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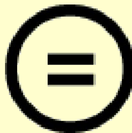

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농학석사 학위논문

Characterizing Antiviral Activity of a Triazine-based Compound Against Plant RNA Viruses

식물 **RNA** 바이러스에 대한 트리아진계 화합물의 항바이러스성 특성 구명

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정 리 라

A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Characterizing Antiviral Activity of a Triazine-based Compound Against Plant RNA Viruses

BY

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ABSTRACT

Characterizing Antiviral Activity of a Triazine-based Compound Against Plant RNA Viruses

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Plant viruses are highly contagious and their effects on plants are often drastic. Plant invasions of viruses cause physiological imbalances in various crops, resulting in significant economic losses caused by reduced crop yields and qualities. Although many catastrophic viral diseases are managed through tolerance or resistance varieties, chemical or biological control of insect vectors, quarantine of infected plants, etc., rapid emergences of plant virus diseases are growing concern due to the expansion of global trade and climate change. In order to effectively control epidemics caused by emerging plant viruses, the need for plant virus control agents is increasing. However, up to date no commercial chemical control agent is available. The purpose of this study is to characterize a triazine-based compound as potential antiviral agent by spraying this compound followed by infecting several plant RNA viruses in *Nicotiana benthamiana*. For this, the triazine-based compound, atrazine, with concentration of 50 μM and 100 μM was sprayed 1 hours after the virus inoculation on the 4-week-old *N. benthamiana*. I tested effect of spraying atrazine against six viruses which belong to different genera, i.e.

Pepper mottle virus (PepMoV; *Potyvirus*), *Potato virus Y* (PVY; *Potyvirus*), *Potato virus X* (PVX; *Potexvirus*), *Cucumber mosaic virus* (CMV; *Cucumovirus*), *Tomato spotted wilt virus* (TSWV; *Tospovirus*) and *Pepper mild mottle virus* (PMMoV; *Tobamovirus*). The inhibitions of virus replication and movement were observed in the systemic leaves of infected plants that sprayed with the atrazine compared to that of control. In case of PMMoV, however, the atrazine treatment did not completely inhibit virus movement to the upper systemic leaves. Interestingly, mild symptoms were observed on atrazine-treated *N. benthamiana*, possibly due to a decrease in the level of accumulation of PMMoV RNAs upon atrazine. To investigate the major signaling pathways associated with atrazine-induced resistance, quantitative RT-PCR was performed using specific primer sets for selected genes involved in the typical signal transduction pathway(s). Expression levels of *R* gene, nitric oxide (NO), salicylic acid (SA) and jasmonic acid-ethylene hormone pathways related genes were significantly increase in the atrazine treated plants compared to those of mock plants. Moreover, the replication and movement of virus was also inhibited in upper systemic leaves of NahG (SA-defective) plants, suggesting atrazine-induced resistance is likely does not related to the SA pathway. Taken together, these results suggest that the atrazine-induced resistance might be involved by other signaling pathways than the SA pathway, that resulted in inhibiting plant virus replications. In conclusion, atrazine could be used as the chemical candidate for controlling or inhibiting plant RNA virus infections.

Keywords: Atrazine, Antiviral activity, Inhibition of virus replication, Plant defense pathways

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INTRODUCTION

Plant viruses consist of the genetic material of either RNA or DNA, the nucleic acid, and the capsid that surround it. Within appropriate host cells, the virus multiply through translation and replication, and the virus progeny is able to spread into adjacent cells through cell-to-cell and long-distance movement to cause systemic infection of plants (Hull 2009; Kim et al. 2018; Meshi et al. 1987).

Plant viruses are highly contagious and their effects on plants are often drastic. Plant invasions of viruses cause physiological imbalances in various crops, resulting in significant economic losses caused by reduced crop yields and qualities (Bos 1982). The damage caused by viral disease is severe worldwide. Recently, the rapid emergences of new diseases and damages caused by plant viruses have been gradually increasing due to the several factors such as agricultural intensification, climate changes and expansion of global trades (Canto et al. 2009; Jones 2009; Kim et al. 2018; Sastry and Zitter 2014), and thus the need for viral disease control agents is also increasing. Many catastrophic viral diseases are mainly managed through i) using tolerant or resistant varieties, ii) applying chemical or biological control agents of insect vectors, iii) quarantine regulation of infected plants or seeds and iv) using cross-protection methods (Islam 2017; Stevens 2013). Broadly, these managements offer no perpetual solution to a virus disease problem (Matthews and Hull 2002). However, unlike other pathogens, for virus diseases no approved or dependable antiviral products is generally available

commercially (Schreinemachers et al. 2015). Although several chemical agents were effective in limiting virus replication, suppressing virus disease symptoms, or inducing host defense mechanisms, such as ribavirin, 2-thiouracil (Bawden and Kassanis 1954; Lerch 1987), unfortunately these chemicals could not be used in fields for virus disease management since these chemicals showed bad effects on humans, animals, and the environment (Waziri 2015). Therefore, identification and development of new antiviral substances are needed for controlling virus diseases in fields.

Atrazine is a triazine-based herbicide that is currently one of the most widely used herbicide in worldwide. It is effective in controlling a broad range of weeds and grasses in corn, sorghum, sugarcane, pineapple, turf and orchards (Kaya et al. 2010).

Plant have the ability to develop resistance responses against pathogen attacks. If the plant immediately recognizes a pathogen infection, recognition often resulted in the onset of the defense-related pathways. Plant defense in response to pathogen is regulate by complex network of signaling pathways. *R* gene-mediated resistance pathways often include the involvement of signaling molecules such as nitric oxide (NO), salicylic acid (SA), jasmonic acid (JA), and ethylene. These pathways are mutually antagonistic, and rather influence each other through a complex network of regulatory interactions (Dong 1998; Feys and Parker 2000; Kachroo et al. 2006; Kunkel and Brooks 2002; Tran et al. 2016).

The aim of this study is to test and characterize the triazine-based compound as potential antiviral agents. The compound was tested against

various plant RNA viruses by observing the inhibition of viral replication and movement. I also investigated induction or suppression of genes related to typical signal transduction pathway(s) upon treatment of the compound by conducting quantitative RT-PCR for determining the genes and/or signaling pathway(s) responsible.

MATERIALS AND METHODS

1. Plant material and virus inoculation

Nicotiana benthamiana that used in this study was grown at 25 ℃ in growth chamber with 16 hours light and 8 hours dark cycles. The plant RNA viruses including pepper mottle virus (PepMoV), potato virus Y (PVY), potato virus X (PVX), cucumber mosaic virus (CMV), tomato spotted wilt virus (TSWV), and pepper mild mottle virus (PMMoV) were mechanically inoculated by using virus-infected saps. A frozen virus-infected leaf tissue was ground using 0.05 M potassium phosphate (pH 7.4). Carborundum was powdered over the leaf surface before inoculation, following by rapid rub the sap all over the leaf surface (Pflieger et al. 2015).

