

Dammarenediol-II Production Confers TMV Tolerance in Transgenic Tobacco Expressing *Panax ginseng* Dammarenediol-II Synthase

Mi-Hyun Lee¹, Jung-Yeon Han¹, Hyun-Jung Kim¹, Yun-Soo Kim¹, Gyung Hye Huh² and Yong-Eui Choi^{1,*}

¹Department of Forest Resources, College of Forest and Environmental Sciences, Kangwon National University, Chunchon 200-701, Republic of Korea

²College of General Education, In Je University, Gimhae 621-749, Republic of Korea

*Corresponding author: E-mail, yechoi@kangwon.ac.kr; Fax, +82-33-244-0155

(Received August 28, 2011; Accepted November 10, 2011)

Panax ginseng is one of the famous medicinal plants. Ginsenosides, a class of tetracyclic triterpene saponins, are mainly responsible for its pharmacological activity. Most ginsenosides are composed of dammarenediol-II aglycone with various sugar moieties. Dammarenediol-II synthase is the first enzyme in the biosynthesis of ginsenosides. Here, we report that transgenic tobacco expressing the *P. ginseng* dammarenediol-II synthase gene (*PgDDS*) produced dammarenediol-II, and conferred resistance to Tobacco mosaic virus (TMV). Upon infection with TMV, lesions developed more rapidly in transgenic tobacco plants, and their size was smaller than those of wild-type plants. Transgenic tobacco plants showed a low level of both the viral titer and mRNA accumulation of TMV coat protein (CP) compared with the wild type. The production of dammarenediol-II in transgenic tobacco stimulated the expression of tobacco pathogenesis-related genes (*PR1* and *PR2*) under both virus-untreated and -treated conditions. When the leaves of wild-type plants were inoculated with a mixture of TMV and dammarenediol-II, the leaves exhibited a reduced viral concentration and TMV-CP expression than those receiving TMV treatment alone. When the leaves of *P. ginseng* were infected with TMV, transcription of *PgDDS* was significantly increased. Transgenic *P. ginseng* plants harboring a β -glucuronidase (*GUS*) gene driven by the *PgDDS* promoter were constructed. The *GUS* expression was activated when the transgenic ginseng plants were treated with TMV. These results indicate that the medicinally important dammarenediol-II can be ectopically produced in tobacco, and the production of dammarenediol-II in tobacco plants allows them to adopt a viral defense system.

Keywords: Dammarenediol-II • Dammarenediol-II synthase • *Nicotiana tabacum* • *Panax ginseng* • TMV tolerance • Tobacco mosaic virus.

Abbreviations: CaMV, Cauliflower mosaic virus; CP, coat protein; DDS, dammarenediol-II synthase; GC-MS, gas chromatography–mass spectrometry; *GUS*, β -glucuronidase;

HIV-1, human immunodeficiency virus type 1; PR gene, pathogenesis-related gene; RT-PCR, reverse transcription–PCR; TMV, Tobacco mosaic virus.

Introduction

Panax ginseng is one of the most widely used medicinal plants, particularly in traditional oriental medicine (Briskin 2000). It has a wide range of pharmacological and physiological activities. The major pharmacologically active constituents of ginseng are triterpene saponins known as ginsenosides. Physiological studies on ginsenosides or their aglycones have shown that they have various activities, including control over the central nervous system and anti-cancer activity (Shibata 2001). Ginsenosides are classified into two groups by their structure, either dammarane-type or oleanane-type. Dammarane-type ginsenosides, including Rb₁–Rg₁, are major constituents, and only ginsenoside Ro is an oleanane-type ginsenoside, which is a minor component. An aglycone derivative of dammarane ginsenosides was shown to have anti-cancer and anti-angiogenic properties and was used as a drug (Shibata 2001). The demand for this aglycone has increased due to the realization of its importance as a human drug.

Triterpene saponins are synthesized via the isoprenoid pathway through farnesyl diphosphate (FPP) by the cyclization of 2,3-oxidosqualene, leading to the formation of oleanane (β -amyrin) or dammarane in the plant kingdom. The first step in the synthesis of dammarane-type ginsenoside is the cyclization of 2,3-oxidosqualene to dammarenediol-II, a reaction catalyzed by the enzyme dammarenediol-II synthase (Fig. 1). Functional characterization of dammarenediol-II synthase proteins (DDS and PNA) was reported in *P. ginseng* and *Centella asiatica* (Tansakul et al. 2006, Han et al. 2006, Kim et al. 2009).

Dammarenediol-II is a ginsenoside precursor. It is also an end-product in some other plant species (Spencer 1981, Huang et al. 2010). Dammarenediol-II is one component of

Plant Cell Physiol. 53(1): 173–182 (2012) doi:10.1093/pcp/pcr160, available online at www.pcp.oxfordjournals.org

© The Author 2011. Published by Oxford University Press on behalf of Japanese Society of Plant Physiologists.

