RESEARCH ARTICLE

Development of a Transient ihpRNA-induced Gene Silencing System for Functional Analysis in Persimmon (*Diospyros kaki* Thunb.)

Rongli Mo[1], Na Zhang[1], Sichao Yang[1], Qinglin Zhang[1,2], and Zhengrong Luo[1,2*]

[1]Key Laboratory of Horticultural Plant Biology, Huazhong Agricultural University, Wuhan 430070, China
[2]Hubei Collaborative Innovation Center for the Characteristic Resources Exploitation of Dabie Mountains, Huanggang 438000, China

*Corresponding author: luozhr@mail.hzau.edu.cn

Abstract

A transient ihpRNA-induced gene silencing system based on *Agrobacterium*-mediated injection infiltration has been established to evaluate candidate genes involved in proanthocyanidin (PAs) biosynthesis in persimmon (*Diospyros kaki* Thunb.). We chose *DkPDS* (*phytoene desaturase*) as a gene-silencing target to evaluate the newly developed transient gene silencing system. Our qRT-PCR analysis indicated that two ihpRNA constructs (pHG-PDS5' and pHG-PDS3') targeted *DkPDS*, which also led to significantly reduce expression of *DkPDS* in 'Mopanshi' persimmon leaves. To further confirm the reliability of the system, we successfully utilized it for *DkLAR* (*leucoanthocyanidin reductase*) gene silencing. The expression levels of *DkLAR* in 'Mopanshi' and 'Eshi 1' leaves were ca. 6-fold and ca. 5-fold lower than those in leaves harboring empty vector (pHG-GFP), respectively. DMACA (4-dimethylaminocinnamaldehyde) staining and the Folin-Ciocalteau assay showed that the accumulation of PAs was markedly inhibited in 'Mopanshi', 'Eshi 1' and 'Youhou' leaves. These results indicate that *DkLAR* plays an important role in the accumulation of PAs in persimmon. The transient ihpRNA-induced gene silencing method developed in this study is a highly efficient and useful tool for functional analysis of persimmon genes involved in PA biosynthesis.

Additional key words: *Agrobacterium tumefaciens*, *DkLAR*, *DkPDS*, proanthocyanidin

Korean J. Hortic. Sci. Technol. 34(2):314-323, 2016
http://dx.doi.org/10.12972/kjhst.20160032

pISSN : 1226-8763
eISSN : 2465-8588

Received: October 13, 2015
Revised: December 14, 2015
Accepted: March 23, 2016

Introduction

Persimmon (*Diospyros kaki* Thunb.) belongs to the genus *Diospyros* in the family Ebenaceae and is recognized as the most important species among fruit crops of this genus. Persimmon is generally classified into two major types, PCNA and non-PCNA, based on the fruit astringency at maturity and genetic characteristic. PCNA-type persimmons include Chinese PCNA (C-PCNA) and Japanese PCNA (J-PCNA), which are further subdivided into three groups, PVNA, PVA and PCA (Akagi et al., 2011). Condensed tannins (also known as proanthocyanidins, PAs) accumulate

in the vacuoles of young and developing fruit. Accordingly, the astringency of persimmon fruit directly depends upon PA levels. In the past decade, our understanding of the metabolic networks of PAs in persimmon was greatly enhanced by the use of transcriptome sequencing technology (Nakagawa et al., 2008; Luo et al., 2014). However, although many candidate genes associated with PA metabolism have been identified, progress in elucidating their functions has been hampered due to the lack of an efficient system for functional characterization of this plant.

Hairpin RNA (hpRNA)-mediated RNA interference (RNAi) technology is an effective approach for gene silencing, thus providing a robust tool for studying gene function. The intron-containing, self-complementary hpRNA (ihpRNA) constructs are more effective for silencing target genes compared to sense and antisense RNAs, as revealed by the higher silencing rates in transgenic lines (Wesley et al., 2001; Smith et al., 2000). Due to its superior characteristics, including the ease of manipulation and the applicability to numerous plant species, *Agrobacterium*-mediated transient ihpRNA silencing has been widely employed to characterize various genes in tobacco (Yan et al., 2012), grapevine (Urso et al., 2013), strawberry (Hoffmann et al., 2006) and Dendrobium (Ratanasut et al., 2015). To the best of our knowledge, transient ihpRNA-induced gene silencing in persimmon has not previously been reported.

PAs are phenolic oligomers or polymers of flavan-3-ol units (catechin and epicatechin). Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), the key enzymes for flavan-3-ol biosynthesis, can convert leucoanthocyanidin to catechin and anthocyanidin to epicatechin, respectively (Xie et al., 2003; Tanner et al., 2003). DkANR converts anthocyanins to both 2,3-cis-flavan-3-ol and 2,3-trans-flavan-3-ol and is involved in PA biosynthesis in persimmon fruit (Akagi et al., 2009). The *DkLAR*, which was isolated from C-PCNA, is closely related to its homologs in other plant species. The expression pattern of *DkLAR* is consistent with tannin cell development in the C-PCNA genotype. *DkLAR* is potentially involved in PA accumulation in persimmon fruit (Wang et al., 2010). Therefore, transient silencing of *DkLAR* in persimmon leaves might allow us to study its function in PA biosythesis in persimmon fruit.