2. Atrazine treatment

Atrazine was dilute to concentrations of 50 μM and 100 μM with 5 % Dimethyl sulfoxide (DMSO). The compounds diluted to each concentration were sprays to foliage one time an hour after the virus inoculation on the 4-week-old *N. benthamiana*.

3. Green fluorescent protein (GFP) imaging

GFP expression during virus replication was observed under the Dark reader™ Hand Lamp HL28T UV Lamp (Clare Chemical Research, Inc., USA) using Nikon D80 digital SLR camera with UV filter (Nikon, Japan).

4. Reverse transcription-polymerase chain reaction (RT-PCR) for virus detection

Total RNA was extracted from systemic leaves using RNAiso Plus (TaKaRa, Japan). About 1 μg of total RNA was used to synthesize complementary DNA (cDNA) with random hexamer and GoScript™ Reverse Transcriptase (Promega, USA) in a reaction volume of 20 μl according to the manufacturer's protocols. RT-PCR was conducted using virus specific primer sets. For RT-PCR, 50 ng of cDNAs were add to the reaction mixture consisted of 2 μl of 10 X PCR buffer, 1.6 μl of dNTP, 0.1 μl of Ex-*Taq* (TaKaRa, Japan), 1 μl each of forward and reverse primers, 1 μl of cDNA (50 ng/ μl) and 13.3 μl of third-distilled H_2O. PCR was performed with the following cycling parameters: 95 ℃ for 3 min; followed by 25 cycles at 95 ℃ for 3 s, annealing temperature for 3 s, 72 ℃ for 1 min; and 72 ℃ for 5 min.

5. Quantitative RT-PCR (qRT-PCR) analysis for determining gene expression

After atrazine treatment with PVX inoculation, the leaves of treated plants were collected at several different time points, which are immediately after chemical treatment, 24, 48, and 120 hours post-treatment. Isolated RNA was reverse transcribed into cDNA. qRT-PCR was performed on a CFX384 real-time PCR system (Bio-Rad, USA). The 10 μl of reaction mixture included 1 μl of cDNA (25 ng/ μl), 5 μl of 2X iQSYBR Green supermix (Bio-Rad, USA), 1 μl of each gene-specific primer sets (Table 1), and 2 μl of third-distilled H_2O. qRT-PCR condition that used in this experiment are 95 ℃ for 3 min (pre-denaturation), followed by 40 cycles of programmed amplification (denaturation 95 ℃ for 10 s; annealing & extension 55 ℃ for 30 s) and melt curve 55 to 95 ℃, for 5s. Relative expression levels of genes were calculated by Bio-Rad CFX Manager software, version 1.6.541.1028 (Bio-Rad, USA), using the 2−ΔΔCt method (Livak and Schmittgen 2001), and were all normalized to the expression levels of *Actin* (ACT) and *Ubiquitin* (UBI3) in each group (Liu et al. 2012; Rotenberg et al. 2006).

6. Statistical analysis

Data was analyzed with IBM SPSS Statistics software, version 25.0 (SPSS Inc., USA). Statistical analyses were performed by means of one-way analysis of variance (ANOVA) followed by sheffe's test. Significant difference from each treatment group compared with the control was considered when *P* values of less than 0.05 (typically $P\text{-}value \leq 0.05$).

Table 1. List of primer sets used for qRT-PCR.

Name	Forward primer (5'–3')	Reverse primer (5'–3')
NbHSP90	ATGGCGGAGGCAGAGACGTT	CGGATCTTGTCCAGAGCATCGGAA
NbSGT1	GCTTTTCGCCGACCGTGCTC	ACAGGCCAACCCCTTACGCA
NbEDS1	TGTTGGCACAGATGAGGTAGCCA	CCCGACGAGTGCCCTGCAAA
NbNDR1	CAATAATACATGCTGCTTTAAGTGCATCC	CTAAAGAGCAAGATGGTTTTGTGGTTCT
NtNPR1	TGCGGCTTCTCCGGAGTTTG	CGGACTCCTCGCCGACAAAA
NbEIN2	GCCGGAGCTATGGGGGAAAT	GGGGGCTTGATTTTTGCTGCC
NbCOI1	TGGCGAAGGGATGCCTTGAA	CGGTCAAGCAAAACCAGCCG
RbohB	CTATGCTTCAGTCTCTTCACCATGCC	GGTTGAGAGCAATGCGTTTG
NbNOA1	TCCTCAAAGTCGTCTCCGTGCT	AATTCGCACGAGGCCTCCCC
NbPDF1.2	CTTCAAGCAAAGCTGCAGCCAAAG	CTATGCACTAAGCCATGTGTGTTTG
PR5	ATGCGCAGCCCCTATTAAC	TGGGTTGTTACATCCACCTTG
ERF1	GTTAACGCCGTCAAGTTGGT	AGAGGCGGCACCTCAAATA
ICS1	GAACCATGTCCTCCTCCCGC	GGGTGAGAAGTTCCGTTGAAGCA
NbPal1	TGGAAAGTGGCAACCCTGCT	CACTCCTCGCCCGGTTGACCG
Nb_actin	CCAGGTATTGCTGATAGAATGAG	CTGAGGGAAGCCAAGATAGAG
Nb_Ubi3	GCCGACTACAACATCCAGAAGG	TGCAACACAGCGAGCTTAACC

RESULTS

1. Effect of atrazine on potyvirus infections in *N. benthamiana*

In order to confirm the effect of atrazine against potyvirus infection, PepMoV and PVY which belonging to the genus *Potyvirus* (Barnett 2012; Kenyon et al. 2014) were use in this study. To test the effect of atrazine against PepMoV, GFP-fused infectious PepMoV DNA clone (pPepMoV::GFP) was use as inoculum. As for testing effect of atrazine treatment against PVY infection, virus-infected leaf tissue was used for inoculation into *N. benthamiana*. The atrazine was sprayed 1 hours after the virus inoculation. In case of pPepMoV::GFP, the expression of GFP was not observed in the systemic leaves of infected plants that sprayed with the atrazine of 50 μM and 100 μM while strong GFP expression was observed with pPepMoV::GFP-inoculated control plants (Fig. 1A). This result suggests that the replication and movement of PepMoV were inhibited by atrazine treatment. As for testing effect against PVY, any symptom developments of PVY was not observed on the plants that were inoculated with PVY and treated with 50 μM and 100 μM of atrazine (Fig. 1B).

To confirm the replication of viral RNA, RT-PCRs were conduct by using each virus specific primer pairs. As expected, I could not detect any amplified DNA from upper systemic leaves of pPepMoV::GFP-infected plants that sprayed with 50 μM and 100 μM of atrazine (Fig. 1C). Similar result was also observed for PVY. No virus-specific amplified DNA was detected by both atrazine concentration treatments (Fig. 1D). These results indicated that

atrazine treatment with 50 μM and 100 μM has inhibitory effect against potyvirus infections.