All rights reserved. For permissions, please email: journals.permissions@oup.com

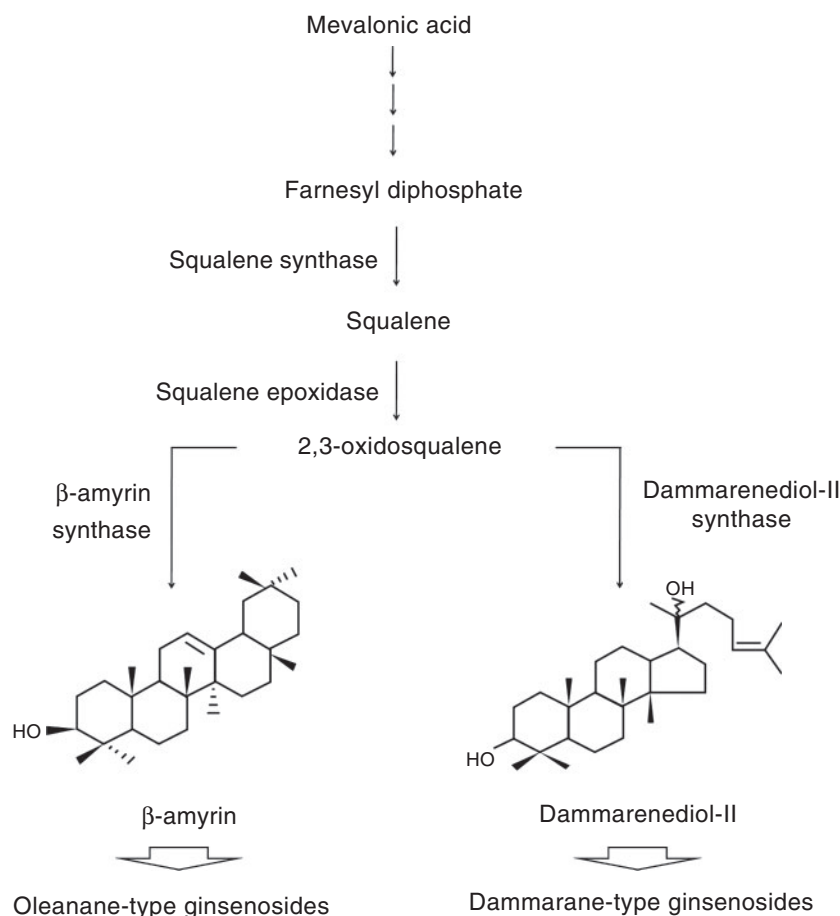


Fig. 1 Biosynthesis of triterpenoid saponins in *Panax ginseng*.

dammar resin in various species of the Dipterocarpaceae family and other species (Mills and Werner 1955, Cheung 1968, Pellicciari *et al.* 1972, Bandaranayake *et al.* 1975, Spencer 1981, Akihisa *et al.* 2004, Huang *et al.* 2010). Notably, approximately a quarter of *Cacalia atriplicifolia* oil is composed of dammarenediol-II. Dammar resin, an exudate of plants composed of triterpenoid compounds, has long been used as a varnish for paintings and is still widely used today. Dammar resin is generally assumed to provide plants with a defense because it seals off injured plant tissue and protects against attack by insects and fungi (Phillips and Croteau 1999). In mammalian and human cells, dammarenediol-II isolated from dammar resin or oil shows potent inhibitory effects against herpes simplex virus type I and II and Epstein–Barr virus (Poehland *et al.* 1987, Akihisa *et al.* 2004).

Dammarenediol-II is likely to be present in trace amounts in *P. ginseng* plants because it is a precursor and because of the rapid flux of intermediates toward the final production of ginsenosides. Therefore, it is nearly impossible to produce commercially attractive yields of dammarenediol-II from cultivated roots of *P. ginseng*. Genetic engineering of dammarenediol-II biosynthesis from other plant species

or microbial hosts might be an option to improve its productivity.

Some triterpenoid saponins have been shown to be capable of deactivating viruses. Maesasaponins have virucidal activity (Apers *et al.* 2001). Oleanolic acid inhibits human immunodeficiency virus type 1 (HIV-1) replication, most probably by inhibiting HIV-1 protease activity (Mengoni *et al.* 2002). The physiological role of triterpene saponin in plants, including dammarenediol-II, is not yet understood. Although dammarenediol-II has an inhibitory effect against some viruses in animal and human cells (Poehland *et al.* 1987, Akihisa *et al.* 2004), no study has addressed its function in plants. In view of its widespread presence in plants, dammarenediol-II may be a component of plant defense systems as a secondary metabolite in many plants.

In the present study, we demonstrate that dammarenediol-II can be produced in model tobacco plants that do not produce dammarenediol-II naturally. Transgenic tobacco producing dammarenediol-II by overexpression of the *PgDDS* gene confers resistance against *Tobacco mosaic virus* (TMV) attack by inhibiting virus replication. β -Glucuronidase (GUS) expression driven by the *PgDDS* promoter in transgenic ginseng plants was activated after TMV infection.

Results

Endogenous dammarenediol-II production in *PgDDS* transgenic tobacco plants

Transgenic tobacco plants constitutively expressing *PgDDS* (AB122080) under the control of the *Cauliflower mosaic virus* (CaMV) 35S promoter were constructed (Fig. 2A). Among the 15 transgenic lines initially produced, three were finally selected. Integration of the *PgDDS* and hygromycin phosphotransferase (*HPT*) genes into the genome of tobacco was confirmed by PCR, while no signal was observed in the wild-type plants (Fig. 2B). Transcription of the *PgDDS* mRNA in transgenic tobacco was confirmed by reverse transcription–PCR (RT–PCR) (Fig. 2C).

To confirm dammarenediol-II production in transgenic tobacco plants expressing *PgDDS*, extracts of leaves were analyzed by gas chromatography–mass spectrometry (GC–MS). Three transgenic lines (T2, T5 and T11 in Fig. 2C) showed a new product (P1), which is not found in the wild type. GC–MS analysis indicated that this product was eluted at a retention time of 38.6 min, which is identical to the retention time of the authentic dammarenediol-II (Fig. 3A). Furthermore, the GC–MS spectra revealed that the fragmentation pattern of dammarenediol-II products with an ion at m/z 426 was completely matched to the MS spectra of authentic dammarenediol-II (Fig. 3B). Accumulation of dammarenediol-II in leaves was about $20\text{--}30\text{ }\mu\text{g g}^{-1}$ DW in the transgenic lines (Fig. 3C). In the wild-type plants, no traceable

dammarenediol-II signal was detected (Fig. 3C). These data reveal that the introduced *PgDDS* was functionally expressed in transgenic tobacco and responsible for the production of dammarenediol-II from endogenous 2,3-oxidosqualene in tobacco.

Dammarenediol-II production in transgenic tobacco plants confers TMV tolerance

To determine the functional role of pathogen resistance of dammarenediol-II in plants, TMV was inoculated into the leaves of the wild type and two transgenic lines (T5 and T11) which showed different levels of dammarenediol-II production. Symptom development was subsequently monitored for 72 h after infection. The results showed that lesions developed more rapidly in T5 and T11 leaves, and their size was smaller than those in the wild-type plants (Fig. 4A). The lesion size at 72 h after TMV infection was determined by measuring the average lesion diameter (Fig. 4B). In wild-type plants, the average lesion size was 3.19 ± 0.27 mm, whereas it was 2.61 ± 0.19 mm and 1.40 ± 1.81 mm in the T5 and T11 transgenic lines, respectively. These results indicate that lesions developed much faster in transgenic lines and were smaller than those in wild-type plants.

