Molecular cloning and characterization of a novel gene encoding limonoid UDP-glucosyltransferase in *Citrus*[1]

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Abstract We isolated a cDNA clone encoding limonoid UDP-glucosyltransferase (limonoid GTase) from the albedo of Satsuma mandarin (*Citrus unshiu* Marc.) and investigated the contribution to limonoid glucoside accumulation in fruit. The isolated cDNA clone (CitLGT) was 1732 bp in length encoding 511 deduced amino acids with a predicted molecular mass of 57.5 kDa. The products of in vitro translation from an expression vector had the limonoid GTase activity. Southern blot analysis of genomic DNA indicated that CitLGT was present as a single copy gene in the *Citrus* genome. The amount of transcript corresponding to CitLGT mRNA changed the same way as the fluctuation of limonin glucoside content during fruit development of navel orange (*Citrus sinensis* Osb.). This indicates that the transcription of CitLGT regulates the conversion of limonoid aglycones to glucosides in citrus fruit.

Key words: Glucosyltransferase; cDNA sequence; Limonoid; LC-MS; Gene-expression; *Citrus*

1. Introduction

There are two components responsible for the bitterness in citrus fruit, the limonoids and the flavanone neohesperidosides [1]. Limonoids are highly oxygenated triterpenes present in Rutaceae and other limited plants such as Meliaceae. So far, 36 limonoids have been isolated from *Citrus* and its closely related genera. Limonoid bitterness occurs gradually after juice processing from sweet orange (*Citrus sinensis* Osb.) such as navel, which is referred to as delayed bitterness [2] 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 [3]. Normally, intact fruit tissue contains a non-bitter precursor of limonin, limonoate A-ring lactone (LARL) [2]. 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 [4]. However, Satsuma mandarin (*Citrus unshiu* Marc.), which is the most popular *Citrus* species as fresh fruit and juice product in Japan, develops fruits with much less delayed limonoid bitterness. The concentration of limonoid aglycone in the juices from Satsuma mandarin was low and there is no problem concerning the delayed bitterness [5]. This debittering of Satsuma mandarin is mainly due to the conversion of LARL to tasteless limonin glucosides, such as limonin 17-β-D-glucopyranoside (LG), in the fruit during maturation from early stage [6]. The pattern of accumulation of limonoid glucoside is different between *C. sinensis* and *C. unshiu*. but the variety of limonoid compounds is the same. These two species are cross compatible to each other and many hybrids between them have been produced for the purpose of breeding. Thus they resemble each other phylogenetically.

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 [7]. Moreover, antifeedant activities against certain insects and invaders are indicated [8]. Recently, the function of limonoids, which is abundant in citrus fruits, is being given attention for the above reason, 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 breast cancer cells in culture [9]. 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 (ca. more than 100 ppm) are contained in many kinds of citrus juice and fresh citrus fruits (Hasegawa, personal communication), 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) [10]. Therefore, limonoid GTase

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is a key enzyme for creating transgenic *Citrus* free from limonoid bitterness as well as for increasing specific limonoid glucoside molecules having anticancer properties.

We have been interested in the molecular regulation of natural debittering and limonoid glucoside accumulation in citrus fruit. For this purpose, the breeding of hybrids between *C. unshiu* and *C. sinensis* has been done by conventional crossing to date. However, molecular research of limonoid GTase has not been reported previously and there is no available molecular information for limonoid GTase so far. As the first step, we have purified the enzyme from navel orange albedo (inner spongy part of rind) [11]. Based on the protein sequences, we first isolated and characterized a cDNA encoding limonoid GTase from navel orange albedo. We discuss the structural features as well as the relationship between the expression of the isolated gene and bitterness and also indicate the pharmacological application of limonoid GTase gene.

2. Materials and methods

2.1. Plant materials

Fruits of Satsuma mandarin (*C. unshiu* Marc. cv. Miyagawa-wase) and navel orange (*C. sinensis* Osb. cv. Washington navel) cultivated at the National Institute of Fruit Tree Science, Okitsu (Shimizu, Shizuoka, Japan) were used. A cDNA for limonoid GTase was prepared from Satsuma mandarin harvested at the mature stage. Satsuma mandarin accumulates a larger amount of limonoid glucoside [6] than navel orange, which indicates that the limonoid GTase gene has a higher redundancy in the cDNA library of Satsuma than that of navel orange. Navel orange was used for enzyme purification and for Southern and Northern blot analyses. All samples were immediately frozen in liquid nitrogen and after lyophilization, they were stored at −80°C until use.