In this study, we established a simple and highly efficient transient gene silencing system for gene functional analysis in persimmon based on ihpRNA-induced gene silencing. To evaluate the efficiency of the transient silencing system, we chose *DkPDS* as a gene-silencing target. *DkLAR* in leaves was also the target of gene silencing induced by T-DNA constructs producing *LAR* ihpRNAs, which led to reduce PA levels. The system developed in this study can be utilized for rapid, high-throughput functional analysis of genes involved in PA accumulation.

Materials and Methods

Plant Materials

Three persimmon (*Diospyros kaki* Thunb.) cultivars, 'Eshi 1' (C-PCNA), 'Youhou' (J-PCNA) and 'Mopanshi' (PCA), grown in the Persimmon Repository of Huazhong Agricultural University (Wuhan, China) were used in this study. Five-week-old leaves were sampled from the trees and used for analysis.

RNA Extraction and Gene Cloning

Total RNA was extracted with an RNA Prep Pure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's

instructions. The cDNA was synthesized from approximately 1 μg of total RNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Two fragments (PDS5' 310 bp and PDS3' 304 bp) of *DkPDS* (GU112527) (Zhao et al., 2011) and a fragment (253 bp) of *DkLAR* (EU747876) (Wang et al., 2010) were amplified from the cDNA of young 'Eshi 1' fruit. The primers used in this study are shown in Table 1.

Table 1. Sequences of the primers used in this study

Target	Gene	Sense primer (5' to 3')	Antisense primer (5' to 3')
Fragment	*GFP*	ATGCTTTTCAAGATACCCAGA	CTTTGATGCCGTTCTTTTGCT
Fragment	*PDS5'*	ACCAGATAAGCCGCTGAAGG	GGGAGTTCAGCCGATTTGAC
Fragment	*PDS3'*	CACATTCAATCACTGGGAGG	TTTTAGCAGAAGTCCCCTTC
Fragment	*LAR*	CCGACCACCCTGTAATTTCCT	TCGGAAGGATTCATCTGGGTA
qRT-PCR	*DkLAR*	GCCATCATCCATCAGTCA	CCTCTCTCAGAAACAACGC
qRT-PCR	*DkACTIN*	CACCACTCAACCCAAAGG	CCAGAATCCAGGACAATAC

Construction of Vectors

The fragments were introduced into the pDON*R*™222 vector using the BP recombination reaction (Invitrogen, USA) and sequenced. After sequence verification, the products were transferred to the pHELLSGATE8 vector containing the *coupled cell division B* (*ccdB*) gene using the LR recombination reaction (Invitrogen, USA) based on Gateway® technology, generating three ihpRNA vectors: pHG-PDS5', pHG-PDS3' and pHG-LAR (Fig. 1). In addition, a fragment (281 bp) of