Enhanced transcription of *PR1* and *PR2* genes in transgenic tobacco

We speculated that the resistance of transgenic tobacco to TMV might have resulted from the enhanced expression of pathogenesis-related (*PR*) genes. Transcriptional *PR1* and *PR2* levels were analyzed at 0 and 24 h after transgenic lines and wild-type plants were or not inoculated with TMV. In transgenic tobacco, the expression of the tobacco pathogenesis-related genes (*PR1* and *PR2*) was elevated even under untreated conditions. Both the *PR1* and *PR2* genes in the wild type and transgenic lines (T5 and T11) responded to TMV infection, and their mRNA levels were clearly increased by TMV treatment (Fig. 4C). Additionally, transcripts of *PR1* and *PR2* were increased in the presence of exogenously applied dammarenediol-II alone (data not shown). These results suggest that the dammarenediol-II activated the expression of *PR* genes of tobacco, which confers the increased resistance against TMV infection.

The appearance of symptoms could be correlated with the presence of virus in systemic tissues. Based on the data of symptom appearance, a virus titer was determined at 72 h after TMV infection. This analysis showed that the average viral titer in transgenic lines (T5 and T11) was significantly (2- to 3-fold) lower than that in wild-type plants (Fig. 4D). TMV coat protein (CP) mRNA was also analyzed at 0 and 72 h by RT–PCR in leaves of wild-type and transgenic plants after TMV infection. TMV infection resulted in equal accumulation of TMV-CP transcripts in both wild-type and transgenic tobacco just after infection (Fig. 4E). After 72 h of TMV infection, TMV-CP mRNA intensity was 2- to 4-fold lower in T5 and T11 leaves

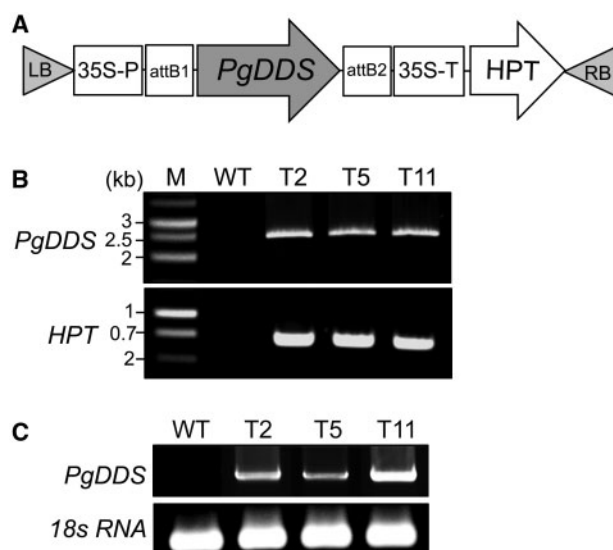


Fig. 2 Expression profile of *PgDDS* transgenic tobacco plants. (A) Schematic diagram of the *PgDDS* cDNA under the control of the CaMV35S promoter, which was inserted into the destination vector pK7WG2.0. (B) Confirmation of the introduced genes by genomic DNA PCR. (C) Expression of *PgDDS* in leaves of wild-type (WT) plants and the indicated transgenic lines (T2, T5 and T11). *18s* RNA was used as a loading control. The wild-type line was not transformed with *Agrobacterium*.

