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Identification of genes involved in proanthocyanidin biosynthesis of persimmon (*Diospyros kaki*) fruit

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Abstract

Persimmon fruit accumulate high-molecular weight proanthocyanidins in 'tannin cells' during their development. Enclosing young persimmon fruit on tree in an ethanol-containing polyethylene bag causes insolubilization of these proanthocyanidins. We examined the effect of this treatment on de novo synthesis of proanthocyanidins. First, we found by RNA blotting that the expression of genes encoding enzymes involved in flavonoid biosynthesis, namely, phenylalanine ammonia-lyase, chalcone synthase, and dihydroflavonol reductase, was down-regulated by the ethanol treatment. Second, we performed suppression subtractive hybridization (SSH) to identify additional genes whose expressions were differentially regulated during the treatment. In addition to six genes for flavonoid biosynthetic enzymes, a gene encoding the key enzyme in proanthocyanidin biosynthesis, anthocyanidin reductase (ANR), and the one for serine carboxypeptidase-like (SCPL) protein were found differentially expressed in astringent and astringency-removed fruit by SSH. These results were confirmed by RNA blottings. We also cloned full-length coding sequences for ANR and SCPL for the first time from persimmon fruit. The present data show that ethanol treatment not only causes direct insolubilization of proanthocyanidins, but also affects the regulation of proanthocyanidin synthesis at the transcriptional level. Our work also demonstrates that persimmon fruit can serve as a profitable material to study proanthocyanidin accumulation at molecular level.

Keywords: Anthocyanidin reductase; Condensed tannin; Ethanol treatment; Persimmon; Serine carboxypeptidase-like protein; Suppression subtractive hybridization

1. Introduction

Condensed tannins, also known as proanthocyanidins (PAs), are colorless flavonoid polymers and accumulate in vacuoles. They are found in barks of many trees, leaves of tea and forage plants, and seeds and fruit of many plants including grapes, cranberries, and persimmon. PAs are important natural products for human beings because of their strong antioxidant activities with beneficial effects on human health [1].

PAs are produced from the condensation of flavan 3-ol units [1–3]. Multiple steps leading to their direct precursors, i.e., leucoanthocyanidins and anthocyanidins, have been elucidated well at molecular level (Fig. 1) [3]. Recently two key enzymes responsible for the synthesis of these starter units have been identified at molecular level (Fig. 1) [2,4,5]. Xie et al. demonstrated that the *BANYULS* (*BAN*) gene encodes anthocyanidin reductase (ANR) which converts anthocyanidin to (−)-catechin [2]. Tanner et al. cloned a gene for leucoanthocyanidin reductase (LAR). LAR converts leucoanthocyanidin to (+)-catechin and is closely related to isoflavone reductases in its primary sequence [5]. Furthermore, several transcription factors, such as WIP domain-containing plant zinc-finger protein (TT1), R2R3 MYB domain-type nuclear protein (TT2), bHLH domain protein (TT8), BSISTER MADS domain protein (TT16), WD40 repeat protein (TTG1), and a zinc finger-like protein of plant-specific WRKY family (TTG2), have been suggested to be involved in PA biosynthesis by genetic studies using *Arabidopsis* [6–8]. In contrast to these findings, however, the mechanism of PA condensation is still not well understood [1].

Young persimmon fruit contain PAs at more than 25% of their dry weight in specialized compartments called ''tannin cells'' [9,10]. The proposed structure of persimmon PAs in