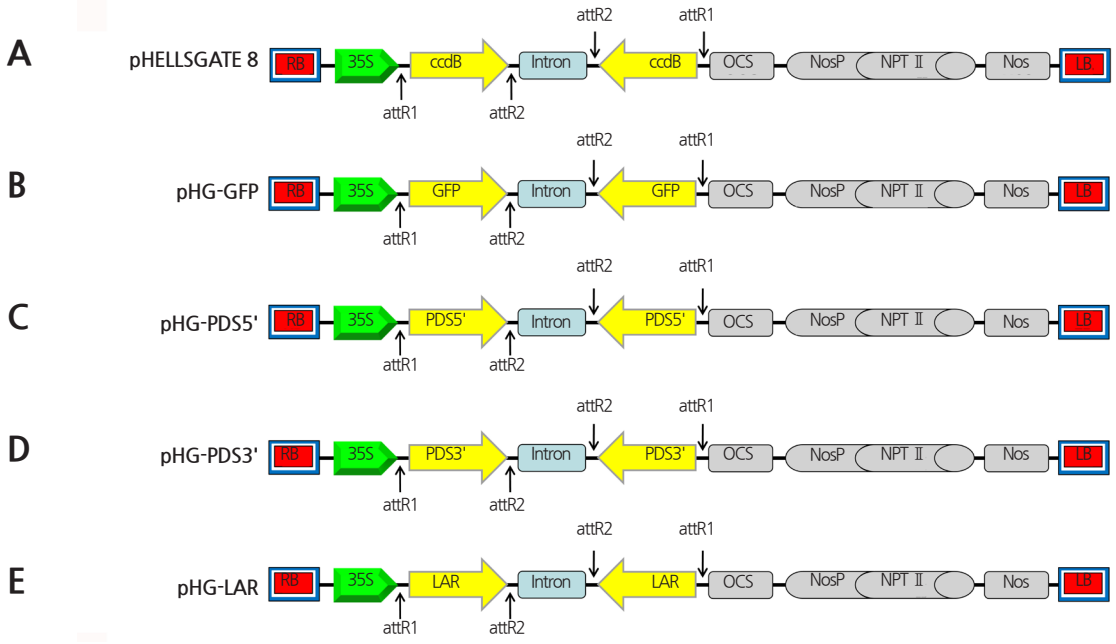

Fig. 1. Schematic maps of ihpRNA expression constructs used for transient gene silencing. (A) pHELLSGATE 8 vector; (B) pHG-GFP containing the *GFP* sequence; (C) pHG-PDS5' and (D) pHG-PDS3' containing the *PDS* sequence; (E) pHG-LAR containing the *LAR* sequence. All constructs contain the CaMV-35S promoter, attR1 and attR2 recombination sites of the Gateway system and octopine synthase (OCS), nopaline synthase (Nos) and kanamycin resistance genes (NosP-NPTII).

GFP was amplified from pCAMBIA1302 and introduced into pHELLSGATE8 to create the pHG-GFP construct, which was used as a control (Fig. 1).

Preparation of *Agrobacterium* Cultures for Infiltration

The vectors were transferred into *Agrobacterium* strain GV3101. To establish *Agrobacterium* suspensions, a single bacterial colony was inoculated in 50 ml of LB medium containing the appropriate concentration of kanamycin and shaken overnight at 250 rpm at 28°C. The cultures were centrifuged at 4,000 × g for 10 min and re-suspended in infiltration medium composed of 10 mM $MgCl_2$, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 150 μM acetosyringone to an optical density (OD) of 0.75 at 600 nm (Mo et al., 2015). The cultures were then incubated at room temperature for 3 h before infiltration. *Agrobacterium*-mediated leaf infiltration of the suspensions was carried out as described by Mo et al. (2015). The infiltrated leaves were collected at 7 or 15 days after agroinfiltration for *DkPDS* expression analysis of 'Mopanshi' transformed with pHG-PDS5', pHG-PDS3' and pHG-GFP, respectively. The pHG-LAR and pHG-GFP vectors were transformed into 'Mopanshi' and 'Eshi 1' leaves, respectively. The leaves were sampled at 10 and 15 days after agroinfiltration.

Expression Analysis by qRT-PCR

The primers used for qRT-PCR analysis are shown in Table 1. The qRT-PCR was performed on a LightCycler® 480 Real-Time PCR Instrument (Roche Diagnostics, Germany) with SYBR Pre-mix Ex-Taq (TaKaRa, Japan), and gene expression was normalized against the expression of the *actin* gene. For each reaction, the average threshold cycle (Ct) was automatically determined by the LightCycler® 480 Real-Time PCR Instrument as the default state. Quantitative RT-PCR was performed with three biological replicates per sample, and data represent means ± SD (n = 3).

Analysis of PA Content

Leaves of three persimmon cultivars, 'Mopanshi', 'Eshi 1' and 'Youhou', transformed with pHG-LAR or pHG-GFP were sampled at 10 and 15 days after agroinfiltration. Histochemical staining with DMACA solution (0.6% DMACA and 1% 6N HCl in methanol) was performed to analyze PA contents in leaves. The infiltrated leaves were decolorized in 30% acetic acid in ethanol for 12–20 h, washed with 75% ethanol and stained blue in DMACA solution for 2 min (Li et al., 1996): the deeper the blue coloring, the higher the PA content. Furthermore, 'Mopanshi' and 'Eshi 1' leaves sampled at 15 days after infiltration were used for tannin content analysis. In addition, soluble and insoluble tannin contents were also measured using the Folin-Ciocalteau method (Oshida et al., 1996). Approximately 0.1 g of leaf tissues was used for soluble and insoluble tannin content analysis, with a total of ten single leaf replicates.

Statistical Analysis

Statistical significance of differences was calculated using Student's test ($p < 0.01$). All statistical analysis was performed with SPSS 17.0.

