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Research Article

Citrus Limonoid Glucosyltransferase: A Key Player For Natural Debittering And Anticancerous Potential

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Abstract

Citrus fruits and juices are rich source of health benefitting phytochemicals which play a vital role in balanced diet and disease prevention. Citrus limonoids and flavonoids are the major phytochemicals which are of great interest in pharmaceutical industries because of their demonstrated anticancerous, antioxidant, anti-inflammatory, hormonal stimulation, antibacterial and antiviral actions. Citrus limonoid biosynthetic pathway contains an important regulatory limonoid glucosyltransferase enzyme (LGT). LGT is the natural debittering enzyme encoded by a single copy gene which has been isolated from different *Citrus spp*. This enzyme is mainly responsible for conversion of all limonoid aglycones (mostly bitter) to their corresponding glucosides (mostly non-bitter) but only during late fruit developmental stage of citrus. Citrus LGT belongs to glycosyltransferase super family whose members are the wide managers to catalyze the transfer of sugar molecules to their acceptor molecules to play several key modifications in plant secondary metabolites. These reveal great significance value in plant cell metabolism especially in detoxification of xenobiotics, production and storage of natural products. Despite to the fact that over expression of *LGT* in citrus will lead to reduce the delayed bitterness caused by limonin (an aglycone) but in addition will enhance the accumulation of limonoid glucosides in fruits. Further, recent studies suggest that citrus limonoids especially glucosides have shown importance against brain, pancreas, colon, and breast cancers. Thus, future studies should be focused on utilizing the potential of *LGT* present in citrus plants in terms of anticancerous properties as well as reducing the delayed bitterness problem important for citrus juice industry.

Keywords: Kinnow Mandarin; Delayed Bitterness; Limonoid Glucosyltransferase; Transcript Expression; Semi-Quantitative PCR; Anticancerous

Highlights

- A brief overview on citrus, its phytochemicals, delayed bitterness problem, and limonoids and flavonoids biosynthesis
- Role of glycosyltransferases in plant metabolism
- Citrus limonoids for anticancerous effects
- Bioinformatics characterization of plant glucosyltransferase
- Molecular cloning and functional expression of limonoid glucosyltransferase

Background

The Citrus is a large genus belongs to family Rutaceae and is the world's most popular and economically important fruit crop. It grows between 40° north and 40° south of equator due to its wide adaptability to the tropical and subtropical conditions but native to Southeast Asia. This genus includes mainly mandarins, limes, lemon, sweet orange, grapefruit and pummelos. Taxonomic classification, history and origins of citrus are full of controversies [1]. However, classification by Swingle (which recognizes 16 species) and Tanaka (which recognizes 162 species) are the most accepted one [2]. Citron, pummelo and mandarin are the three ancient Citrus spp. and all other hybrids available today are originated from intercrossing of these [3]. Most of the Citrus spp. are diploid and having basic chromosome number x=9 while the genome size varies from 367 Mb for sweet orange [4] to 383 Mb for Pummelo [5].

Citrus fruits possess health promoting phytochemicals like flavonoids, limonoids, coumarins and carotenoids with varied concentrations (Table 1). These phytochemicals are naturally occurring bioactive compounds which are known to show antioxidant, hormonal, stimulation, antibacterial actions and several effects (www.phtochemicals.info). Out of these, citrus limonoids are highly oxygenated triterpenes that occur as bitter, water insoluble limonoid aglycones, and non-bitter, water soluble limonoid glucosides. Despite to the health promoting properties, citrus limonoids (limonin) has been a main cause of delayed bitterness in citrus juices and its products. Delayed bitterness has been affecting the citrus fruit and juice industry with significant economic losses.

