Indian J. Genet. Plant Breed., (2025); 85(3): 512-520
https://doi.org/10.31742/ISGPB.85.3.17
ISSN: 0975-6906
www.isgpb.org

RESEARCH ARTICLE

# Molecular insights to discover the nucleotide variations related to limonin-associated delayed bitterness in new interspecific citrus hybrids

**Narendra Singh[†], Radha Mohan Sharma[*], Anil Kumar Dubey[††], Om Prakash Awasthi, Nimisha Sharma, Supradip Saha[1], Chellapilla Bharadwaj[2], Amitha Mithra Sevanthi[3], Jogendra Singh[4], Mukesh Shivran and Amrender Kumar[5]**

## Abstract

In the present study, molecular analysis of limonoid-associated delayed bitterness was carried out in 16 newly developed inter-specific citrus scion hybrids (*Citrus maxima* Merr. × *Citrus sinensis* (L.) Osbeck) along with their parental genotypes. The Limonoid UDP-glucosyltransferase, a key enzyme responsible for debittering through glucosylation, was targeted for *de novo* primer design. However, PCR amplification using these primers could not decipher polymorphism based on the amplicon size among the 16 citrus hybrids and their parental genotypes. Therefore, five genotypes having contrasting bitterness properties (Low bitterness: SCSH-9-11/12, SCSH-11-9/13; high bitterness: SCSH-17-8/14 and parents White Fleshed Pummelo, and Mosambi) were selected for Sanger sequencing of PCR amplified products to decipher the variation at the nucleotide level. The analysis of variants and their annotation in the genomic region of the reference genome (*Citrus sinensis* (L) Osbeck) indicated a total of 19 missense variants corresponding to high limonin content and 12 missense variants for low limonin content. The transition to transversion ratio in the studied genotypes was found to be 0.83 and 0.29 for the high and low limonin groups, respectively. The changes in amino acids with respect to nucleotide variants in low limonin were identified. The identified nucleotide variations were exploited to design bitterness-specific primers in *citrus* sp., which serve as a reference SNV dataset. Further, this resource could be utilized to develop bitterness-specific markers for marker-assisted breeding in perennial citrus fruits.

**Keywords**: Delayed bitterness, Inter-specific hybrids, limonin, Nucleotide variant, Sweet orange.

## Introduction

The citrus fruits rank 2nd in terms of human nutrition, quantum of production (124.24 million tons), and global trade among the cultivated fruit crops (FAO 2020). In India, next to mango and banana, citrus fruits occupy the highest area of 1.07 million hectares with an annual production of 14.7 million tons (Anonymous 2023). Being a rich source of bioactive compounds such as organic acids, carotenoids, flavonoids, essential minerals, vitamins, and metabolites, citrus fruits have gained the scientific and commercial attention of researchers and growers in recent years. The health-promoting properties of citrus juice are contributed to by the biochemical constituents such as organic acids, minerals, vitamins, phenols, flavonoids, and limonoids (Peterson et al. 2006; Kruger et al. 2014; Huang et al. 2021; Raghavan and Gurunathan 2021). The simplest way to exploit the biological properties of these health-promoting compounds is through daily dietary intake with food; however, developing elite cultivars enriched in health-promoting bioactive compounds must consider the potential limitations of consumer acceptance, as these compounds are also known to impart bitterness, making the juice less desirable. Therefore, breeding novel genotypes with reduced bitterness properties is one of the key objectives in citrus crop improvement programs (Guadagni et al. 1973; Raithore et al. 2016; Gupta et al. 2023).

The bitterness caused by the limonin is known as delayed bitterness because the bitterness of the juice increases with time after the fruit is cut. The biochemical basis for delayed bitterness has been extensively studied and revealed that in intact fruit, it is present as a non-bitter form of Limonoate A-ring lactone (LARL), which slowly converts to the bitter limonin compound spontaneously under aerobic and acidic conditions (below pH 6.5), and also mediated by an enzyme, limonoid D-ring lactone hydrolase (Chandler and Kefford 1966; Maier et al. 1969; Rouseff 1982; Zaare-Nahandi et al. 2008; Kore and Chakraborthy 2015; Nishad et al. 2018; Pardo et al. 2021; Deterre et al. 2021). Several biochemical approaches have been applied to eliminate the delayed bitterness and produce an acceptable-quality juice;

however, developing genotype(s) with naturally reduced bitterness properties is the most sustainable method (Puri et al. 1996; Raithore et al. 2016; Gupta et al. 2023). The natural debittering process has been studied in different citrus species, and it was reported that glucosylation of limonoids leads to the formation of the non-bitter principle, which is catalyzed by limonoid UDP-glucosyltransferase (Limonoid GTase) enzyme (Fong et al. 1989; Karim and Hashinaga 2001; Zaare-Nahandi et al. 2008). Therefore, limonoid UDP-glucosyltransferase is identified as a key enzyme for citrus without limonoid bitterness and increases specific glucoside molecules. Enhancing non-bitter limonoid glucosides offers a sustainable approach to addressing delayed bitterness while improving health benefits, making them more suitable for the processing industry and consumers.