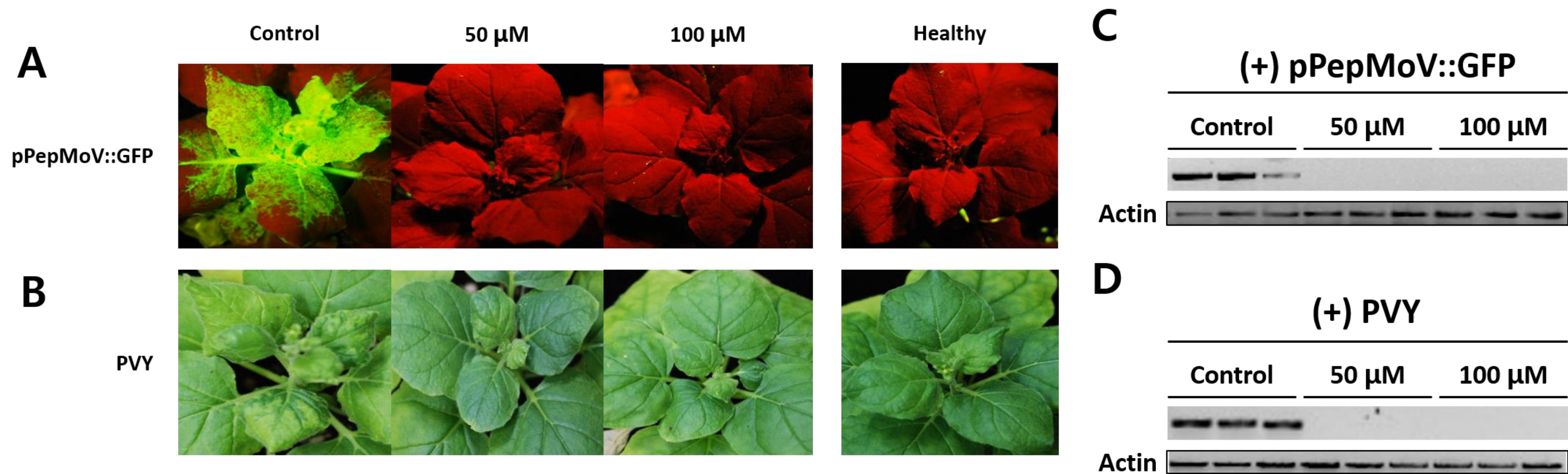


Figure 1. Effect of atrazine on potyvirus infections in *Ncotiana benthamiana*. pPepMoV::GFP (A) and PVY (B) were inoculated on *N. benthamiana*. The plants that were inoculated using GFP-fused infectious clone was observed under UV lamp (A). The plants inoculated by infected leaf tissue were observed under normal light (B). The viral RNA of PepMoV and PVY in systemic leaves was detected by RT-PCR (C and D).

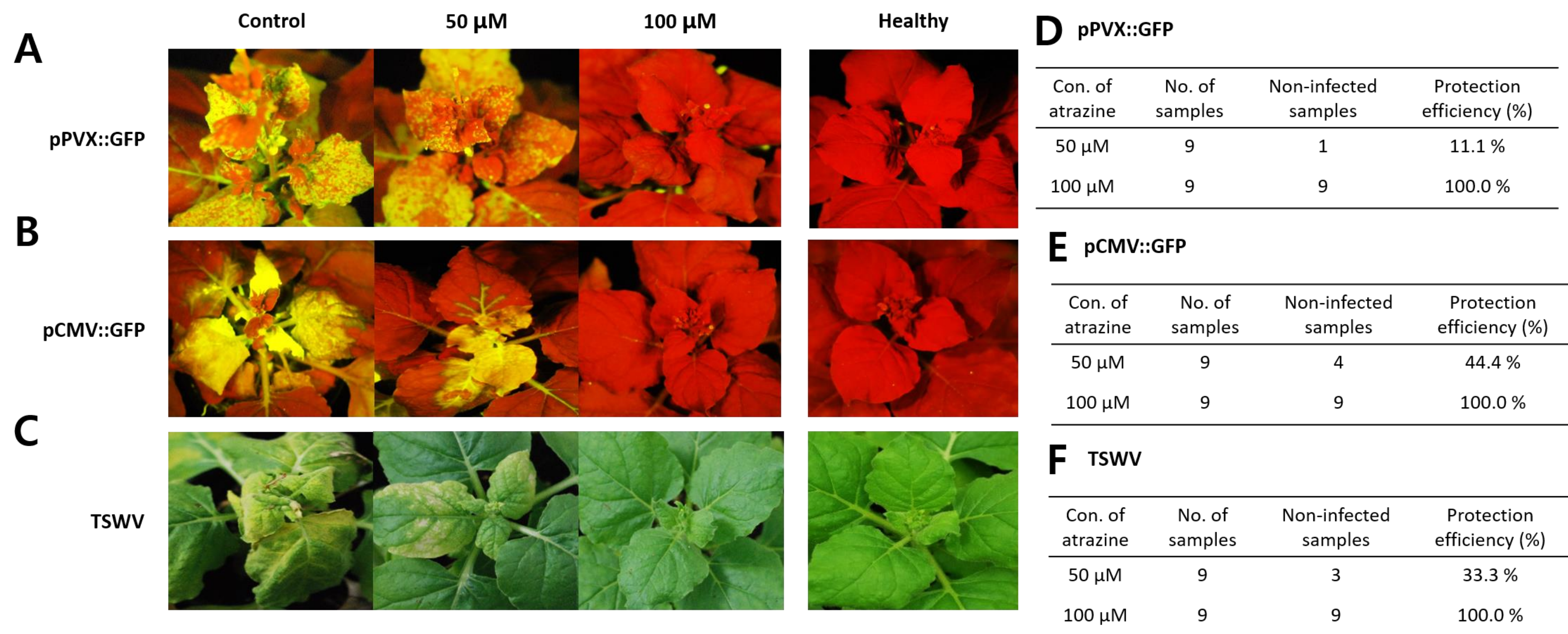
2. Effect of atrazine on viral infections from other plant RNA viruses in *N. benthamiana*

As described above, atrazine treatment with 50 μM and 100 μM was showed strong inhibitory activity against potyvirus infections including PepMoV and PVY. To further investigate that the efficacy of the atrazine against several other plant RNA viruses, PVX (*Potexvirus*) (King et al. 2011), CMV (*Cucumovirus*) (Gibbs and Harrison 1970), TSWV (*Tospovirus*) (Fauquet CM et al. 2005), and PMMoV (*Tobamovirus*) (Kenyon et al. 2014) that are belong to different genera were used. While full-length infectious clones expressing GFP for PVX (pPVX::GFP) and CMV (pCMV::GFP) were used, each virus-infected leaf tissue was used for TSWV and PMMoV inoculations into *N. benthamiana*.

In case of pPVX::GFP and pCMV::GFP-infected plants, the expression of GFP was not observed on the upper systemic leaves treated with 100 μM (Fig. 2A and B). For the TSWV test, plants treated with 100 μM atrazine did not show any symptoms of TSWV, while control plants showed typical specific symptoms of TSWV (Fig. 2C). Protection efficacy against these three viruses by atrazine treatment in *N. benthamiana* were ranged 11 to 44 % at 50 μM concentration, whereas a 100 % protection was observed with 100 μM atrazine concentration treatment (Fig. 2D, E and F). These results indicate atrazine treatment of 100 μM was very effective in preventing PVX, CMV, and TSWV infections in *N. benthamiana* plants.