Table (1): Major Phytochemicals present in citrus fruits and their concentration

Phytochemicals	**Constituents**
Limonoids (mg/100g)	80-320
Flavonoids (mg/100g)	17-48
Coumarins (mg/100g)	2.2
Carotenoids (mg/100g)	Traces to 300

Although, the intact citrus fruit tissue does not contain bitter limonin but it contains non-bitter precursor, limonoate A ring lactone (LARL). In juice, this precursor is converted to limonin [6]. Freeze damage or physical damage to citrus fruit, including juicing converts this precursor (monolactone) into bitter limonin (dilactone). This process proceeds under acidic conditions (<pH 6.5) prevailing in citrus fruits plucked during early to mid fruit developmental stages, and is accelerated by the action of limonin D-ring lactone hydrolase (LLH) [7-8]. Although, a natural debittering process also exists which is catalyzed by limonoid glucosyltransferase enzyme (LGT) but this occurs only during mature fruit developmental stage in citrus

[9]. For these two important regulatory steps of citrus limonoid biosynthesis, only genes encoding LGT has been isolated from mandarin, lime, navel orange [10-12] and recently from Kinnow mandarin (KP306791). But there is no report of overexpression of *LGT* gene in citrus. Instead of limonoids, flavonoid (naringenin) also causes the immediate/primary bitterness while tasting the citrus fruit juices.

In India, studies have been done on debittering citrus juices using physiochemical [13-14] and enzymatic methods [15], but molecular research works related to citrus limonoid and its metabolic engineering using biotechnological tools are lacking. Efforts have been done on using immobilized enzyme technology i.e. limonoid metabolizing/catabolizing enzymes from bacteria and fungi for clarification and debittering of citrus juice [16–18]. But all these methods lack economical viableness, ease of operation, preservation of natural nutritional characteristics of citrus juices [17] and thus, are not suitable for large scale purposes. There is a need to develop an acceptable and versatile debittering method that can substantially remove or mitigate this bitterness of citrus fruits and juices [19].

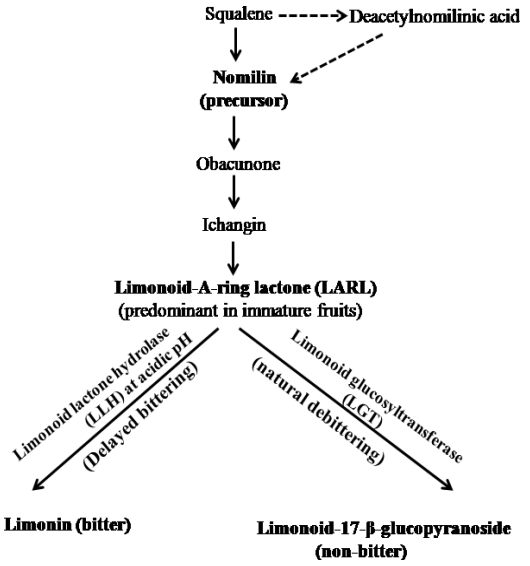
Despite to the cause of bitterness, citrus limonoids and flavonoids are also valued for health benefits especially for their strong antioxidant and anticancerous properties. Limonoid glucoside which is the ultimate end product of LGT in citrus fruits has great importance against several types of cancers. Citrus limonoids especially glucosides show importance against brain [20] pancreas, colon, and breast cancers [21-23]. Recently,
[24] have reviewed the importance of citrus bioactive molecules against several types of human cancers. Also, citrus limonoids and their derivatives possess good bioavailability and have non-toxic effects in animal models and humans [25]. Thus, LGT is a key player for natural debittering as well as anticancerous potential in citrus fruits.

Citrus limonoids and flavonoids biosynthesis

Citrus fruits have been considered as rich source of limonoids and flavonoids which are important health promoting and diseases preventive phytochemicals. Both of these secondary metabolites have been seen important for preventing several kinds of cancers.