2.2. Isolation of limonoid GTase cDNA

Purified limonoid GTase was obtained from navel orange (*C. sinensis* Osb.) albedo following the method of Hasegawa et al. [11]. The albedo of Satsuma mandarin may have a higher activity of limonoid GTase than navel orange. However, it is difficult to collect albedo sample from Satsuma mandarin because it is much thinner at a mature stage than that of navel orange. So, we used the albedo of navel orange at a mature stage for enzyme purification. In addition to the N-terminal amino acid sequences, the internal sequences were determined by the gel cleavage method [12] to synthesize two degenerated oligonucleotide primer pairs, sense Llg: 5′-GGNACNG-ARWSNYTNGTNCAYGT-3′ and antisense GT-19R: 5′-GTNG-GYTCRTANGTRAA-3′ for the cloning of the partial limonoid GTase gene.

To obtain limonoid GTase cDNA, we prepared the first strand cDNA and a cDNA library for mRNA of the albedo of Satsuma mandarin at a mature stage. The first strand cDNA was synthesized with a Ready-To-Go first strand synthesis kit (Amersham-Pharmacia) on the purified poly(A)$^+$RNA with oligotex-dT30 (Takara) from total RNA, which was prepared from Satsuma mandarin fruit according to Ikoma et al. [13]. PCR was performed on the first strand cDNA under the following conditions: 1 min at 94°C, 1 min at 45°C and 2 min at 72°C to obtain a partial fragment with the degenerated primers. A cDNA library from albedo of Satsuma mandarin at a mature stage was constructed in a Lambda Uni-ZAP XR vector (Stratagene), according to the manufacturer's instructions. The phage was packaged using a Gigapack Gold packaging kit (Stratagene). Using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim), the candidates of limonoid GTase cDNA were screened from the library with the probe of a partial limonoid GTase obtained by RT-PCR.

The nucleotide sequences of the candidates were determined with a Taq Dye Primer Sequencing Kit (Applied Biosystems Instrument). Comparison with nucleic acid sequences deposited in DDBJ, GenBank and EMBL databases were made using the program FASTA [14] and the sequence alignment and homology were analyzed with the GENETYX-MAC software (Software Development).

2.3. Detection of limonoid glucoside by liquid chromatography mass spectrometry (LC-MS)

The coding region of the cDNA for limonoid GTase gene was ligated with the expression vector of pGEX4T-1 (Amersham-Pharmacia). Following the manufacturer's instructions, a fusion protein combined with glutathione *S*-transferase (GST) was produced and extracted. The fusion protein was incubated in a reaction mixture of 50 mM MES buffer containing 100 μM UDP-glucose, 50 μM $MnCl_2$ and 10 mM limonoate under weakly acidic conditions (pH 6.6) at 37°C for 3 h. The reaction mixture was filtrated with Sep-Pak-C18 cartridge (Waters) and the adsorbate was eluted with methanol and lyophilized. The samples resolved in 50% methanol were analyzed by LC-MS using an atmospheric pressure chemical ionization (APCI) mode in order to confirm the presence of LG. For the reaction mixtures, an ODS-hypersil column (Hewlett Packard, 125 mm in length, 4 mm in diameter) was used with a gradient mobile phase of A, 0.5% acetic acid, and B, methanol. The gradient condition was as follows: at 0 min, the mobile phase consisted of phase A/phase B (90:10). At 5 min, the linear gradient system started. At 30 min, the mobile phase was changed to 85:15 (A/B). The flow rate was 1.0 ml/min. A mass spectrometer (model JMS-700, JEOL) was used in the negative ion APCI mode.

2.4. Southern blot and Northern blot analyses

For Southern blot analysis, total genomic DNA was isolated from the mature leaves of navel orange according to Dellaporta et al. [15]. Ten micrograms of the total DNA was digested with *Eco*RI, *Dra*I or *Xba*I. After electrophoresis on 1.0% agarose gel, they were transferred to nylon membrane (Hybond-NX, Amersham-Pharmacia) and hybridized with the probe of the entire coding region for limonoid GTase cDNA clone (designated as CitLGT) labeled by the same procedure as described for the partial cDNA labeling. Hybridization was performed at 65°C and the membrane was washed with 0.1×SSC containing 0.1% SDS at 65°C twice for 15 min and then, exposed to X-ray film (RX-U, Fuji film).

