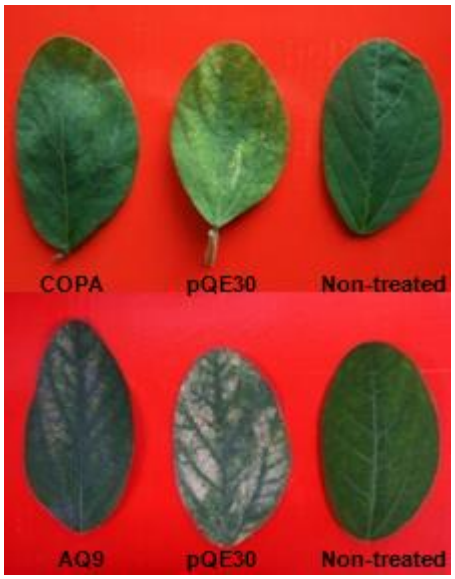

Fig. 4. Comparison of representative feeding damages by *T. urticae* between the soybean leaves agroinfiltrated with *T. urticae* COPA or AQ9 gene and the control leaves agroinfiltrated with pQE30 fragment.

3.4 Downregulation of COPA and AQ9 transcripts in *T. urticae* fed agroinfiltrated soybean plants expressing COPA and AQ9 hairpin RNA

qPCR analysis was performed to measure the extent of COPA and AQ9 gene silencing in *T. urticae* fed soybean plants expressing COPA and AQ9 hairpin RNAs. The COPA transcript levels were reduced in *T. urticae* fed soybean plants expressing COPA hairpin RNA from 2 days post-infestation. Compared to 0 day post-infestation (control), the COPA transcript levels were reduced significantly 23.8-, 20.7- and 18.8-fold at 2, 4 and 6 days post-infestation, respectively ($P < 0.001$). The AQ9 transcript levels were also reduced significantly 4.09-, 4.56- and

3.13-fold at 2, 4 and 6 days post-infestation in *T. urticae* fed soybean plants expressing AQ9 hairpin RNA, respectively ($P < 0.01$) (Fig. 5).

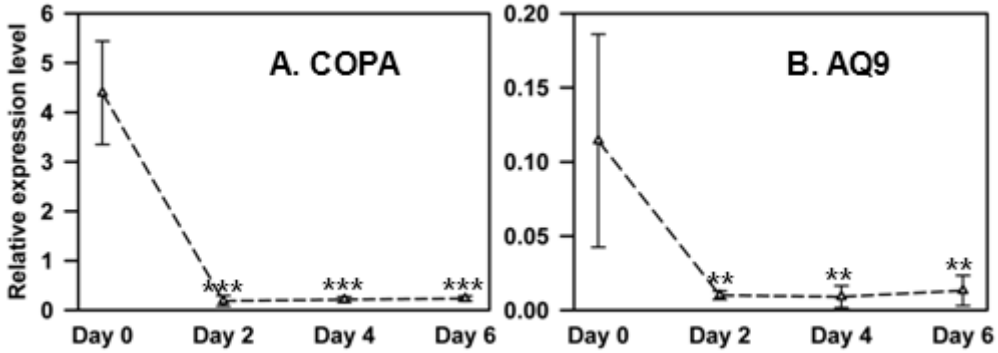

Fig. 5. Extent of COPA and AQ9 gene knockdown in *T. urticae* fed agroinfiltrated soybean plants expressing *T. urticae* COPA (A) or AQ9 (B) hairpin RNA.

4. Discussion

Here, *T. urticae* COPA and AQ9 genes were selected as target genes for agroinfiltration because COPA and AQ9 dsRNA delivery via leaf disc-mediated ingestion exhibited high mortality (Kwon, Park et al. 2013). When soybean plants were agroinfiltrated with COPA and AQ9 hairpin RNA cassettes, *T. urticae* mortality increased to 55.0 ± 14.2% on COPA plants and 46.8 ± 24.5% on AQ9 plants at 6 days post-infestation (Fig. 3). The population density of the second generation *T. urticae* was considerably lower in the COPA-agroinfiltrated soybean plants, whereas it increased rapidly on control plants agroinfiltrated with pQE30

vector; similar results were observed for AQ9-agroinfiltrated plants (personal observation, U Lee and SH Lee).