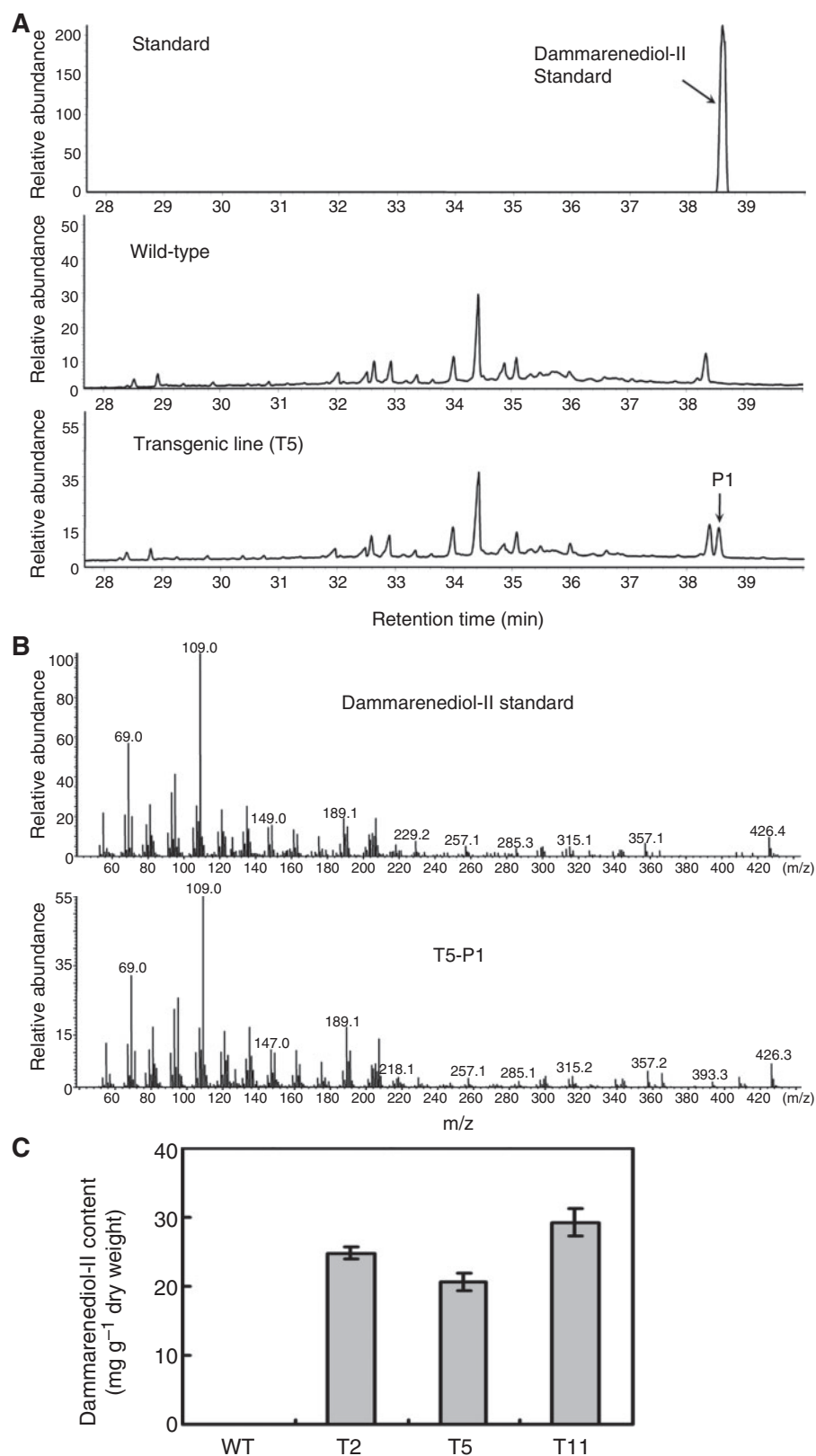


Fig. 3 GC-MS analysis of *PgDDS* transgenic tobacco lines. (A) GC chromatogram of standard dammarenediol-II (upper, Standard), and leaf extract from a wild-type plant (middle, wild-type) and a transgenic line (lower, T5). (B) MS spectrum of the standard dammarenediol-II (upper) and identified dammarenediol-II peaks (lower, T5-P1) obtained from a transgenic line (T5). (C) Production of dammarenediol-II. Leaf samples from wild-type and transgenic lines (T2, T5 and T11) were processed to isolate and measure dammarenediol-II. Data are mean values with the standard deviation obtained from three independent plants.

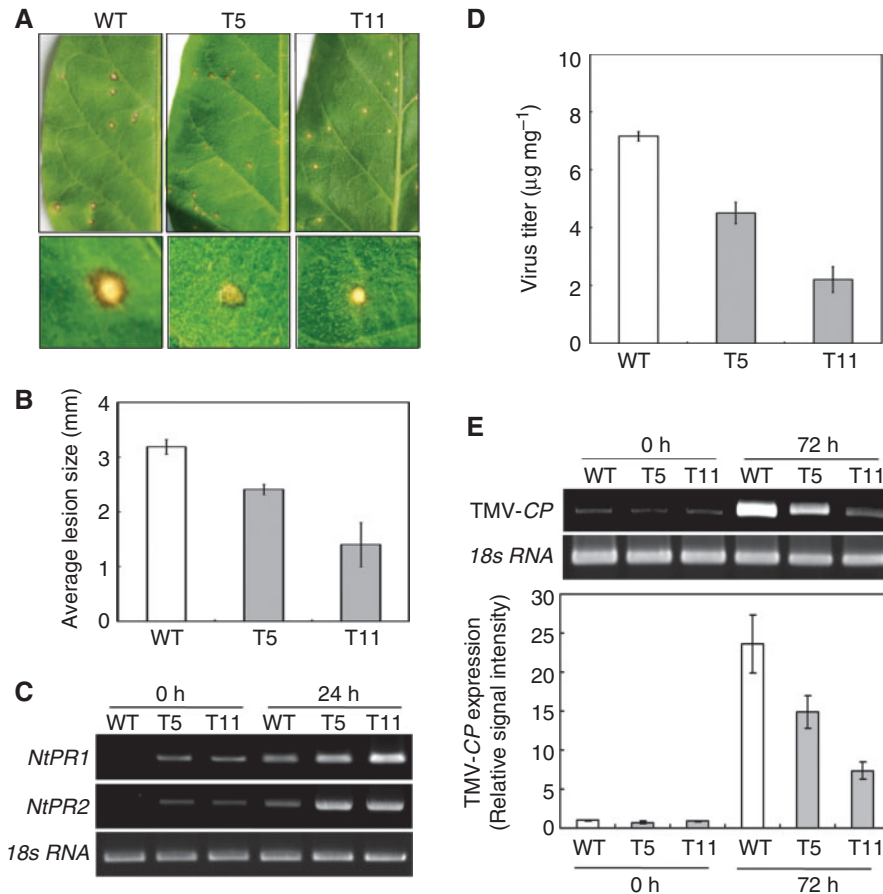


Fig. 4 Resistance against TMV infection. (A) Lesion formation. Healthy leaves from a wild-type plant and transgenic lines (T5 and T11) were detached, treated with TMV and kept at 20°C for 72 h. Mock treatment was performed with buffer only. For analysis, nine leaves of three wild-type, three T5 and three T11 plants were infected. The experiment was performed in three independent sets. (B) Lesion size. The size of lesions was measured under a microscope at 72 h after TMV infection. Values are the mean from 50 lesions with standard deviations. (C) Expression of *NtPR1* and *NtPR2* in wild-type and transgenic lines (T5 and T11) either infected with TMV or mock treated. Mock treatment was performed with buffer only. As the equal loading control, 18s RNA was used. (D) Virus concentrations were measured in infected plants at 72 h after TMV infection. The graph depicts the average viral concentrations (six leaves from each line) as calculated from three independent repeats. (E) Viral RNA detection by RT-PCR. RNA samples from TMV-infected tissues amplified with TMV-CP-specific primers. RT-PCR was repeated in five independent samples. The intensity of the bands was quantified by using NIH image-J software and normalized to 18s RNA. Relative signal intensity represents intensity relative to the basal intensity obtained from 0 h of the wild type. The data shown are averages of five independent repetitions. 18s RNA was used as a loading control.

compared with that in wild-type leaves (Fig. 4E). These experiments demonstrated that endogenous dammareniol-II was able to act as a signaling molecule for the hypersensitive response to TMV infection and to suppress virus replication significantly.

Effects of exogenously applied dammareniol-II

The effect on TMV resistance was examined for exogenously applied dammareniol-II. A TMV solution ($10 \mu\text{g ml}^{-1}$) was mixed with 0, 20 and $200 \mu\text{g ml}^{-1}$ dammareniol-II, and kept at 25°C for 24 h. Leaves of wild-type tobacco plants were infected with a TMV and dammareniol-II mixture. The average viral titer in leaves treated with the mixture was significantly lower than in leaves treated with TMV alone at 72 h

(Fig. 5A). TMV-CP expression was also decreased by approximately 50% in leaves treated with the TMV and dammareniol-II mixture compared with leaves treated with TMV alone (Fig. 5B). The leaves treated with the TMV and dammareniol-II mixture showed a clear reduction of the viral titer and TMV-CP expression compared with those treated with TMV alone.