The limonoids have also been reported as excellent chemotaxonomic markers [26] because they are found in limited plants belonging to the Meliaceae (Neem) and Rutaceae (Citrus) families. Limonoids are the tetracyclic secondary metabolite derivatives. The term 'limonoids' was derived from limonin which is reported as the first tetranortriterpenoid obtained from citrus bitter principles. The biosynthesis of limonoids begins with deacetylnomilinic acid which gets converted to nomilin in stem [19]. (Figure 1). Further, nomilin is transferred to other tissues including leaves, fruits (including peel, seeds), and roots where nomilin is capable of synthesizing other limonoids [27]. Thus, nomilin is regarded as an ultimate precursor for forming all other limonoids in Citrus and related species. It has been reported that LGT is the only enzyme that is able to convert all limonoid aglycones such as limonin, nomilin and obacunone into their respective glucosides such as limonin, nomilin and obacunone into their respective glucosides such as limonin glucopyranoside, nomilin glucopyranoside, obacunone glucopyranosides through a natural debittering process in citrus fruits during fruit maturation [28].

Figure (1): Limonoid biosynthetic pathway operating in Citrus plants. Nomilin is the initial precursor for synthesizing all other limonoids. The biosynthesis of limonoids begins with deacetylnomilinic acid which gets converted to nomilin in stem. In citrus juices, LARL gets converted to limonin under acidic conditions prevailing in immature fruits by LLH enzyme which is referred as delayed bitterness.



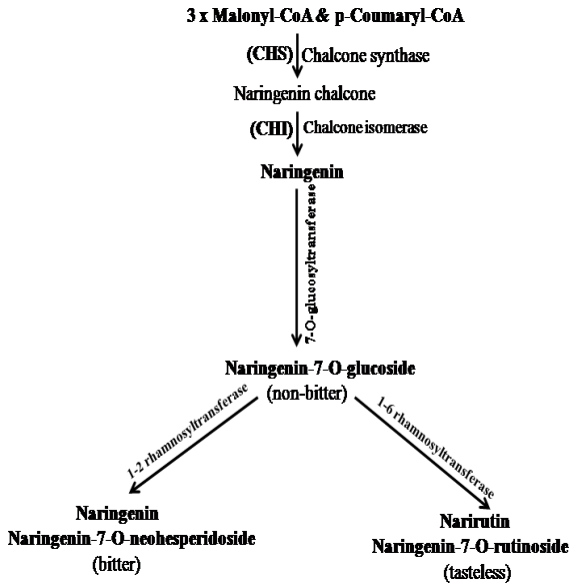
LLH and LGT are the two important regulatory enzymes which compete each other for the newly biosynthesized LARL in when the fruit tissues are damaged [29]. Therefore, limonoids are found in two forms as aglycones (bitter taste) and glucosides (non-bitter) [30]. Till date there are 62 limonoids reported from citrus and its related genera out of which 44 are aglycones and 18 are glucosides [31-32]. A large amount of limonin is accumulated in citrus seeds whereas intact fruit tissues possess a non- bitter precursor of limonin i. e. LARL [6]. When any physical damage to citrus fruits occur this non-bitter precursor gets chemically converted into limonin in irreversible way under acidic conditions (below pH 6.5) exhibiting delayed bitterness problem [33] by the LLH [7]. The LGT gene is present as single copy in citrus genome and thus is very importance for the regulation of delayed bitterness in citrus products [10]. But after 2000, After 2000, *LGT* gene has been isolated from Satsuma mandarin, lime, navel orange [10-12], and recently from Kinnow mandarin (KP306791) from our laboratory.

In addition to limonoids, flavonoids are also present in abundance in citrus fruits. The flavonoids are polyphenolic compounds having typical carbon skeleton with two aromatic rings enclosing a heterocyclic six-membered ring with oxygen, characterized by common benzo-gamma-pyrone structure [36]. These possess several antioxidant properties such as antiradical, antilipoperoxidators and as metal chelators [37]. Flavonoids are the secondary products which show specific roles in plants such as providing protection against biotic and abiotic stresses, ultraviolet rays protection, attraction of pollinators and seed dispersal while in humans these are important for fighting against some diseases and thus can improve the health. Citrus flavonoids have been suggested to be responsible for the prevention of cancer and degenerative diseases [38]. Flavonoids are divided into different subgroups such as flavanones, flavones, flavonols, leucoanthocyanidins, anthocyanins and isoflavonoids which are abundant in flowers, fruits and leaves and comprise a diverse set of functions in different plants [39]. Citrus fruits are rich source of flavanones which are synthesized naturally in fruits and are available in their aglycone and glucoside forms [40]. Flavanones are the predominant flavonoid found in citrus juices that we consume [41-42].