qPCR analysis confirmed that these significant increases in mortality resulted from the knockdown of COPA and AQ9 transcripts (Fig. 5). The difference in mortality observed between COPA and AQ9 agroinfiltrated soybean plants was in agreement with the results of earlier screening for potentially lethal genes via leaf disc-mediated dsRNA delivery (Kwon, Park et al. 2013, Lee, Dubey et al. 2016). It had been suggested that delivering hairpin RNA to herbivorous insects via transgenic plants would result in a 40-70% reduction of targeted mRNA expression (Zha, Peng et al. 2011).

Success of *in planta* RNAi relies primarily on the efficient expression of hairpin RNA. Use of the 35S constitutive promoter in the hairpin RNA vector appears to effectively produce sufficient COPA and AQ9 hairpin transcripts to induce *T. urticae* mortality, although it is difficult to accurately measure the amounts of target hairpin RNA produced in agroinfiltrated soybean plants. siRNA silencing amplification by RNA-dependent RNA polymerase (RdRp) is common in plants, and siRNA is a major form that is translocated between plant tissues (Dunoyer and Voinnet 2005). However, it is unclear yet whether hairpin/dsRNAs or siRNAs of COPA and AQ9 were predominantly ingested by *T. urticae* and eventually taken up by *T. urticae* cells. Previously, intact, long dsRNA produced in

transgenic plants was reported to effectively suppress insect gene expression (Dunoyer and Voinnet 2005).

Since COPA is involved in the formation of coatomer-coated vesicles that are responsible for vesicular traffic between the endoplasmic reticulum and Golgi complex in eukaryotic cells (Kirchhausen 2000), RNAi-mediated COPA knockdown may cause endomembrane system disintegration. Consequently, the RNAi-mediated down-regulation of the COPA protein may disrupt several physiological functions (e.g., autophagy, protein sorting and trafficking), thereby ultimately resulting in *T. urticae* death. On the other hand, the AQ9 gene product is primarily involved in osmoregulation, meaning that AQ9 knockdown may disrupt intracellular water and solute homeostasis, leading to *T. urticae* death (Spring, Robichaux et al. 2009). Sequence alignment of *T. urticae* COPA with orthologous genes from a variety of non-target species, including arthropods (*Drosophila melanogaster*, *Tribolium castaneum*, *Metaseiulus occidentalis*, *Apis mellifera* and *Bombyx mori*), mammals (*Bos taurus* and *Homo sapiens*) and the experimental host plant (*Glycine max*), revealed that there are 68-75% sequence identities across these species but no continuous identical sequence stretch of 20-24 bp was found (Supplementary Table 1). No orthologous genes to *T. urticae* AQ9 were identified in these non-target species. Therefore, the potential non-target effects by RNAi based on *T. urticae* COPA or AQ9 would be expected to be

minimal but experimental verification remains to be done.

Agroinfiltration has been widely employed as a rapid and cost-effective method for transient gene expression (Marion, Bach et al. 2008, Wroblewski, Caldwell et al. 2009, Chen, Equi et al. 2010). However, this study is the first to report the use of agroinfiltration for confirming the lethality of the hairpin RNAs targeting arthropod genes. Agroinfiltrated soybean plants expressing target gene hairpin RNA also appear to be a very effective means of inducing transient RNAi and screening lethal genes. All of the experimental, agroinfiltrated soybean plants were normal with respect to their overall biological traits, confirming the specificity of *T. urticae* COPA and AQ9 gene targeting and the absence of negative effects on the host plants. Considerable time and cost are required to produce and screen transgenic plants exhibiting desirable phenotypes, and it is particularly difficult to generate transgenic soybean plants because legumes have poor *in vitro* regeneration capacity (VELTCHEVA and Lilova SVETLEVA 2005). With these facts in mind, our findings support the idea that agroinfiltration can be used as a rapid tool for identifying lethal arthropod gene targets for effective RNAi prior to producing transgenic plants expressing target gene hairpin RNA.

