

# Molecular approach to citrus flavonoid and limonoid biosynthesis

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Received 5 September 2002, accepted 3 January 2003.

## Abstract

Citrus fruits are rich in secondary metabolites such as carotenoids, flavonoids and limonoids. Recent studies show that these secondary metabolites possess potential health benefits for humans, including anti-inflammatory and anti-cancer properties. Due to these properties, biochemical studies of citrus secondary metabolites have been intensively investigated. However, our knowledge on the molecular aspects of the biosynthesis of these compounds has been limited. Some of the genes involved in the flavonoid and limonoid biosynthesis have been isolated from citrus. In this review, we report on the expression of flavonoid- and limonoid-biosynthetic genes in citrus towards pharmacological applications and breeding using a molecular technique.

**Key words:** Bitterness, citrus, flavonoid, gene expression, limonoid.

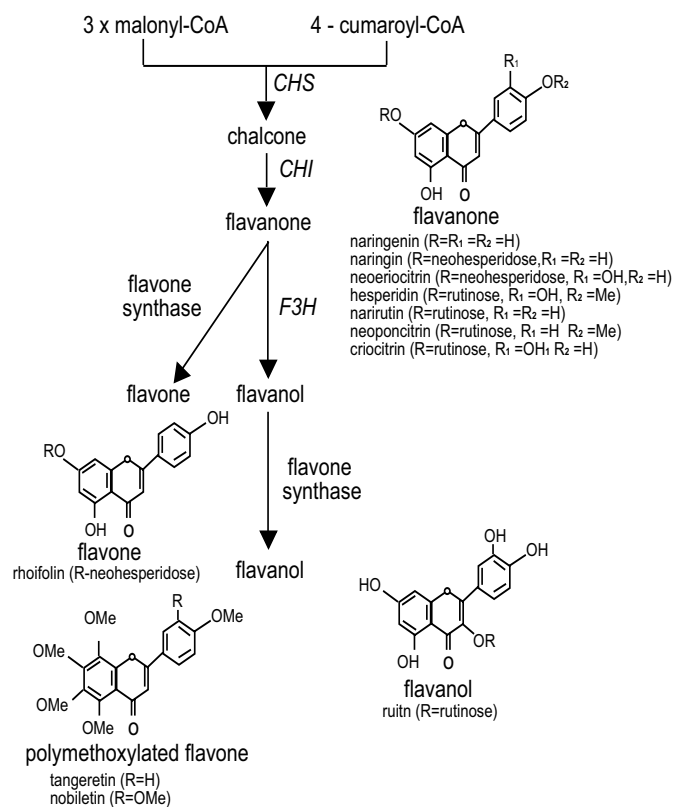
## Introduction

Citrus fruits are one of the important horticultural crops, with worldwide agricultural production over 100 million metric tons per year. They supply humans with many constituents, such as simple sugars, vitamin C, carotenoids, flavonoids, limonoids, fiber, folic acid and potassium, which have important effects on health. Almost half of the citrus fruits produced are processed into juices, concentrates, jams and other food products. Bitterness has been one of the problems in citrus products because bitterness reduces the quality and value of citrus juices and thus has a significant negative impact on the citrus industry. Bitterness in citrus fruits and juices is mainly caused by two different phytochemical families: flavonoids and limonoids. Limonoid bitterness occurs gradually in certain varieties of winter citrus after juice processing, which is referred to as "delayed bitterness"<sup>21</sup>, and in the fruits after freezing or mechanical damage. On the other hand, bitter flavonoids are accumulated in the fruit tissues of species related to pummelo<sup>10</sup>. Pharmacological investigations have shown that certain citrus flavonoids and limonoids have potential health benefits - they possess anti-inflammatory and anti-cancer properties. Flavonols such as rutin accumulate to relatively high levels in citrus leaves and fruits have been shown to be efficient nitric oxide scavengers, a property that ameliorates nitric oxide-induced tissue damage<sup>8</sup>. A recent study indicates that nobiletin, a polymethoxylated-flavone which is accumulated in Shiikuwasha fruits (*C. depressa* Hayata), exhibits anti-carcinogenic activity. Citrus limonoids have been also shown to induce detoxification enzymes such as glutathione S-transferase and quinone reductase and to inhibit the formation of chemically induced neoplasia in the forestomach, small intestine, colon, lung, skin and oral cavity of laboratory animals<sup>18, 22</sup>, and of human breast cancer cells in culture<sup>9</sup>. They also exhibit anti-feedant activity against insects<sup>1</sup>. Although many studies on flavonoid and limonoid biosynthesis in citrus have been done from the biochemical point of view<sup>9, 10</sup>, molecular studies have been limited in spite of their importance. Within the past few years, the genes encoding the key enzymes required

for the biosynthesis of flavonoids and limonoids in citrus have been identified. In this review, we describe recent findings regarding the expressions of genes involved in the biosynthesis of flavonoids and limonoids in citrus fruit.