To investigate the change in the transcription level of the gene during fruit development, we performed Northern blot analysis. Total RNAs were isolated from the juice sac/pulp segment and albedo of navel orange at 40, 70, 100, 130, 160, 190 and 220 days after flowering (DAF). At 40 DAF, the whole fruit was used for RNA isolation because of the difficulty of separating each part. Ten micrograms of the total RNA was electrophoresed on 1.2% agarose gel containing formaldehyde and then transferred to the Hybond-NX. Hybridization was carried out at 42°C under denaturing conditions with formamide. The membrane was washed as described for the Southern blot analysis and exposed to X-ray film.

3. Results

3.1. Molecular cloning and nucleotide sequencing

Using the pair of degenerated oligonucleotide primers synthesized based on N-terminal and internal amino acid sequences of the purified limonoid GTase, we successfully amplified the 177-bp fragment by PCR toward the first strand cDNA of Satsuma mandarin. The fragment was used as a probe to isolate the clones with the complete coding region for limonoid GTase from Satsuma mandarin cDNA library. One candidate clone (CitLGT) was isolated by screening a 2×10^4 pfu cDNA library.

CitLGT was 1732 bp in length with a poly (A) tail and contained a 1536-bp open reading frame which started at the 50th ATG, terminated at the 1585th TGA and encoded 511 deduced amino acid residues (Fig. 1). The mature protein (511 deduced amino acid residues) had a calculated molecular mass of 57.5 kDa, which is in agreement with that of the previously reported limonoid GTase [11]. The deduced amino acid sequence of the CitLGT coding region was confirmed to have two conserved regions of glucosyltransferases from other plants by a homology search [16]. One is registered in a PROSITE database as a glucosyltransferase signature sequence

[17], which is the region for the UDP-glucose binding domain with 44 amino acid residues. Fig. 2 shows an alignment of the putative UDP-glucose binding domain of CitLGT. This region has 43 to 68% identity in amino acid sequences to previously reported glucosyltransferases, indicating that the isolated clone conserved the features of the glycosyltransferase family. Moehs et al. [18] reported another conserved domain at the amino terminal region in plant glycosyltransferases. The CitLGT has 30 to 45% identity to other plant glucosyltransferases in this region. Except for two domain regions, limonoid GTase did not have a high similarity to the registered plant and animal glycosyltransferases. Potential glycosylation sites were located at the 54th and 363rd amino acid of mature protein and the UDP-glucosyltransferase signature site was located at the 341st amino acid.