There is a need to develop citrus varieties with superior fruit quality (thin peel, high juice content, seedlessness, and enhanced aroma and flavour), enhanced health-promoting properties, and reduced bitterness. Along with traditional desirable traits such as citrus, breeders increasingly focused on high nutraceutical properties and reduced bitterness (Salonia et al. 2020; Anticona et al. 2022). Keeping in view, the citrus hybridization program has been initiated at the Division of Fruits and Horticultural Technology, ICAR-IARI, New Delhi, in which interspecific citrus hybrids have been developed between the pummelo (*C. maxima* Merr.) and sweet orange cv. Mosambi (*C. sinensis* (L.) Osbeck) with the aim to improve the nutritional value of citrus fruits, and low bitterness in the sweet citrus varieties.

Crop improvement in perennial fruit species is time-consuming and tedious due to the long juvenile phase, high heterozygosity, and large tree size, apart from several genetic barriers such as polyembryony, self and cross incompatibility, recalcitrant seed, etc. It may take 7 to 9 years to observe fruit characteristics after crossing/hybridization. To fasten the breeding program marker, assisted breeding holds promise for the future, with the translation of trait loci and whole-genome sequences into diagnostic genetic markers that are effective and affordable for use by breeders. The availability of genomic resources, advanced sequencing techniques, and analytical tools offered the opportunity to find sequence variants potentially useful as molecular markers (De Mori and Cipriani 2023). Among the different markers, single-nucleotide polymorphisms (SNPs) are becoming more widespread in the genomic era, and various technologies have been developed for SNP detection, most of which are also helpful for detecting small insertions/deletions (indels). They can be used to trace the induced/natural variant pedigree and tagging. Consequently, closely linked markers of specific traits can be used for MAS, pyramiding, and cloning of mutant genes (Lamo et al. 2017). Therefore, the present study aimed to find the single-nucleotide variations (SNVs) associated with limonin-associated delayed bitterness in newly developed citrus hybrids, which is a major problem in citrus juice processing and storage.

Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.

[1]Division of Agricultural Chemicals, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.

[2]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi, 110 012, India.

[3]ICAR-National Institute of Plant Biotechnology, Pusa Campus, New Delhi 110 012, India.

[4]Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.

[5]Agricultural Knowledge Management Unit, ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India.

[†]Present Address: School of Crop Science, ICAR-Indian Agricultural Research Institute, Hazaribag, 825 405, Jharkhand, India.

[††]Present address: ICAR-Central Soil Salinity Research Institute, Regional Research Station, Lucknow, 226002, Uttar Pradesh, India.

***Corresponding Author:** R.M. Sharma, Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute, New Delhi, India, E-Mail: rmsharma345@gmail.com

**How to cite this article:** Singh N., Sharma R.M., Dubey A.K., Awasthi O.P., Sharma N., Saha S., Bharadwaj C., Sevanthi A. M., Singh J., Shivran M. and Kumar A. 2025. Molecular insights to discover the nucleotide variations related to Limon in-associated delayed bitterness in new interspecific citrus hybrids. Indian J. Genet. Plant Breed., **85**(3): 512-520.

**Source of support:** Nil

**Conflict of interest:** None.

**Received:** Feb. 2025 **Revised:** July 2025 **Accepted:** Aug. 2025

## Materials and methods

### *Plant material*

The hybridization between the pummelo and sweet oranges was carried out during the flowering months (March) of 2012 and 2013 using the conventional method of emasculation followed by controlled pollination. Since the female parent pummelo is a typical monoembryonic species (Wu et al. 2018), all the progeny raised were interspecific hybrids. The hybrid progeny was established at 5 m × 5 m spacing at the experimental orchards of the Division of Fruits and Horticultural Technology, ICAR-Indian Agricultural Research Institute (IARI), New Delhi (28°64′ N and 77°15′ E; 228 m above the mean sea level). The trees were subjected to uniform management practices. The hybrid progeny started to fruit in the year 2016-17. Out of the large hybrid population (>400 hybrids), based on the physical, biochemical, and nutritional value, 16 superior hybrids (Table 1) were selected for processing traits in terms of limonin content (in the fresh and stored juice) and sensory evaluation as per the method described by Raithore et al. (2016). The limonin content

