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Review

Citrus limonoids: mechanism, function and its metabolic engineering for human health

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Summary

Introduction – **Citrus fruits are a rich and cheap source of several bioactive phytochemicals including limonoids which are present in glucoside and aglycone forms. Considerable attention has been given on citrus limonoid aglycones, mainly limonin, as a major cause of delayed bitterness problem affecting citrus industry all over the world. Recently, the clinical studies have demonstrated that citrus limonoid, especially its glucoside forms, are very effective against colon, breast, brain and pancreas cancers in animal models and cell line levels.** *Materials and methods* – **The present review emphasizes the double importance of citrus limonoids in terms of bitterness and anticancerous properties. Limonoids are present in a very high amount in citrus seeds and peel and thus their extractions can have commercial value.** *Results and discussion* – **The waste/byproduct left in citrus juice industry can be efficiently utilized to separate these bioactive compounds to use them as nutraceutical and functional food for human health. In addition, citrus limonoid glucosyltransferase which is a key player for natural debitterness and anticancerous potential, can be utilized for metabolic engineering of citrus limonoids to get rid of delayed bitterness problem along with enhanced limonoid glucoside molecules.** *Conclusion* – **The future research should focus on citrus limonoid metabolic engineering, and extraction as well as utilization of bioactive limonoids for health promoting and disease-preventing benefits.**

Keywords
India, *Citrus* spp., *Citrus reticulata*, fruit quality, human health, limonoid metabolism, metabolic engineering

Significance of this study

What is already known on this subject?

- Citrus limonoid aglycones, especially limonin, is the major cause of delayed bitterness problem affecting citrus industry all over the world. Only one regulatory enzyme encoding gene, limonoid glucosyltransferase (*LGT*) in citrus limonoid biosynthetic pathway has been isolated from different citrus species.

What are the new findings?

- The waste/byproduct left in citrus juice industry can be utilized efficiently to separate limonoids as bioactive compounds to use them as nutraceutical and functional food for human health. Citrus limonoid glucosyltransferase can be a key player for both natural debittering and anticancerous potential. Metabolic engineering of citrus limonoids to get rid of delayed bitterness problem along with enhanced limonoid glucoside molecules having anticancerous properties will be valuable.

What is the expected impact on horticulture?

- The presence or absence of *LGT* can serve as a molecular indicator for determining the level of accumulation of limonoid glucoside and may reflect ultimately the possibility of delayed bitterness in available citrus germplasm.

Résumé

Les limonoïdes des agrumes: mécanisme, fonction et ingénierie métabolique pour la santé humaine.

Introduction – **Les agrumes sont une source riche et bon marché de plusieurs composés phytochimiques bioactifs, y compris les limonoïdes qui sont présents sous les formes glucoside et aglycone. Une attention considérable a été accordée aux limonoïdes aglycones d'agrumes principalement la limonine, principale responsable des problèmes d'amertume tardive affectant l'industrie des agrumes dans le monde entier. Récemment, les études cliniques ont démontré que les limonoïdes d'agrumes en particulier ses formes glucosidiques sont très efficaces contre les cancers du côlon, du sein, du cerveau et du pancréas dans les modèles animaux et les gradients de lignées cellulaires.** *Matériel et methodes* – **La présente revue souligne la double importance des limonoïdes des agrumes en termes d'amertume et de propriétés anticancéreuses. Les limonoïdes sont présents en très grande quantité dans les pépins et les peaux des agrumes et leur extraction peut donc avoir une valeur commerciale.** *Résultats et discussion* – **Les déchets/sous-produits issus de l'industrie des jus d'agrumes peuvent être utilisés efficacement pour séparer ces composés bioactifs afin de les utiliser comme aliments nutraceutiques et fonctionnels pour la santé humaine. A l'avenir, l'enzyme citrus limonoid glucosyl transférase qui est un facteur clé de contrôle de l'amertume naturelle et du potentiel anticancérogène, pourrait être utilisé en ingénierie métabolique des limonoïdes d'agrumes pour se débarrasser du problème de l'amertume retardée à base de molécules de glucosides de limonoïdes améliorées.**

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Conclusion – **Les recherches futures devraient être axées sur l'ingénierie métabolique des limonoïdes d'agrumes, ainsi que sur l'extraction et l'utilisation de limonoïdes bioactifs pour la promotion de la santé et la prévention des maladies.**

Mots-clés
agrumes, *Citrus reticulata*, qualité du fruit, métabolisme des limonoïdes, santé humaine, ingénierie métabolique

Introduction

Citrus is one of the major nutritional and most traded fruit crops of the world. Citrus fruits are appreciated for their refreshing juice and health benefits and are consumed as fresh or utilized for processed products and by-products. *Citrus* is a large genus belonging to *Rutaceae* family and is native to southeast Asia. It is grown between 40° North and 40° South of equator due to its wide adaptability to the tropical and subtropical conditions. Citrus fruits generally include sweet orange, mandarin, lime, lemon, pummelo, and grapefruit. India produces 8.7 Mt citrus fruits and ranks sixth in the world after China, Brazil, USA, Spain and Mexico (ICAR-NRCC, India).

Taxonomic classification, history, and origins of citrus are full of controversies (Liu *et al*., 2012). However, classification by Swingle (which recognizes 16 species) and Tanaka (which recognizes 162 species) are the most accepted ones (Nicolosi, 2007). Citron, pummelo and mandarin are the three ancient *Citrus* spp. and all other hybrids available today originated from intercrossing of these (Swingle, 1967). Although, most of the *Citrus* spp. are diploid and having basic chromosome number $x=9$ (Frost, 1925; Nakamura, 1929) but genome size varies from 367 Mb of sweet orange (Xu *et al*., 2013) to 383 Mb of pummelo (Ollitrault *et al*., 2003). In north-western India 'Kinnow' mandarin (*Citrus reticulata* Blanco) is a very popular commercially important citrus variety in Punjab which occupies 61.5% of total fruit area and ranks first in fruit production (DH, India). It is the result of a hybrid cross between King and Willow mandarins (*Citrus nobilis* Lour. × *C. deliciosa* Tenora).

Health promoting and disease preventing benefits of citrus fruits are linked with their containing high amount of bioactive phytochemicals, mainly limonoids and flavonoids, which have been attributed to numerous therapeutic properties such as anticancer, antiviral, antioxidant, antitumor, anti-inflammatory, and more recently, protection against cardiovascular and degenerative diseases (Tadeo *et al*., 2008; Cirmi *et al*., 2016). Recent attention has been given to the citrus limonoids for human nutrition studies because these possess substantial anticancer activity especially against brain (Poulose *et al*., 2006), colon, pancreas and breast cancers (Kim *et al*., 2012a; Chidambara Murthy *et al*., 2013; Tundis *et al*., 2014). These induce programmed cell death (PCD) in cancer cells and activate the detoxifying enzyme system of the human body that facilitates the removal of complex chemical wastes including carcinogenic compounds.

Among the phytochemicals, citrus limonoids are highly oxygenated triterpenes occurring as water-insoluble aglycones and water-soluble glucosides forms. Certain forms of the limonoid aglycones can be extremely bitter, especially the relatively abundant limonin (in its closed ring form), but not all forms. While only a few limonoid glycosides have been isolated and characterized which generally seem to be tasteless. Instead of health promoting properties, citrus juices and citrus by-products are significantly affected by delayed bitterness problem, which is mainly due to the high amount of limonoid aglycones, especially limonin. Even in 'Kinnow' mandarin a high amount (2,500 ppm) of limonin is present in its seeds, while its peel and juice contain 80 and 20 ppm limonin, respectively (Mahajan *et al*., 2011), what is well above the threshold level. Although, bitter limonoid aglycones are endogenously converted into non-bitter limonoid glucoside derivatives through a natural debittering process in citrus fruits (Endo *et al*., 2002) and this inter-conversion is catalyzed by two regulatory enzymes namely limonoid lactone hydrolase (LLH) and limonoid glucosyltransferase (LGT) (Hasegawa *et al*., 1991). Out of these two, the only gene encoding for LGT has been isolated from 'Satsuma' mandarin, navel orange, lime (Kita *et al*., 2000, 2003; Zaare-Nahandi *et al*., 2008) and recently from 'Kinnow' mandarin (NCBI accession number KP306791). Since *LGT* gene is present as a single copy in the citrus genome, it is very important with respect to the regulation of delayed bitterness (Kita *et al*., 2000). Enhanced activity of *LGT* in citrus fruits may increase the glucoside level, which in turn will reduce the bitterness problem also. This can be achieved by regulating this enzyme activity at the molecular level. Thus, cloning of gene encoding *LGT* is crucial for creating transgenic citrus free from limonoid bitterness by reducing bitter limonoid aglycone accumulation in fruit tissues as well as increasing the specific limonoid glucoside molecules having anticancer properties (Hsu *et al*., 1998; Kim *et al*., 2012a; Chidambara Murthy *et al*., 2013). By this the citrus limonoid glucosyltransferase can serve as a key player for natural debittering and anticancerous potential (Patel *et al*., 2017). Further, citrus limonoids and their derivatives are having good bioavailability, and have non-toxic effects in animals and humans.