In present study, we have established the utility of COPA and AQ9 as potential genes for plant host-mediated RNAi control of *T. urticae*. We also demonstrated that agroinfiltration could be used to rapidly screen lethal gene targets against

arthropod pests before producing transgenic plants. Furthermore, our findings represent proof of concept that hairpin RNA expressed in plant hosts can also induce RNAi and eventually kill *T. urticae*, a sap-sucking pest. However, several questions remain to be addressed: e.g., how much target hairpin RNA was transcribed and processed to dsRNA (or siRNA), and how much of that RNA was translocated into the phloem sap and available for *T. urticae* ingestion. Since *T. urticae* often feeds on cell chloroplasts (Park and Lee 2002), enhancement of target hairpin RNA expression in host plant by transplastomic transformation would improve the efficacy of RNAi-based *T. urticae* control .

CHAPTER 3.

Transgenic Arabidopsis for two spotted spider mite resistance

Transgenic Arabidopsis for two spotted spider mite resistance

Abstract

Recently, RNA interference (RNAi) technology has been emerged as a potent tool for pest control strategy. Based on the previous studies on RNAi via leaf disc-mediated systemic delivery of dsRNA and in planta expression of hairpin RNA by agroinfiltration, COPA(coatomer subunit alpha) gene has been found to be a crucial target for RNAi against Tetranychus urticae. In current study, transgenic plants of Arabidopsis thaliana expressing COPA hairpin RNA were generated by the floral dip method. Putative transgenic plants were screened by PCR and positive transformants were subjected to bioassay using age-synchronized and host-adapted *T. urticae*. *T. urticae* feeding on plants expressing dsRNA/siRNA showed more than 60% corrected mortality as compared to the mites feeding on control plants at 6 days post-infestation. Our data shows that in planta expression of hairpin gene such as COPA may serve as an effective way for the control of this important pest in ornamental and economically important plants.

1. Introduction

Management of agricultural pests has undergone through a considerable change as a number of new approaches have been implemented in pest control. Trangenic

plants that defend against insect pests by expressing insect-specific toxins, such as *Bacillus thuringensis* (Bt) endtoxins toxin, have reduced crop damages by insect pests. In addition to the Bt transgenic crops, transgenic crops expressing the hairpin RNA of a target gene have recently been developed under the principle of RNA interference (RNAi)-induced lethality against Coleopteran insects. Nevertheless, both Bt and RNAi transgenic crops target mostly chewing pests, such as Coleopteran and Lepidopteran pests. To date, no transgenic crops based on either Bt toxin or RNAi have been developed to control sucking pests yet.

The two spotted-spider mite, *Tetranychus urticae Koch,* is one of the most notorious polyphagous sucking pests that cause serious damage to a wide variety of crop species. It quickly adapts to a variety of hosts and develops pesticide resistance, thereby being the most hard-to-control pest. As an alternative control strategy based on RNAi that can mitigate the acaricide resistance problem, a protocol for feeding RNAi against *T. urticae* via the leaf disc-mediated systematic delivery has been recently developed by using the coatomer subunits ε, β and α (COPE, COPB and COPA, respectively) of coatomer I (COPI) genes [1]. The COPI complex, which is composed of seven subunits (α, β, β′, γ, δ, ε and ζ), plays a crucial role in the retrograde transport from *trans*-Golgi apparatus to *cis*-Golgi and endoplasmic reticulum [10], hypothesizing that downregulation of this gene, disrupt several physiological functions (e.g., autophagy, protein sorting and

trafficking), thereby ultimately results in *T. urticae* death. More recently, the agroinfiltration was attempted for the transient *in planta* expression of COPA hairpin RNA in soybean and kidney bean and the ingestion RNAi-mediated silencing of COPA has been demonstrated to be effective in evaluating target gene's RNAi lethality against *T. urticae* as a pre-screening tool prior to the generation of transgenic crops. Nevertheless, no actual transgenic plant expressing hairpin RNA has been developed yet.