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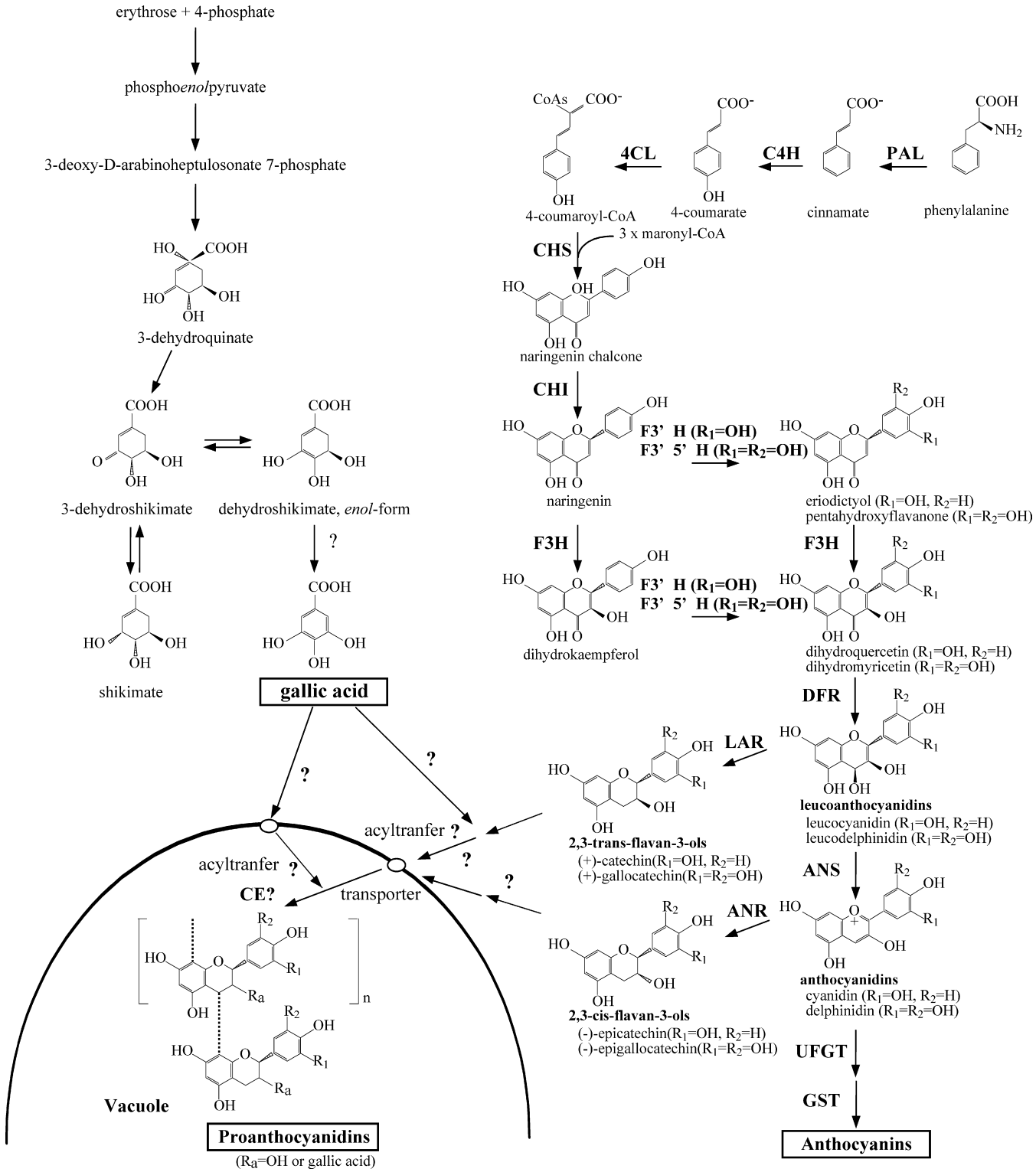

Fig. 1. Scheme of the proanthocyanidin biosynthetic pathway. ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; CE, condensing enzyme; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate: coenzyme A ligase; DFR, dihydroflavonol reductase; F3H, flavonol 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; GST, glutathione *S*-transferase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia-lyase; UFGT, UDP-glucose flavonoid 3-*O*-glucosyltransferease.

'Hiratanenashi' consists of catechin, catechin 3-*O*-gallate, gallocatechin, and gallocatechin 3-*O*-gallate as repeating units at a molar ratio of 1:1:2:2, which are linked through the C-6 or C-8 position with C-4 [11,12]. The average molecular weight of the methylated derivatives of persimmon PAs was estimated to be 1.38×10^4 Da based on comparison with a polystyrene standard [11]. These data suggest that gallic acid is essential for PA accumulation in persimmon fruit.