Biochemical composition of citrus juices

Citrus fruits and juices contain a range of nutrients and phytochemicals. Major nutrients like vitamin C, vitamin A, potassium, folate, calcium and phytochemicals like flavonoids, limonoids and carotenoids are present. Citrus fruits are low in fat, energy, and salts, and contain good amounts of carbohydrates such as sucrose, glucose and fructose (Liu *et al*., 2012). In citrus, juice content of mandarin is 51.82% which is much higher than that of lemon (42.83%) and orange (45.26%). Its total soluble solids (TSS) to titratable acidity (TA) ratio is also higher which is 11.75. Different chemical constituents present in the citrus fruits are listed in Table 1. 'Kinnow' mandarin juice is a good source of carotenoids, flavonoids, limonoids and vitamin C (Sharma, 2003) but it also contains 15-20 ppm limonin (Mahajan *et al*., 2011) which is actually responsible for delayed bitterness problem. Carbohydrates present in 'Kinnow' mandarin juice are simple sugars like glucose, fructose, and sucrose while non-starch polysaccharides like dietary fibers in the amount of 1.8 g 100 g^{-1} are also present in fruit. These are beneficial as they are slowly digested and reduce the uptake of glucose and give satiety (Turner and Burri, 2013). In addition to this, the amount of potassium and sodium present in citrus fruits are 102 and 0–2 mg 100 g^{-1}, respectively. This low sodium to potassium ratio saves from many chronic diseases in humans. In addition, the waste/byproduct left after extracting the citrus juice possesses good nutrition quality and can act as feedstuff for livestock. The citrus waste has high calcium (0.92%) and high antioxidant property (Kour *et al*., 2014).

Table 1. Chemical constituents of citrus fruit juices and their composition. Source: modified after Liu *et al.* (2012).

Constituents	Concentrations
Juice (%)	42–52
TSS (%)	8–16
Acidity (%)	0.3–7.0
Energy (kCal)	29–53
Limonoids (mg mL^{-1})	80–320
Vitamin C (mg 100 g^{-1})	26–49
Vitamin A (µg 100 g^{-1})	1–46
Potassium (mg 100 g^{-1})	102–181
Folate (µg 100 g^{-1})	10–30
Flavonoids (mg 100 g^{-1})	17–48
Dietary fibers (g 100 g^{-1})	1.8–2.8
Carotenoids (µg 100 g^{-1})	traces to 300
Calcium (mg 100 g^{-1})	12–40

Thus, the citrus fruit residue can act as low-cost potential nutraceutical resource (Negro *et al.*, 2016).

Mechanisms of citrus limonoids to influence human health

Nutrition research on the health benefits has recently advanced to a new stage beyond the vitamin deficiency diseases. Cancer is the leading cause of morbidity and mortality worldwide, with approximately 8.8 million deaths in 2015. The number of new cases of cancer is expected to rise by about 70%, *i.e.*, 14 million in 2012–2022 (WHO, U.S.). Citrus limonoids aglycones and glucosides present in citrus fruits both have been shown several advantages, especially protection against various types of cancer. These have health-promoting properties and show disease-preventing mechanisms (Miller *et al.*, 2000) (Figure 1). Different limonoid aglycones such as limonin, nomilin, obacunone, isoobacunoic acid and ichangin and their corresponding glucosides are strong inducers of glutathione *S*-transferase (GST) activity and phase II detoxification enzymes in liver and intestinal mucosa (Lam *et al.*, 1989, 1994; Chidambara Murthy *et al.*, 2015). GST acts as a detoxifying enzyme system that facilitates the removal of complex chemical waste including carcinogens from the cell (Miller *et al.*, 2004).

Limonoids also inhibit many types of chemically induced neoplasia which has been demonstrated in animals and cultured mammalian cells (Lam and Hasegawa, 1989). In animal models, these inhibit neoplasia in chemically induced cancers of the colon (Tanaka *et al.*, 2000a, b, 2001), stomach (Lam *et al.*, 1989), buccal pouch (Miller *et al.*, 1992, 2004), and blood (Pettit *et al.*, 1983). In one example, topical application of limonin was found to reduce 60% tumor burden in 7,12-dimethylbenz[a]anthracene induced oral carcinogenesis in hamsters (Miller *et al.*, 1989). A similar application of limonin glucoside to the same oral tumors showed 55% reduction in tumor burden (Miller *et al.*, 1992).

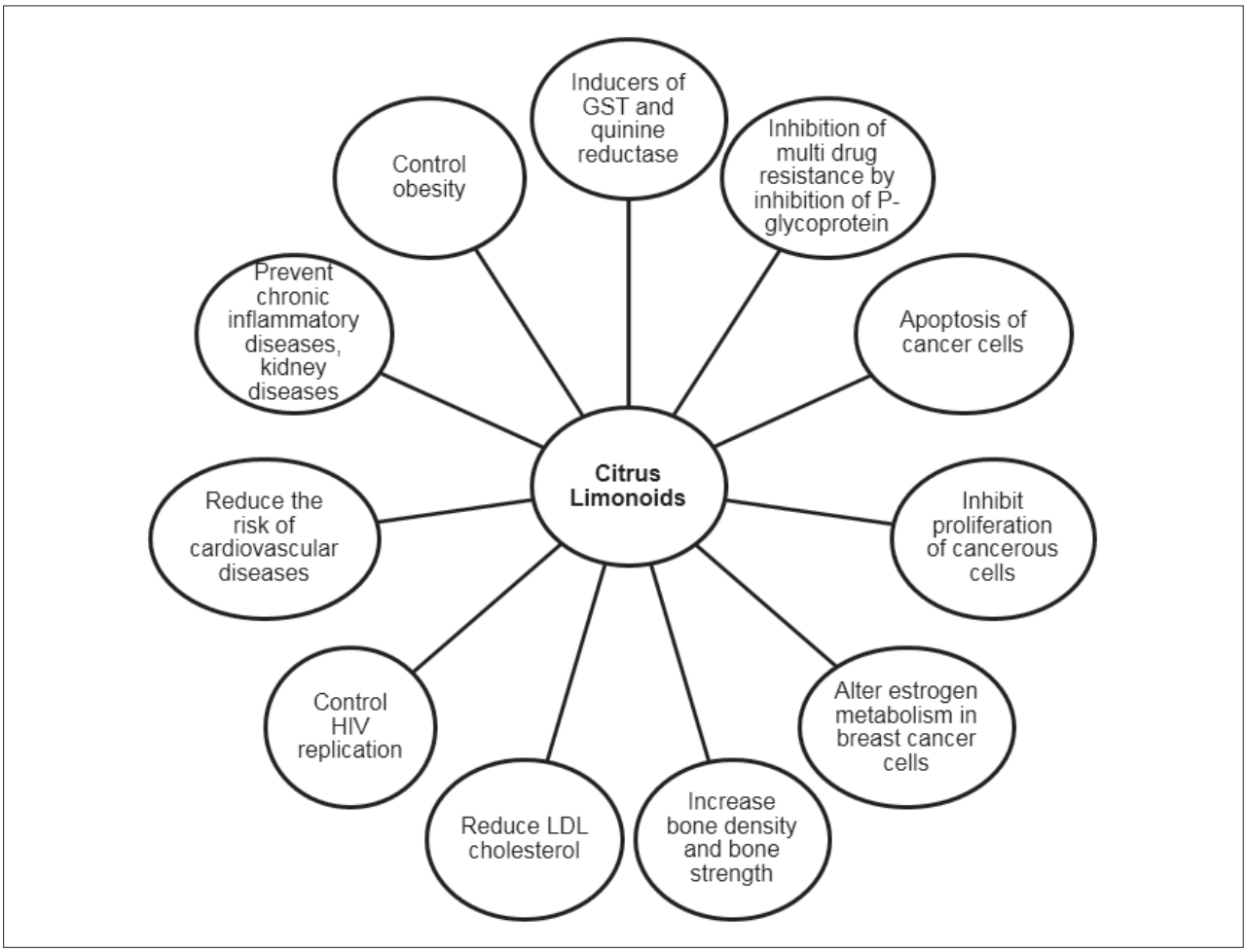

Figure 1. Disease prevention mechanisms of citrus limonoids. Source: Kim *et al.* (2012a).