Here, as a proof of concept, *Arabidopsis* plants were transformed *via* floral-dip method, thereby constitutively expressing a hairpin RNA homologous to a part of the *T. urticae* COPA gene. When infested with *T. urticae*, the transgenic *Arabidopsis* resulted in *T. urticae* mortality and transcript downregulation. Our findings strongly demonstrate that sap-feeding *T. urticae* can be actually killed by *in planta* expression of target lethal gene, such as COPA, and RNAi-based transgenic plants can be exploited as a novel altertantive mean to control sucking pests *T. urticae*, as well.

2. Materials and methods

2.1 Plant materials and growth conditions

The potting media was prepared by mixing sand, soil and farmyard manure in a

1:1:1 ratio. Earthen pots and the soil mixture were sterilized by autoclaving for 1 h at 121 C and 15 psi, and the Arabidopsis seeds were surface-sterilized for 10 min in a 10% (v/v) bleach solution and then rinsed twice with sterile water. Seedlings were grown in shallow pots (10-cm diameter and 8-cm deep) in a plant growth chamber [16 h, 25 C 16:8 (L: D) photoperiod].

2.2 *T. urticae* strain and rearing

The PyriF strain, which has the most similar genetic background to the green-type *T. urticae*, has been reared on kidney bean plants (*Phaseolus vulgaris*) in an insectary (25 ± 1°C, 55 ± 5% RH, 16:8 (L: D) photoperiod) since 2007 and was then adapted to *Arabidopsis* plants for three months prior to use for bioassay.

2.3 Hairpin RNAi vector construction

The hairpin RNA-expressing RNAi construct was prepared by inserting partial sense and antisense COPA gene fragments into an RNAi vector (pPZP: PIN). The RNAi vector was prepared by inserting the entire hairpin-forming cassette from the T-vector insert into pPZP: PIN. Partial COPA cDNA sequences were amplified in sense and antisense orientation by reverse transcriptase using gene-specific primers and then cloned into the pGEM-T easy vector (Promega, Madison, WI). The clones in the intermediate vector were confirmed by sequencing and

restriction digestion, and the confirmed COPA gene sequences and GUS intron were then cloned into the RNAi vector using *Xho*I and *BamH*I restriction sites.

2.4 *Agrobacterium tumefaciens* transformation

A. tumefaciens competent cells were transformed using an electroporation system (Bio-Rad Laboratories, Inc., Hercules, CA). Purified binary vector (50-100 ng) containing a cloned gene was added to the top of 50 μl frozen competent cells. The mixture of competent cells and vector was incubated on ice for 2 min and then transferred into an ice-cold 0.2 cm electroporation cuvette. The electroporation parameters were as follows: 25 μF and 200Ω, using a 2.5 KV pulse for duration of 5 ms. YEP media (1 ml) was added to the electroporation mixture; the resulting mixture was then transferred to a sterile tube and incubated with shaking (28°C, 200 rpm, 2-4 h). Cultures (200 μl) were plated on YEP medium containing spectinomycin, rifampicin and chloramphenicol (50, 30 and 25 μg/ml, respectively) and grown at 28°C for 2 days.

2.5 Floral- Dip Method

A. tumefaciens strain EHA 105 carrying the binary plasmid pPZP: PIN was used in all experiments. Agrobacterium was grown to stationary phase in liquid culture at 25–28°C, 200 rpm. in sterilized YEP. Cultures were typically started from a

1:100 dilution of smaller overnight cultures and grown for roughly 20–24 h. Cells were harvested by centrifugation for 10 min at room temperature at 5000 × *g and* then resuspended in infiltration medium to a final OD600 of approximately 1.5 prior to use. The inoculation medium consisted of 1/2 strength Murashighe & Skoog's Basal Medium, 5.0% sucrose, 44 mM benzyl amino purine, 0.005% Tween-20, pH adjusted to 5.6.

For the floral dip, plants were placed in an inverted way into the inoculum medium in a beaker and tissues above ground level were submerged for 30 sec. The infiltrated plants were removed from the beaker, placed in a plastic tray and covered with plastic wrap to maintain humidity. Plants were left in a low light and dark condition for overnight and returned to the greenhouse the next day. Plants were grown for a further 3–5 weeks until siliques were matured, keeping the bolts from each pot together and separated from neighboring pots. Seeds were harvested over a piece of clean paper. The majority of the stem and pod material was removed from the paper by gentle blowing and seeds were stored in microfuge tubes and kept at 4°C under desiccation.