## Citrus Flavonoids

**Features of flavonoid pathways in citrus:** Chalcone synthase (CHS) is the first enzyme in the biosynthesis of all classes of flavonoids in plants (figure 1). It catalyzes the stepwise condensation of three acetate residues from malonyl CoA with *p*-coumaroyl CoA. The latter *p*-coumaroyl CoA is supplied from the phenylpropanoid pathway, which converts phenylalanine into a myriad of phenolic secondary metabolites in plants. Naringenin chalcone, the product of the CHS reaction, is then converted into a flavanone form by an intra-molecular reaction in which the C-ring is closed by the enzyme chalcone isomerase (CHI). These two forms of naringenin -- the chalcone form and the flavanone form -- appear to be the precursors for all the myriad compounds produced by plants with this related structure. By 3 $\beta$ -hydroxylation, flavanone 3 $\beta$ -hydroxylase (F3H) catalyzes the conversion of (2*S*)-flavanones to (2*R*, 3*R*)-dihydroflavonols, which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins. Citrus species are of great interest because they accumulate large amounts of flavonoids, especially flavanone glycosides<sup>15</sup>. Flavanone is probably modified in a stepwise fashion to the various derivatives by hydroxylation, methylation, glucosylation and then rhamnosylation<sup>20</sup>. The most common glycosidic group attached to the flavonoids in citrus is rhamnose-glucose diglycoside. This sugar group is present in two isomeric forms, neohesperidose and rutinose. The isomers differ only in the position at which rhamnose is attached to glucose<sup>10</sup>. Flavanone neohesperidosides are accumulated (sometimes along with the rutinoides) in citrus species related to the pummelo, such as grapefruit, sour orange and natsudaidai, and they give a bitter taste to citrus fruits<sup>15, 27</sup>. Major flavanone neohesperidosides detected in these species are naringin, poncirin, neoeriocitrin and neohesperidin. Other species related to citron and mandarin



**Figure 1.** Biosynthesis of flavonoids in citrus (partially modified from Moriguchi et al.,<sup>24</sup>). Major flavonoids accumulated in *Citrus unshiu* are also indicated.

orange accumulate the tasteless flavanone rutinosides narirutin and hesperidin<sup>25</sup>. Interestingly, the major flavonoids accumulated differ according to the tissues. For example, fruits of satsuma mandarin (*Citrus unshiu* Marc.) intensively accumulate hesperidin and narirutin, whereas leaves accumulate hesperidin and rutin instead of narirutin<sup>25</sup>.

**Biochemical studies of flavonoid biosynthesis:** The flavonoid pigments of plants have been intensively investigated, and flavonoid pigments provide good taxonomic markers because of their diversification from species to species. In addition to a taxonomic interest, bitterness is a major problem for the citrus industry due to the lower market value of bitter juice. Biochemical investigations of the accumulation patterns of flavonoids have been done. High levels of the flavanone glycoside naringin are associated with very young tissue, and lower levels are found in older tissues in grapefruit (*C. paradisi* Macf., cv 'Duncan')<sup>15</sup>. Radioactively labeled acetate and phenylalanine fed to detached immature grapefruit fruits resulted in the accumulation of labeled naringin<sup>4</sup>. Similar experiments using intact and detached tissues of grapefruit seedlings showed that naringin is synthesized in young, rapidly growing leaves and then is transported to other parts of the plants. The radiolabeled naringin is not synthesized in detached stems and roots when fed labeled acetate<sup>5</sup>. Lewinsohn et al.<sup>19</sup> showed that grapefruit suspension cells are able to specifically *O*-glucosylate exogenous naringenin and hesperitin at position 7. They also proved that flavanones can be hydroxylated, methylated, glucosylated and then rhamnosylated in a stepwise fashion in cell-free extracts from citrus<sup>20</sup>. Collectively, these biochemical studies indicate that the biosynthesis of flavanone glycosides occurs constitutively during the cell stages of cell division and differentiation and not during

periods of cell elongation and subsequent maturation. This constitutive biosynthetic pathway is highly regulated in citrus. Another interest lies in identifying the genes expressed with the induction of somatic embryogenesis and the regulation of flavonoid production in citrus cell cultures. It has been generally reported that grapefruit suspension cells<sup>19</sup> and undifferentiated calli of grapefruit and orange<sup>2</sup> lose the ability to produce flavonoids. Lime (*C. aurantifolia* (Christm.) Swing.) cultures are an exception<sup>3</sup>. For the relationship between enzyme activities and anthocyanin production in carrot suspension-cell cultures, Gleitz and Seitz<sup>7</sup> reported two CHS forms with different molecular subunit weights and different isoelectric points, and they showed the differential accumulation of these proteins during the course of anthocyanin synthesis. Ozeki et al.<sup>26</sup> showed that CHS mRNA in carrot cells was induced when anthocyanin synthesis was initiated. In contrast, the amount of enzyme protein and the amount of mRNA for CHS were below detectable levels when anthocyanin synthesis was repressed<sup>26</sup>. These reports imply that the production of anthocyanin in carrot-cultured cells is regulated by CHS genes.