The flavonoid biosynthetic pathway in citrus is similar to that of other plants [43]. The latest update of citrus flavonoids biosynthetic pathway is given by [44]. The condensation of three molecules of malonyl-CoA and one molecule of p-coumaryl-CoA results in formation of naringenin chalcone and this step is catalyzed by chalcone synthase (CHS). The naringenin chalcone is then converted into naringenin by closing the stereospecific ring and is catalyzed by chalcone isomerase (CHI) (Figure 2).

Figure (2): Flavonoid biosynthetic pathway in Citrus plants. The condensation of three molecules of malonyl-CoA and one molecule of p-coumaryl-CoA is catalyzed by CHS enzyme. The CHS and CHI catalyse the key

initiating regulatory steps in flavonoid biosynthetic pathway, which is then followed by the process of glycosylation resulting in formation of naringenin-7-o-glucoside and rhamnosylation resulting in formation of



naringenin and narirutin.

These two regulatory steps are followed by the addition of hydroxyl and/or methyl groups to produce different flavanone aglycones and glucosides [40]. These citrus flavanones are glucosylated at position 7 to create flavanone-7-O-glucosides such as naringenin glucosides, which are further glycosylated by either a 1–6 rhamnosyltransferase to yield tasteless 7-O-rutinosides (such as narirutin) or a 1–2 rhamnosyltransferase to yield bitter 7-O-neohesperidosides (such as naringin) [45- 46], [40]. Flavonoids are usually present as diglycosides form exhibiting typical taste and flavour to citrus fruits. Out of these aglycone forms naringenin is the most important in terms of producing primary or immediate bitterness in citrus juices [47]. Test of cell- free extracts of citrus for UDP-glucose: flavanone-7-O-glucosyltransferase activity was done with certain enzymatic preparations of aglycones such as naringenin and hesperetin with UDP-glucose which resulted in synthesis of UDP-rhamnose. [44] Tried for reducing the naringenin in grapefruit (*Citrus paradisi* Macf) which is responsible for lowering down the palatability of this fruit. For this, RNA interference approach was used to manipulate the aglycone levels. The sense and antisense constructs corresponding to different genes of flavonoid biosynthetic pathway in grapefruit were employed for transformation. This had resulted in fruits with lowered flavanone levels for better consumption. [48] identified and predicted several related putative genes from Citrus EST (CitEST) database of *Citrus sinensis* (L.) Osbeck which can be involved in general phenylpropanoid and main flavonoid biosynthetic pathways. This study seems to be important to essential insights concerning the flavonoid biosynthesis and regulation in citrus. But further checking and validation all that information with protein translation and assembly as well as protein activity and function is awaited yet. Flavonoids have been shown to be of great importance to human nutrition and health.

Role of glycosyltransferases in plant metabolism

Limonoid glucosyltransferase present in citrus belongs to the glycosyltransferase super family which play several important roles in plant metabolism. Glycosyltransferases recognize a wide range of carbohydrate acceptor molecules and are involved in detoxification of biotic toxins, xenobiotics, herbicides, pesticides and pollutants [49]. These glycosyltransferase enzymes are involved in transferring the carbohydrate group from UDP-sugar to different acceptor molecules. There is transfer of single or multiple sugar units from activated sugars (UDPG-uridine diphosphate glucose) forming glycosidic bonds [50]. Thus, glycosyltransferases are the managers of small molecules which use the process of glycosylation to maintain the cellular homeostasis and housekeeping in plant cells and help in providing tolerance against several biotic and abiotic stresses on plants.

In most of the places, the glycosylation is the last step in the plant secondary metabolite biosynthetic pathways of flavonoids, terpenoids, alkaloids and saponin etc. Glycosyltransferases have been classified into 91 distinct families [51] which comprise over 30,000 glycosyltransferase sequences identified across all kingdoms [52]. These are the very prevalent enzyme type, representing 1–2% of the genomes sequenced yet. There are many different glucosyltransferases reported from different agronomic crops. A table representing different glucosyltransferases is given Table (2).