Enhanced transcriptional activity of *PgDDS* and the *PgDDS* promoter induced by TMV

The transcriptional activity of *PgDDS* against viral infection in *P. ginseng* was analyzed by RT-PCR. Transcripts were significantly accumulated after *P. ginseng* leaves were infected with TMV (Fig. 6A). TMV-CP mRNA was also detected in

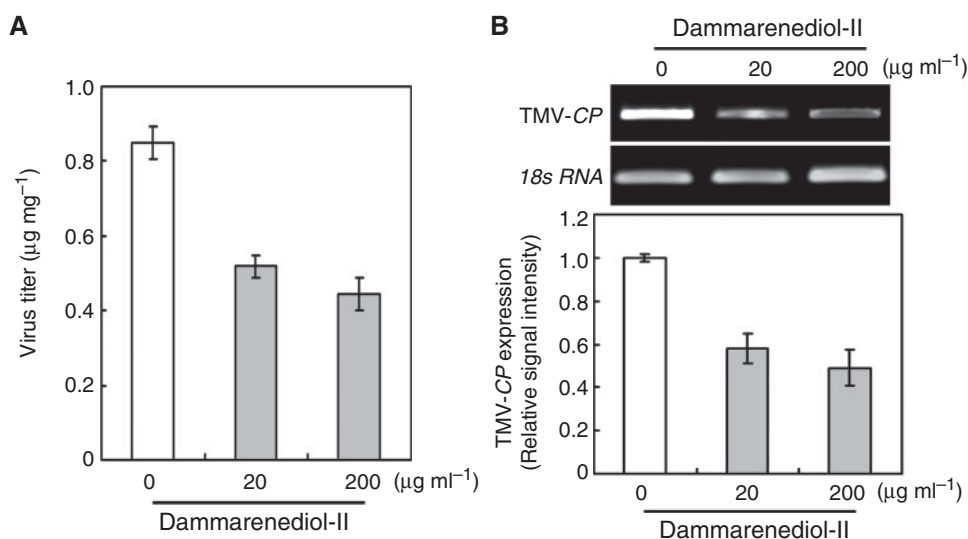


Fig. 5 Effect of exogenously applied dammarenediol-II. TMV solution ($10 \mu\text{g ml}^{-1}$) was added to 0, 20 and $200 \mu\text{g ml}^{-1}$ dammarenediol-II, and the mixture was kept at 25°C for 24 h. Healthy leaves from a wild-type plants were detached and treated with TMV with 0, 20 and $200 \mu\text{g ml}^{-1}$ dammarenediol-II. (A) Virus concentrations were measured in infected plants at 72 h. The data shown are the average of viral concentrations (three leaves from each line) as calculated from three independent repeats. (B) Viral RNA detection by RT-PCR. RNA samples from TMV-infected tissues amplified with TMV-CP-specific primers. RT-PCR was repeated in five independent samples. The intensity of the bands was quantified by using NIH image-J software and normalized to 18s RNA. Relative signal intensity represents intensity relative to the basal intensity obtained from $0 \mu\text{g ml}^{-1}$ dammarenediol-II. The data shown are averages of five independent repetitions. 18s RNA was used as a loading control.

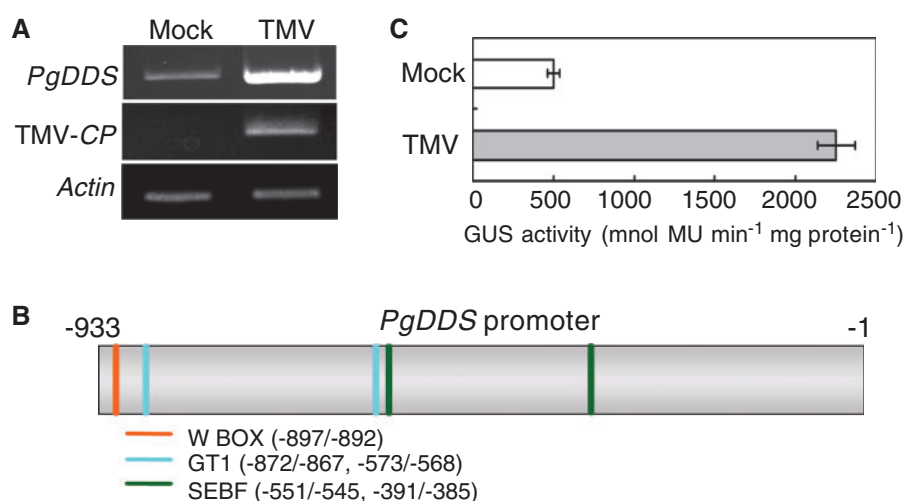


Fig. 6 TMV inducibility of the *PgDDS* promoter. (A) The accumulation of transcripts for *PgDDS* and TMV-CP in TMV-infected leaves. RNA samples from TMV-infected leaves were amplified with *PgDDS*- and TMV-CP-specific primers. Actin was used as a loading control. (B) Schematic diagram of the *PgDDS* promoter. The W box (TTGAC), GT-1 (GAAAAA) and SEBF (TGTCNC) are indicated by colored bars. (C) GUS activity of *PgDDS* promoter::GUS plants subjected to TMV or mock treatment. The experiment was performed in three independent sets.

TMV-infected leaves, but no signal was detected in the control leaves (**Fig. 6A**). This result demonstrates that the *PgDDS* gene responded to viral infection.

To determine the response of the promoter of the *PgDDS* gene to TMV infection, the promoter region of *PgDDS* was isolated. A total of 933 bp of 5'-flanking sequence at the promoter site of *PgDDS* was obtained (AB638615). Putative *cis*-elements related to pathogens, a W box (TTGAC[C/T]),

GT-1 (GTTTTT) and SEBF (TGTCNC) were identified in the upstream sequence using the PLACE database (**Fig. 6B**). The presence of these putative *cis*-elements in the promoter region suggests that *PgDDS* may respond to pathogens and/or stresses. The transcriptional activity of the *PgDDS* promoter was examined in response to TMV infection after transgenic plants expressing the GUS reporter gene driven by the *PgDDS* promoter were constructed. When transgenic ginseng leaves

were inoculated with TMV, GUS activity was greatly enhanced at >4-fold that of the control (Fig. 6C), indicating that the promoter contains a sequence necessary for TMV defense and activates *PgDDS* gene expression.