**Expression analysis of chalcone synthase, chalcone isomerase and flavanone 3 $\beta$ -hydroxylase during embryogenesis in citrus cell cultures or during citrus fruit development:**

Clones of two CHS (CitCHS1 and CitCHS2), one CHI (CitCHI) and one F3H (CitF3H) were isolated from the cDNA library of Valencia orange (*C. sinensis* Osbeck) seeds<sup>23,24</sup>. During citrus somatic embryogenesis<sup>23</sup>, both transcripts corresponding to CitCHS1 and CitCHS2 mRNAs were hardly detectable in the undifferentiated calli. When the calli were transferred onto the medium for the induction of somatic embryogenesis, the accumulation of transcripts corresponding to CitCHS2 and CitCHI mRNAs was induced and continued up to the formation of small green plantlets. Unlike CitCHS2 and CitCHI, the transcript corresponding to CitCHS1 mRNA hardly accumulated in either proliferation or the somatic embryogenesis process. Interestingly, the transcript for CitF3H was detectable even in the undifferentiated calli. Flavonoid contents were below the detection level in the undifferentiated citrus calli and were detected in greenish embryoids<sup>23</sup>. The major flavonoids detected in the greenish embryoids and in the leaf were neodiosmin and hesperidin, respectively, indicating the changes in the flavonoid metabolic pathway during morphogenesis. Thorpe et al.<sup>28</sup> reported that phenylalanine ammonia-lyase (PAL) activity is detected in grapefruit callus cultures. They also showed that although the callus possesses the ability to convert phenylalanine to cinnamic acid, there is no detectable formation of naringenin and its glycosides, unlike in intact grapefruit<sup>28</sup>. These results indicate that CitCHS2 may be a primary key enzyme of the flavonoid biosynthetic pathway for flavonoid accumulation in citrus cell cultures. Expression profiles in *C. unshiu* have been investigated using Northern blot analysis of a preparation of the total RNA of the albedo (inner spongy part of the rind), flavedo (outer part of the rind) and juice sacs/segment epidermis (edible part) from various stages of development, as well as from flowers (balloon stage) and young and mature leaves<sup>24</sup>. The transcript levels of CitCHS1, CitCHS2, CitCHI and CitF3H were high in young active tissues such as young leaves and fruitlets at 26 days after flowering (DAF),

decreasing and disappearing in senescent tissues and/or towards fruit development. Major flavanone glycosides were narirutin and hesperidin in the flowers, albedo, juice sacs/segment epidermis and flavedo of satsuma mandarin, accounting for more than 91% of the total flavonoids<sup>24</sup>. In both young and mature leaves, hesperidin and the flavonol rutin, instead of narirutin, mainly accumulated. The flavonoid contents in the flavedo and juice sacs/segment epidermis were highest in the young tissues, decreasing with ripening<sup>24</sup>. Therefore, taking into consideration the patterns of gene expression and flavonoid accumulation, flavonoids are synthesized in the early developmental stage of citrus as indicated by the previous biochemical studies.