In contrast, plants infected with PMMoV treated with 50 μM and 100 μM atrazine did not completely inhibit the movement of the virus to the upper

systemic leaves. Mild symptoms, however, were observed on atrazine-treated *N. benthamiana*. Observed symptoms are weaker than the one in the virus only infected plants (control) (Fig. 3A). Virus specific amplified DNA was detected in the upper systemic leaves of plants infected with PMMoV by RT-PCR analysis (Fig. 3B), indicating movement and replication of the virus in the systemic leaves. To determine whether the atrazine treatment caused milder symptom development either by retarding virus movement or RNA replication level, qRT-PCR was performed. For this analysis, the upper systemic leaves of treated plants were collected at 3, 6, and 10 days post inoculation. The accumulation level of PMMoV RNAs were significantly reduced in the plants treated with 50 μM and 100 μM atrazine compared to untreated plants (control) (Fig. 3C). These results suggest that treatment of 50 μM and 100 μM atrazine affected negatively for the replication of PMMoV RNA and thus delayed symptom development.



D pPVX::GFP

Con. of atrazine	No. of samples	Non-infected samples	Protection efficiency (%)
50 μM	9	1	11.1 %
100 μM	9	9	100.0 %

E pCMV::GFP

Con. of atrazine	No. of samples	Non-infected samples	Protection efficiency (%)
50 μM	9	4	44.4 %
100 μM	9	9	100.0 %

F TSWV

Con. of atrazine	No. of samples	Non-infected samples	Protection efficiency (%)
50 μM	9	3	33.3 %
100 μM	9	9	100.0 %

Figure 2. Effect of atrazine on other plant RNA virus infections in *N. benthamiana*. pPVX::GFP (A); pCMV::GFP (B); and TSWV (C) were inoculated on *N. benthamiana*. The plants that were inoculated using GFP-fused infectious clones were observed under UV lamp (A and B). The plants inoculated by infected leaf tissue were observed under normal light (C). Protection efficiency when each virus was inoculated and treated with atrazine (D, E, and F).

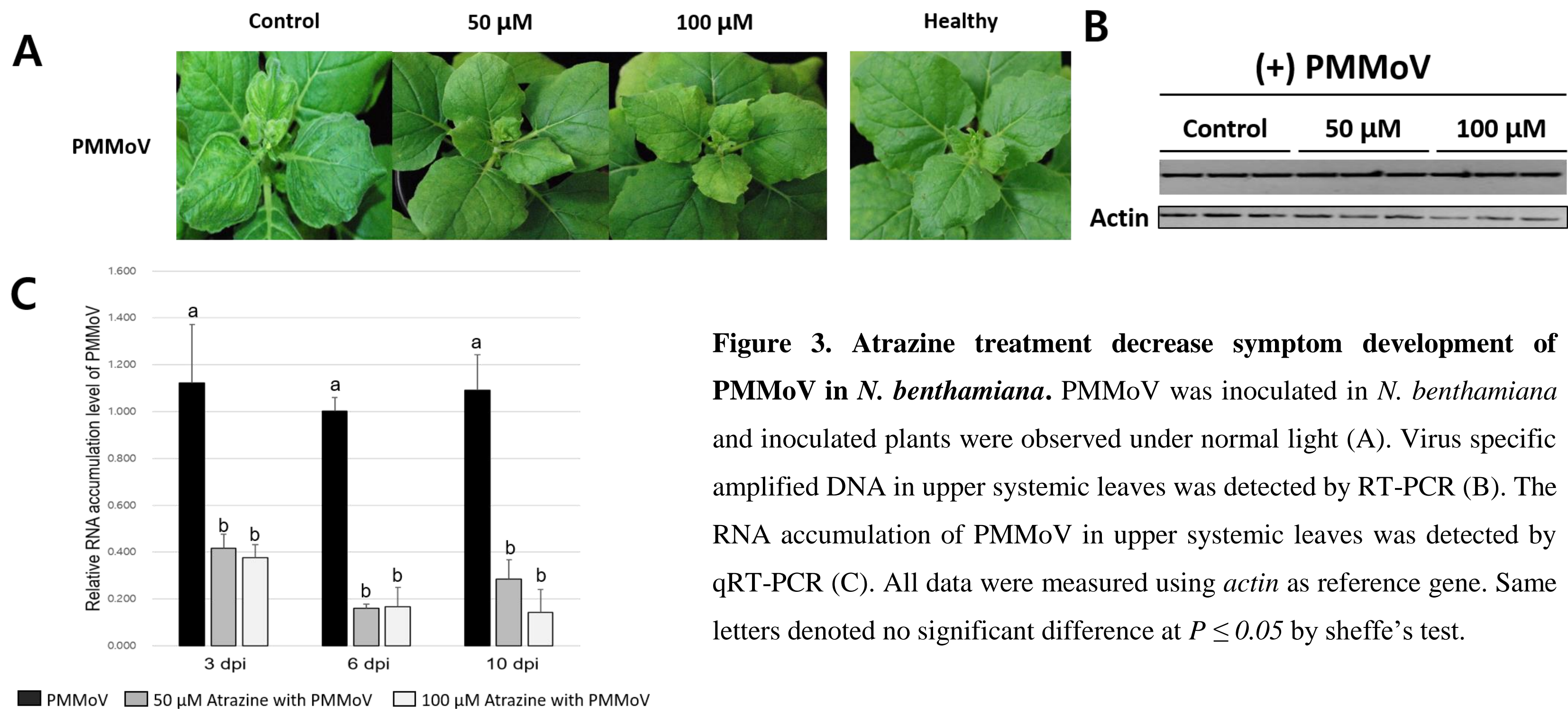


Figure 3. Atrazine treatment decrease symptom development of PMMoV in *N. benthamiana*. PMMoV was inoculated in *N. benthamiana* and inoculated plants were observed under normal light (A). Virus specific amplified DNA in upper systemic leaves was detected by RT-PCR (B). The RNA accumulation of PMMoV in upper systemic leaves was detected by qRT-PCR (C). All data were measured using *actin* as reference gene. Same letters denoted no significant difference at $P \leq 0.05$ by sheffe's test.

3. Minimal inhibitory concentration of atrazine treatment to inhibit PepMoV infection

In order to determine the minimum inhibitory concentration of atrazine, tested conducted additional experiments with pPepMoV::GFP, which showed strong inhibition on viral RNA replication as well as movement by 50 μM and 100 μM concentrations of atrazine in previous results. Atrazine was diluted to 10, 20, 30, and 40 μM and sprayed on plants. As shown in Fig. 4, the plants inoculated with the pPepMoV::GFP and treated with the 10 and 20 μM of atrazine showed GFP expression from the virus replication in upper systemic leaves that were not significantly different compared to the control plants. However, in plants treated with 30 and 40 μM of atrazine, GFP expression of the virus was not observed in the upper systemic leaves. These results suggest that atrazine treatment is effective in inhibiting replication and movement of PepMoV when used with 30 μM or higher concentration.

