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BRIEF COMMUNICATION

# Isolation and expression of gene encoding leucoanthocyanidin reductase from *Diospyros kaki* during fruit development

Y. WANG, Q.L. ZHANG and Z.R. LUO*

*Department of Pomology, Key Laboratory for Horticultural Plant Biology, Huazhong Agricultural University, Wuhan 430070, P.R. China*

## Abstract

Leucoanthocyanidin reductase (LAR) converts leucoanthocyanidin to (+)-catechin, a precursor of proanthocyanidins abundant in Japanese persimmon (*Diospyros kaki* Thunb.) fruits. A putative *LAR* gene (*DkLAR*) was isolated by rapid amplification of cDNA ends from young fruits. The full-length cDNA of *DkLAR* gene was 1 356 bp long and encoded an open reading frame of 349 residues. The deduced DkLAR protein was closely related to the homolog in other plant species. The expression of the *DkLAR* gene in Chinese pollination-constant non-astringent (PCNA) genotype was coincident with the tannin cell development, but was not in Japanese PCNA and Chinese pollination-variant astringent (PCA) genotypes.

*Additional key words*: condensed tannin, Japanese persimmon, real-time RT-PCR.

Proanthocyanidins (PAs), also named condensed tannins (CTs), are plant secondary metabolites and involved in a wide range of functions in plants (Shimoji and Yamasaki 2005, Jaakola *et al*. 2008). CTs also contribute to the astringency and taste in many plants, such as tea and grape. Japanese persimmon (*Diospyros kaki* Thunb.) fruits accumulate abundant CTs and are classified into four types depending on the effect of pollination on flesh colour and their patterns of astringency-loss: pollination-constant non-astringent (PCNA), pollination-variant non-astringent PVNA, pollination-variant astringent (PVA) and pollination-constant astringent (PCA). Among these types, only PCNA type is commercially desirable because the fruits can lose astringency naturally on the tree. However, there are only a few PCNA cultivars, and its trait of astringency-loss is recessive to non-PCNA types (Ikeda *et al*. 1985). Recently, in a Chinese PCNA persimmon cv. Luotian-tianshi trait of astringency-loss was dominant (Ikegami *et al*. 2006). These results suggested that mechanism of CTs biosynthesis was different in Chinese and Japanese PCNA types.

CTs are oligomers and polymers of flavan-3-ol units [(+)-catechin, (-)-epicatechin]. The key enzymes for flavan-3-ol biosynthesis are leucoanthocyanidin reductase (LAR), which converts leucoanthocyanidin to (+)-catechin, and anthocyanidin reductase (ANR), which converts anthocyanidin to (-)-epicatechin (Tanner *et al*. 2003, Xie *et al*. 2003). LAR activity has been reported in several plants and its activity correlated with CTs accumulation (Joseph *et al*. 1998, Marles *et al*. 2003). Several genes in the CTs biosynthesis pathway including *DkANR* have been reported in Japanese persimmon fruits (Ikegami *et al*. 2005a,b). However, the LAR enzyme activity in Japanese persimmon fruits has not yet been studied. In this study, the putative *LAR* gene (*DkLAR*) was isolated and mRNA levels were analyzed in different de-astringent types for better understanding the mechanism of CTs accumulation.

Fruits of persimmon (*Diospyros kaki* Thunb.) cvs. Luotian-tianshi (PCNA, Chinese origin), Suruga (PCNA, Japanese origin) and Mopanshi (PCA, Chinese origin) were collected in the Persimmon Repository, Huazhong Agricultural University, Wuhan, China. To measure tannin cell size and CTs content per fruit, ten fruits of each

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*Abbreviations*: ANR - anthocyanidin reductase; CTAB - cetyl-trimethyl-ammonium bromide; CT - condensed tannin; IFR - isoflavone reductase-like proteins; LAR - leucoanthocyanidin reductase; PA - proanthocyanidin; PCA - pollination-constant astringent; PCNA - pollination-constant non-astringent; PVA - pollination-variant astringent; PVNA - pollination variant non-astringent; RACE - rapid amplification of cDNA ends; RT-PCR - reverse transcriptase polymerase chain reaction.