Limonoid aglycones and a mixture of limonoid glucosides were when administered in human breast cancer cell lines. It was demonstrated that limonoids were more potent than tamoxifen for estrogen-independent breast cancer cells and equally potent as tamoxifen for estrogen-dependent breast cancer cells (Guthrie *et al*., 2000).

Different limonoids operate by different mechanisms of action (Lam *et al*., 1989, 1994) as was evident in the case of skin carcinogenesis. In this case, nomilin inhibit carcinogenesis in the initiation phase and limonin during the promotional phase (Lam *et al*., 2000). This difference could be because nomilin has one large A ring while limonin has separate A and A' ring. Changes in the A ring of the limonoid nucleus can lead to a loss of anticancer activity. Despite to A ring, changes in the D ring can be tolerated without any apparent loss of biological activity (Miller *et al*., 2004). Further, Poulose *et al*. (2005) also demonstrated that superior quenching and apoptosis-inducing potential are linked with A-ring configuration only. Limonin and its glucoside have an additive effect on inhibition of colon cancer when combined with curcumin (Chidambara Murthy *et al*., 2013). This is possible because these induce PCD in cancer cells (Tian *et al*., 2001; Poulose *et al*., 2005) and activate the phase II detoxifying enzymes in cancer-induced animals (Tanaka *et al*., 2001). Another limonoid obacunone inhibits adenocarcinoma in colonic Caco-2 cells (Guthrie *et al*., 2000) and neuroblastoma SH-SY5Y cells (Poulose *et al*., 2006). The possible mechanisms for obacunone mediated colon cancer inhibition are blocking the development of a precursor lesion, induction of GST and quinine reductase reducing the number of aberrant crypt foci and inducing apoptosis (Tanaka *et al*., 2000b, 2001). Further, in case of pancreas Panc-28 cells obacunone treatment induce apoptosis by activating caspase-3,9 and decreasing Bcl_2/Bax expression and activation of tumor suppressor protein p-53. Bcl_2 and Bax are PCD related genes. Increase in the level of Bax expression directs the cell to apoptosis or PCD. Also, obacunone decreases nuclear factor-kappa B (NF-κB) and expression of cyclooxygenase (COX-2) in cancerous cells, which is the vital inflammatory mediator during cancer (Chidambara Murthy *et al*., 2011a; Kim *et al*., 2014). Thus, decrease in their level will activate the anti-inflammatory pathways. In breast cancer cells obacunone inhibits p38 MAPK signaling pathway, thus resulting in cell cycle arrest and apoptosis (Figure 1). In addition, in cardiovascular models it activates the same p38 MAPK pathway (Kim *et al*., 2011), thus preventing cardiovascular diseases unlike tamoxifen, letrozole (AI) and anastrazole (AI) (Johnston *et al*., 2003; Burstein *et al*., 2010). Obacunone glucoside is also reported to prevent proliferation of human adenocarcinoma (SW480 cells) and inhibits neuroblastoma cells even at 50 μM or less concentration (Poulose *et al*., 2005, 2006). Induction of p21 by obacunone and obacunone glucoside was observed, which was supported by cell cycle arrest in G2/M phase by both compounds (Chidambara Murthy *et al*., 2011b). Its possible mechanisms are reduction of DNA synthesis and induction of caspase 3/7 activity (Poulose *et al*., 2005, 2006). Obacunone and its glucosides activate Bcl_2 associated PCD in human prostate cancer cells. They induce intrinsic apoptosis by activating caspase-9, caspase-3 and cytosolic cytochrome-c in a time-dependent manner (Chidambara Murthy *et al*., 2015).

The citrus limonoids such as obacunone, limonin, nomilin and their glucosides and some aglycones are found to have cytotoxic effects against lung, oral and skin cancers in animal test system and human breast cancer cells (Berhow *et al*., 2000; Tanaka *et al*., 2000a, 2001; Nakagawa *et al*., 2001; Silalahi, 2002; Manners *et al*., 2003; Kim *et al*., 2013). However, cancer cell lines such as HL-60, NCI-SNU-1, HeLa, SCOV-3, and HepG2 were less sensitive to limonoid glucosides (Tian *et al*., 2001). While SH-SY5Y neuroblastoma cells were more sensitive to limonin, nomilin, deacetylnomilin and obacunone and their glucosides than Caco-2 colonic adenocarcinoma cell lines (Kim *et al*., 2009). Although, at micromolar levels, both aglycones and glucosides arrested the cell growth, but according to biochemical and morphological data glucosides induced a more rapid cell death in cancer cells. Further, aglycone toxicity was dose-dependent but below the killing potential of glucosides (Tundis *et al*., 2014). Therefore, it seems that limonoid glucosides are more effective as compared to their respective aglycones forms.

In addition to limonoids, flavonoids, abundantly present in citrus fruits, also have antioxidant, anti-inflammatory, cholesterol-lowering and anticancerous properties (Turner and Burri, 2013). Citrus flavonoids and limonoids such as limonin 17- beta-D-glucopyranoside, and other limonoid glucosides in orange juices found to prevent colon tumour (Miyagi *et al*., 2000). Recent evidence shows that limonin glucosides reduce the circulating concentrations of liver proteins (GGT, ALT, ALP and complement C3) and save from a number of chronic inflammatory diseases, chronic kidney disease and cancers (Kelley *et al*., 2015). Keeping the importance of citrus limonoids for human health, these can serve as nutraceuticals having no side effects.

Limonoid metabolism

Limonoid metabolism includes limonoid biosynthesis in plants and limonoid degradation in both plants as well as in microorganisms.

Limonoid biosynthesis in plants

Limonoids are highly oxygenated triterpenes present mostly in *Rutaceae* and other limited plants of *Meliaceae* and *Simaroubaceae* (Roy and Saraf, 2006). The term 'limonoid' is derived from limonin, which was first identified as the bitter constituent of *Citrus* seeds in 1841 (Tundis *et al*., 2014). Based on radioactive tracer work, it has been shown that only phloem regions of the stem are the site for biosynthesis of limonoids from acetate, mevalonate and/or furanesyl pyrophosphate in *Citrus limon*. Deacetylnomilinic acid also gets converted into nomilin in the stem (Roy and Saraf, 2006). Further, stem nomilin formed is translocated to other tissues including leaves, fruits including peel, seeds, and roots, where nomilin or deacetylnomilin are further capable of biosynthesizing other limonoids in most of the citrus (Ou *et al*., 1988). Although, seed and fruit tissues are capable of biosynthesizing other limonoids starting from nomilin independently by at least four different pathways (Endo *et al*., 2002; Moriguchi *et al*., 2003). Thus, nomilin is considered to be the precursor of all other limonoids accumulated in *Citrus* and related species except in cortex and inner core (Ou *et al*., 1988) (Figure 2A).