Results and Discussion

Transient ihpRNA-induced *DkPDS* Gene Silencing via Agroinfiltration

In order to develop an ihpRNA-based transient gene silencing system in persimmon leaves *in vivo*, we chose *DkPDS* as the gene-silencing target. Silencing PDS (encoding a key enzyme in the carotenoid biosynthesis pathway) (Zhao et al., 2011) caused photobleaching in plants. We cloned two fragments of *DkPDS* into the ihpRNA interference vector pHELLSGATE8 to examine the effects of different sequence fragments on transient silencing efficiency. Therefore, the *DkPDS* gene was targeted by two ihpRNA constructs, pHG-PDS5' and pHG-PDS3', under the control of the CaMV 35S promoter. These vectors were transiently transformed into 'Mopanshi' (PCA) leaves by agroinfiltration. As shown by qRT-PCR analysis, *DkPDS* expression in leaves infiltrated with either construct was reduced three-fold compared to empty vector at 7 days after infiltration (Fig. 2). These results indicate that *Agrobacterium*-mediated transient transformation of leaves with ihpRNA successfully induced *DkPDS* gene silencing, with no difference in silencing efficiency between constructs. However, we observed extensive leaf drop in plants infiltrated with pHG-PDS5' and pHG-PDS3' at 10 days after agroinfiltration (data not show). This observation indicates that *DkPDS* was successfully silenced, which resulted in dysfunctional leaf photosynthesis and led to leaf abscission. Therefore, we were unable to observe a photobleaching phenotype in these plants.

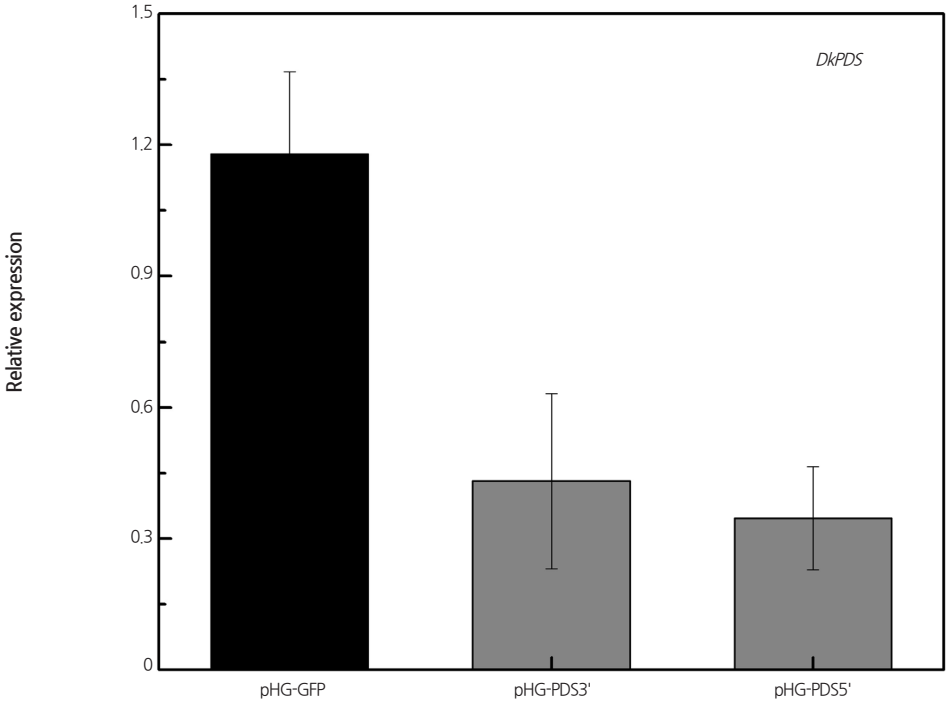

Fig. 2. Expression analysis of *DkPDS* in 'Mopanshi' persimmon leaves. Leaves transformed with the pHG-GFP, pHG-PDS5' and pHG-PDS3' ihpRNA vector, respectively, were sampled for qRT-PCR analysis at 7 days after agroinfiltration. Error bars indicate SEs from three biological replicates.

