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Delay expression of limonoid UDP-glucosyltransferase makes delayed bitterness in citrus

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ABSTRACT

Genes encoding limonoid UDP-glucosyltransferase from albedo of six *Citrus* species with different levels of delayed bitterness are isolated and cloned in vector pTZ57R/T. Our results indicate that gene sequence of sweet lime (with intense juice delayed bitterness) have complete identity with Satsuma mandarin (without distinctive juice delayed bitterness). Also gene sequence of Marsh seedless grapefruit, local orange and Thompson navel orange (with mild juice delayed bitterness) have very similarity with Satsuma mandarin. On the other hand, this gene started to express 60, 120, and 210 days after full blooming in albedo of Satsuma mandarin, sweet oranges and sour orange, and both grapefruit and sweet lime, respectively. Expression pattern of limonoid glucosyltransferase gene in leaves was quite different with albedo. Thus, we supposed the delayed bitterness in this species was related to delay in expression of limonoid glucosyltransferase gene in albedo and lower limonoid glucoside accumulation in fruits.

Limonoids, the bitter constituents of citrus juice, are a group of highly oxygenated triterpenoids observed in the Rutaceae, Meliaceae, and related plant families. Limonin is a major component of limonoids in citrus and lowers the market value of fruit juice. So far, 36 limonoids have been isolated from Citrus and its closely related genera. Limonoid bitterness occurs gradually after juice processing from some citruses, which are referred to as delayed bitterness [1] and also in the fruits after freezing or mechanical damage. Limonin is the major bitter component of limonoid that causes delayed bitterness. Many biochemical approaches have been applied to eliminate the delayed bitterness and to produce the acceptable quality of juice [2]. Normally, intact fruit tissue contains a non-bitter precursor of limonin, limonoate A-ring lactone (LARL) [1]. The conversion of LARL to limonin in the juice proceeds under acidic conditions below pH 6.5 and is enhanced by the action of an enzyme, limonoid D-ring lactone hydrolase [3]. However, Satsuma mandarin (*Citrus unshiu* Marc.), which is the most popular *Citrus* species as fresh fruit and juice product in East Asia, develops fruits with much less delayed limonoid bitterness. The concentration of limonoid aglycone in the juices from Satsuma mandarin was low and due to that delayed bitterness has not been observed [4]. Conversion of LARL to tasteless limonin glucosides, such as limonin 17-L-D-glucopyranoside (LG), in the fruit during maturation from early stage is the main debittering factor in Satsuma mandarin [5]. The pattern of accumulation of limonoid glucoside is different between *Citrus* species, but the variety of limonoid compounds is the same. The limonoid content in citrus fruits reportedly decreases during the process of ripening [6]. This decrease is considered to be due to conversion of limonoid to a corresponding glucoside. In citrus juice, the glucoside levels are much higher than the free limonoids [7]. The free limonoids cause a bitterness problem after juice preparation [8].

In addition to the bitter property, limonoids have potential biological functions. Limonoids inhibit chemically induced carcinogenesis in mice, hamsters and cultured human breast cancer cells [9]. Moreover, antifeedant activities against certain insects and invaders have been reported [10]. Recently, due to the above function of limonoids, which is abundant in citrus fruits, a wide attention has been directed to them, thereby resulting in an increase in demand commercially. The easiest ways of limonoid intake are by drinking juice and eating the fruit itself but bitter limonoids such as limonin, nomilin, and obacunone obstruct their uptake.

On the other hand, non-bitter citrus limonoid glucosides, which are tasteless and water-soluble, have also been shown to exhibit anticancer activity in oral carcinogenesis in hamsters and human

Abbreviations: DAF, days after full blooming; LARL, limonoate a-ring lactone; LG, limonin 17-L-D-glucopyranoside.