Sr. no	Plant	Glucosyltransferase	Size of encoding gene
1	*Citrus reticulata* (Kinnow mandarin)	Limonoid glucosyltransferase (KP306791)	1533 bp
2	*Citrus unshiu* (Satsuma mandarin)	Limonoid glucosyltransferase (AB033758.1)	1732 bp
3	*Citrus sinensis* (navel orange)	Limonoid glucosyltransferase (EU531465.1)	1536 bp
4	*Citrus ichangensis*	Ichang papeda limonoid UDP glucosyltransferase gene (EU254210.1)	846 bp
5	*Citrus aurantium*	Lime haplotype 2 limonoid UDP glucosyltransferase gene (EU254186.1)	1536 bp
6	*Citrus limettioides* (lime)	Limonoid glucosyltransferase (EU531463.1)	1536 bp
7	*Citrus maxima*	Limonoid glucosyltransferase	1536 bp
8	*Citrus x paradisi*	Limonoid glucosyltransferase	1536 bp
9	*Citrus unshiux Citrus sinensis* x *Citrus reticulata*	Limonoid glucosyltransferase (EF119744.1)	1509 bp
10	*Medicago truncatula*	UDP-glucosyltransferase family protein (AY747627.1)	1896 bp
11	*Oryza sativa*	UGT706C1	1425 bp
12	*Oryza sativa*	UGT706D1	1416 bp
13	*Oryza sativa*	UGT707A3	1443 bp
14	*Oryza sativa*	UGT709A4	1425 bp
15	*Arabidopsis thaliana*	UDP-Glucosyltransferase 75B1 (NM_001331554.1)	2136bp
16	*Brassica napus*	UDP-glycosyltransferase 84A2-like (NM_001315569.1)	1757bp
17	*Dorotheanthus bellidiformis*	Betanidin-5-O-glucosyltransferase (Y18871.1)	1468bp

18	*Gentiana triflora*	Anthocyanin 30-glucosyltransferase (AB076697.1)	1447bp
19	Nicotiana tabacum	Immediate-early salicylate-induced glucosyltransferase (U32643.1)	1429bp
20	*Sorghum bicolor*	UDP-glucose glucosyltransferase (AF199453.1)	1477bp
21	*Malus domestica*	UDP glucose:flavonoid 3-O-glucosyltransferase (AF117267.1)	1450bp
22	*Vitis vinifera*	UDPglucose: flavonoid 3-O-glucosyltransferase (AB047099.1)	1369bp
23	*Petunia hybrida*	Anthocyanidin 3-O-glucosyltransferase (AB027454.1)	1345bp
24	*Forsythia intermedia*	Flavonoid 3-O-glucosyltransferase (AF127218.1)	1363bp
25	*Hordeum vulgare* subsp. vulgare	UDPglucose flavonol 3-O-glucosyl transferase (X15694.1)	1366bp
26	*Zea mays*	UDP-glucose flavonoid-3-O-glucosyltransferase (AY167675.1)	1414bp
27	*Iris hollandica*	Anthocyanin 5-O-glucosyltransferase (AB113664.1)	1390bp
28	*Nicotiana tabacum*	Glucosyltransferase NTGT2 (U32643.1)	1411bp
29	Torenia hybrid cultivar	Anthocyanin 5-glucosyltransferase (AB076698.1)	1435bp
30	*Perilla frutescens* var. crispa	UDP-glucose:anthocysnin 5-O-glucosyltransferase (AB013596.1)	1381bp
31	*Scutellaria baicalensis*	UDP-glucose: flavonoid 7-O-glucosyltransferase (AB031274.1)	1429bp
32	*Alium cepa*	flavonoid glucosyl-transferase (AY262063.1)	1408bp
33	*Sesamum indicum*	UDP-glucose:sesaminol 2'-O-glucoside-O-glucosyltransferase (AB333799.1)	1408bp