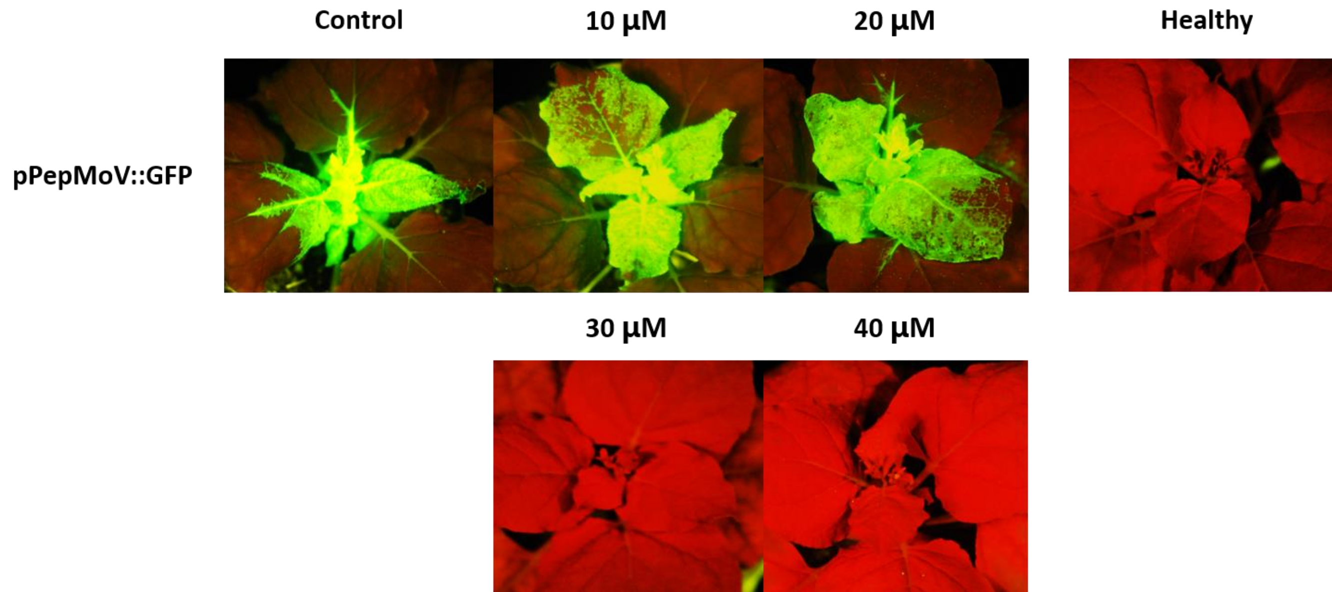


Figure 4. Minimal inhibitory concentration of atrazine. pPepMoV::GFP was inoculated on *N. benthamiana* and inoculated plants were observed under UV lamp. Atrazine was diluted in 10, 20, 30, and 40 μM, respectively, and sprayed to foliage.

4. Effects of treatment with the atrazine on expression of several genes related to major signal transduction pathways

To explore the possible involvement of major signaling pathways in atrazine-induced resistance to plant RNA viruses, qRT-PCR was performed using specific primer sets to detect several genes related to major signal transduction pathways. Among the major signal transduction pathways, genes related to *R* gene, hypersensitive response (HR), nitric oxide (NO) pathway, salicylic acid (SA) and jasmonic acid-ethylene (JA/ET) pathway respectively were select. To investigate difference of expression levels of these selected genes, after atrazine treatment, the leaves of treated plants were collected at several different time points, which are immediately after the treatment, 24 hours, 48 hours, and 120 hours post treatment. At each time point, total RNAs were extracted and used for qRT-PCR analysis.

In *N. benthamiana*, atrazine treatment significantly increased in the expression of *SGT1* and *NDR1* related to *R* gene and *NOA1* related to NO pathway compared with the mock group (Fig. 5A and B). The expression level of *SGT1* increased by a 2.6-fold at 48 h after the atrazine treatment compared with that observed in the mock group. The expression of *NDR1* was increased 2.5-, and 3-fold in 48 h and 120 h after the treatment compared with that in the mock. In case of SA pathway, expression of *ICS1*, *PAL1*, and *PR5* genes also showed significant increase between mock and treated plants (Fig. 5D). In gene of *ICS1*, expression level was much higher than that in mock (1.5-fold higher). The expression level of *PAL1* gene increased from 24 h to 10-fold, and then increased 2.3- to 8-fold in 48 h and 120 h. The atrazine treatment

resulted in upregulation of *PR5*, which was increased approximately 2- to 3.8-fold compared with the mock. In addition, expression levels of *ERF1*, *COI1*, *EIN2*, and *PDF1.2* upregulated compared with mock group in JA/ET pathways after atrazine treatment (Fig. 5E). In the atrazine treatment, *ERF1* expression was enhanced 2- to 3.4-fold compared with that in the mock plants in 24 h, 48 h, and 120 h. The expressions of *COI1* were promoted in atrazine treatment, with approximately increase of 1.5-fold compared with the mock group. In case of *EIN2*, the expression level increased 2-fold in 48h and 120 h, respectively, compared to that in the mock-treated group. The expression level of *PDF1.2* was increased 1.5- to 2.2- fold compared with that in mock.

However, *EDS1* related to *R* gene and *RbohB* related to HR were much lower compared with that in the mock group (Fig. 5A and C). These results indicate that expressions of many genes related to several signaling pathways such as *R* gene, NO, SA and JA/ET were upregulated following atrazine treatment, which suggests that atrazine treatment could induce expression of many genes related with plant defense signaling pathways.