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breast cancer cells in culture [11]. The biological activity of non-bitter limonoid glucosides is equal to the value of bitter limonoids and the water solubility is an important factor for medical application. Moreover, since a large amount of limonoid glucosides (more than 100 ppm) are contained in many kinds of citrus juice and fresh citrus fruits [12], it is easy to take limonoid glucosides as anticancer components in the diet. Because humans have been consuming citrus fruits from ancient times as a food, it can be said that citrus limonoids are very safe compounds for human health. Thus, not only for the processing industry, but also for the consumer, these limonoid glucosides are important compounds. The glucosylation of limonoids is catalyzed by UDP-D-glucose:limonoid glucosyltransferase (limonoid GTase) [13]. UDP-glycosyltransferases (UGT) are a superfamily of enzymes that catalyzes the addition of the glycosyl group from a UTP-sugar to a small hydrophobic molecule [14]. These enzymes share a conserved domain of about 50 amino acid residues located in their C-terminal section and from which a pattern has been extracted to detect them. Therefore, limonoid glucosyltransferase is a key enzyme for creating transgenic citrus without limonoid bitterness and increasing specific limonoid glucoside molecules. Molecular mechanism of delayed bitterness has been remained as a main question in citrus studies. However, analysis of limonoid glucosyltransferase expression at different time after full blooming has not been reported and there is no available data for sequence comparison among *Citrus* species.

The main goal of this study was the effect of gene expression on natural debittering and limonoid glucoside accumulation in citrus fruit. For this purpose, we amplified limonoid glucosyltransferase gene in six citrus fruit with different level of delayed bitterness. Total RNA is extracted and expression of limonoid glucosyltransferase gene in albedo (inner spongy part of rind) and leaves during fruit development is investigated by RT-PCR. We discuss the structural features as well as the relationship between the expressions of the isolated genes.

Materials and methods

Materials. Fruit and leaf of 'Shahsavari' sweet orange (a local cultivar in north of Iran) (*C. sinensis* [L.] Osb.) was obtained from a private orchard in Chaboksar city at Gilan province. Also plant materials of Thompson navel orange (*C. sinensis* [L.] Osb. cv. Thompson navel), Satsuma mandarin (*C. unshiu* Marc. cv. Owari), Sweet Lime (The Indian or Palestine sweet lime) (*C. limettioides* Tan), Marsh seedless grapefruit (*C. paradisi* Macfadyen cv. Marsh seedless) and common sour orange (*C. aurantium* L.) cultivated at Iran citrus research institute (Ramsar, Iran) were used. All samples were immediately frozen in liquid nitrogen and they were stored at −80 °C until use. Fruit of these trees were harvested at 30-day intervals starting 60 days after flowering (DAF).

PCR amplification and cloning. Total DNA were extracted from fully expanded fresh leaves according to Murray and Thompson [15] with minor modifications as described by Asadi-Abkenar and Isshiki [16]. Since limonoid glucosyltransferase has no intron in the genome [17], PCR was performed using the total DNA to obtain the coding region of the limonoid glucosyltransferase gene under the following conditions: forward primer, LGT1 (5′-ATGGGAACTGAATCTCTTGTTCAT-3′), and reverse primer, LGT2 (5′-TCAATACTGTACACGTGTCCGTCG-3′). The reaction was performed for 30 cycles (1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C). The amplified fragments were analyzed on 1% agarose gel electrophoresis. The amplified fragment were cloned into a pTZ57R/T vector with a TA cloning system (Fermentas, Burlington, Canada) into competent cells of *Escherichia coli* XL1-Blue (CinnaGen, Tehran, Iran) and sequenced to detect nucleotide substitutions among the limonoid glucosyltransferases using MWG automatic DNA Sequencing Service (Germany).

Sequence analysis. In this study, the following programs and databases were used: BLAST and Genbank at NCBI, Motifscan, and Prosite at the ExPASy Server, ClustalW at EBI server and Genebee. Regions of local similarity between sequences were found using BLAST and our sequences are submitted to Genbank under different accession number (*C. limettioides*/EU531463; *C. sinensis* cv. Thompson navel/EU531467; *C. sinensis* cv. Shahsavari/EU531465; *C. aurantium*/EU531466; *C. paradisi* cv. Marsh seedless/EU531464). All possible motifs that occur in each sequence were found by Motifscan and Prosite. We used ClustalW for multiple sequence alignment for DNA and proteins and phylogenetics relationship among species are evaluated using Genbee and phylogenetics trees are drawn by PhyloDraw program.