34	*Avena sativa*	UDP-glucose:sterol glucosyltransferase (Z83832.1)	1825bp

A lot of literature is there which suggest the use of glucosyltrasferases in pharmacological and agronomic importance. But very few glucosyltransferases reported so far have been characterized till date. Metabolic engineering of cyanogenic pathway in *Arapidopsis* revealed the potential use of glucosyltransferase as biocatalysts for the biotransformation [53]. This led to the generation of first glucosyltransferase identified in connection with the glycosyltaion of cyanidin 3-O-glucosides which are responsible for red pigmentation [54-55] reported the first molecular and biochemical characterization of 3-O glucosyltransferase engaged in the formation of blue anthocyanin gentiodelphin from Gentiana triflora (clustered gentian). This study proved the novel foundation for pigmentation in different flower species. The extent of glycosylation and its properties was attempted in Stevia rebaudiana which produces a low calorie sweetener (steviosides) consisting of mixture of diterpenoid glycosides [56].

Another important example of cloning of glucosyltransferase was from yellow saffron (*Crocus sativus*). Its dry stigma possesses apocarotenoids which are responsible for colour, bitter taste but having neutraceutical properties [57]. The cloning of terpenoid glucosyltransferase (saffron apocarotenoidcrocetin UDP glucosyltransferase (UGT) from yellow saffron was reported which is highly expressed in stigma of crocin accumulating species. This recombinant enzyme can be a good tool for the alternative production of crocin in heterologous systems [58].

There are certain biological roles of glycosyltransferases or glucosyltransferases like involvement in hypersentitive responses during biotic stresses on plants. Tobacco glucosyltransferases have showed hypersensitive responses by upregulating the salicyclic acid [59] and it was determined by its downregulation in tobacco mosaic virus infection. This resulted in more susceptibility of plants to viral infection [60] while their overexpression resulted in increased resistance against Potato virus Y [61].

UGTs are responsible for the inactivation and detoxification of xenobiotics in conjugation with cytochromes P450 [62]. In this context, glucosyltransferases has showed its role in plant-fungal interactions which resulted in resistance against deoxynivalenol (DON) which is a myotoxin generated by pathogenic fungi Fusarium. These myotoxins when present in food can deteriorate our health. A UGT73C5 from Arabidopsis which catalyzed the transfer of glucose to C3-OH position of DON and was overexpressed in plant showed the enhanced tolerance to myotoxin [63].

Because of the action of LLH there is reduction in quality and value of their citrus fruit juices especially during early to mid fruit developmental stages. As both of these enzymes utilize the precursor LARL in citrus, overexpression of LGT can easily speed up the natural debittering process as compared to delayed bitterness process in Kinnow mandarin [64]. The glucosyltransferases have been found to be localized in cytoplasm [65-67] and golgi apparatus [68]. These enzymes are characterized by presence of a highly conserved motif or signature sequence called as plant secondary product glucosyltransferase (PSPG) box [69] at their C-terminal end [12] which is involved in transferring the sugar moiety to acceptor [49]. This C-terminal domain interacts with UDP while N-terminal domain interacts with the acceptor molecule [70].

Citrus limonoids for anticancerous effects

Citrus fruits are rich source of limonoids which have been shown several protections against various types of cancer.

Limonoid aglycones and glucosides both have health promoting as well as disease preventing properties [71]. For their non- bitter and water soluble characteristics, limonoid glucosides are considered as the most important. Citrus limonoids have been reported to show the anticancer activity in laboratory animals and activity at cell line levels. But different limonoids operate by different mechanisms of action. Generally the limonoids are strong inducers of glutathione S-transferase (GST) activity and phase II detoxification enzymes in animal and human body [72-73]. Citrus limonoids possess substantial anticancer activity especially against brain [20], colon, pancreas and breast cancers [21-23]. Citrus limonoids like obacunone, limonin, nomilin and their glucosides reported to possess cytotoxic effect in series of human cancer against lungs, oral and skin cancer in animal system and human breast cancer cells [26,74-77,25]. Citrus limonoid aglycones and glucoside mixture were showed more potent effect than tamoxifen for estrogen independent breast cancer cells and equally potent as tamoxifen for estrogen dependent breast cancer cells [75]. Further, citrus limonoids and their derivates, having good bioavailability and having non-toxic effects in animals and human [25], are of great importance to human nutrition studies. At micromolar levels, both aglycones and glucosides arrested the cell growth, but according to the biochemical and morphological data glucosides induce a more rapid cell death in cancer cells. Further, aglycone toxicity was dose-dependent but below the killing potential of glucosides [23].