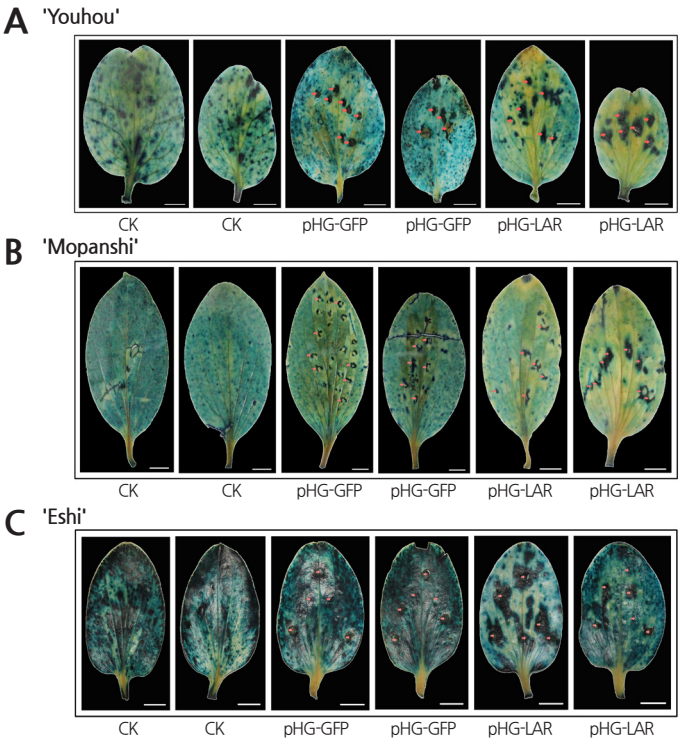

Fig. 3. Analysis of PA content in 'Youhou' (A), 'Mopanshi' (B) and 'Eshi 1' (C) persimmon leaves, as revealed by DMACA staining at 10 days after agroinfiltration. CK represents untreated leaves. pHG-GFP and pHG-LAR represent leaves transformed with vectors pHG-GFP and pHG-LAR ihpRNA, respectively. Injection holes are marked with pink arrows. Scale bar = 1 cm.

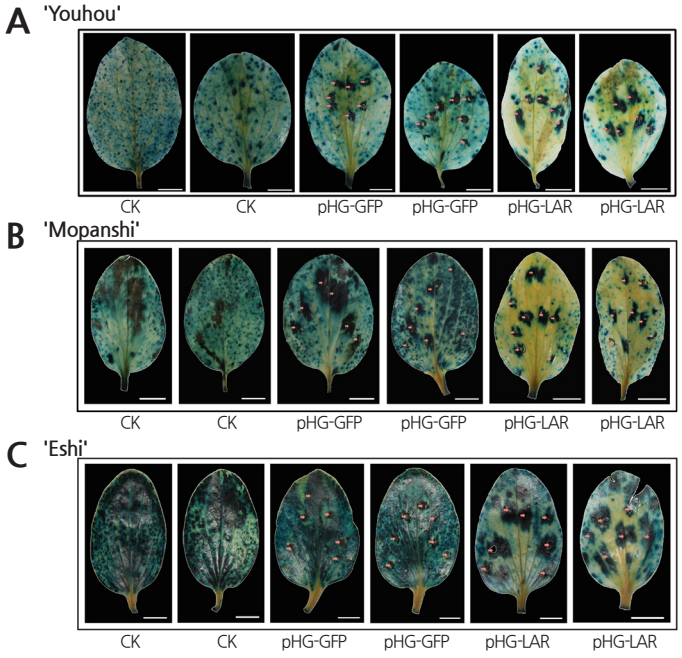

Fig. 4. Analysis of PA content in 'Youhou' (A), 'Mopanshi' (B) and 'Eshi 1' (C) persimmon leaves, as revealed by DMACA staining at 15 days after agroinfiltration. CK represents untreated leaves. pHG-GFP and pHG-LAR represent leaves transformed with vectors pHG-GFP and pHG-LAR ihpRNA, respectively. Injection holes are marked with pink arrows. Scale bar = 1 cm.

Transient Silencing of *DkLAR* Reduces PA Levels in Persimmon Leaves

As described above, silencing of *DkPDS* caused leaf abscission. Therefore, we chose *DkLAR*, which is potentially involved in the accumulation of persimmon PAs (Wang et al., 2010), for transient silencing to evaluate the stability and reliability of the transient ihpRNA-mediated gene silencing system in persimmon. The ihpRNA interference vector pHG-LAR, which is targeted to the *leucoanthocyanidin reductase* (*LAR*) gene, was transformed into 'Mopanshi' (PCA), 'Youhou' (J-PCNA) and 'Eshi 1' (C-PCNA) leaves *in vivo* by agroinfiltration. DMACA staining revealed significantly reduced PA levels in leaves infiltrated with pHG-LAR compared to the control (Figs. 3 and 4). These results suggest that the transient silencing of *DkLAR* inhibits PA accumulation in persimmon leaves. Moreover, lower PA levels were detected in infiltrated leaves sampled at 15 days versus 10 days after agroinfiltration (Figs. 3 and 4). Therefore, our *Agrobacterium*-mediated transient ihpRNA-inducing gene silencing system is stable and should be appropriate for persimmon cultivars with different types of astringency.

We also collected 'Mopanshi' and 'Eshi 1' leaves transformed with pHG-LAR for further experimental analysis at 15 days after agroinfiltration to confirm the notion that *DkLAR* is involved in PA accumulation (Wang et al., 2010). The qRT-PCR analysis showed that the expression of *DkLAR* was ca. 6-fold and ca. 5-fold lower in 'Mopanshi' and 'Eshi 1' leaves than in the positive control transformed with empty vector (pHG-GFP), respectively