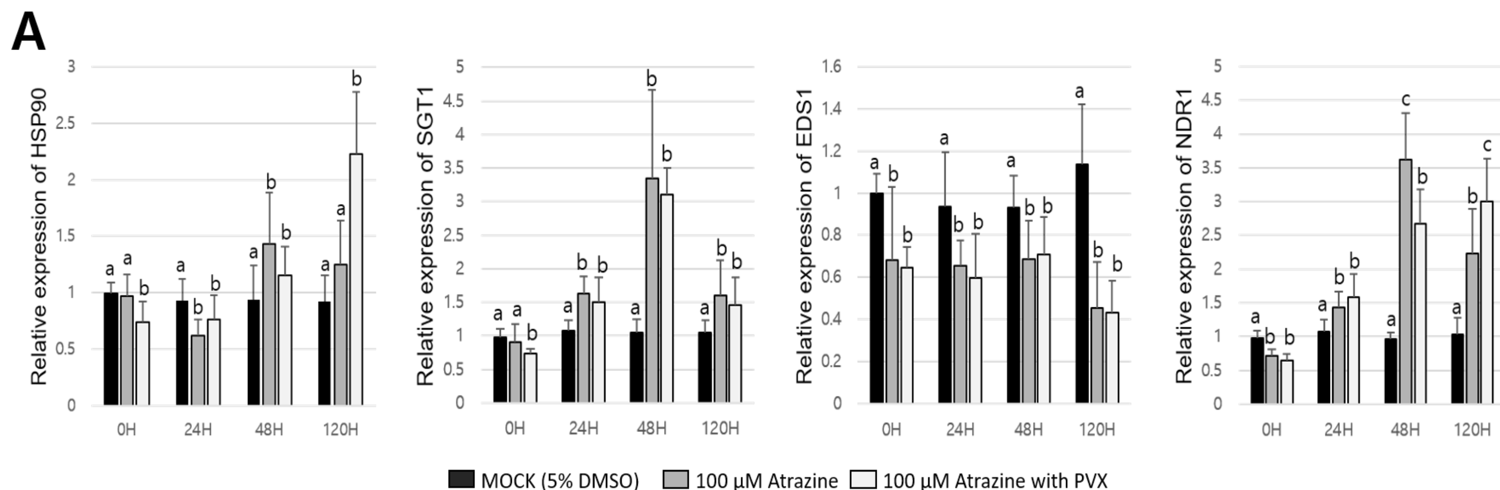


Figure 5. The relative expression of genes related to HR, *R* gene, NO, SA, and JA/ET pathways in *N. benthamiana* detected by qRT-PCR. Mock plants were pretreated with 5 % DMSO. The experimental groups were divided into two groups which are group of plants that were only treated with 100 μM atrazine and the other group is plants that were treated with 100 μM atrazine 1 hours after PVX inoculation. (A) The relative expression levels of *HSP90*, *SGT1*, *EDS1* and *NDR1*, host factors related to *R* gene. (B) The relative expression level of *NOA1*, a gene related to NO pathway. (C) The relative expression level of *RbohB*, a gene related to HR. (D) The relative expression levels of *ICS1*, *PAL1*, *NPR1* and *PR5* genes related to SA pathway. (E) The relative expression levels of *ERF1*, *COI1*, *EIN2* and *PDF1.2* genes related to JA/ET pathway. All data were measured using *actin* and *ubiquitin* as reference gene. Values were presented as means ± SD from three independent measurements. Same letters denoted no significant difference at $P \leq 0.05$ by sheffe's test.