Bioinformatics characterization of plant glucosyltransferase

A significant goal in the post genome era is to relate the annotated genome sequence to the physical functions in a cell. Certain computational methods have been developed to interpret the results and predict the optimal performance of these sequences.

[12] reported that gene sequence of sweet lime had complete identity with LGT gene sequence of Satsuma mandarin which were depicted by the PCR amplification, nucleotide sequencing, phylogenetic analysis, motifs analysis, Prosite domain structure map carried out for high scoring of the sequence and to detect similarity between species of limonoid glucosyltransferase.

In silico analysis of genes involved in flavonoids biosynthesis in Citrus sinensis was reported [48] analyzed and predicted the putative genes involved and playing key roles in phenylpropanoid and flavonoid biosynthetic pathways. The major goal of this work was to identify the related Expressed Sequence Tags (ESTs) of *Citrus sinensis* from CitEST database. By this work some interesting and novel informations of flavonoid metabolite pathway such as protein translation and assembly, and some protein activity were found out [50] revelaed in silico motif diversity analysis of glycon of plant secondary product glycosyltransferases. The reaction carried out by UGTs is critical in regulating the levels of secondary metabolites involving signalling and hormonal compounds.

For analysis, glucosyltransferases (GTases from diverse plant species whose genes were sorted out from the databases. The degree of homology was detected by aligning all the GT sequences by ClustalW followed by certain phylogenetic tools. It was resulted that highly considerable motif in their C-terminus named PSPG box was observed in all the sequences using motif discovery tools. Hence, wide range of gene sequences was analyzed in systematic manner to determine the structure, function of PSPG box motif at C-terminal [78] identified 137 UDP glycosyltransferase genes with multigene family from flax (*Linum usitatissimum L.*) by taking help of its draft genome sequence. All these glycosyltransferases possessed conserved signature motifs and were showed the similarity of 36-98%. All the sequences were aligned using ClustalW tool and phylogenetic

tree was constructed for determining their evolutionary status. This study could be useful for seed specific glycosylated metabolite accumulation and other processes in plants.

Molecular cloning and functional expression of limonoid glucosyltransferase

Citrus limonoid biosynthetic pathway involves two important regulatory steps which are catalyzed by LGT and LLH enzymes. Although, the delayed bitterness and its cause was known but only the molecular cloning of *LGT* gene from different *Citrus spp* was started after 2000. LGT has been reported as single copy in the citrus genome and thus its cloning and functional expression studies are appeared to be very important to avoid delayed bitterness in citrus fruits. The expression of *LGT* produces limonoid glucoside molecules which are recently known to possess anticancerous properties in animal models and thus, this will serve its importance in pharmaceuticals and neutraceutical industries in future. For this, functional expression of *LGT* in a suitable host is very important for the production of its stable and efficient protein.

Different full length *LGT* genes were isolated from Satsuma mandarin (AB033758), lime (EU531463), navel orange (EU531465), sour orange (EU531466), grapefruit (EU531464) [10-12], Kinnow mandarin (KP306791) and pummelo (EU304828). The isolated 1732bp cDNA clone encoding LGT from albedo of Satsuma mandarin was showed its contribution to limonoid glucoside accumulation in fruit. This full length gene showed 511 deduced amino acids with a predicted molecular mass of 57.5 kDa during its *in vitro* translation along with the limonoid GTase activity were investigated. It was indicated that the transcription of *LGT* regulates the conversion of limonoid aglycones to glucosides in navel orange fruit [10].