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* Corresponding author; fax: (+86) 27 87282010, e-mail: luozhr@mail.hzau.edu.cn

sample were collected monthly from June to October 2007. Another ten fruits of each sample in early June, late August and early October 2007 were picked respectively, peeled and diced, and then were immediately frozen in liquid nitrogen and stored at -80 °C for RNA isolation.

Tannin cell size was measured as described by Ikegami *et al*. (2005b). Images of tannin cell were taken with *OLYMPUS BX61* (*Olympus*, Tokyo, Japan), and the areas of 100 tannin cells from each sample were measured with *IPP5.0* software (*Media Cybernetics*, Bethesda, USA). Soluble tannin content and insoluble tannin content were measured by the Folin-Ciocalteu method (Oshida *et al*. 1996).

Total RNA was extracted from Luotian-tianshi young fruits by the modified cetyl-trimethyl-ammonium bromide (CTAB) method (Shan *et al*. 2002). First-strand cDNA was synthesized according to the manufacturer's instructions of *ReverTra Ace-α*$^{TM}$ kit (*Toyobo*, Tokyo, Japan). The fragment of *LAR* gene was amplified with the degenerate primers DLARF (5′-AAGAGGTTTYTK CCNTCDGARTTYGGDCAYG-3′) and DLARR (5′-GCA GCCGTTGATGAAGATRTCRTGNGT- 3′) which were designed based on the conserved regions of known LAR proteins. To obtain longer sequence information, rapid amplification of cDNA ends (RACE) was carried out as the description in the manufacture's instructions of *BD Smart™ RACE cDNA Amplification* Kit (*Palo Alto*, CA, USA). For RACE-PCR, gene specific primers were designed from the sequenced fragments (5′ RACE, first PCR primer, 5′-GCTTTGACGCTGCCATCGCCATAA-3′, nested PCR primer, 5′-TGGAAGTGGTCAAGGGGAGG AAGAAC-3′; 3′ RACE, first PCR primer, 5′-GGCTCG GACATCGGGAAATTCACG-3′, nested PCR primer, 5′-AGCCTCCCCAGAGTTACCGTCACCGA-3′). The full-length *DkLAR* gene was amplified by RT-PCR with the primers 5′-CAAAAAGCAGAAATGACTGTG-3′ (start codon was underlined) and 5′-AGAACGCTC CCTCATAAACC-3′. Phylogenetic tree was constructed using the Neighbor-joining method by the *MEGA4* program. The pI and molecular mass of the DkLAR protein were calculated using the pI/molecular mass calculation tools at www.expasy.org.

For real-time PCR, cDNA was synthesized as described above; specific primers (5′-GCCATCATC CATCAGTCA-3′ and 5′-CCTCTCTCAGAAACAA CGC-3′) were designed based on the sequence of the 3′ untranslated regions of *DkLAR* gene. Real-time RT-PCR was performed using the *ABI 7500* Real Time System (*PE Applied Biosystems*, Foster City, CA, USA). *Actin* (5′-CACCACTCAACCCAAAGG-3′ and 5′-CCAGAA TCCAGGACAATAC-3′) and the primers for *DkLAR* gene were diluted in the *Syber Green PCR Master Mix* (*PE Applied Biosystems*) and 0.015 cm$^3$ of the reaction mix were added to each well. The thermal cycling conditions were an initial incubation at 50 °C for 2 min and at 95 °C for 1 min, and then cycled at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. Gene expression was quantified with *ABI 7500 Sequence Detector Version 1.3.1* (*PE Applied Biosystems*) and normalized against the expression of *actin* gene.

In cv. Suruga, tannin accumulation stopped at the early growth stage of the fruit. Tannin cell size changed slightly after July (Fig. 1*A*) and tannin content per fruit decreased with the growing of the fruit (Fig. 1*B*). In Luotian-tianshi, changes pattern of the tannin cell size was similar to that in Suruga, but tannin content per fruit increased gradually during the fruit growth (Fig. 1*A*,*B*). In Mopanshi, both tannin cell size and tannin content per fruit increased stably throughout the growth of the fruit (Fig. 1*A*,*B*). These results indicated that Chinese PCNA genotype was different from Japanese PCNA and Chinese PCA genotypes in tannin biosynthesis.