Expression analysis of limonoid glucosyltransferase gene using RT-PCR. To investigate the change in the transcription level of the gene during fruit development, we performed RT-PCR analysis. Total RNAs were isolated from leaves and albedo of species at 60, 90, 120, 150, 180, and 210 days after flowering (DAF). Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The RNA was analyzed by 1.0% agarose gel electrophoresis. The concentration of extracted RNA was determined by UV absorption spectroscopy at 260 nm. The first strand cDNA was synthesized at 42 °C for 60 min in the presence of 200 U M-MuLV Reverse transcriptase (Fermentas, Burlington, Canada), 20 U RNase inhibitor, dNTP mix (final concentration each at 1 mM), and reverse primer (LGT2). The PCR amplification of cDNA was carried out by use of first strand cDNA and under previous PCR condition. The cDNA fragments coding for our species insert into pTZ57R/T vector and ligation mixture were transformed into of *E. coli* XL1-Blue and then sequenced as reported earlier [18].

Results

PCR amplification and nucleotide sequencing

Using the primers for amplification of the limonoid glucosyltransferase coding region [17], fragments of 1536 bp, as the same size as reported earlier [19] for Satsuma mandarin, local sweet orange, sour orange, sweet lime and grapefruit, and a fragment with 1530 bp in Thompson navel orange were obtained (Fig. 1). Sequencing results showed that Satsuma mandarin, sweet lime and sour orange have quite similar sequences. Sequence of other species had more than 98% identity with these species. In Thompson navel, orange position of UDP-glycosyltransferase domain (a suitable candidate for enzymatic activity) was similar to other species.

Phylogenetic and motif analysis

Phylogenetic analysis using amino acid sequence data showed high similarity among species. Thompson navel orange showed close relationship with pummelo. Other species have much similarity together (Fig. 2).

Related sequence and domain structure

When predicted amino acid sequence was used as the input sequence for computer-based searches for similarity, high scoring related sequences were found between our species limonoid glucosyltransferase, and some other limonoid glucosyltransferases in citrus, grape, strawberry, rice, and other species. Using ProSite a domain structure map for the predicted amino acid sequence of these species protein is deduced. Fig. 3 illustrates these results and Table 1

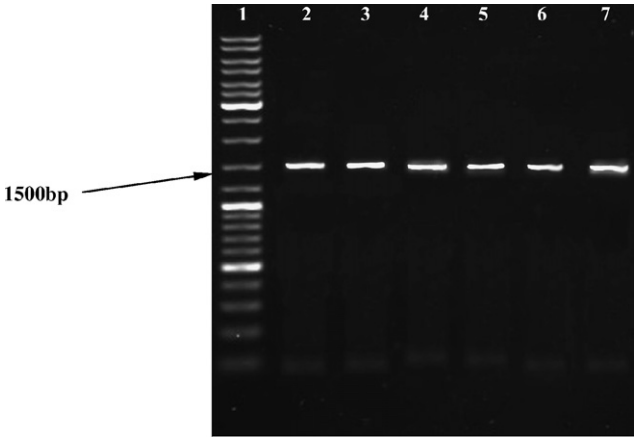

Fig. 1. PCR product of UDP-limonoid glucosyltransferase gene on 1% agarose gel electrophoresis. Lane 1, size marker; lane 2, *C. unshiu*; lane 3, *C. paradisi* cv. Marsh seedless; lane 4, *C. sinensis* cv. Shahsavari; lane 5, *C. sinensis* cv. Thompson navel; lane 6, *C. limettioides*, and lane 7, *C. aurantium*.

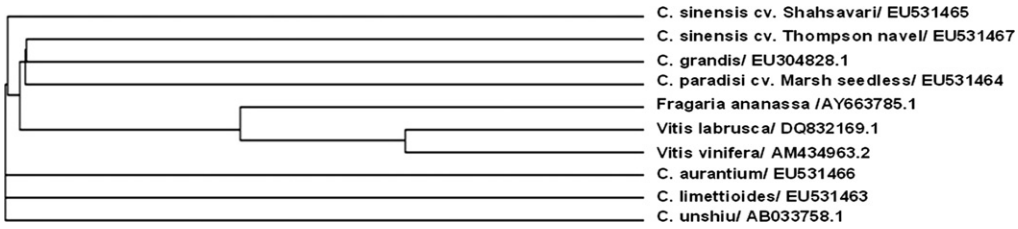

Fig. 2. A phylogenetic tree for aligned amino acid sequences of the citrus UDP-*limonoid glucosyltransferase.*