2.6 Initial screening of putative transformants by using an glufosinate and PCR ·

Seeds were treated with 95% ethanol for 30–60 sec, then with 50% bleach (2.625% sodium hypochlorite, final volume) containing 0.05% Tween 20 for 5

min, followed by three rinses with sterile water for surface sterilization. To select for transformed plants, sterilized seeds were suspended in 0.1% sterile agarose and plated on 10 μg/ml glufosinate selection plates at a density of approximately 3000 seeds per 150 × 15 mm^2 plate, cold-treated for 2 days, and then grown for 7–10 days.

2.7 Quantitative real-time PCR (qPCR) detection of dsRNA transcripts in transgenic Arabidopsis

qPCR was performed to quantify the hairpin/dsRNA in transgenic *Arabidopsis* plants. Total RNA was isolated using the Trizol method (Invitrogen, Carlsbad, CA) from transgenic leaves expressing COPA. cDNA was synthesized from total RNA (1 μg) using random hexamer primers and the SuperScript cDNA synthesis kit (Invitrogen). qPCR assays were performed using a Roche Light cycler 96 under the following conditions: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and then 55°C for 30 s.

2.8 Total RNA extraction from *T. urticae*

Total RNA was extracted from female mites using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Briefly, 50 female mites were homogenized in 100 μl of TRI reagent

using a 0.5 ml glass-glass micro homogenizer (Kontes glass company, Vineland, NJ) and then centrifuged at 12,000 × g. The supernatants were then collected and precipitated with isopropanol. The resulting pellets were washed twice with 75% ethanol and then dissolved in nuclease-free water. Any contaminating genomic DNA was removed by treating the total RNA (20 μg) with DNase I (1.25 units; Takara Korea Biomedical Inc., Seoul, South Korea) for 20 min at 37°C. The total RNA was then precipitated with 3 M sodium acetate (pH 5.2) and pre-chilled absolute ethanol. The purified RNA (1 μg) was then used for random hexamer-primed cDNA synthesis using the SuperScript cDNA synthesis kit (Invitrogen, Carlsbad, CA).

2.9 Quantitative real-time PCR (qPCR) analysis

qPCR was performed to quantify the hairpin/dsRNA in transgenic *Arabidopsis* plants. Total RNA was isolated using the Trizol method (Invitrogen, Carlsbad, CA) from transgenic leaves expressing COPA. cDNA was synthesized from total RNA (1 μg) using random hexamer primers and the SuperScript cDNA synthesis kit (Invitrogen). qPCR assays were performed using a Roche Light cycler 96 under the following conditions: 95°C for 30 s, followed by 45 cycles of 95°C for 5 s and then 55°C for 30 s.

2.10 Bioassay and mortality evaluation

Mites (PyriF) were adapted to *Arabidopsis* plants and maintained. The mite populations were synchronized prior to infesting on transgenic *Arabidopsis* plants. Twenty –twenty five female mites were inoculated onto individual transgenic plants, and their mortality was evaluated at 2, 4 and 6 post-inoculation days.

3. Results

3.1 Development of gene construct for the expression of hairpin RNA in transgenic Arabidopsis for targeting COPA gene of *Tetranychus urticae*

The same gene costruct as described above were used in this study. The result was described in Chapter 1.