Cloning of *LGT* in bacterial expression vector is a preliminary step to test its functionality in prokaryote before studying its overexpression in citrus. For this *E. coli* is the most popular heterologous expression host for the production of recombinant proteins due to its advantages such as low cost, high productivity, well characterized genetics, simple growth requirements and rapid growth [79]. It is also termed as cell- factory because it has most popular expression platform [80]. Although, a lot of literature is there on the expression of plant glucosyltransferase gene in bacteria but very few studies are there on the expression of LGT cloned from citrus plants. [81] expressed different cDNA fragments encoding sterol glucosyltransferase from oat and Arabidopsis in *E. coli* and exhibited sterol glucosyltransferase activity in homogenates of transformed bacterial cells using enzymatic assay. *E. coli* strains are highly important for recombinant expression of eukaryotic genes. Some *E. coli* facilitates certain rare codons, easy disulfide bond formation, tuneable gene expression, improved tolerance of toxic gene products. There are many expression strains out of which BL21 (DE3) is an outstanding strain which have been mostly used by the experts. These strains are deficient in most harmful natural proteases and have the capacity to maintain the plasmid expression stably. For prokaryotic expression BL21 (DE3) strain of *E. coli* is the most common host [82].

Plant GTase are reported to have molecular weight in the range of 40 to 60kDa [34]. [83] isolated and characterized LGT from albedo tissue of pummelo. This enzyme was purified to 180 fold by combination of $(NH_4)_2SO_4$ fractionation ion exchange chromatography on DEAE cellulose and DEAE toyopearl. SDS-PAGE analysis of its expressed protein revealed 55kDa with displayed activity at pH 7.8 and 37°C with Km values of

65 and 200mM for limonin and UDPG. In addition of Mn^{+2} and Co^{+2} stimulated the enzyme activity while EDTA inhibited it. One interesting fact was that this enzyme was

stable at 4°C for 6 months in Tris HCL buffer, pH7.5 [84] reported cloning of four genes encoding.

UDP-dependent glucosyltransferases from rice and were expressed in *E. coli*. It was found that these enzymes could use flavonoids like apigenin, daidzein, genistein, kaempferol, luteolin, naringenin and quercetin as potential glucose acceptor. [85] reported high level of LGT expression in *E. coli* which resulted in formation of insoluble aggregates as inclusion bodies. These dynamic structures or elementary bodies formed by unbalanced equilibrium between aggregated and soluble proteins by *E. coli* and hence are localized in both cytoplasmic and periplasmic spaces of *E. coli* during high level expression of heterologus protein. Further, the soluble LGT protein was able to isolate by renaturation of these inclusion bodies with beta-cyclodextrin treatment after protein denaturation by urea. In the same year, [86] cloned *LGT* gene from different Jeju Citrus spp. and the deduced glucosyltransferase proteins harboured a highly conserved PSPG motif within C terminal region.

Conclusion

Limonoid glucosyltransferase catalyses the regulatory step of limonoid biosynthesis in citrus plants and this can be useful in dual ways. First, overexpression of gene encoding LGT will utilize more amount of precursor, LARL and thus, leaving no or very less amount of free limonoids for the conversion of bitter limonin in case of delayed bitterness, after citrus juice extraction. Secondly, this will also accumulate limonoid glucoside molecules which have been shown strong anticancerous potential in animal and cell line levels during clinical trials. As the citrus fruits are rich and cheap source of various health promoting bioactive compounds especially in the Southeast Asia region. Citrus consumption with the enhanced health benefits can be increased up to a great level by implementing the metabolic engineering of citrus limonoids which has not realized till date. Keeping the importance of citrus limonoid glucosides against different types of cancers, the future research studies should be focused on to utilize them as most important component of today's healthy human diet. This review finally concludes that glycosyltransferases are the good biotechnological targets as their encoding genes can be inserted into a variety of plants to improve the food and crop quality.

Conflicts of Interest

The authors declare no conflicts of interest for this manuscript.

Authors Contributions

EP and PM designed and wrote the paper. GSS contributed critical suggestions in writing the manuscript.

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