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

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**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 ginsenosides 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...
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.

Fig. 1 Biosynthesis of triterpenoid saponins in Panax ginseng.
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 20–30 μg 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. 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.
compared with that in wild-type leaves (Fig. 4E). These experiments demonstrated that endogenous dammarenediol-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 dammarenediol-II

The effect on TMV resistance was examined for exogenously applied dammarenediol-II. A TMV solution (10 μg ml⁻¹) was mixed with 0, 20 and 200 μg ml⁻¹ dammarenediol-II, and kept at 25°C for 24 h. Leaves of wild-type tobacco plants were infected with a TMV and dammarenediol-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 dammarenediol-II mixture compared with leaves treated with TMV alone (Fig. 5B). The leaves treated with the TMV and dammarenediol-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
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 maesasaponins 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 (OD 260) 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′-CA AGTTGAGGCCAGCCAGG-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′-ATGTCTTATAGATCCTACTCCACATCT-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 Dral, EcoRV, PvuII, and Stul, 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-Dral, L2-EcoRV, L3-PvuII and L4-Stul). 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′-GTAATACGACTCACTATAGGG-3′ and GSP7 (a PgDDS gene-specific primer, 5′-TCCTTCTGTGAGTACCCACGC-3′), followed by a second PCR with nested AP2 (5′-ACT ATAGGGCACGCTGTG-3′) and nested GSP6 (PgDDS gene-specific primer, 5′-TTTTTGTCTGGCAAAAAGTGTAG TC-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. SalI/BamHI fragments of the PgDDS promoter (AB638615) were synthesized by PCR with forward (5′-ACTATAGGGACGCTGTTG-3′) and reverse (5′-CGGGATCCTGGCCCCAAAACCCCAT-3′) primers. The PCR products were recloned into the SalI/BamHI site of pBI1010 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.

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References