3.2 Development of transgenic Arabidopsis lines

Independent transgenic lines were generated which expressed hairpin RNA complementary to mRNA of *COPA* of *T. urticae* (five lines). Integration of the gene in T_0 transgenic lines was confirmed by PCR (Fig. 1)**.** The leaf disc bioassay showed all five transgenic lines of COPA to be lethal to the infested mites. These lines were selected for detailed study. The T_0 lines were self-pollinated, and seeds collected. The T_1 and T_2 lines were screened for glufosinateresistance to

glufosinate. All glufosinate-resistant lines showed to possess the *COPA gene cassette.* Lethality of T_1 and T_2 lines to *T. urticae* was evaluated (Fig. 2). Lethality to *T. urticae* in T_0 generation, the presence of glufosinate resistance, the amplification of transgene and the lethality to *T. urticae* observed in the T_1 and T_2 generation plants indicated the stable integration of *COPA gene cassette into Arabidosis genome*. No apparent difference in growth and morphology was observed between the transgenic and control plants

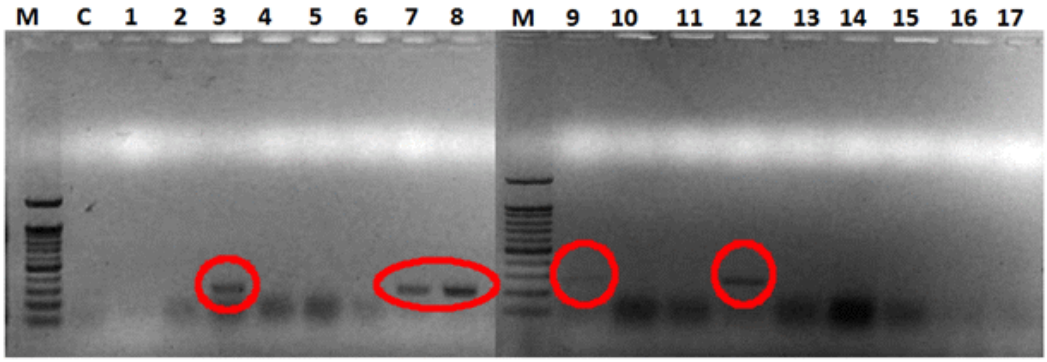

Fig. 1. PCR results confirmed by COPA primer

(M:100 bp DNA ladder. C: Untransformed plant with same genetic background. 1-17: Expected transgenic plants for COPA transgene. Plant 3,7,8,9 and 12 are positive transgenic for COPA gene)

3.3 Lethality of Arabidopsis expressing COPA hairpin RNA against *T. urticae*

72 h after the infestation on Arabidopsis expressing COPA hairpin RNA (T_2), a low level of *T. urticae* mortality (16.8 ±13.19%) was observed. The mortality

increased to 52.4 ± 11.9% and 61.7 ± 24.5% at 96 and 144 h post-infestation, respectively (Fig. 2).

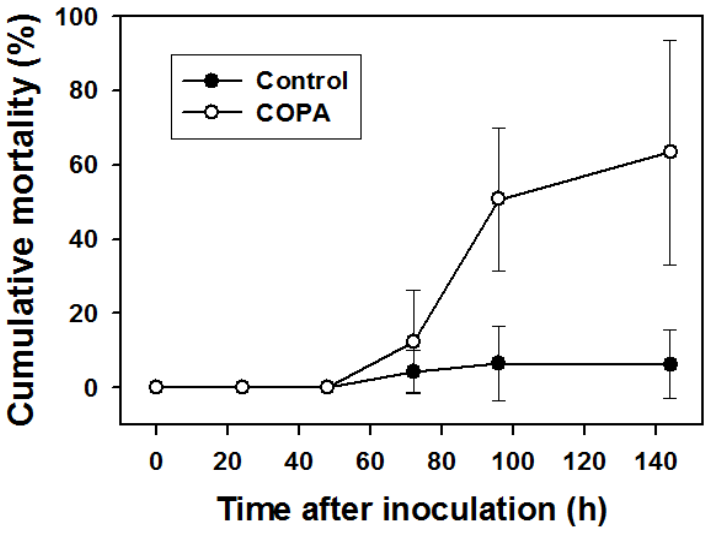

Fig. 2. Toxicity test of *A. thaliana* expressing *T. urticae* COPA hairpin RNA against *T. urticae*

3.4 Downregulation of COPA transcripts in *T. urticae* fed Arabidopsis expressing COPA hairpin RNA

qPCR analysis was performed to measure the extent of COPA gene silencing in *T. urticae* fed Arabidopsis plants expressing COPA hairpin RNA. The COPA transcript levels were reduced in *T. urticae* fed soybean plants expressing COPA hairpin RNA from 0 days to 6 days post-infestation. Compared to 0 day post-infestation (control), the COPA transcript levels were reduced 1.35-fold at 4 days post-infestation.