### Citrus Limonoids

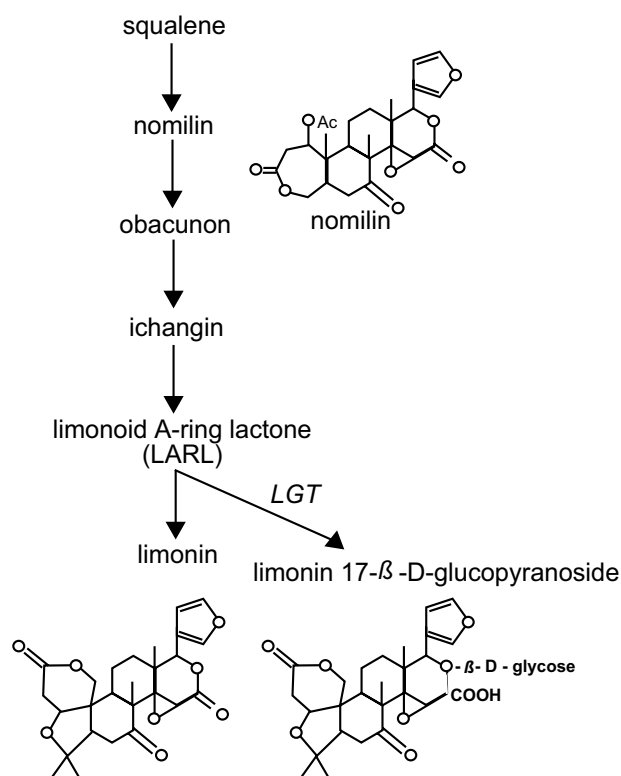
**Features of limonoid biosynthesis in citrus:** Limonoids are highly oxygenated triterpenes present in Rutaceae and other limited plants such as Meliaceae. Significant progress has been made in understanding where, when and how limonoids and limonoid glucosides are biosynthesized and accumulated. The citrus limonoids occur as limonoid aglycones and limonoid glucosides. So far, 36 limonoid aglycones and 17 limonoid glucosides have been isolated from citrus and its closely related genera<sup>12</sup>. These aglycones are classified into four taxonomic groups: the *Citrus* group (19 limonoids), the *Fortunella* group (12 limonoids), the *Papedocitrus* group (one limonoid) and the *Poncirus* group (four limonoids). The biosynthetic pathways of each of these groups have been elucidated based on radioactive tracer work, and the details have been reviewed recently<sup>9</sup>. Nomilin is most likely the initial precursor of all the known limonoids in citrus. Nomilin has been shown to be biosynthesized from acetate, mevalonate and/or farnesyl pyrophosphate in the phloem region of stems. This precursor then migrates to other tissues such as leaves, fruit tissues and seeds, where other limonoids are biosynthesized from this compound independently. Limonoid aglycones are converted to non-bitter 17 $\beta$ -D-glucopyranoside derivatives such as limonin 17 $\beta$ -D-glucopyranoside (LG) during maturation. This natural debittering process is catalyzed by the enzyme UDP-D-glucose: limonoid glucosyltransferase (limonoid glucosyltransferase) (figure 2). A single enzyme appears to be responsible for the glucosidation of all the limonoid aglycones to their respective glucosides. Limonoid glucosides are major secondary metabolites and accumulate in the fruit tissues and seeds in significant quantities. Limonoid glucosyltransferase (GTase) has been identified and chosen for possible genetic manipulation to create transgenic citrus fruit trees that have fruit free from limonin bitterness.

**Molecular cloning and expression analysis of limonoid glucosyltransferase:** Since debitterness is catalyzed by limonoid GTase<sup>13</sup>, the isolation of limonoid GTase is crucial for creating transgenic citrus free from limonoid bitterness as well as for increasing specific limonoid glucoside molecules having anti-cancer properties. As the first step, Kita et al.<sup>17</sup> isolated limonoid GTase based on the purified protein sequences of N-terminal and internal regions<sup>13</sup> and confirmed that the isolated limonoid GTase (CitLGT) possesses limonoid GTase activity using the recombinant fusion protein. They also showed that CitLGT is a single copy without introns by Southern blot analysis and the genomic sequence amplified by PCR. Using the cloned CitLGT, Kita et al.<sup>17</sup> showed that

CitLGT is transcribed strongly in the juice sacs/segment epidermis and albedo at the later developmental stage of navel orange fruit. In navel orange juice, the limonoid bitterness decreases as the fruit matures<sup>11</sup>. LARL is converted to nonbitter LG, which increases sharply in the juice sacs at the late stage of maturation<sup>14</sup>. Since the increase of the CitLGT transcript level is parallel to fruit maturation, the conversion of LARL to LG and mRNA accumulation could probably take place simultaneously in the juice sacs/segment epidermis. Furthermore, in albedo, the onset and increase of transcription occur simultaneously with LG production. No evidence of translocation of LG has been found in fruit tissues from other organs and the site of the metabolization of limonoid aglycon to glucosides is limited to the seed and mature fruit<sup>6,14</sup>. Therefore, the close correlation between mRNA accumulation and LG content in these tissues suggests that the transcription level of the CitLGT gene regulates the LG accumulation during citrus fruit maturation.

### Future Prospects

Bitterness is an important determining factor for the quality and values of citrus fruits and their processed products. Particularly, delayed bitterness due to limonin is still a major problem in the citrus industry worldwide. An Ongoing molecular biology study aimed at the creation of transgenic citrus fruit certainly provides a way to eliminate the bitterness problems. Significant progress has been made in understanding the biological activities of citrus flavonoids and limonoids in animal systems. Further comprehensive research to define the



**Figure 2.** Biosynthesis of limonoids in citrus with structures of the major limonoids.

role of these compounds in human health is necessary. Nonetheless, there already has been commercial interest in utilization of these compounds. Since the by-products of citrus juice processing contain high concentrations of these compounds, these by-products may represent new profitable commercial products for the citrus industry. Using the isolated genes, the mass production of the useful flavonoids and limonoids in *E. coli* or citrus cell culture systems could be expected. Overall, these genes would contribute not only to citrus breeding but also to pharmacological applications.

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