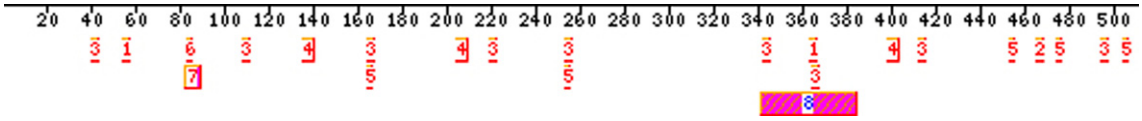

Fig. 3. Putative UDP-glucosyltransferases domain in different *Citrus* species.

Table 1
Different motifs and sites in citrus *UDP-limonoid glucosyltransferase*

Motif function	*C. paradisi* cv. Marsh seedless	*C. unshiu*, *C. limettioides*, *C. aurantium*	*C. sinensis* cv. Shahsavari	*C. sinensis cv.* Thompson navel
N-glycosylation site	54 NFTY 57 363 NSTM 366	54 NFTY 57 363 NSTM 366	54 NFTY 57 363 NSTM 366	54 NFTY 57 363 NSTM 366
cAMP- and cGMP-dependent protein kinase phosphorylation site	465 RRRS 468	465 RRTS 468	465 RRTS 468	465 RRRS 468
Casein kinase II phosphorylation site	40 TTPE 43 108 SAEE 111 164 SEKE 167 219 TFYE 222 253 TVRD 256 342 SPQE 345 364 STME 367 412 SRDE 415 494 TAND 497	40 TTPE 43 108 SAEE 111 164 SEKE 167 219 TFYE 222 253 TVRD 256 342 SPQE 345 364 STME 367 412 SRDE 415 467 TSVE 470 494 TAND 497	40 TTPE 43 108 SAEE 111 164 SEKE 167 219 TFYE 222 253 TVRD 256 342 SPQE 345 364 STME 367 412 SRDE 415 467 TSVE 470 494 TAND 497	40 TTPE 43 108 SAEE 111 164 SEKE 167 219 TFYE 222 253 TVRD 256 342 SPQE 345 364 STME 367 412 SRDE 415 492 TANE 495
N-myristoylation site	135 GLPSAM 140 204 GQYENL 209 398 GLRLCR 403	135 GLPSAM 140 204 GQYENL 209 398 GLRLCR 403	135 GLPSAM 140 204 GQYENL 209 398 GLRLCR 403	135 GLPSAM 140 204 GQYENL 209 398 GLRLCR 403
Protein kinase C phosphorylation site	164 SEK 166 253 TVR 255 453 SDR 455 474 SSK 476 504 SRR 506	164 SEK 166 253 TVR 255 453 SDR 455 474 SSK 476 504 SRR 506	164 SEK 166 253 TVR 255 453 SDR 455 474 SSK 476 504 SRR 506	164 SEK 166 253 TVR 255 436 SLK 438 453 SDR 455 474 SSK 476
Cell attachment sequence	83 RGD 85	—	—	83 RGD 85
Tyrosine kinase phosphorylation site	82 RRGDLDqY 89	82 RREDLDqY 89	82 RREDLDqY 89	82 RREDLDqY 89
UDP-glycosyltransferases signature	341…384	341…384	341…384	341…384

defines the sites, different motifs, and their functions in our species. All genotypes have UDP-glycosyltransferases signature. The putative UDP-glycosyltransferases domain in different *Citrus* species was found as 341-WspQekVLahpsvaCFVTHCGwnStmeSLasgvPVitfPqw gDQ-384 and is highly conserved among the various *Citrus* species.

Expression of limonoid glucosyltransferase gene during the fruit development

The expression patterns of the limonoid glucosyltransferase gene during fruit development were evaluated by RT-PCR analysis with total RNA from the leaves and albedo of species. The transcripts for the limonoid glucosyltransferase gene were detected in both leaves and albedo at the predicted fragment size by RT-PCR. Limonoid glucosyltransferase gene was transcribed in leaves from 120 DAF, and it was transcribed much earlier (60 DAF) in albedo of Satsuma mandarin. Transcription in other species started later (Table 2).