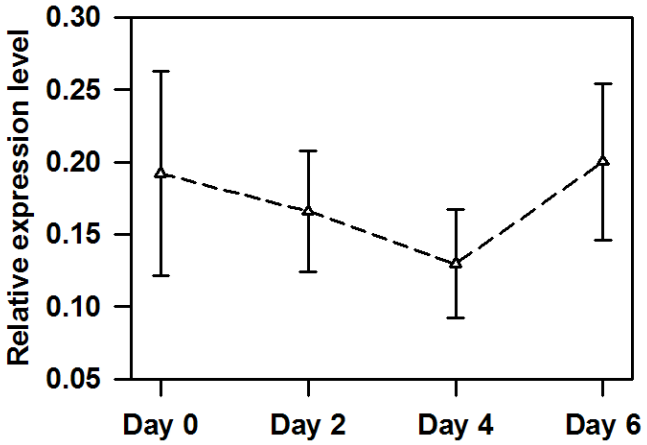

Fig. 2. Extent of COPA gene knockdown in *T. urticae* fed *A. thaliana* expressing *T. urticae* COPA hairpin RNA

4. Discussion

Success of *in planta* RNAi relies primarily on the efficient expression of hairpin RNA. In current study, hairpin RNA was determined to be expressed constitutively in transgenic *Arabidopsis* under the control of CaMV 35S promoter. It is unclear whether the hairpin RNA expression is tissue-specific or general in most tissues and how the expressed hairpin RNA is processed. As a substantial level of mortality of *T. urticae* was observed on the transgenic Arabidopsis, the expressed hairpin RNA (and/or its processed siRNA) appears to be translocated to the phloem syetem and delivered to *T. urticae* when sucking the phloem sap. To

maximize the delivery of expressed hairpin RNA to *T. urticae*, its tissue-specific expression in the sieve tube cells would be desirable, where expressed foreign transgene is directly translocated into the phloem sap, thereby facilitating the uptake of hairpin RNA into *T. urticae*. Since *T. urticae* is known to consume chloroplasts when sucking plant sap (Park and Lee 2002),, expression of target hairpin RNA in chloroplasts would also enhance the efficacy of RNAi-based *T. urticae* control significantlyas recently demonstrated in the case of RNAi against Colorado potato beetle, which was mediated by hairpin RNA expressed in chloroplasts

Selection of the target gene is of utmost importance for the effectiveness of RNAi. Since COPA is involved in the formation of coatomer-coated vesicles that are responsible for vesicular traffic between the endoplasmic reticulum and Golgi complex in eukaryotic cells. RNAi-mediated COPA knockdown may cause endomembrane system disintegration. Consequently, the RNAi-mediated down-regulation of the COPA protein may disrupt several physiological functions (e.g., autophagy, protein sorting and trafficking), thereby ultimately resulting in *T. urticae* death. Nevertheless, more potent lethal gene can be still identified from the *T. urticae* genome, as the initial identification of target gene (i.e., COPA) was based on a small scale screening. Since the ingestion RNAi mediated by *in planta* expression of lethal target gene was demonstrated to kill *T. urticae*, an extensive

screening of more potent lethal genes from *T. urticae* merits further investigation. Since the alimentary track tissues are the first contact point to the uptaken hairpin RNA, any housekeeping genes with essential functions in these tissues would be the primary candidates.

Variation in hairpin RNA expression can occur depending on the integration site in the plant genome. Although it is not determined yet the precise location of the COPA transgene cassette in the Arabidopsis genome, it is likely to be in the intergenic region as no apparent negative aspects were noticed in the transgenic lines

In summary, our study provides a proof of concept that hairpin RNA expressed in plant hosts can induce RNAi and eventually kill *T. urticae,* a sap-sucking pest, as well. Our results also provide a framework for the ingestion RNAi against other sucking arthropod pests, including aphids, thrips, whiteflies, etc. Nevertheless, several questions remain to be addressed: how much target hairpin RNA was transcribed and processed to dsRNA (or siRNA), and how much of the processed RNA was translocated into the phloem sap and became available to *T. urticae* ingestion. Searching for more potent lethal genes and more efficient expression of hairpin RNA in plant tissues either via tissue-specific or organelle-specific transformation would be desirable.