Discussion

Dammarenediol-II production in tobacco

Dammarenediol-II synthase is one of the key enzymes involved in ginsenoside saponin biosynthesis in *P. ginseng* (Han et al. 2006, Tansakul et al. 2006). Dammarenediol-II is a valuable compound in the pharmaceutical field (Huang and Qi 2005, Usami et al. 2008). Despite its high medicinal value, dammarenediol-II is present in only trace amounts in *P. ginseng* because it is an intermediate compound. It is produced by a complicated process involving the chemical hydrolyzation of ginsenosides extracted from ginseng roots (Shibuya et al. 2006). In this report, dammarenediol-II was produced in transgenic tobacco plants overexpressing the *PgDDS* gene. This result indicates that the introduced *PgDDS* gene was responsible for the production of dammarenediol-II via the conversion of intrinsically existing 2,3-oxidosqualene to dammarenediol-II and could be applicable for efficient production of dammarenediol-II in other plants for medicinal purposes.

Antiviral activity of dammarenediol-II

In the present work we demonstrated that transgenic tobacco plants producing dammarenediol-II exhibited resistance against TMV attack. Virus-induced lesions developed much faster in transgenic lines overexpressing the *PgDDS* gene and were smaller than those in wild-type plants. One of the most effective defense mechanisms in plants against viruses is the role of resistance (*R*) genes by triggering localized areas of cell death around the infected cell, which are called lesions, to stop the infection from spreading (Dinesh-Kumar et al. 2000). The present study showed that *PR* genes are transcriptionally activated in transgenic tobacco plants producing dammarenediol-II even under unstressed conditions, resulting in resistance to TMV infection. This is consistent with the previous findings that endogenous caffeine in tobacco plants activated the *PR* genes encoding *PR1* and *PR2*, and conferred resistance to infection by both TMV and the bacterial pathogen *Pseudomonas syringae* (Kim and Sano 2008). A correlation between the activation of defense responses and the accumulation of high levels of *PR* proteins has been observed in a number of transgenic plants (Waller et al. 2006, Lee and Sano 2007, Kim and Sano 2008). The resistance to TMV in transgenic tobacco overexpressing *PgDDS* is possibly the result of constitutive activation of the defense response, accumulation of *PR* proteins, and enhancing the hypersensitive response to virus.

Some triterpene saponins and sapogenins have been shown to deactivate viruses in animal systems; for example, a purified saponin mixture from *Maesa lanceolata* (Sindambiwe et al. 1998), and maesaponins had virucidal activity (Apers et al.

2001). The triterpenoid sapogenin oleanolic acid inhibits HIV-1 virus replication probably by inhibiting HIV-1 protease activity (Mengoni et al. 2002). Some triterpenoid saponins from *Ilex oblonga* exhibited inhibitory activities against TMV replication in leaf disk culture of tobacco (Wu et al. 2007a, Wu et al. 2007b). Dammarenediol-II isolated from dammar resin or oil also showed potent inhibitory effects against viruses in an animal system (Poehland et al. 1987, Akihisa et al. 2004). These results suggest that dammarenediol-II can affect the viral life cycle, such as viral infection, replication or particle release to the outside of host cells.

Upon infection with TMV, transgenic tobacco plants overexpressing *PgDDS* showed a low level of both the viral titer and mRNA accumulation of TMV-CP compared with that in the wild type. When the leaves of wild-type plants were inoculated with a mixture of TMV and dammarenediol-II, the leaves exhibited a reduced viral concentration and TMV-CP expression compared with TMV treatment alone. These results mean that dammarenediol-II exhibited an anti-viral effect on TMV, possibly inhibiting TMV replication, although the detailed inhibition mechanism is unclear. Further investigations are in progress to study the mechanism of the anti-viral activities of dammarenediol-II.

Enhanced transcription of the *PgDDS* gene driven by the *PgDDS* promoter by TMV treatment

Transcription of the *PgDDS* gene was significantly enhanced after *P. ginseng* leaves were infected with TMV. TMV-CP transcripts were also detected in TMV-infected leaves, but no signal was detected in the control leaves. The transcriptional activation of the *PgDDS* gene was further demonstrated by *PgDDS* promoter analysis. GUS expression driven by the *PgDDS* promoter in transgenic ginseng leaves was clearly enhanced by TMV infection. The enhanced transcriptional activity of *PgDDS* mRNA and the *PgDDS* promoter against viral infection in *P. ginseng* suggests that ginsenoside triterpene biosynthesis might be up-regulated by virus infection and involved in the virus defense mechanism.

In conclusion, transgenic tobacco plants expressing the *PgDDS* gene produced medicinally valuable dammarenediol-II and showed increased resistance to TMV. Dammarenediol-II also acts as a secondary metabolite promoting pathogen resistance in tobacco and could be a useful compound for enhancing the viral resistance of plants. The *PgDDS* gene could be a new candidate for genetic engineering to strengthen viral defense in plants.

Materials and Methods

Plant material and TMV treatment

Wild-type and transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a growth chamber at 23°C under a 14/10 h light/dark cycle. TMV (10 µg ml⁻¹ in phosphate buffer)

inoculation was performed by mechanically inoculating detached leaves of 2-month-old plants by rubbing with carborundum (600 mesh), keeping them for 20°C for 72 h. For exogenously applied dammarenediol-II, the TMV solution (10 µg ml⁻¹) was added to 0, 20 and 200 µg ml⁻¹ dammarenediol-II, and the mixtures were kept at 25°C for 24 h. Healthy leaves from wild-type plants were detached and treated with TMV solution containing 0, 20 and 200 µg ml⁻¹ dammarenediol-II. Mock treatment was performed with carborundum and buffer only. Three leaves from each plant were assayed for one series of experiments, and, in total, at least three series were performed. Lesion development was assessed at 72 h after TMV infection.

Analysis of viral titers

For the analysis of the viral titer, six leaves from wild-type and transgenic lines (T5 and T11) were assayed at 72 h after TMV infection. The viral RNA and virus concentration was analyzed as previously described (Kathiria *et al.* 2010). TMV was isolated from infected tobacco leaves that were homogenized in 0.5 M phosphate buffer pH 7.0, 143 mM β-mercaptoethanol (1 ml g⁻¹ of fresh tissue). Two volumes of chloroform/butanol (50:50) were added and the samples were centrifuged for 15 min at 3,000 × g. Viral particles were precipitated using 50 µl of 40% PEG-6000 and a 10% NaCl solution for 10 min on ice. Virus was collected by centrifugation in a microfuge, and resuspended in 20 µl of 10 mM phosphate buffer pH 7.0 lacking β-mercaptoethanol. The viral titer was estimated by measuring the optical density at 260 nm (OD₂₆₀) using a spectrophotometer. The titer is determined as mg of TMV per 10 mg FW. Viral titer analysis was repeated in three independent samples.

cDNA synthesis and RT-PCR

Total RNA was extracted from wild-type and transgenic leaves using an RNeasy Plant Mini Kit (Qiagen) and treated with DNase I before the synthesis of cDNA from 0.5 µg aliquots using Improm-II reverse transcriptase (Promega). RT-PCR was performed with the synthesized cDNAs and templates using primer pairs. All primers used in this study are listed in **Supplementary Table S1**.