Limonoids occur in two forms, as water insoluble aglycones and as water soluble glucosides in fruits and seeds of *Citrus* spp. (Manners and Breksa III, 2004). Till date, 62 limonoids, including 44 aglycones and 18 glucosides have been isolated from citrus and its closely related genera, and still, the number is increasing (Jayaprakasha *et al*., 2008; Kim *et al*., 2012b). Although, citrus seeds accumulate a large amount of limonin (dilactone), whereas the intact citrus fruit tissues possess predominantly a non-bitter precursor of limonin, *i.e*., limonoate A-ring lactone (LARL) (monolactone)

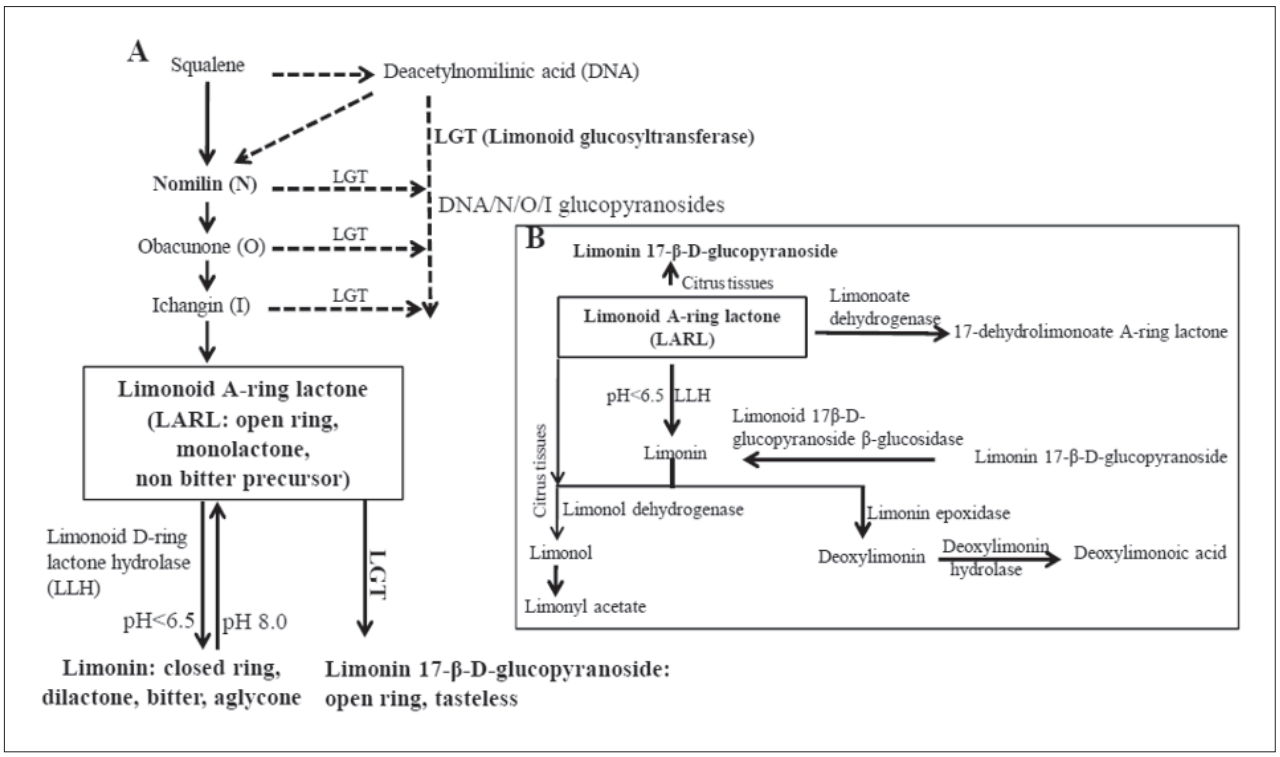

Figure 2. The limonoid metabolism pathway in citrus and microorganisms. (A) Limonoid biosynthesis pathway in citrus. Nomilin is the initial precursor of all limonoids synthesized in stem. LGT is the only single regulatory enzyme that is able to convert all limonoid aglycones such as nomilin, obacunone, ichangin present in *Citrus* spp. to their respective glucosides such as nomilin/nomilinic acid/obacunone/ichangin glucopyranosides, in addition to LARL to limonin glucopyranoside. While during the juicing process, freeze damage or physical damage to the citrus fruit, bitter limonin is immediately produced from precursor LARL by LLH regulatory enzyme at the prevailing acidic conditions especially in early to mid-stage developing fruit. (B) Limonoid degradation pathways in microorganisms and plants where hydrolase and dehydrogenases play their roles.

(Maier and Beverly, 1968). The concentration of LARL decreases as fruit maturity progresses (Maier *et al*., 1980). The presence of limonoid glucosides, limonin, dehydo forms of LARL suggests about the fate of LARL. When physical damage or freeze damage to citrus fruits occurs or juices are prepared from citrus fruits, LARL is chemically converted into limonin in a reversible manner under the produced acidic conditions (below pH 6.5), generating the delayed bitterness problem (Maier *et al*., 1969; Merino *et al*., 1996). This conversion of LARL to limonin is enhanced by the action of an enzyme LLH isolated from citrus seeds (Maier *et al*., 1969) (Figure 2A). But in different bacterial species and orange fruit tissues, an enzyme called limonoate dehydrogenase irreversibly converts LARL into its dehydro forms, thus avoiding limonin production (Hasegawa and Maier, 1990).

In addition, citrus limonoid aglycones are glucosidated by LGT in maturing fruit tissues and seeds. LGT is a single enzyme that appears to be responsible for the glucosidation of all the limonoid aglycones to their respective glucosides (Moriguchi *et al*., 2003). Bitter limonoid aglycones are endogenously converted into non-bitter limonoid glucosides derivatives through a natural debittering process in citrus fruits during fruit maturation and this inter-conversion is catalyzed by two regulatory enzymes, *i.e*., LLH and LGT (Hasegawa *et al*., 1991; Endo *et al*., 2002). These two enzymes compete with each other for the newly biosynthesized LARL as monolactones in the fruit tissues.

Molecular cloning of gene(s) related to the limonoid biosynthetic pathway in citrus was started after 2000. Out of two important regulatory genes in limonoid biosynthetic pathway gene encoding LGT has been isolated from different *Citrus* spp. such as 'Satsuma' mandarin (*C. unshiu*) (AB033758.1), navel orange (*C. sinensis*) (EU531465.1) (Kita *et al*., 2000, 2003), limes (*C. limettioides*) (EU531463.1) (Zaare-Nahandi *et al*., 2008) and recently from 'Kinnow' mandarin (KP306791). It has been reported that LGT is the only enzyme able to convert all limonoid aglycones such as limonin, nomilin, obacunone present in *Citrus* spp. to their respective glucosides such as limonin glucopyranoside, nomilin and nomilinic acid glucopyranoside, obacunone glucopyranosides (Hasegawa *et al*., 1991, 1997; Tian *et al*., 2001) (Figures 2A and 3). The presence of limonoid glucosides, especially 320 ppm of total limonoid glucosides in commercial orange juices suggests that LARL is metabolized to its glucoside during late stages of fruit growth and maturation (Hasegawa *et al*., 1991). Although, the limonoid glucosides in citrus fruit tissues are stable (Herman *et al*., 1991) but in seeds, they are hydrolyzed during germination to liberate the glucose molecules by catalytic action of an enzyme, limonoid 17β-D-glucopyranoside β-glucosidase (Ronneberg *et al*., 1995). In juice, the glucosides are generally stable, except nomilin glucoside which converts to nomilinic acid glucoside below pH 3.5 or above 8.0 (Herman *et al*., 1991). Limonoid aglycones are present as the open D-ring form in citrus fruit tissues, leaves and stems. In seeds, limonoid aglycones are present in both open and closed D-ring forms (Hasegawa *et al*., 1996). Alkaline conditions open the D-ring, whereas acidic conditions close it (Maier *et al*., 1969).

Limonoid degradation in citrus tissues

Limonoid metabolites are degraded under the action of a variety of enzymes. The limonoid glucosides formed are converted into limonoid aglycones by the catalytic action of limonoid 17 β-D-glucopyranoside β-glucosidase in citrus germinating and dormant seeds. In this process, glucose molecule is liberated (Ronneberg *et al*., 1995; Berhow *et al*., 2000). But, limonoid glucosides in other citrus fruit tissues are stable (Herman *et al*., 1991). Limonoid β-glucosidase activity is also found in several bacterial species from soil (Hasegawa *et al*., 1989) and from human gastrointestinal tract (Hasegawa, 1999). Other enzymes such as limonoid dehydrogenase in navel orange albedo tissue (Hasegawa *et al*., 1974a), and LLH has been discovered in citrus seeds (Hasegawa, 1976). LLH converts LARL into limonin under acidic condition while limonin dehydrogenase converts LARL into its dehydro forms. In addition, the fate of LARL is not so simple as it has been shown to be converted into minor limonoids such as 17-dehydrolimonate A-ring lactone (Hsu *et al*., 1973; Hasegawa *et al*., 1974b), deoxylimonin and deoxylimonoate (Hasegawa *et al*., 1980) and possibly, limonol and limonyl acetate (Figure 2B). These conversions are very minor and thus, alone cannot justify the decrease of LARL during late stages of fruit growth. But the LARL as monolactone is predominant limonoid present in leaves, stem and fruits tissues of citrus (Fong *et al*., 1991).