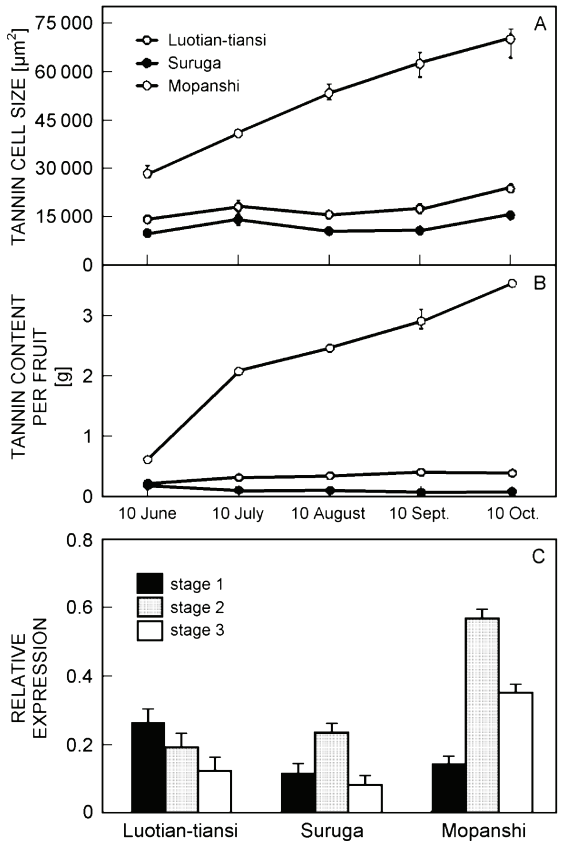


Fig. 1. Development of tannin cell and transcript level analysis of *DkLAR* gene in cv. Luotian-tianshi (Chinese PCNA genotype), Suruga (Japanese PCNA genotype) and Mopanshi (Chinese PCA genotype). *A* - Seasonal changes of tannin cell size. The areas of 100 tannin cells from each sample were measured with *IPP5.0* soft (*Media Cybernetics*, USA). *B* - Seasonal changes of tannin content per fruit. *C* - Transcript levels of *DkLAR* gene were measured by real-time RT-PCR. Stage 1 - early June, stage 2 - late August, stage 3 - early October. Value means ± SE (*A*, *B*, $n = 3$; *C*, $n = 4$).

The full length cDNA of *DkLAR* gene was isolated from young fruit of Luotian-tianshi and registered in NCBI (GenBank accession number EU747876). The 1356 bp long *DkLAR* cDNA contained a 1047 bp open reading frame (ORF) and sequence motifs common to the reductase-epimerase-dehydrogenase (RED) family of

protein. These include the Rossmann dinucleotide-binding domain characterized by the motif GXXGXXG starting from $Gly^{20}$, which may interact with NAD(P), and three residues, $Ser^{118}$, $Tyr^{137}$ and $Lys^{140}$, critical to the catalytic site (Bottoms *et al*. 2002). The deduced DkLAR protein contains 349 amino acids with a predicted molecular mass of 38 kDa and pI of 5.47, and has 71 % identity with LAR protein from *Vitis vinifera*. The deduced sequence revealed that LAR is most closely related to the isoflavone reductase (IFR) group of enzymes that are common in plants and is a member of the larger and more widespread RED family of proteins (Labesse *et al*. 1994, Jornvall *et al*. 1995, Tanner *et al*. 2003). The DkLAR protein contained the RFLP, ICCN, and THD motifs which conserved in LAR protein but absent in the related IFR proteins (Bogs *et al*. 2005). Neighbor-joining analysis clearly showed that the DkLAR clustered with verified LARs from tea, grape and apple and distracted from the IFR cluster (Fig. 2). These results supported that the clone we obtained indeed encoded DkLAR protein.