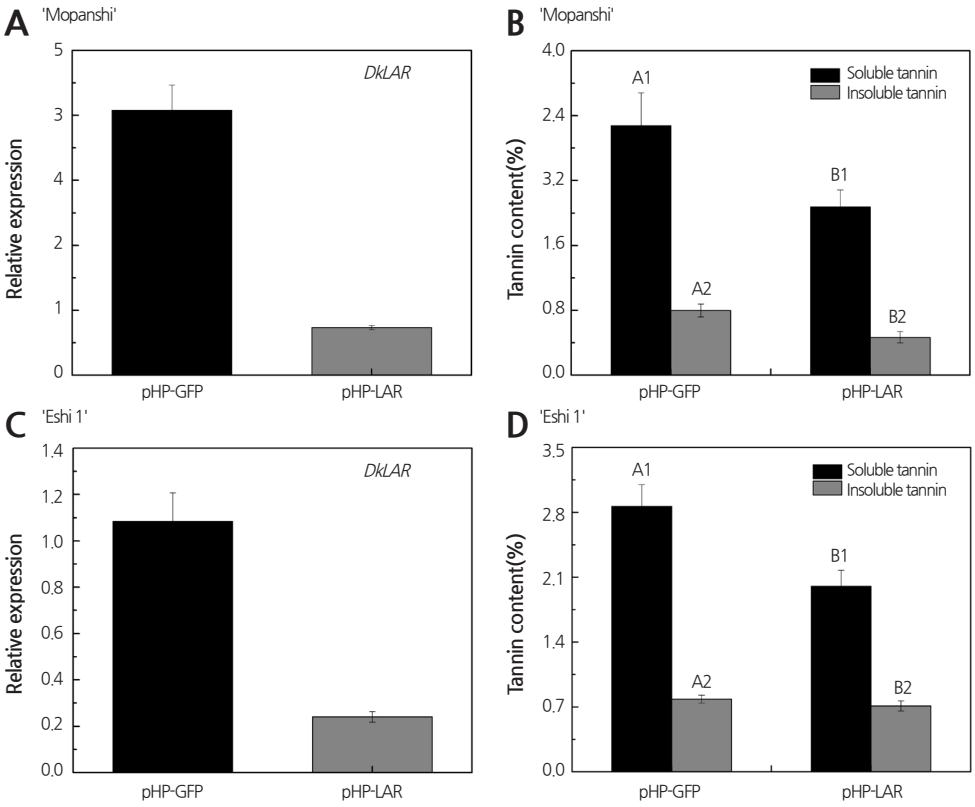

Fig. 5. Transient silencing of *DkLAR* in 'Mopanshi' and 'Eshi 1' persimmon leaves. (A) and (C) Analysis of expression levels of *DkLAR* in leaves at 15 days after agroinfiltration. Error bars indicate SEs from three biological replicates. (B) and (D) Detection of soluble and insoluble tannin contents in persimmon leaves by the Folin-Ciocalteau method. Error bars indicate SEs from ten biological replicates. pHG-GFP and pHG-LAR represent leaves transformed with vectors pHG-GFP and pHG-LAR ihpRNA, respectively.

(Figs. 5A and 5C). In addition, the Folin-Ciocalteau assay revealed extremely significant reductions in the levels of soluble tannin in leaves of both persimmon cultivars infiltrated with pHG-LAR (Figs. 5B and 5D). The same tendencies were observed for insoluble tannin contents in 'Mopanshi' persimmon leaves, which were significantly reduced compared to the empty vector control (Fig. 5B). However, a slight reduction in insoluble tannin levels was observed in 'Eshi 1' persimmon leaves (Fig. 5D). These results indicate that *DkLAR* gene silencing leads to reduce PA levels in persimmon leaves, which is consistent with the results of DMACA staining (Fig. 4). LAR activity is clearly associated with PA accumulation (Joseph et al., 1998; Pang et al., 2007). To date, *LAR* has been isolated from many plant species, including grapevine (Bogs et al., 2005; Gagne et al., 2009) and poplar (Yuan et al., 2012; Wang et al., 2013). Previous studies have confirmed that *VvLAR*, which shares 71% sequence identity with *DkLAR* (Wang et al., 2010), contributes to PA accumulation in grape (Bogs et al., 2005). The overexpression of *PtrLAR1* (Wang et al., 2013) and *PtrLAR3* (Yuan et al., 2012) also causes strong accumulation of PAs in poplar. Together, these findings suggest that *DkLAR*, an LAR homolog, is required for PA accumulation in persimmon.

Functional studies of genes involved in PA biosynthesis in Arabidopsis are often based on the lose-of-function *tt* (*transparent testa*) mutants (Debeaujon et al., 2000; Devic et al., 1999), which can be easily identified using DMACA staining (Abrahams et al., 2002). However, it is not currently possible to create mutants in perennial woody plants such as persimmon for functional analysis of PA biosynthesis genes. Persimmon leaves can accumulate a large number of PAs. Thus, combined with DMACA staining, the in vivo transient gene silencing system for persimmon leaves developed in this study can serve as a model method for other perennial woody plants for rapid functional analysis of genes involved in PA biosynthesis.