Limonoid degradation by microorganisms

Different enzymes have been discovered in different bacterial species which degrade the limonoid metabolites. Limonoate dehydrogenase from *Arthrobacter* and *Pseudomonas* (Brewster *et al*., 1976) catalyze the irreversible conversion of LARL into its dehydro form (Humanes *et al*., 1997). Another enzyme LLH reversibly converts LARL into limonin (Maier *et al*., 1969) depending upon pH of the reaction conditions which has been found in *Pseudomonas* (Hasegawa, 1976). At pH 6.0 D-ring of LARL is lactonized to produce limonin, while at pH 8.0 D-ring of limonin is hydrolyzed to produce LARL via 17-dehydrolimonoid pathway (Hasegawa, 1976). Limonin is converted into deoxylimonin by enzyme limonin epoxidase via deoxylimonoid pathway (Hasegawa *et al*., 1974a) in *Acinetobacter* sp. (Vaks and Lifshitz, 1981) which has been isolated from soil. Deoxylimonin is further converted to deoxylimonoic acid by deoxylimonin hydrolase which has been isolated from *Pseudomonas* 329-18 (Hasegawa *et al*., 1974b) (Figure 2B). Microorganisms such as *Arthrobacter globiformis* (Hasegawa *et al*., 1972), *Pseudomonas* 321-18 (Hasegawa *et al*., 1974b) use limonin as single carbon source. The *Corynebacterium fascians* (Hasegawa and King, 1983) which metabolize limonoids through 17-dehydrolimonoid pathway has been isolated from soil by enrichment with 3-furoic acid, but it also uses trans-19-hydroxyobacunoic acid pathway (Hasegawa and Bennett, 1983). Bac-

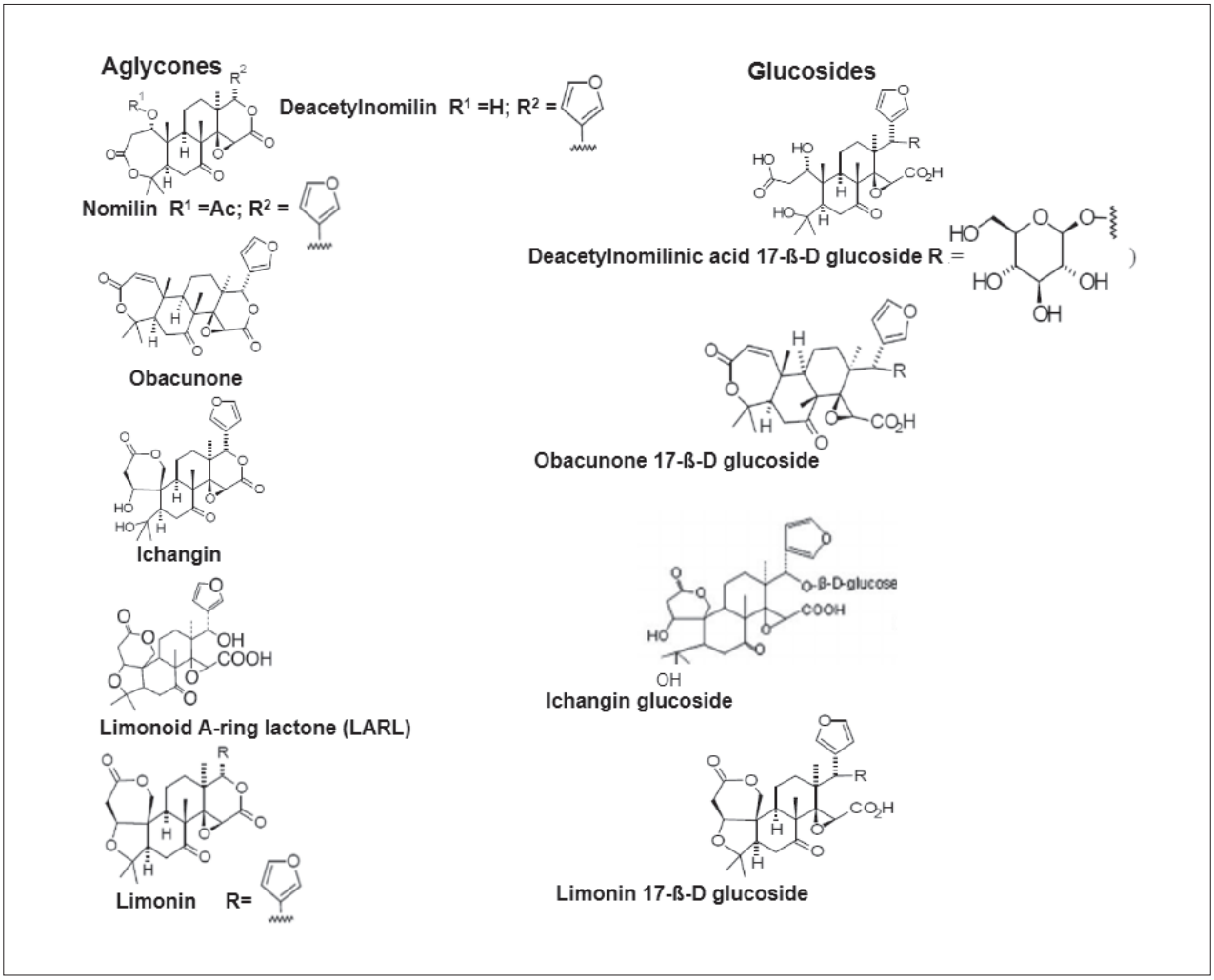

Figure 3. Molecular structures of citrus aglycones and glucosides. Source: Gualdani *et al.* (2016).

teria such as *Rhodococcus fascians* (Martinez-Madrid *et al*., 1989) and *C. fascians* offer an advantage as they produce constitutive enzymes for limonin degradation without utilizing exogenous limonoid as an inducer (Hasegawa *et al*., 1980). Another bacterium, *Arthrobacter globiformis II*, metabolizes bitter limonin to another non-bitter metabolite like limonol when the juice is treated on a column packed with immobilized cells (Hasegawa *et al*., 1983). Bacteria such as *Acinetobacter* spp. has been immobilized in dialysis sacs for debittering the citrus juices (Vaks and Lifshitz, 1981).

Characteristics of limonoid metabolism enzymes and genes

Several limonoid metabolism enzymes have been described in plants and from microorganisms. In limonoid biosynthesis pathway two major regulatory enzymes are there; one is limonoid-A-ring lactone hydrolase (LLH) and another is limonoid 17-β-D glucosyltransferase (LGT). Although, there is no information available on the characterization of LLH except its pH-dependent activity which has been discussed earlier in limonoid metabolism part of this review. But a lot of information is available on LGT. Among limonoid degrading enzymes such as limonin dehydrogenase which shows optimum activity at pH 8.0 and temperature 40 °C and requires zinc ions and sulfhydryl groups for catalytic action (Puri *et al*., 2002).