As shown in Table 2, transcription in sweet lime (with intense juice delayed bitterness) started too late, but transcription in Satsuma mandarin (without distinctive juice delayed bitterness and with same gene sequence) started soon. Expression of this gene in leaves is started 150 DAF in sour orange and Satsuma mandarin. In 210 DAF, limonoid glucosyltransferase transcripts were observed in both leaves and albedo of all species.

Discussion

The glucosylation of limonoids is catalyzed by UDP-D-glucose: limonoid glucosyltransferase. UDP-glycosyltransferases (UGT) are a superfamily of enzymes that catalyzes the addition of the glycosyl group from a UTP-sugar to a small hydrophobic molecule [14]. We evaluated existence and expression pattern of this gene in six different species. In all species, UDP-glycosyltransferases signature was observed (Fig. 3). Therefore, it seems that all six species have the ability to synthesis limonoid glucosyltransferase. The cause of

Table 2
Expression pattern of limonoid glucosyltransferasegene in albedo (A) and leaves (L)

	60 DAF		90 DAF		120 DAF		150 DAF		180 DAF		210 DAF	
	A	L	A	L	A	L	A	L	A	L	A	L
C. aurantium					*		*	*	*	*	*	*
C. sinensis cv. Shahsavari					*		*		*	*	*	*
C. sinensis cv. Thompson navel					*		*		*	*	*	*
C. unshiu	*		*		*		*	*	*	*	*	*
C. limettioides										*	*	*
C. paradisi cv. Marsh seedless										*	*	*

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C. limettioides                HRVKELVEKTATATANDKVELVESRRTRVQY 511
C. unshiu                      HRVKELVEKTATATANDKVELVESRRTRVQY 511
C. aurantium                   HRVKELVEKTATATANDKVELVESRRTRVQY 511
C. paradisi cv. Marsh seedless HRVKELVEKTATATANDKVELVESRRTRVQY 511
C. sinensis cv. Shahsavari     HRVKELVEKTATATANDKVELVESRRTRVQY 511
C. sinensis cv. Thompson navel HRVKELVEK--TATANEKVELVESRRTRVQY 509
                               *********  *****:***************
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Fig. 4. Multiple sequence alignment for C-terminus of *limonoid glucosyltransferase* using ClustalW.

delayed bitterness in *Citrus* species has been attributed to lack of limonoid glucosyltransferases or presence of mutant forms. Our results also indicate that gene sequence of sweet lime (with intense juice delayed bitterness) has complete identity with Satsuma mandarin (without distinctive juice delayed bitterness). Therefore, it seems the delayed bitterness in these species was not due to lack of gene or presence of a mutant gene. On the other hand, this gene started to express 60, 120, and 210 days after full blooming in albedo of Satsuma mandarin, sweet oranges and sour orange, and both grapefruit and sweet lime, respectively (Table 2). Therefore, there is no possibility of synthesizing of limonoid glucosyltransferase in sweet lime until end ripening period of fruits. This may cause association of LARL in mature fruit and make this fruit susceptible to delayed bitterness [12]. However, in Satsuma mandarin fruits, expression of limonoid glucosyltransferase genes take place from 60 DAF to end of ripening period of fruits. It has been indicated in Satsuma mandarin most of LARL convert to LG and concentration of LARL in juice of fruits is too low [20]. This fruit is not susceptible to delayed bitterness. As indicated in Table 2 other species (with mild delayed bitterness) show delayed expression in both albedo and leaves (see Fig. 4).

Previous investigations have not shown the expression of limonoid glucosyltransferase in leaves. The expression profile of limonoid glucosyltransferase in leaves of *Citrus* species is reported (Table 2). As indicated in Table 2, the expression pattern of limonoid glucosyltransferase in leaves is not closely related to bitterness level of fruits. The lack of limonoid glucoside (LG) transportation from leaf to fruit has also reported [21,22].

In conclusion, results presented in this manuscript show intense juice delayed bitterness in some *Citrus* species like sweet lime might be due to delay in expression of limonoid glucosyltransferase. It may be concluded, the content of glucosylated LARL in these species is lower than non-bitter fruits. However, some other reasons which need more investigation can be considered. Rate of gene expression, post translational modification, and expression of an active product in fruits with intense juice delayed bitterness has to be investigated.

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