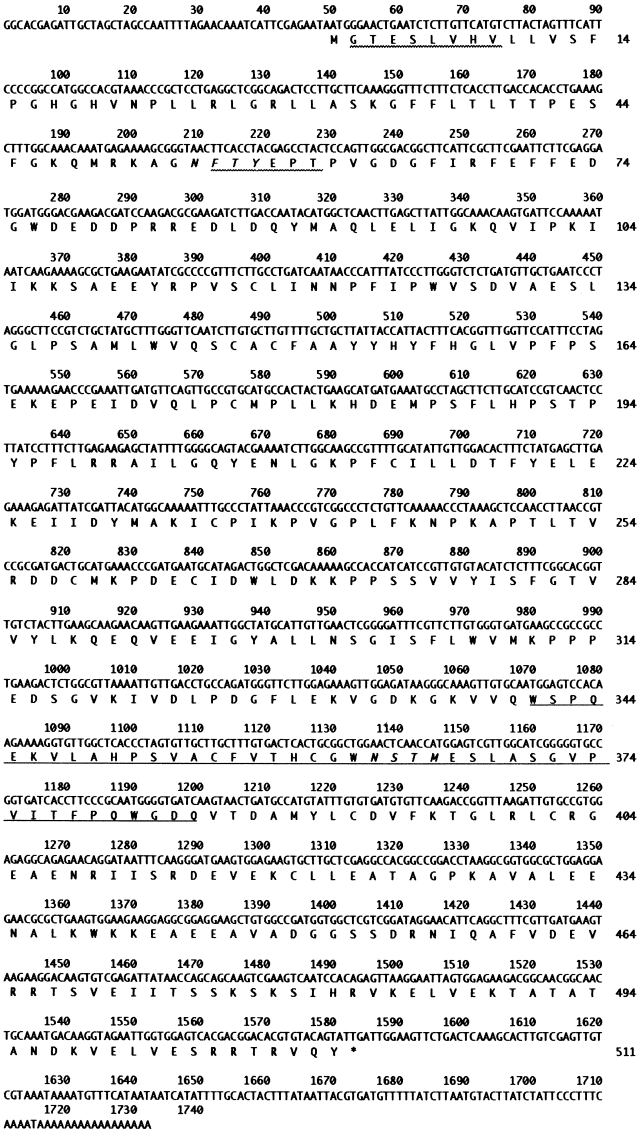

Fig. 1. The nucleotide sequence of the cDNA encoding limonoid GTase and the deduced amino acid sequences. A single open reading frame, beginning at the first ATG codon is shown directly below the nucleotide sequence which is numbered in the 5′–3′ direction. Nucleotides are numbered above the sequence and the deduced amino acid residues at the right side. The potential glycosylation sites are indicated in italics. The UDP-glucose binding domain is underlined. The primer sites used in this study are wave underlined.

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CitLGT          WSPQEKVLAHPSVACFVTHCGWNSTMESLASGVPVITFPQWGDQ
STSGT (U82367)  WVPQLTIMEHSATGGFMTHCGTNSVLEAITFGVPMITWPLYADQ
CASGT (X77462)  WSPQIHIMSHPSVGVFLSHCGWNSVLESITAGVPIIAWPIYAEQ
IAAGT (U81293)  WCDQFRVLNHRSIGCFVTHCGWNSTLESLVSGVPVVAFRQWNDQ
IPFGT (AF028237) WAPQVQVLSHPGVGAFVTHCGWNSTLEAISFGVCLICRPFYGDQ
PLZGT (AF101972) WAPQLEILSHSSTGGFMSHCGWNSCLESITMGVPIATWPMHSDQ
                *  *  ** *    ***  *  *   **   ***   *  ***
PROSITE         WXXQXXLLXHXXXXAFLSXSGXXSXXXSLXXXLPLXXXPLLSDQ
                      I         IT T   T   TI   I I    IITE
                      V         VA A   A   AV   V V    VVA
                      M         MG G   G   GM   M M    MMG
                                                       FF
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Fig. 2. Comparison of the amino acid sequences of the UDP binding domain of UDP-glucosyltransferases. CitLGT is compared with solanidine UDP-glucosyltransferase from potato (STSGT), UDP-glucosyltransferase from cassava (CASGT), IAA glucosyltransferase from *Arabidopsis thaliana* (IAAGT), flavonoid 3-*O*-glucosyltransferase from *Ipomoea purpurea* (IPFGT) and zeatin *O*-glucosyltransferase from *Phaseolus lunatus* (PLZGT). PROSITE: proposed glucosyltransferase signature sequence on the PROSITE database. Accession numbers in the database are shown in parentheses.

In order to verify the isolated CitLGT, we induced the recombinant fusion protein with GST in *Escherichia coli*. The extracted fusion protein had the predicted molecular weight and it showed a response signal on Western blot analysis when probed with a monoclonal antibody against the limonoid GTase (data not shown).

The limonoid GTase activity was identified by LC-MS analysis of the reaction mixture after in vitro incubation of UDP-glucose and LARL as the substrates with the expressed fusion protein in *E. coli*. The presence of LG in the reaction mixture was confirmed by LC-MS using a negative APCI mode. Fig. 3 shows mass chromatograms at *m*/*z* 649 ([M-H]-) of both authentic LG and the reaction mixture. The mass chromatogram obtained from the reaction mixture of the fusion protein with a CitLGT cDNA translate showed a peak at approximately 20 min which corresponds to the authentic LG. Otherwise, the peak was not detectable in the control plasmid, pGEX4T-1 having only the GST coding sequence (data not shown). Fig. 3b depicts the APCI/LC-MS spectrum of the peak at 20 min obtained with the reaction mixture. It was identical to that of authentic LG, indicating that the isolated CitLGT was the real cDNA encoding the limonoid GTase gene.