Limonoid glucosyltransferases belong to the family of glycosyltransferases. Glycosyltransferases are the enzymes involved in transferring the carbohydrate group from UDP-sugar to different acceptor molecules. Although, glycosyltransferases recognize a wide range of carbohydrate acceptor molecules and are involved in detoxification of biotic toxins, xenobiotics, herbicides, pesticides and pollutants (Bowles *et al*., 2005). But glycosyltransferases identified in *Arabidopsis thaliana* genome can recognize acceptors like auxins, cytokinins, salicylic acid, phenylpropanoids, trichlorinated phenol (Messner *et al*., 2003) and flavonoids (Lim *et al*., 2004). While some glycosyltransferases such as 1,2 rhamnosyltransferase are also involved in controlling the flavor related properties. Rhamnosyltransferases transfer rhamnose from uridine diphosphate-rhamnose to naringenin-7-*O* glucoside and thus producing bitter naringenin-7-*O*-neohesperidoside (Frydman *et al*., 2004). Some glucosyltransferases are involved in hypersensitive responses during defense mechanism against pathogens as evident by Tobacco glucosyltransferase (TOGT) (Fraissinet-Tachet *et al*., 1998). It has been reported that downregulation of these genes resulted in susceptibility of the plants to Tobacco Mosaic Virus (Chong *et al*., 2002) while their overexpression resulted in increased resistance against Potato virus Y (Matros and Mock, 2004). This shows that the products of plant glucosyltransferase may have antiviral properties (Tadeo *et al*., 2008). Glycosyltransferase enzymes have been classified into 91 distinct families (Wang and Hou, 2009) and over 30,000 glycosyltransferases are known in all kingdoms (Rini *et al*., 2009). In plants, these enzymes are localized in the cytoplasm (Ross *et al*., 2001). However, an enzyme like UDP-glucose: sterol glucosyltransferase has been found to be localized in the plasma membrane (Ullmann *et al*., 1993), vacuolar membrane (Verhoek *et al*., 1983) and golgi apparatus (Dupèron and Dupèron, 1987). These enzymes transfer glycosyl group to acceptor molecule and alter its properties. These enzymes have a conserved domain of 50 amino acids at their C-terminal end (Zaare-Nahandi *et al*., 2008) and this sequence is involved in transferring the sugar moiety to acceptor (Bowles *et al*., 2005). This domain is also called as plant secondary product glycosyltransferase (PSPG) box in family 1 glycosyltransferases (Hughes and Hughes, 1994). This conserved domain serves as a signature sequence as it helps in identifying the homologous sequences in other species and organisms (Veach *et al*., 2003; Grubb *et al*., 2004; Hou *et al*., 2004; Richman *et al*., 2005). The C-terminal domain interacts with UDP while N-terminal domain interacts with the acceptor molecule (Lim and Bowles, 2004; Wang and Hou, 2009).

Plant GTases are reported to have a molecular weight in the range of 40 to 60 kDa. LGT from 'Satsuma' mandarin has been purified with a molecular weight of 57.5 kDa. It possesses two conserved domains; one is registered in PROSITE database as a glucosyltransferase signature sequence. UDP-glucosyltransferase signature site was detected at 341st amino acid and *N*-linked glycosylation sites were found at 54th and 363rd amino acid (asparagine) of the mature protein (Kita *et al*., 2000). The signature sequences serve as a site for UDP-glucose binding and possess 43 to 68% identity in amino acid sequences to earlier reported glucosyltransferases from other species. Second conserved domain at the amino terminal end has 30 to 45% identity with other plant glucosyltransferases (Moehs *et al*., 1997; Kita *et al*., 2000). However, excluding these above-conserved regions, all other amino acid sequence in LGT is unique and did not show any similarity to the registered plant and animal glucosyltransferases.

LGT from albedo tissues of navel orange has been reported to have a molecular weight in the range of 56-58 kDa (Hasegawa *et al*., 1997). LGT from albedo tissues of pummelo has also been characterized biochemically. The enzyme is composed of the single polypeptide chain, with molecular weight 55 kDa similar to flavonone glucosyltransferase from *Citrus paradisi* seedlings (McIntosh *et al*., 1990). Its activity is optimum at pH 7.8 which is similar to *Citrus paradisi* flavonone glucosyltransferase, navel orange limonoid GTase and indoxyl-UDPG-glucosyltransferase (McIntosh *et al*., 1990; Hasegawa *et al*., 1997). Also, temperature optimum for this enzyme activity was reported at 37 °C which is similar to that of naringenin UDP-glucosyltransferase from grapefruit seedlings (McIntosh and Mansell, 1990). Further, Mn^{2+} (5 mM) and Co^{2+} (5 mM) are shown to increase the activity of pummelo LGT, while Cu^{2+} and Hg^{2+} are reported to inhibit the enzyme activity (Karim and Hashinaga, 2002). Inhibition of activity by these two ions suggests that thiol, carboxyl groups or histidine residues are directly or indirectly involved in the catalytic mechanism of this enzyme (Kundu and Das, 1970). Further, evidence for histidine and tyrosine as catalytic residue is provided by the inhibitory effect of amino-acid modifying agent diethylpyrocarbonate. Similarly, inhibitory effect of Citraconic anhydride suggests the lysine or arginine may be involved in catalytic activity of pummelo LGT (Karim and Hashinaga, 2002). Based on the crystal studies of several UDP-glycosyltransferase (UGTs), it has been observed that their N and C terminal domains possess similar Rossman-type folds which are involved in interaction with donor and acceptors (Shao *et al*., 2005).

Earlier, Owens and Mcintosh (2009) identified a flavonol 3-*O*-glucosyltransferase clone from *Citrus paradisi* by utilizing the primers designed against a predicted flavonoid glucosyltransferase gene (AY519364) from *Citrus sinensis*. This encoded *C. paradise* protein was 51.2 kDa with a predicted pI of 6.27. Later, it was clear that the putative glucosyltransferases were not constitutively expressed and there were variable degrees of putative natural product glucosyltransferase (PGTs) expression between different tissues and stages of development of *Citrus paradisi* (Daniel *et al*., 2011). Recent-

ly, nine putative natural PGTs from *Citrus paradisi* have been identified and their full length coding regions were recombinantly expressed in *Escherichia coli*. These PGT proteins then tested for activity with suitable substrates including flavonoids, simple phenolic, coumarin, and/or limonoid compounds as well (Devaiah *et al*., 2016).

In limonoid biosynthesis pathway, genes encoding LGT have been reported so far from different *Citrus* spp. These include 1.7 kb gene sequence from 'Satsuma' mandarin (*C. unshiu*) (AB033758.1), 1.5 kb sequence each from navel orange (*C. sinensis*) (EU531465.1) (Kita *et al*., 2000, 2003), sour orange (*Citrus aurantium*) (EU531466), lime (*C. limettioides*) (EU531463.1) (Zaare-Nahandi *et al*., 2008) and pomelo (*Citrus maxima*) (EU304828). Glycosyltransferases family genes generally contain very less number of intron or no introns at all. The *LGT* genes possess no intron and exist as a single copy in the citrus genome (Kita *et al*., 2000). Similarly, Zeatin *O*-glucosyltransferase from *Phaseolus lunatus* is lacking intron (Martin *et al*., 1999). But in *Arabidopsis thaliana* glycosyltransferase genes contain maximum two introns, but the majority of GTases contain no introns at all (Ross *et al*., 2001). Kita *et al*. (2003) demonstrated two alleles (CitLGT1/CitLGT2) of *LGT* at a single locus which are related to the delayed bitterness in citrus. The navel orange is homozygous for CitLGT1 while 'Satsuma' mandarin is heterozygous possessing both alleles (CitLGT1/CitLGT2). The CitLGT2 differs from CitLGT1 by 15 nucleotide substitutions and these substitutions are scattered throughout the coding region of the gene. Also, the resulting amino acid sequence differs only by 12 amino acids and thus, 3 out of 15 nucleotide substitutions do not affect the translation product.

Recently our lab at Punjab Agricultural University Ludhiana has also cloned 1533 bp full-length intronless *LGT* gene from fruit tissue of 'Kinnow' mandarin (*C. reticulata* Blanco) (KP306791) and it has been shown to translate the largest ORF encoding 211 amino acids. Upon BLAST homology search it has resulted into 98% identity with already reported *LGT* gene sequences from citrus. This *LGT* gene sequence is 200 bp shorter than the earlier reported *LGT* from 'Satsuma' mandarin. Comparison with other *LGT* nucleotide sequences, it showed several indels (Arora *et al*., 2018).

Tissue-specific expression of limonoid glucosyltransferase gene(s) in citrus

LGT is one of the important regulatory enzymes of limonoid metabolic pathway in *Citrus* spp. So, the expression pattern of its encoding gene has been determined by reverse transcription-PCR and northern blot analysis in many *Citrus* spp. Expression of *LGT* is variable in different tissues of different *Citrus* spp. *LGT* gene transcript starts accumulating first in juice sac/pulp segment 130 days after flowering (DAF) and then in albedo (190 DAF) in navel oranges (Kita *et al*., 2000). As the fruits start reaching maturity stage, *LGT* transcript accumulation also increases (Kita *et al*., 2000) and similar is the case with limonin 17-β-D glucopyranoside (LG) accumulation (Herman *et al*., 1991). Thus, there exist parallelism between developmental stage, *LGT* transcript expression, and LG accumulation. However, *LGT* transcript accumulation occurs at a very young stage (40 DAF) in flowers and fruits but LG is not detected in these parts of navel orange. This was due to the conversion of LG to limonin by glucosidase (Hasegawa and Ifukul, 1994). Further, LG once synthesized cannot be translocated to any other tissue part. Limonoid aglycones are also converted to their respective glucosides in seeds and mature fruit (Herman *et al*., 1991; Fong *et al*., 1992).