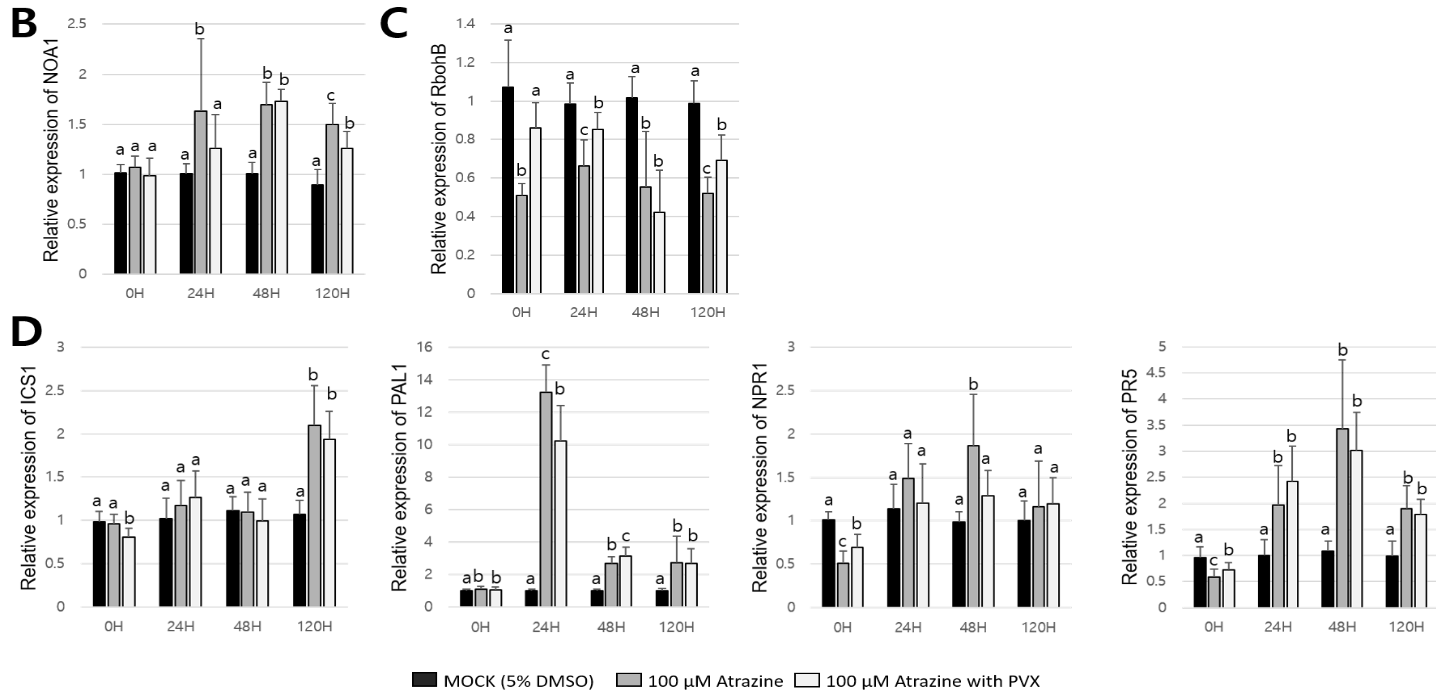


Figure 5. - continued

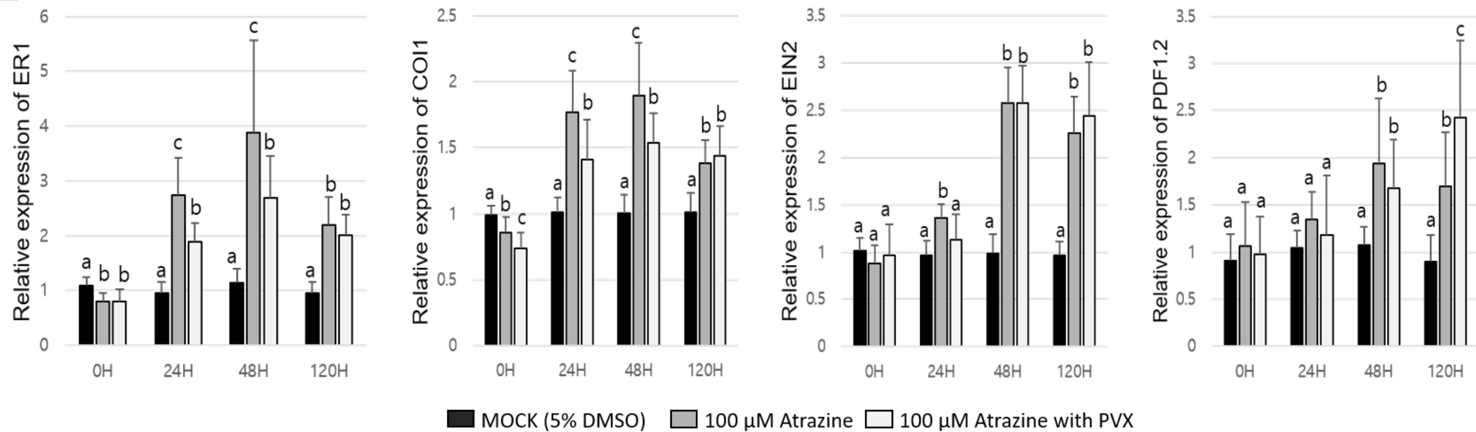


Figure 5. - continued

5. Effect of atrazine on PepMoV infection in NahG plants

In order to explore the signaling pathways involved in atrazine treatment-induced inhibitory effect to plant RNA viruses in *N. benthamiana*, wild type and NahG, SA signaling-defective mutant, plants were used. In NahG plants, the GFP expression of PepMoV was inhibited in upper systemic leaves of sprayed with atrazine of 50 μM compared to that of control untreated plants (Fig. 6A). The similar results were also observed with wild type plants (Fig. 6B). In NahG and wild type plants, viral RNA was not detected in the upper systemic leaves of plants infected with PepMoV as can be seen in the RT-PCR results (Fig. 6C and D). These results indicate that the replication and movement of PepMoV were successfully inhibited by atrazine treatment in SA signaling-defective mutant plants. Therefore, these might suggest that the inhibitory effect of atrazine in plant RNA viruses may not be mainly due to onset of genes related with the SA pathway.

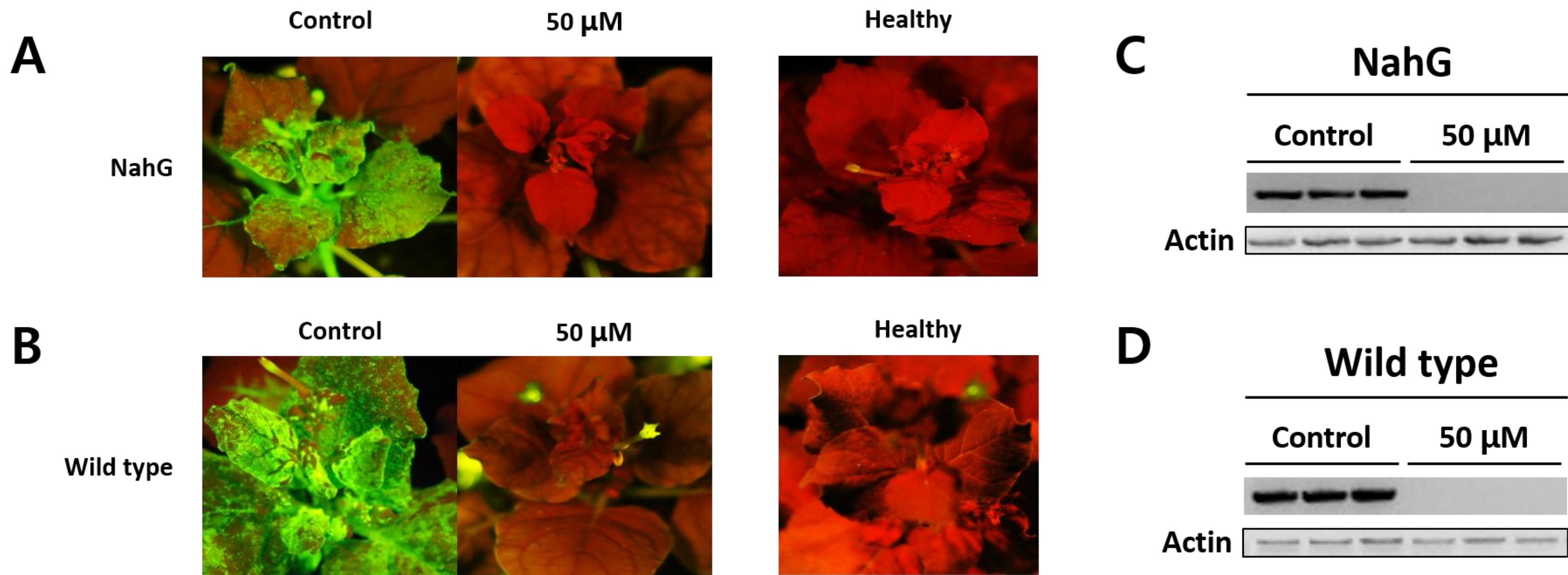


Figure 6. Inhibitory effect of atrazine treatment on PepMoV infection in wild type and NahG plants. PepMoV was inoculated in NahG (A) and wild type (B) plants. The plants that were inoculated using GFP fused infectious clone were observed under UV lamp (A and B). The viral RNA of PepMoV in systemic leaves was detected by RT-PCR (C and D).

DISCUSSION

Emerging plant viruses that spread through international travel and trade of seed, seedlings, cuttings, and fruit pose a serious threat to worldwide agricultural production and food security that caused catastrophic losses to crop and affected quality (Rojas and Gilbertson 2008). Although it is very difficult to give apparent figures on the economic impact of plant viruses in agriculture, the production losses due to plant virus infections are estimated to cost worldwide more than $30 billion every year (Nicaise 2014). Despite huge efforts in plant health management, no antiviral chemical product is commercially available for managing plant virus diseases, and thus viral diseases remain notoriously difficult to control or eradicate (Hanssen et al. 2010; Schreinemachers et al. 2015). Therefore, this study aims to explore and develop new antiviral compound that applicable to control and/or manage viral diseases. The compound used for this study is the atrazine, which is one of the most widely used herbicide. At the first screening, when treated with 50 μM and 100 μM of atrazine in testing inhibition of potyvirus infections or replications including PepMoV and PVY, no symptoms caused by the virus infection were observed in the upper systemic leaves of plants treated with both concentrations of atrazine, and no viral RNA was detected (Fig. 1). These results indicate that treatment of atrazine is an effective method in inhibiting the replication and movement of potyviruses in *N. benthamiana*.

In addition, treatment of 100 μM of atrazine for PVX, CMV, and TSWV, belonging to genus other than potyvirus, also inhibited virus replication and

movement with protection efficiency of 100% (Fig. 2). In case of PMMoV, however, virus-specific amplified DNA was detected. Weak symptoms were observed in the upper systemic leaves of plants treated with atrazine compared to that of control, and these could be caused by that the reduced accumulation level of PMMoV RNAs upon atrazine treatment (Fig. 3). These results indicate that the atrazine-induced resistance suppressed symptom development and reduced virus replication in treated plants, but could not completely inhibit upper systemic spread of PMMoV. Taken together, these results suggest that the resistance to plant RNA virus infections may be explained by an inhibition of virus replication and of upper systemic movement (Fraser 2012). These results also suggest that atrazine treatment could be used as one of an effective virus disease management strategy and that atrazine can be used as broad-spectrum antiviral agent for managing many plant RNA viruses species.