3.2. Genomic structure of limonoid GTase gene in Citrus

Genomic DNA isolated from mature leaves of navel orange was digested with *Eco*RI, *Dra*I or *Xba*I. The CitLGT probe was hybridized to only one or two fragments (Fig. 4). When Southern blot analysis was performed under a low stringency, there was no additional band (data not shown). The single *Dra*I fragment was estimated to be approximately 1.9 kb, which corresponded closely to the cDNA length. When the genomic PCR fragment was amplified by using the primer pairs synthesized based on the N and C terminals of the CitLGT coding region, the generated fragments were identical in length to the coding sequence of CitLGT. The genomic PCR sequence did not harbor any additional sequences to that of cDNA (data not shown). This indicated that the limonoid GTase gene lacked introns in the genome. The cDNA sequence and the same genomic sequence had one *Eco*RI site and no *Dra*I site, corresponding to the fragments on the blots. This means that the gene is a single copy in the citrus genome.

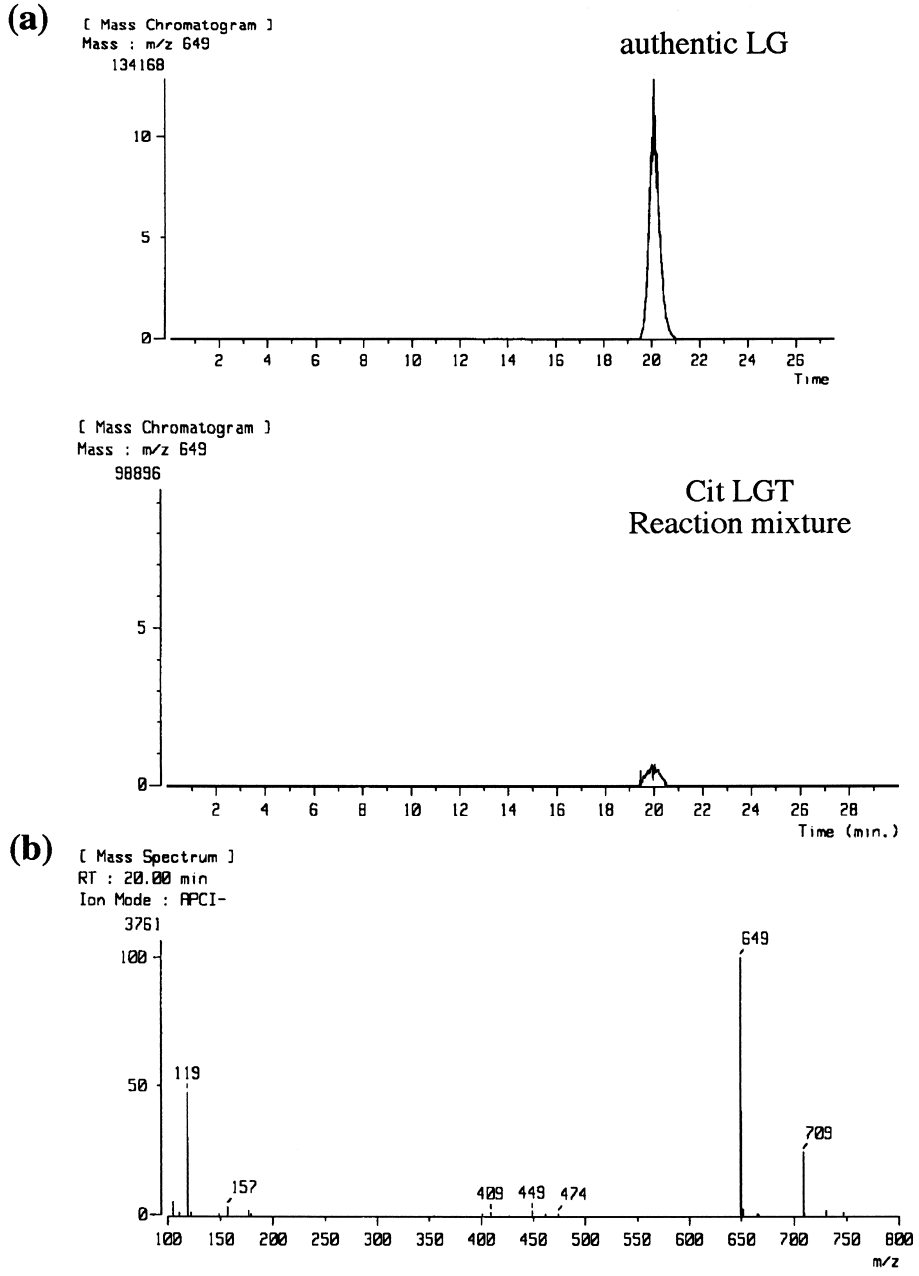

Fig. 3. APCI/LC-MS chromatogram (*m/z* 649) of the authentic LG and the reaction mixture of CitLGT and substrates (a). Mass spectrum of the peak at 20 min of the APCI/LC-MS chromatogram of the reaction mixture (b).