There are two alleles for CitLGT and their expression pattern has been studied in navel orange and 'Satsuma' mandarin. In navel orange fruits, CitLGT1 transcript starts expressing 150 DAF while no expression was observed of CitLGT2. While in 'Satsuma' mandarin (with no delayed bitterness problem) CitLGT2 transcript accumulates at all stages of fruit development. Thus presence or absence of CitLGT2 serves as a molecular indicator for determining the level of accumulation of LG and may reflect ultimately the delayed bitterness (Kita *et al*., 2003).

Later on, Zaare-Nahandi *et al*. (2008) analyzed the expression pattern of *LGT* in leaves and albedo of different *Citrus* spp., including navel oranges and 'Satsuma' mandarin. In 'Satsuma' mandarin *LGT* starts expressing very early (60 DAF) in albedo tissue. While in leaf tissues it expresses somewhat late, *i.e.*, 120 DAF. In *Citrus aurantium* and *C. sinensis* transcript expression starts from 120 DAF in albedo tissue while in their leaves it starts expression 180 DAF. In *C. limettoides* and *C. paradisi* (which are very sour in nature), *LGT* expression starts very late 180 DAF in leaves and 210 DAF in albedo. Hence, this finding suggested that the delayed bitterness arises due to the delay in expression of *LGT* in different tissues of *Citrus* spp.

Strategies for limonin reduction

Bitterness due to limonin has been the major problem that affected the citrus industry worldwide. Several physiochemical and enzymatic attempts have been made to reduce the bitter limonin content in different citrus juices. The use of polyamides (Griffith, 1969) for 'Washington navel' orange, adsorbents (Johnson and Chandler, 1988) for grapefruits, soluble 0.5% β-cyclodextrin for grapefruits (Konno *et al*., 1982), polystyrene-DVB resins for grapefruits (Manlan *et al*., 1990), Amberlite XAD-16HP and Dowex-L-285 and polyvinyl chloride beads for 'Washington navel' orange (Fayoux *et al*., 2007; Kola *et al*., 2010) have been attempted. Kaushal and Thakur (2001) demonstrated the use of adsorbent Amberlite XAD-16 packed in glass column to debitter the 'Kinnow' orange juice. Aggarwal and Sandhu (2004) have studied the effect of four different hydrocolloids such as carboxymethylcellulose, gum acacia, pectin and sodium alginate on reducing limonin content of 'Kinnow' mandarin juice. Application of ethylene (20 pg mL^{-1}) (Maier *et al*., 1973) and carbon dioxide under pressure (Kimball, 1987) have also been reported to reduce the limonin bitterness in navel orange, grapefruit and lemon fruits up to some extent. In addition, several bitterness suppressing agents such as sucrose, citric acid, neohesperidin dihydrochalcone, hesperidin dihydrochalcone, aspartylphenylalanine methyl ester and neodismin have been added in citrus juices to avoid from bitterness. But all these methods are non-specific in nature, lack reproducibility, economical viableness, ease of operation, and change the chemical composition of juices and remove the nutrients and also affect the flavor of the juices (Puri *et al*., 1996, 2008). An attempt has also been made to develop an economic process using debittering resin to reduce the bitter component limonin from sweet orange juice (Siddiqui *et al*., 2013).

Secondly, several soluble enzymes or immobilized enzymes from different microorganisms have been isolated and were employed for debittering the citrus juices. In the 1970's and 1980's, efforts have been done on utilizing soluble, immobilized enzymes such as limonin dehydrogenase, deoxylimonin hydrolase, limonol dehydrogenase from bacteria like *Arthrobacter globiformis*, *Pseudomonas*, *Corynebacterium*

(Hasegawa *et al*., 1985), *Acinetobacter* sp. (Vaks and Lifshitz, 1981; Puri, 1993) and fungi like *Aspergillus* and *Penicillium* (Puri, 1993) capable of metabolizing limonin and naringin. Iborra *et al*. (1994) entrapped *Rhodococcus fascians* cells in the k-carrageenan matrix to use it in continuous stirred reactor to degrade the limonin. Debittering the 'Kinnow' juice using enzymatic method is rare except immobilized *Arthrobacter globiformis* (Premi *et al*., 1995) and *Rhodococcus fascians* cells (Marwaha *et al*., 1994). Although debittering enzymes by microbes preserve the natural color, the flavor of juices and are cost effective and energy saving but due to the use of unclarified juice, the permeability of limonin and naringin or their limited solubility under operational conditions seem to be affected. Also, clogged columns and drop in pressure necessitated the need of molecular approaches for debittering the citrus juices (Puri *et al*., 2002).

The third approach is genetic engineering, where different strategies can potentially be used to reduce the limonin content. All above methods require the juices to be extracted from citrus fruits. But as soon as the citrus fruit is physically damaged, the endogenously present inactive LLH enzyme is activated at prevailing acidic pH and juice becomes bitter (Maier *et al*., 1969). Thus, all the earlier debittering approaches seem to be less effective. But by metabolic engineering of limonoid biosynthetic pathway limonin formation can be reduced or prevented altogether in the citrus plant itself. However, molecular cloning of gene(s) related to the limonoid biosynthetic pathway in citrus was not started before 2000. In this context, recently, we have cloned an important regulatory 1533 bp full-length *LGT* gene (KP306791) from fruits of 'Kinnow' mandarin which could be utilized for producing limonin free citrus plants. Metabolic engineering of limonoid biosynthesis can be attempted by two ways: Cloning of *LLH* gene and its silencing through RNA interference, and/or cloning of *LGT* gene and its overexpression in citrus plants itself. But here, the later strategy seems to have a greater advantage (Arora *et al*., 2016). Because, in addition to the reduction in bitterness, overexpression of *LGT* gene will also increase the specific limonoid glucoside molecules which are effective anticancerous agents and may serve as important neutraceuticals (Mohanpuria *et al.*, 2015). Thus, transgenic citrus free from delayed bitterness could be created. To avoid from biosafety issues, Cis-genic approach where the native promoter of *LGT* could be used in plant transforming marker-free vector construction. Earlier, Endo *et al.* (2002) reported the transformation of a cDNA encoding LGT in callus cells of citrus which results in the production of limonoid glucosides.

Further, limonoid degradation pathway mediated by hydrolase and dehydrogenases is known in bacteria, fungi, and yeast (Hasegawa *et al*., 1985; Puri, 1993), such gene(s) from either a microorganism or plants can be overexpressed in citrus to enhance the limonin degradation. However, the limonoid degradation pathway and its enzymatic steps have not been elucidated clearly in citrus so far. Only the presence of limonoid dehydrogenase in navel orange albedo tissue (Hasegawa *et al*., 1974a), LLH in citrus seeds (Hasegawa, 1976), and limonoid 17 β-D-glucopyranoside β-glucosidase has been reported in germinating and dormant citrus seeds (Ronneberg *et al*., 1995; Berhow *et al*., 2000). Nevertheless, no gene encoding these limonoid degradative enzymes has been reported from citrus so far.

Feasibility of citrus improvement through genetic modifications

Citrus is highly nutritious and an industrially important fruit crop of the world, and there is a major thrust in its improvement because of international market competition, disease, pest pressure and several abiotic and biotic stresses (Dutt and Grosser, 2010). But the genetic improvement of citrus through conventional breeding is a difficult task (Gong and Liu, 2013). *Citrus* has several inherent limitations like large plant size, nucellar polyembryony, apomixes, long juvenile period (which may extend from 5 to 21 years), high heterozygosity and sexual incompatibility in terms of pollen or ovule sterility and thus, transgenic technology has potential to improve its nutritional quality and maintaining its yield (Singh and Rajam, 2009; Gong and Liu, 2013). In addition, due to the lack of sufficient knowledge about the pattern of inheritance of horticultural traits breeding efforts in citrus cultivars have been greatly affected. With the advances in transgenic techniques, it has become feasible to introduce novel characteristics in the plant genome. But for efficient plant regeneration, an optimized tissue culture system is very important. In this context, micropropagation has gained popularity in *Citrus* to obtain a large number of genetically identical, physiologically uniform and developmentally normal plantlets (Singh, 2002). Thus, it overcomes several constraints and can increase citrus fruit quality and disease resistance. Once the regeneration conditions are standardized they can subsequently be used for transformation experiments successfully.