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KOREAN ABSTRACT

흡즙성 해충인 점박이응애에 대한 섭식 RNA 간섭에 의한 치사력 검증 체계 구축

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초록

특정유전자의 발현 조절을 위해 보편적으로 사용되는 RNA 간섭 기술은 최근 해충방제용 형질전환 식물체에 접목되어 미래의 해충 방제 전략으로 간주된다. 섭식 RNA간섭에 의한 치사력 검증 체계 구축의 일환으로서 이미 치사력이 검증된 점박이응애 유래 COPA(Coatomer subunit of coatomer protein complex) 유전자의 hairpin RNA를 일시적으로 식물체에서 발현할 수 있는 agroinfiltration 체계를 최적화하여 RNA간섭 효과를 검정하였다. Agroinfiltration의 방법에 따른 hairpin RNA 발현효율은 Carborundum을 이용한 agroinfiltration과

비교하였을 때, 대두에서는 바다모래 사용시 2.7배, 강낭콩에서는 주사기 사용시 2.8배의 더 많은 hairpin RNA 발현량\을 나타내었다. 일시적으로 발현된 hairpin RNA는 대두와 강낭콩 모두에서 agroinfiltration 이 후 24시간에서 제일 높은 상대적 발현량을 보였다. Agroinfiltration된 기주 내에서 발현된 hairpin RNA는 인접한 비처리 잎으로 미미한 수준이 전달되는 것을 대두와 강낭콩에서 모두 확인하였다.

COPA 및 AQ9(Aquaporin 9) hairpin RNA를 발현하는 대두의 응애에 대한 치사력검정은 pQE30 hairpin RNA를 발현하는 대두를 음성대조군으로 사용하여 수행하였다. Agroinfiltration후 48간 이후에서의 hairpin RNA의 상대적 발현량은 항존유전자인 actin과 비교하였을 때 COPA는 23.7배, AQ9은 23.9배로 나타났다. 이에 따른 점박이응애의 평균누적사충률은 접종 후 144시간에서 COPA는 64.1%, AQ9은 39.5% 그리고 pQE30는 14.3% 보였으며 통계적으로 유의하였다. 점박이응애 내의 RNA간섭현상 여부를 확인하고자 qPCR를 수행하였고, COPA와 AQ9 모두 144시간까지 해당 유전자의 발현이 유의하게 감소하는 것을 확인하였다.

이를 토대로 애기장대를 floral-dip 방법을 이용하여 형질전환체를 개발하였다. PCR에 의해 정상적으로 COPA hairpin RNA를 발현하는 개체를 선발하였고, 이를 T_1, T_2세대를 대상으로 생물검정을 실시하였다. 생물검정 결과, 평균누적사충률은 접종 후 144시간에서 COPA는 52.4%, 그리고 대조군은 11.9% 보였다. 점박이응애 내의 RNA간섭현상 여부를

확인하고자 qPCR를 수행하였고, COPA의 유전자 발현이 접종 후 96시간에서 제일 많이 감소하는 것을 확인하였다.

본 실험에서는 형질전환체 개발 전 섭식 RNA간섭 체계를 구축함으로써, 짧은 시간 안에 해당유전자의 치사력을 검증할 수 있었다. 또한 모델식물인 애기장대를 대상으로 형질전환체의 치사력을 검증할 수 있었다. 앞으로 더 높은 치사력을 갖는 유전자의 선별과 hairpin RNA의 발현량을 증대시킬 수 있는 방법이 접목된다면 흡즙성 해충의 방제에 큰 기여를 할 수 있을 것으로 사료된다.

검색어: 점박이응애, Agroinfiltration, RNA 간섭, 섭식 RNAi 시스템,

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