Viral coat protein RNA detection

A mixture of mRNA and 1 mM of a 3' CP-specific primer (5'-CAAGTTGCAGGACCAGAGG-3') was prepared. The RNA-primer mixture was incubated for 10 min at 70°C and then a reverse transcription reaction solution was added. The reactions were incubated for 50 min at 50°C and chilled on ice. For PCRs, 2 µl of reverse transcription reaction products were added to 25 µl of a PCR mixture. For TMV-CP amplification, the 3' primer was the same as the one used for the reverse transcription reaction and the 5' primer was 5'-ATGTCTTATAGTACTACTCCATCT-3'. After loading on an agarose gel for electrophoresis, products were identified by visualization with ethidium bromide staining. The intensity of bands was

quantified by using NIH image-J software and normalized to 18s RNA. RT-PCR was repeated in five independent samples.

GC-MS analysis of dammarenediol-II

Quantitative analysis of dammarenediol-II was performed using a gas chromatograph (Agilent 7890A) linked to an MSD system (Agilent 5975C). Milled powder (1 g) of freeze-dried leaves was soaked in 80% (v/v) methanol at 60°C. After evaporation, the residue was dissolved in H₂O and washed twice, followed by extraction with H₂O-saturated *n*-butanol. A 2 µl aliquot of the solution was analyzed by GC-MS as described in our previous report (Kim *et al.* 2011). Authentic dammarenediol-II was directly subjected to the same conditions. The representative fragmentation ion values by GC-MS of authentic dammarenediol-II exhibited *m/z* 426. For each experiment, three plants of the wild type and transformed lines (T2, T5 and T11) were tested in three independent experimental series.

Cloning of the PgDDS promoter

Genomic DNA was purified from cultured ginseng roots according to the instructions of the DNeasy Plant Maxi prep kit (Qiagen). Genomic DNA was digested with *DraI*, *EcoRV*, *PvuII*, and *StuI*, which are six-cutter enzymes that leave a blunt end at the cutting site, and adaptor ligation was subsequently used to construct four genomic libraries (L1-*DraI*, L2-*EcoRV*, L3-*PvuII* and L4-*StuI*). To obtain the 5'-flanking region of the *PgDDS* gene, genome walking was performed according to the manufacturer's instructions (Clontech). The primary PCR was carried out using AP1 (an adaptor primer, 5'-GTAATACGACTCACTATAGGGC-3') and GSP7 (a *PgDDS* gene-specific primer, 5'-TCCCTCTCTGGAGTACCAGCATC G-3'), followed by a second PCR with nested AP2 (5'-ACT ATAGGGCAGCGTGCT-3') and nested GSP6 (*PgDDS* gene-specific primer, 5'-TATTGTCTGCAAACAAAGTTGTTAG TGCT-3') primers. The two non-overlapping gene-specific primers were designed based on the conserved region of *PgDDS* cDNA sequences. The PCR product was cloned into the pGEM-T Easy vector (Promega) and sequenced. The promoter region was screened for *cis*-elements using the PLACE software (Higo *et al.* 1999).

Construction of plasmids

To construct an expression plasmid for tobacco, full-length *PgDDS* (AB122080) was cloned into the pCR 8.0 vector (Invitrogen) and transferred to the destination vector pH7WG2. *PgDDS* promoter constructs to drive *GUS* gene expression were prepared as follows. *Sall/BamHI* fragments of the *PgDDS* promoter (AB638615) were synthesized by PCR with forward (5'-ACTATAGGGCAGCGTGCT-3') and reverse (5'-CGGGATCCTGCCCCAAACTTCCCAT-3') primers. The PCR products were recombined into the *Sall/BamHI* site of pBI101 bearing the *GUS* reporter gene. The resulting constructs were used to transform ginseng using *Agrobacterium* strain GV3101 cells.

Plant transformation

Transgenic tobacco plants were produced as previously described (Yap et al. 2002). Genetic transformation of *P. ginseng* was performed as described in our previous reports (Choi et al. 2001).

Quantitative measurement of GUS activity

GUS activity was measured using 4-methylumbelliferyl- β -D-glucuronide (4-MUG) as described previously (Oh et al. 2003). At least three series were performed.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Science & Technology (MEST), Republic of Korea [WCU project (R33-10157)]; the Rural Development Administration, Republic of Korea [Next-Generation BioGreen 21 Program (Pj008088)].