Moreover, the current results show that the efficiency of transient silencing differs depending on genotype (Figs. 3-5). In general, syringe-mediated agroinfiltration requires forcing the bacterial suspension through stomata and into air spaces in the leaf (Sparkes et al., 2006). Leaf architecture traits including high density of palisade and spongy mesophyll cells, low density and/or small aperture of stomatal pores and overall fragility of leaf tissue often lead to poor infiltration (Simmons et al., 2009, 2012). Thus, we speculate that the difference in transient silencing efficiency in different persimmon genotypes is due to differences in leaf architecture. Similar results have been reported in other plant species (Wroblewski et al., 2005; Manavella and Chan, 2009; Andrieu et al., 2012).

In conclusion, we developed a simple, stable and efficient transient ihpRNA-induced gene silencing system based on *Agrobacterium* infiltration in persimmon leaves *in vivo* that can be utilized for rapid and high-throughput functional analyses of persimmon genes. Silencing of *DkLAR* using this system inhibited PA accumulation in persimmon.

Acknowledegment: This study was financially supported by the Natural Science Foundation of China (31471861) and the Special Fund for Agro-scientific Research in the Public Interest (201203047).

Literature Cited

Abrahams S, Tanner GJ, Larkin PJ, Ashton AR (2002) Identification and biochemical characterization of mutants in the proanthocyanidin pathway in Arabidopsis. Plant Physiol 130:561-576 doi:10.1104/pp.006189

Akagi T, Ikegami AK, Suzuki Y, Yoshida J, Yamada M, Sato A, Yonemori K (2009) Expression balances of structural genes in shikimate and flavonoid biosynthesis cause a difference in proanthocyanidin accumulation in persimmon (*Diospyros*

kaki Thunb.) fruit. Planta 230:899-915. doi:10.1007/s00425-009-0991-6

Akagi T, Ikegami AK, Yonemori K (2011) Proanthocyanidin biosynthesis of persimmon (*Diospyros kaki* Thunb.) fruit. Sci Hortic 130:373-380. doi:10.1016/j.scienta.2011.07.021

Andrieu A, Breitler JC, Sirè C, Meynard D, Gantet P, Guiderdoni E (2012) An in planta, *Agrobacterium*-mediated transient gene expression method for inducing gene silencing in rice (*Oryza sativa* L.) leaves. Rice 5:1-13. doi:10.1186/1939-8433-5-23

Bogs J, Downey MO, Harvey JS, Ashton AR, Tanner GT, Robinson SP (2005) Proanthocyanidin synthesis and expression of genes encoding leucoanthocyanidin reductase and anthocyanidin reductase in developing grape berries and grapevine leaves. Plant Physiol 139:652-663. doi:10.1104/pp.105.064238

Debeaujon I, Leon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination and longevity in Arabidopsis. Plant Physiol 122:403-413. doi:10.1104/pp.122.2.403

Devic M, Guilleminot J, Debeaujon I, Bechtold N, Bensaude E, Koornneef M, Pelletier G, Delseny M (1999) The BANYULS gene encodes a DFR-like protein and is a marker of early seed coat development. Plant J 19:387-398. doi:10.1046/j.1365-313X.1999.00529.x

Gagne S, Lacampagne S, Claisse O, Geny L (2009) Leucoanthocyanidin reductase and anthocyanidin reductase gene expression and activity in flowers, young berries and skins of *Vitis vinifera* L. cv. Cabernet-Sauvignon during development. Plant Physiol Biochem 47:282-290. doi:10.1016/j.plaphy.2008.12.004

Hoffmann T, Kalinowski G, Schwab W (2006) RNAi-induced silencing of gene expression in strawberry fruit (*Fragaria × ananassa*) by agroinfiltration: a rapid assay for gene function analysis. Plant J 48:818-826. doi:10.1111/j.1365-313X.2006.02913.x

Joseph R, Tanner G, Larkin P (1998) Proanthocyanidin synthesis in the forage legume Onobrychis vicifolia: a study of chalcone synthase, dihydroflavonol 4-reductase and leucoanthocyanidin 4-reductase in developing leaves. Aust J Plant Physiol 25:271-278. doi:10.1071/PP97068

Li YG, Tanner G, Larkin P (1996) The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. J Sci Food Agric 70:89-101

Luo C, Zhang QL, Luo ZR (2014) Genome-wide transcriptome analysis of Chinese pollination-constant nonastringent persimmon fruit treated with ethanol. BMC Genomics 15:112. doi:10.1186/1471-2164-15-112

Manavella PA, Chan RL (2009) Transient transformation of sunflower leaf discs via an *Agrobacterium*-mediated method: applications for gene expression and silencing studies. Nat Protoc 4:1699-1707. doi:10.1038/nprot.2009.178