3.3. Expression of CitLGT during the fruit development

The expression patterns of the CitLGT gene during fruit development were evaluated by the Northern blot analysis with total RNA from the juice sac/pulp segment and albedo of navel orange. As shown in Fig. 5, the transcripts for the CitLGT gene were detected in both the juice sac/pulp segment and albedo at the predicted fragment size on the Northern blot. CitLGT was transcribed strongly in the juice sac/pulp segment from mid-season (130 DAF∼) of maturation, and it was transcribed much later (190 DAF∼) in albedo. The transcription tended to be up-regulated toward maturation in both tissues, but there were clear differences in the strength and timing of CitLGT transcripts between the juice sac/pulp segment and albedo.

4. Discussion

A number of UDP-glucosyltransferases from different plant species have been studied to control the production of secondary metabolites and plant hormone. Some of the genes have already been isolated [18,19], but the genes for the other triterpenoid-glucoside related to taste have not. Two enzymes are involved in the flavonoid pathway to convert taste-related substances in *Citrus* called UDP-glucose:flavanone-7-*O*-glucosyltransferase and UDP-rhamnose:flavanone rhamnosyl transferase. By adding sugar to non-bitter naringenin and hesperetin, they produce bitter-tasting naringin and neohesperidin, respectively. These two enzymes have been isolated and characterized [20–22], but the genes have not been iso-

lated. Therefore, the limonoid GTase gene is the first citrus gene proved to be involved in the conversion of bitter tasting limonin to non-bitter tasting limonoid glucoside. Moreover, to produce and obtain limonoid glucosides containing many functional components efficiently, it is necessary to collect some information about the limonoid GTase gene.

Firstly, the identity of the isolated cDNA encoding limonoid GTase is characterized through the sequence similar to the previously reported UDP-glycosyltransferases in plants and the similarity of molecular mass with limonoid GTase from navel orange albedo. In addition, performing the Western blot analysis and the reaction activity of the fusion protein, we were able to determine that the isolated clone encodes limonoid GTase. The genomic sequence having no introns is not specific to the limonoid GTase gene because the genomic sequence of zeatin *O*-glucosyltransferase from *Phaseolus lunatus* also lacks an intron [19]. The significance of the absence of intron in these genes related to the secondary metabolism is not clear yet, but the feature of a gene without an intron in a locus might provide a key to the origin of the bio-diversity of plant glucosyltransferases for secondary metabolism.

Southern blot analysis revealed no additional signals even under low stringency conditions which indicates CitLGT as a single copy in the *Citrus* genome. If a gene of high homology which has a similar function to limonoid GTase, exists in the *Citrus* genome, additional signals should appear. This suggests the absence of other sequences similar to CitLGT. We confirmed that the limonoid GTase in *Citrus* was produced only from the CitLGT locus. Therefore, we investigated the CitLGT expression in *Citrus* through fruit maturation to elucidate its relation to glucosylation of limonoids. In navel orange juice, the limonoid bitterness decreases as the fruit matures [10]. LARL is converted to non-bitter LG, which increases sharply in the juice sac at the late stage of maturation [23]. In this study, Northern blot analysis revealed that increasing the transcription level of the CitLGT gene is parallel to the fruit maturation. Therefore, the conversion of LARL to LG and the mRNA accumulation could probably take place simultaneously in the juice sac/pulp segment. Also in albedo tissue, the onset and increase of transcription occurred simultaneously with LG production. Furthermore, the mRNA for CitLGT in albedo started to accumulate later than that in the juice sac/pulp segment because the onset of mRNA transcription was delayed. This synchronized increase of mRNA accumulation, which is parallel to the time difference of maturation between the juice sac/pulp segment and albedo, also indicates that the CitLGT activity regulates the conversion of LARL to LG. No evidence of translocation of LG has been found in fruit tissues from other organs and the site of metabolization of limonoid aglycon to glucosides is limited to the seed and the mature fruit [23,24]. Therefore, the close correlation between mRNA accumulation and LG content in these tissues suggests that the transcription level of the CitLGT gene regulates the LG accumulation during fruit maturation in citrus fruit.