References

- Akihisa, T., Tokuda, H., Ukiya, M., Suzuki, T., Enjo, F., Koike, K. et al. (2004) 3-Epicabraheahydroxylactone and other triterpenoids from Camellia oil and their inhibitory effects on Epstein–Barr virus activation. *Chem. Pharm. Bull.* 52: 153–156.
- Apers, S., Baronikova, S., Sindambiwe, J.B., Witvrouw, M., De Clercq, E., Vanden Berghe, D. et al. (2001) Antiviral, haemolytic and molluscicidal activities of triterpenoid saponins from *Maesa lanceolata*: establishment of structure–activity relationships. *Planta Med.* 67: 528–532.
- Bandaranayake, W.M., Gunasekera, S.P., Karumanayake, S.B., Sotheeswaran, S. and Sultanbawa, M.U.S. (1975) Terpenes of *Dipterocarpus* and *Doona* species. *Phytochemistry* 14: 2043–2048.
- Briskin, D.P. (2000) Medicinal plants and phytomedicines. Linking plant biochemistry and physiology to human health. *Plant Physiol.* 124: 507–514.
- Cheung, H.T. (1968) Constituents of dipterocarpaceas resins. 2. Structure of dryobalanone from *Dryobalanops aromatica*. *J. Chem. Soc.* 21: 2686–2697.
- Choi, Y.E., Yang, D.C., Kusano, T. and Sano, H. (2001) Rapid and efficient *Agrobacterium*-mediated genetic transformation by plasmolyzing pretreatment of cotyledons in *Panax ginseng*. *Plant Cell Rep.* 20: 616–621.
- Dinesh-Kumar, S.P., Tham, W.H. and Baker, B.J. (2000) Structure–function analysis of the tobacco mosaic virus resistance gene N. *Proc. Natl Acad. Sci. USA* 97: 14789–94.
- Han, J.Y., Kwon, Y.S., Yang, D.C., Jung, Y.R. and Choi, Y.E. (2006) Expression and RNA interference-induced silencing of the dammarenediol-II synthase gene in *Panax ginseng*. *Plant Cell Physiol.* 47: 1653–1662.
- Higo, K., Ugawa, Y., Iwamoto, M. and Korenaga, T. (1999) Plant cis-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res.* 27: 297–300.
- Huang, D. and Qi, D.F. (2005) Dammarane saponin, their use as anti-cancer agents, and a process for producing same. United States Patent Application No. 09/982,018.
- Huang, X., Yin, Z., Ye, W. and Shen, W. (2010) Chemical constituents from fruits of *Ligustrum lucidum*. *Zhongguo Zhong Yao Za Zhi* 35: 861–864.
- Kathiria, P., Sidle, C., Golubov, A., Kalischuk, M., Kawchuk, L.M. and Kovalchuk, I. (2010) Tobacco mosaic virus infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants. *Plant Physiol.* 153: 1859–1870.
- Kim, T.D., Han, J.Y., Huh, G.H. and Choi, Y.E. (2011) Expression and functional characterization of three squalene synthase genes associated with saponin biosynthesis in *Panax ginseng*. *Plant Cell Physiol.* 52: 125–137.
- Kim, O.T., Lee, J.W., Ban, J.H., Kim, Y.C., Hyun, D.Y., Cha, S.W. et al. (2009) Characterization of a dammarenediol-II synthase in *Centella asiatica* (L.) Urban. *Plant Physiol Biochem.* 47: 998–1002.
- Kim, Y.S. and Sano, H. (2008) Pathogen resistance of transgenic tobacco plants producing caffeine. *Phytochemistry* 69: 882–888.
- Mills, J.S. and Werner, A.E.A. (1955) The chemistry of dammar resin. *J. Chem. Soc.* 3132–3140.
- Mengoni, F., Lichtner, M., Battinelli, L., Marzi, M., Mastroianni, C.M., Vullo, V. et al. (2002) In vitro anti-HIV activity of oleanolic acid on infected human mononuclear cells. *Planta Med.* 68: 111–114.
- Lee, M.H. and Sano, H. (2007) Attenuation of the hypersensitive response by an ATPase associated with various cellular activities (AAA) protein through suppression of a small GTPase, ADP ribosylation factor, in tobacco plants. *Plant J.* 51: 127–139.
- Oh, D.H., Kwon, C.S., Sano, H., Chung, W.I. and Koizumi, N. (2003) Conservation between animals and plants of the cis-acting element involved in the unfolded protein response. *Biochem. Biophys. Res. Commun.* 301: 225–230.
- Pellicciari, R., Ardon, A. and Bellavita, V. (1972) Triterpenes from *Pouteria caimoto*. *Planta Med.* 22: 196–200.
- Phillips, M.A. and Croteau, R.B. (1999) Resin-based defense in conifers. *Trends Plant Science* 4: 184–190.
- Poehland, B.L., Carte, B.K., Francis, T.A., Hyland, L.J. and Allaudeen, H.S. (1987) In vitro antiviral activity of dammar resin triterpenoids. *J. Nat. Prod.* 50: 706–713.
- Shibata, S. (2001) Chemistry and cancer preventing activities of ginseng saponins and some related triterpenoid compounds. *J. Korean Med. Sci.* 16: S28–37.
- Shibuya, M., Hoshino, M., Katsube, Y., Hayashi, H., Kushiro, T. and Ebizuka, Y. (2006) Identification of beta-amyrin and sophoradiol 24-hydroxylase by expressed sequence tag mining and functional expression assay. *FEBS Lett.* 273: 948–959.
- Sindambiwe, J.B., Calomme, M., Geerts, S., Pieters, L., Vlietinck, A.J. and Vanden Berghe, D.A. (1998) Evaluation of biological activities of triterpenoid saponins from *Maesa lanceolata*. *J. Nat. Prod.* 61: 585–590.
- Spencer, G.F. (1981) Dammarenediol-II II esters from *Cacalia atriplicifolia* L. seed oil. *J. Nat. Prod.* 44: 166–168.
- Tansakul, P., Shibuya, M., Kushiro, T. and Ebizuka, Y. (2006) Dammarenediol-II synthase, the first dedicated enzyme for ginsenoside biosynthesis, in *Panax ginseng*. *FEBS Lett.* 580: 5143–5149.

- Usami, Y., Liu, Y.N., Lin, A.S., Shibano, M., Akiyama, T., Itokawa, H. et al. (2008) Antitumor agents. 261. 20(S)-protopanaxadiol and 20(S)-protopanaxatriol as antiangiogenic agents and total assignment of (1)H NMR spectra. *J. Nat. Prod.* 71: 478–481.
- Waller, F., Muller, A., Chung, K.M., Yap, U.L., Nakamura, K., Weiler, E. et al. (2006) Expression of a WIPK-activated transcription factor results in increase of endogenous salicylic acid and pathogen resistance in tobacco plants. *Plant Cell Physiol.* 47: 1167–1174.
- Wu, Z.J., Ouyang, M.A., Wang, C.Z. and Zhang, Z.K. (2007a) Six new triterpenoid saponins from the leaves of *Ilex oblonga* and their inhibitory activities against TMV replication. *Chem. Pharm. Bull.* 55: 422–427.
- Wu, Z.J., Ouyang, M.A., Wang, C.Z., Zhang, Z.K. and Shen, J.G. (2007b) Anti-tobacco mosaic virus (TMV) triterpenoid saponin from the leaves of *Ilex oblonga*. *J. Agric. Food Chem.* 55: 1712–1717.
- Yap, Y.K., Kakamu, K., Yamaguchi, Y., Koizumi, N. and Sano, H. (2002) Promoter analysis of WIPK, a gene coding a tobacco MAPKinase with reference to wounding and tobacco mosaic virus infection. *J. Plant Physiol.* 159: 77–83.