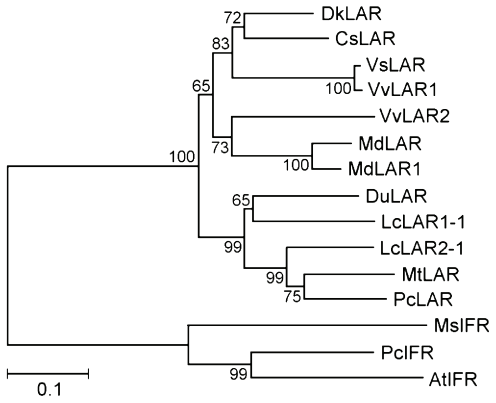


Fig. 2. Phylogenetic tree of the LAR and IFR proteins. The tree was constructed by the Neighbor-joining method. The proteins are as follows: *Camellia sinensis* (CsLAR, ABM88784), *Vitis shuttleworthii* (VsLAR, CAI56326), *Vitis vinifera* (VvLAR1, CAI26310, VvLAR2, CAI26308), *Malus* × *domestica* (MdLAR, AAZ79365, *MdLAR*1, AAZ79364), *Desmodium uncinatum* (DuLAR, Q84V83), *Lotus corniculatus* (LcLAR1-1, ABC71326, LcLAR2-1, ABC71330), *Medicago truncatula* (MtLAR, CAI56327), *Phaseolus coccineus* (PcLAR, CAI56322), *Medicago sativa* (MsIFR, CAA41106), *Pyrus communis* (PcIFR, AAC24001), *Arabidopsis thaliana* (AtIFR, NP_177664). Numbers indicate bootstrap values.

The mRNA levels of *DkLAR* were determined by real-time RT-PCR in the three stages according to the developing characters of tannin cell (Fig. 1*C*). The transcript level of *DkLAR* gene in Luotian-tianshi was detected throughout the fruit growth and decreased gradually with the development of the fruit (Fig. 1*C*). In Suruga, transcript level of *DkLAR* gene was also high during growing of the fruit, but was higher at middle stage, despite the termination of tannin cell enlargement, than at the early stage (Fig. 1*A*,*C*). Mopanshi was similar to Suruga in transcript pattern of the *DkLAR* gene, and the transcript level in Mopanshi was higher than those in Luotian-tianshi and Suruga (Fig. 1*C*). These results showed that the Chinese PCNA genotype was different from those of Japanese PCNA and Chinese PCA genotypes in transcript pattern of the *DkLAR* gene during the biosynthesis of the tannin cells.

Tannin content per fruit in Luotian-tianshi increased gradually with the developing of the fruits. In addition, transcript levels of the upstream genes in CTs biosynthesis pathway retained relatively high till the late stage (Ikegami *et al*. 2005b). These results suggested that tannin cell biosynthesis continued to the late stage. Catechin is a starter unit produced by the leucoanthocyanidin catalyzed by LAR enzyme. Transcript pattern of the *DkLAR* gene in Luotian-tianshi was coincident with tannin cell synthesis. These results suggested that *DkLAR* gene play an important role in tannin accumulation in Chinese PCNA genotype. In Suruga, catechin was detected throughout the growing period of the fruit (Yonemori *et al*. 1983), and the *DkLAR* gene transcript level was relatively high through the growing season of the fruit. However, the tannin cell biosynthesis ceased at the early stage in July. It suggested the mRNA level of *DkLAR* gene was closely related with catechin synthesis but was not with CTs accumulation in Japanese PCNA genotype. We found that there was a difference between Chinese and Japanese PCNA genotypes in the mechanism of tannin biosynthesis. In previous studies, Luotian-tianshi was separated from Japanese PCNA cultivars by the genetic relationships analysis (Guo *et al*. 2006), and the trait of natural deastringency was distinct in Chinese and Japanese PCNA genotypes (Ikegami *et al*. 2006). In Mopanshi, the *DkLAR* gene transcript level was higher than those in Chinese and Japanese PCNA genotypes. However catechin disappeared rapidly in June and gallic acid was major component of tannin in Japanese PCA cultivars (Yonemori *et al*. 1983). Maybe, the tannin components are different in Chinese and Japanese PCA.

The full length cDNA of *DkLAR* gene was successfully isolated from the Luotian-tianshi young fruits and its expression was coincident with tannin cell development (Chinese PCNA type), but not with that in Suruga (Japanese PCNA type) and Mopanshi (Chinese PCA type). The mechanism of biosynthesis of CTs in different de-astringent fruits requires further study.

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