Mo RL, Huang YM, Yang SC, Zhang QL, Luo ZR (2015) Development of *Agrobacterium*-mediated transient transformation in persimmon (*Diospyros kaki* Thunb.). Sci Hortic 192:29-37. doi:10.1016/j.scienta.2015.05.013

Nakagawa T, Nakatsuka A, Yano K, Yasugahira S, Nakamura R, Sun N, Itai A, Suzuki T, Itamura H (2008) Expressed sequence tags from persimmon at different developmental stages. Plant Cell Rep 27:931-938. doi:10.1007/s00299-008-0518-9

Oshida M, Yonemori K, Sugiura A (1996) On the nature of coagulated tannins in astringent-type persimmon fruit after an artificial treatment of astringency removal. Postharvest Biol Technol 8:317-327. doi:10.1016/0925-5214(96)00016-6

Pang YZ, Peel GJ, Wright E, Wang ZY, Dixon RA (2007) Early steps in proanthocyanidin biosynthesis in the model legume *Medicago truncatula*. Plant Physiol 145:601-615. doi:10.1104/pp.107.107326

Ratanasut K, Monmai C, Piluk P (2015) Transient hairpin RNAi-induced silencing in floral tissues of *Dendrobium* Sonia 'Earsakul' by agroinfiltration for rapid assay of flower colour modification. Plant Cell Tissue Organ Cult 120:643-654. doi:10.1007/s11240-014-0632-z

Simmons, C.W., J.S. VanderGheynst, and S.K. Upadhyaya. 2009. A model of *Agrobacterium tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. Biotechnol. Bioeng. 102:965-970. doi:10.1002/bit.22118

Simmons CW, Nitin N, VanderGheynst JS (2012) Rapid, in situ detection of *Agrobacterium tumefacien*s attachment to leaf tissue. Biotechnol Prog 28:1321-1328. doi:10.1002/btpr.1608

Smith NA, Singh SP, Wang MB, Stoutjesdijk PA, Green AG, Waterhouse PM (2000) Total silencing by intron-spliced hairpin RNAs. Nature 407:319-320. doi:10.1038/35030305

Sparkes IA, Runions J, Kearns A, Hawes C (2006) Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generations of stably transformed plants. Nat Protoc 1:2019-2025. doi:10.1038/nprot.2006.286

Tanner GJ, Francki KT, Abrahams S, Watson M, Larkin PJ, Ashton AR (2003) Proanthocyanidin biosynthesis in plants. J Biol Chem 278:31647-31656. doi:10.1074/jbc.M302783200

Urso S, Zottini M, Ruberti C, Schiavo FL, Stanca AM, Cattivelli L, Valè G (2013) An *Agrobacterium tumefaciens*-mediated gene silencing system for functional analysis in grapevine. Plant Cell Tissue Organ Cult 114:49-60. doi:10.1007/s11240-013-0305-3

Wang LJ, Jiang YZ, Yuan L, Lu WX, Yang L, Karim A, Luo KM (2013) Isolation and characterization of cDNAs encoding leucoanthocyanidin reductase and anthocyanidin reductase from *Populus trichocarpa*. PLoS One 8:e64664. doi:10.1371/journal.pone.0064664

Wang Y, Zhang QL, Luo ZR (2010) Isolation and expression of gene encoding leucoanthocyanidin reductase from *Diospyros kaki* during fruit development. Biol Plant 54:707-710. doi:10.1007/s10535-010-0125-9

Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT, Liu Q, Gooding PS, Singh SP, Abbott D, Stoutjesdijk PA, et al

(2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581-590. doi:10.1046/j.1365-313X.2001.01105.x

Wroblewski T, Tomczak A, Michelmore R (2005) Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato, and Arabidopsis. Plant Biotechnol J 3:259-273. doi:10.1111/j.1467-7652.2005.00123.x

Xie DY, Sharma BS, Paiva NL, Ferreira D, Dixon RA (2003) Role of anthocyanidin reductase, encoded by *BANYULS* in plant flavonoid biosynthesis. Science 299:396-399. doi:10.1126/science.1078540

Yan P, Shen WT, Gao XZ, Li XY, Zhou P, Duan J (2012) High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. PLoS One 7:e38186. doi:10.1371/journal.pone.0038186

Yuan L, Wang LJ, Han ZJ, Jiang YZ, Zhao LL, Liu H, Yang L, Luo KM (2012) Molecular cloning and characterization of *PtrLAR3*, a gene encoding leucoanthocyanidin reductase from *Populus trichocarpa*, and its constitutive expression enhances fungal resistance in transgenic plants. J Exp Bot 63:2513-2524. doi:10.1093/jxb/err425

Zhao DQ, Zhou CH, Kong F, Tao J (2011) Cloning of phytoene desaturase and expression analysis of carotenogenic genes in persimmon (*Diospyros kaki* L.) fruits. Mol Biol Rep 38:3935-3943. doi:10.1007/s11033-010-0510-7