The minimum inhibition concentration of the atrazine was 30 μM or higher (Fig. 4). For any pesticide, the road from the laboratory to commercial application can be a long one, with many hurdles. Relatively high prices, one of the biggest factors, make it difficult for most farmers to use pesticides (Singh 2010). That is why the ideal compound should be available at a reasonable cost, and in addition, it is better if they have a broad-spectrum inhibitory effect against a wide range of viruses. From a commercial point of view, a broad-spectrum antiviral chemical would find a much wider market (Hansen and Stace-Smith 1989). Thus, based on the above results, atrazine is

considering as more competitive as a candidate for chemical control agent against plant viral diseases.

The plants gained resistance by evolving signaling mechanisms that ultimately limit the ingress and spread of viral pathogens (Kachroo et al. 2006). After pathogen invasion, *R* gene–mediated pathogen recognition often involves the accumulation of various signaling molecules, including NO, SA, JA and/or ET, the expression of pathogenesis-related (PR) genes and the development of a HR (Cooley et al. 2000; Kachroo et al. 2006; Kunkel and Brooks 2002). To investigate the expression level of genes associated with defense hormone pathways by treatment with atrazine, qRT-PCR was performed. As shown Fig. 5, many genes related to *R* gene, NO, SA, and JA/ET pathways were significantly induced compared to the mock group after atrazine treatment. The most notably induced genes were *PAL1*, *PR5*, and *ERF1* with the expression level increased approximately 2 times or more compared to mock at 24, 48, and 120 hours after atrazine treatment. These results suggest that after treatment of atrazine, not only the *R* gene is induced, but also the NO, SA, and JA/ET pathways are induced and thus turning on general immune response against virus infections. In addition, compare to the mock, the low expression level of *RbohB*, a gene associated with HR, can be explained by previous data that HR is often associated with resistance but not necessary or sufficient for resistance (Kachroo et al. 2006). Since tested specific genes involved in SA pathway, NahG plants which is SA defective mutant were used and were inoculated with PepMoV to determine whether SA signaling pathway contributes as the major signal pathway for atrazine-

induced resistance against plant RNA viruses. Likewise results of wild type plants, the replication and movement of PepMoV was also inhibited in upper systemic leaves of NahG plants treated with 50 μM atrazine (Fig. 6). The treatment of atrazine suggests that atrazine-induced resistance may require involvement of the other signaling pathways than the SA pathway. Since signaling network pathways in plants are highly complex, further research is required to understand the resistance response of plants caused by atrazine.

In conclusion, atrazine treatment 1 hours after inoculation with the virus induced resistance to different species of plant RNA viruses including PepMoV, PVY, PVX, CMV, and TSWV in *N. benthamiana*. Although atrazine did not show similar strong inhibitory effects against PMMoV infection, which belong to the genus *Tobamovirus*, atrazine treatment did show negative effect on PMMoV replication and movement. Since atrazine treatment induced many genes that play roles in resistance via *R* gene, NO, SA, and JA/ET pathways, atrazine treatment might convert plants into immune status to pathogen infections including viruses. Replication and movement of viruses in atrazine treated NahG (SA-defective) plants, however, did not show any difference in virus replication as well as movement as compared to wild-type *N. benthamiana* plants suggesting that resistance to virus infections by atrazine treatment might require involvement of more than one signal transduction pathways.

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식물 RNA 바이러스에 대한 트리아진계 화합물의 항바이러스성 특성 구명

정리라

초록

식물바이러스병은 다양한 작물에서 생리학적 불균형을 일으켜 작물의 수확량과 품질을 떨어뜨리고 경제적 손실을 초래한다. 식물바이러스병의 방제방법으로는 내병성 또는 저항성 품종을 사용하거나 바이러스 병을 매개하는 매개충 방제, 감염된 식물이나 씨앗의 제거 등이 있다. 최근 기후 변화 및 세계 무역의 확대로 인하여 식물바이러스병 발생률이 증가하고 있으며 이에 따라 화학적 방제제의 필요성이 더욱 대두되고 있다. 그러나, 지금까지 식물바이러스병 방제를 위한 상업적으로 이용할 수 있는 제제가 없다. 따라서 본 연구는 트리아진계 화합물인 아트라진의 항바이러스성 특성을 구명하고 잠재적인 항바이러스 제제로의 개발 가능성을 확인하고자 하였다. 이를 위해, *Nicotiana benthamiana* 에 바이러스 접종 1 시간 후 50 μM 과 100 μM 농도의 아트라진을 경엽처리하였고 7 일, 10 일, 14 일 경과 후 바이러스의 증식 및 이동을 관찰하였다. 사용된 바이러스는 각각 다른 속에 속하는 6 종이며, *Potato virus Y* (PVY; *Potyvirus*), *Pepper mottle virus* (PepMoV; *Potyvirus*), *Potato virus X* (PVX; *Potexvirus*), *Cucumber mosaic virus* (CMV;

Cucumovirus), *Tomato spotted wilt virus* (TSWV; *Tospovirus*) 그리고 *Pepper mild mottle virus* (PMMoV; *Tobamovirus*)이다. 연구 결과, GFP 가 삽입된 infectious clone 을 사용한 PepMoV, PVX, CMV 의 경우, 대조군과 비교하여 아트라진을 처리한 식물의 상엽에서 바이러스의 GFP 발현이 관찰되지 않았으며 이는 바이러스의 증식과 이동이 억제되었음을 뜻한다. PVY 와 PMMoV 는 바이러스에 감염된 조직을 접종원으로 사용하였다. PVY 의 경우, 아트라진을 처리한 식물에서 증상발생이 관찰되지 않았으며, RT-PCR 결과에서 바이러스의 RNA 가 검출되지 않았다. 그러나, PMMoV 의 경우, 아트라진 처리 시 상엽으로의 바이러스 이동이 억제되지 않았으며, 대조군에 비하여 증상 발생 정도가 약화된 것을 관찰할 수 있었다. 이는 qRT-PCR 결과에서 PMMoV RNA 축적 감소를 통해 확인할 수 있다. 이러한 아트라진에 의해 유도되는 저항성과 관련된 주요 신호 경로를 조사하기 위해, 식물의 신호전달경로에 관련된 유전자의 특이적 프라이머 세트를 사용하여 qRT-PCR 을 수행하였다. 그 결과, 식물의 방어반응과 관련된 R gene, nitric oxide, salicylic acid, jasmonic acid-ethylene 경로 관련 유전자의 발현 수준이 mock 과 비교하여 아트라진을 처리한 식물에서 유의미하게 증가하였다. 또한, NahG (salicylic acid-defective) 식물의 상엽에서 바이러스의 증식 및 이동이 억제되었으며 아트라진-유도 저항성이 salicylic acid 경로와

관련성이 낮다는 것을 시사한다. 종합하자면, 아트라진-유도 저항성이 salicylic acid 경로 이외의 다른 신호전달경로와 관련되어 식물바이러스 증식을 억제함을 도출할 수 있었다. 결론적으로, 아트라진은 식물 RNA 바이러스의 감염을 제어하거나 억제하기 위한 화학적 방제제 후보 물질로의 이용가능성을 제시하였다.

주요어: 아트라진, 항바이러스성 특성, 바이러스 증식 억제, 식물방어기작

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