Citrus has immense potential for genetic improvement because a number of scientists have worked successfully on its tissue culture and transformation. Genetic transformation of *Citrus* is a promising tool that enables the introduction of desirable traits without altering the genetic background. It has been noted that citrus tissue culture is highly genotype-dependent (Gutiérrez *et al*., 1997). Singh *et al.* (1994*)* propagated *Citrus reticulata* Blanco cv. Khasi mandarin and *Citrus limon* cv. Assam lemon under *in vitro* conditions. Multiple shoots were obtained from shoot tips when two *Citrus* spp. were cultured on MS medium supplemented with BAP (1.0 mg L^{-1}), kinetin (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}). In addition, root induction was observed when 7-weeks-old single shoots (2 cm long) of both the species were cultured on MS medium supplemented with BAP (0.25 mg L^{-1}), NAA (0.5 mg L^{-1}) and IBA (0.5 mg L^{-1}). Mohanty *et al.* (1998) micropropagated *Citrus sinensis* cv. Mosambi using nutrient liquid medium composed of MS and MT (Murashige and Tucker) basal medium supplemented with different concentrations of vitamins and plant growth regulators. The average number of shoots developed was found the maximum in medium supplemented with BAP and IAA and root development was found optimum in medium containing NAA + IBA. Kumar *et al*. (2001a, b) studied *in vitro* plant regeneration in 'Kinnow' mandarin and sweet orange cv. Mosambi using epicotyls segments obtained from *in vitro* grown nucellar seedlings.

Optimization of transformation efficiency, reproducibility, and regeneration are very critical factors for successful transgenesis in any crop. Although, citrus is recalcitrant to *Agrobacterium*-mediated transformation (Spolaore *et al*., 2001) but the use of healthy explants, suitable selection marker genes and co-cultivation conditions, composition of culture media and, most importantly, super-virulent strains of *Agrobacterium* (mostly EHA105) (Cervera *et al*., 1998) ensure production of transgenic plants in good numbers.

Success of citrus transformation has been evident from the attempts on several species and hybrids, including *Carrizo citrange* (Moore *et al*., 1992), *Poncirus trifoliate* (Kaneyoshi *et al*., 1994), 'Washington navel' orange (Bond and Roose, 1998), Mexican lime (Peña *et al*., 1997), grapefruit (Luth and Moore, 1999; Yang *et al*., 2000), sour orange (Gutiérrez *et al*., 1997), sweet orange (Yu *et al*., 2002), pomelo (Yang *et al*., 2006), swingle citrumelo (a very popular rootstock in the US and Brazil) (Molinari *et al*., 2004) and *Citrus reticulata* (Khawale *et al*., 2006).

Genetic transformation of citrus through *Agrobacterium* has been attempted by several workers using different explants such as seeds, epicotyls (Kaneyoshi *et al*., 1994; Luth and Moore, 1999; Yang *et al*., 2000; Almeida *et al*., 2002; Yu *et al*., 2002), embryogenic cells (Yao *et al*., 1996), nodal and internodal stem segments (Moore *et al*., 1992; Chávez-Vela *et al*., 2003) and callus (Hidaka *et al*., 1990). Out of this epicotyl of *in vitro* germinated citrus seedlings are the most responsive explant and thus, it has been widely used in transformation experiments (Moore *et al*., 1992). In addition, there are few studies performed by direct gene transfer methods such as biolistic and electroporation in citrus. There are no published reports of transgenic plant regeneration through biolistic transformation in citrus. Only Wu *et al*. (2016) have attempted biolistic transformation of *in vitro* derived epicotyl explant of 'Carrizo' rootstock and succeeded in producing transgenic shoots. Subsequently, hardening and rooting of these citrus microshoots was found difficult, what is frequent in woody plants. Recently produced 'Carrizo' transgenic shoots were successfully micrografted onto immature 'Carrizo' rootstocks (Wu *et al*., 2016). Micro-grafting seems to be a very good technique to observe scion and stock influence for any desirable characters and serves important way to shorten the total time required and to avoid several limitations in woody plants for varietal improvement through transgenic approach.

Electroporation is also an effective direct gene transfer system used for *Citrus sinensis* L. Osb. (Fleming *et al*., 2000; Niedz *et al*., 2003) and *Citrus reticulata* Blanco (Hidaka and Omura, 1993) transformation where protoplast was used as an explant. Somatic cell hybridization through protoplast fusion has also been used as an integral part of citrus variety improvement worldwide in order to overcome citrus reproductive biology complications (Grosser *et al*., 2000; Khan, 2007).

In addition to all above studies, transformation for gene silencing purposes was also attempted in citrus. The sweet orange was transformed with intron-containing hairpin RNA (ihpRNA) construct using Citrus psorosis virus (CPsV), *54K* and *24K cp* genes and resulted transgenic plants showed a high level of virus resistance (Reyes *et al*., 2011). In another report Mexican lime (*Citrus aurantifolia*) was transformed with ihpRNA construct containing full-length untranslatable versions of *p20*, *p23* and *p25* genes (which code for silencing suppressor proteins to overcome the host antiviral defense) from Citrus tristeza virus (CTV) strain T36 to silence these genes in CTV-infected cells. The resulted three transgenic lines showed complete resistance to viral infection (Soler *et al*., 2012).

Recently, genome editing technique which is very popular and powerful tool for crop improvement, has also been implemented to produce disease-resistant citrus plants. First successful targeted genome editing was attempted in sweet orange by employing *Xanthomonas citri* ssp. citri (Xcc)-facilitated agroinfiltration to deliver the Cas9, along with single guide RNA (sgRNA) which targeted the *CsPDS* gene (encode for phytoene desaturase) with a mutation rate of 3.2–3.9% without any off-target effects (Jia and Wang, 2014). Later on, Jia *et al.* (2017) also employed the CRISPR/Cas9/sgRNA technology in 'Duncan' grapefruit to modify the canker susceptibility gene (*CsLOB1*), a member of Lateral Organ Boundaries Domain gene family of plant transcription factors for disrupting its portion of a 1st exon in both the alleles. This had resulted in six independent lines with a maximum mutation rate of 88.79 to 89.36% in two lines which did not develop any typical canker symptoms. Thus, the CRISPR technology can provide a promising way to generate several stable, desirable disease-resistant citrus varieties in future.

Conclusion

This manuscript is basically an overview of the citrus limonoids, its biosynthesis and degradation, mechanisms of anticancerous effects and feasibility of citrus improvement through genetic engineering and CRISPR/Cas9 technique. We have tried to show the health benefits of natural bioactive compounds, especially limonoids, which are present in a very high amount in citrus seeds and peel. The extractions of these citrus limonoids have commercial value. Thus, why the waste/byproduct left in the citrus juice industry should not be utilized efficiently to separate these bioactive compounds to use them as nutraceutical and functional food for human health and also as an important ingredient of feedstuff for our livestock?

Despite to the health benefits, citrus limonoids (limonin) are also the cause of delayed bitterness problem and thus is important for citrus fruit industry. As the natural de-bittering enzyme encoding *LGT* gene of limonoid biosynthesis pathway has already been isolated from different *Citrus* spp. even from 'Kinnow' mandarin (Arora, 2016), we think for the metabolic engineering of citrus limonoids by overexpressing *LGT* gene and/or silencing of *LLH* gene in citrus through RNAi. However, the key questions arises that what will happen when we silence or modify the *LLH* gene through CRISPR/Cas9 or RNAi? Also, will there be any effects on citrus limonoid biosynthesis or not? In addition, the enhanced expression of *LGT* only in citrus fruits juice sac is really a challenge. The possibility is there to produce transgenic citrus, free from delayed bitterness along with enhanced specific limonoid glucosides molecules which have been known effective against several types of cancers and diseases in humans. The citrus limonoid glucosyltransferase can serve as a key player for natural de-bittering and anti-cancerous potential.

Citrus fruits are a rich and cheap source of various health promoting bioactive compounds especially in the Southeast Asia region. Citrus consumption 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 to utilize them as most important component of today's healthy human diet. A successful citrus metabolic engineering is important to enhance the quality of citrus fruits for good human health.

Conflicts of interest

The authors declare no conflicts of interest.

Authors' contributions

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

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