A high transcription of CitLGT mRNA was observed in the flower and the fruit at a young stage (40 DAF) but LG was not detectable in those organs of navel orange [25]. A high glucosidase activity may result in the reduction of the

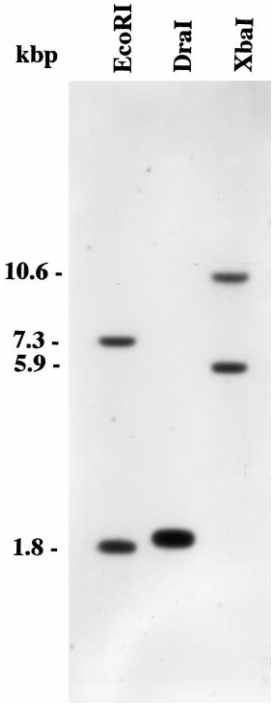

Fig. 4. Southern blot analysis of the gene for CitLGT against the genomic DNA of navel orange. Total DNA (10 μg) was digested with *Eco*RI, *Dra*I or *Xba*I, and fractionated on a 1.0% agarose gel, then blotted on nylon membrane (Hybond-NX, Amersham-Pharmacia). The blot was hybridized with Dig-11-dUTP labeled cDNA and washed twice with 0.1×SSC and 0.1% SDS at 65°C for 15 min and exposed to X-ray film (RX-U, Fuji).

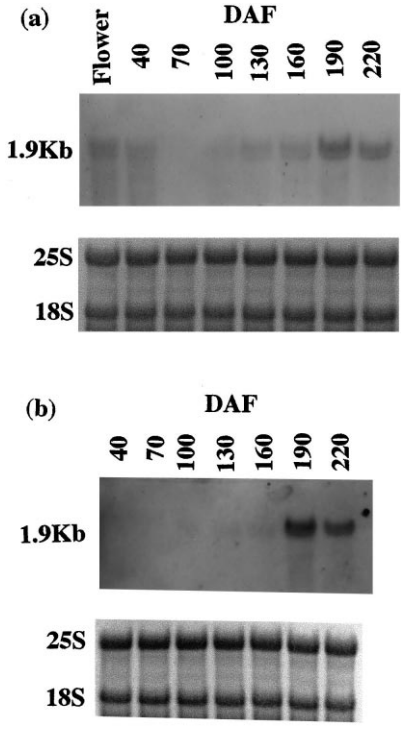

Fig. 5. Northern blot analysis of CitLGT in juice sacs/pulp segment (a) and in albedo (b). Total RNA (10 μg) was fractioned on a 1.2% agarose gel containing formaldehyde, then blotted on nylon membrane (Hybond-NX, Amersham-Pharmacia). Hybridization was performed at 42°C and washed twice with 0.1×SSC and 0.1% SDS at 65°C for 15 min and then exposed to X-ray film (RX-U, Fuji). In the lower panel, RNA on the membrane was stained with methylene blue.

glucoside content in these tissues as in young seedlings in which limonoid glucosides were not accumulated because of a high glucosidase activity [25].

We could confirm the function of cloned cDNA for limonoid GTase only by using the sensitive detection system of LC-MS, because the enzymatic activity of raw fusion protein (GST+limonoid GTase) from the in vivo translation system in *E. coli* was quite low. The low activity might be due to the enzymatic nature of the glucosyltransferase. Glucosyltransferases are generally localized to the membrane and the activity is unstable when they are solubilized [26,27]. Nakazawa et al. [27] reported that the *V*max value of mammalian membrane-bound galactosyltransferase expressed in *E. coli* was lower than that of the native enzyme. Moreover, most of the proteins that penetrate the cytoplasmic membrane receive enzymatic ornamentation such as *N*-linked glycosylation or *O*-linked glycosylation during synthesis [28]. CitLGT contained two potential sites for *N*-linked glycosylation at Asn54 and Asn363. The posttranslational modification of limonoid GTase, such as glycosylation may be related to the regulation of gene expression in *Citrus*. The isolation of CitLGT would provide a tool to elucidate the molecular mechanism of natural debittering, such as posttranslational glycosylation and phosphorylation.

By application of genetic transformation and the regeneration system of *Citrus* [29,30], the function of CitLGT in transgenic *Citrus* should be investigated more in detail. Further studies of the genes for CitLGT would contribute not only to citrus breeding but also to pharmacological application. Mass production of LG in *E. coli* harboring the limonoid GTase gene could be expected when subcultured with the LARL as substrate and transgenic *Citrus* free from limonoid bitterness could be more valuable pharmacologically.

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