**Table 1.** Details of newly developed citrus hybrids and parents used for molecular study

| Genotypes | Pedigree |
|---|---|
| Parental Genotypes | |
| White Fleshed Pummelo | *Citrus maxima* Merr. |
| Red Fleshed Pummelo | *Citrus maxima* Merr.) |
| Sweet orange cv. Mosambi | *Citrus sinensis* (L) Osbeck |
| *Hybrid Genotypes* | |
| (1) SCSH-5-10/12, (2) SCSH-7-2/12, (3) SCSH-7-7/13, (4) SCSH-9-6/12, (5) SCSH-9-10/12, (6) SCSH-9-11/12, (7) SCSH-9-17/12, (8) SCSH-11-9/13, (9) SCSH-11-11/12, (10) SCSH-11-15/12, (11) SCSH-13-4/13, (12) SCSH-13-17/12, (13) SCSH-15-7/12, (14) SCSH-17-8/14, (15) SCSH-17-19/13 | White Fleshed Pummelo × Mosambi |
| (16) SCSH-9-2/12 | Red Fleshed Pummelo × Mosambi |

in fruit juice was analyzed using the advanced analytical technique UPLC-QTOF-ESI-MS at two stages, i.e., fresh juice and stored juice (24 hours of storage at 4°C).

### *DNA isolation and PCR amplification*

The total genomic DNA was extracted from the young leaves of each genotype (Table 1) using the method of Doyle and Doyle (1987). Agarose gel electrophoresis (0.8%) and a Nanodrop spectrophotometer were used to assess the purity and quantification of the DNA samples. The working standard concentration of DNA was adjusted to 30 ng/µl using MilliQ water, and the remaining stock genomic DNA was stored at -20°C for further use.

To study the molecular basis for limonin-associated delayed bitterness, the nucleotide sequence of Limonoid UDP-glucosyltransferase was accessed from the NCBI reference genome of sweet orange (*Citrus sinensis*) (*LOC*102630581; Gene ID: 102630581) (https://www.ncbi.nlm.nih.gov/gene/102630581) for the development of primers. A total of 9gene-specific primers were designed *de novo* using Batchprimer 3 software (www.frodo.wi/mit.edu/primer3). The details of primers and their sequences are presented in Table 2. The primers were designed to amplify the target gene and decipher the polymorphism among these 16 interspecific hybrids. Further, amplicon from five contrasting genotypes having low and high limonin-associated delayed bitterness were sequenced using the Sanger platform (Table 3).

### *Variant calling and annotation of sequenced fragments*

*Limonoid UDP-glucosyltransferase* [LOC102630581] gene sequence for *C. sinensis* organism present at chromosome 8 [NC_023053.1:c2698177-2696241] was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/gene/102630581) and indexed using BWA software (version 0.7.17). The BWA MEM algorithm mapped raw reads of selected samples to the above gene sequence. The output .sam files were converted to their binary format, i.e., bam, using samtools (version 1.11) and sorted based on coordinates. Variants were called for the individual samples after mapping them against the gene sequence using samtools (version 0.1.19) mpileup function using default parameters and bcftools (version 0.1.19).

Variant annotation was done using snpEff (version 4.3t, http://pcingola.github.io/SnpEff/ ). Genetic coordinates were converted to genomic coordinates for variant annotation using in-house Perl scripts. It annotates and predicts the effects of genetic variants on genes and proteins. Sniplay (https://sniplay.southgreen.fr/cgi-bin/home.cgi), a web-based software, was used to visualize basic statistics of variants. The root mean square mapping quality [MQ] over all the reads at the site was used to filter reliable SNVs.

The reference protein sequence for *limonoid UDP-*

**Table 2.** Details of primer sets derived from Limonoid UDP-glucosyltransferase gene sequences

| Primer | Forward | Reverse | Annealing temperature (°C) |
|---|---|---|---|
| NCLGS 1 | GTGATCACCTTCCCGCAATG | ATGTTCCTATCCGACGAGCC | 59.1 |
| NCLGS 2 | CCCTAGTGTTGCTTGCTTTGT | ATCCTGTTCTCTGCCTCTCC | 56.2 |
| NCLGS 3 | TGTTGCTTGCTTTGTGACTCA | GCACTTCTCCACTTCATCCC | 59.1 |
| NCLGS 4 | ATGCATTGTTGAACTCGGGG | CGATGCCAACGACTCCATG | 57.0 |
| NCLGS 5 | AAAGCCACCATCATCCGTTG | TGGACTCCATTGCACAACTT | 59.1 |
| NCLGS 6 | TGTTGAACTCGGGGATTTCG | CAAAGCAAGCAACACTAGGGT | 58.1 |
| NCLPS1 | TCTTGCATCCGTCAACTCCT | TTGTCGAGCCAGTCTATGCA | 58.1 |
| NCLPS2 | GCTATTTTGGGGCAGTACGA | CAACGGATGATGGTGGCTTT | 57.0 |
| NCLPS3 | TGGGGCAGTACGAAAATCTTG | TAGACAACCGTGCCGAAAGA | 57.6 |
| NCLOW2 | GGCTGGATAATCAGGCAAGC | ATTCCAAGCCTAGGGCCAAT | 59.1 |
| NCLOW3 | AAGCATTGTGCATGGTGTCC | TCGTTGACTTTGACCCTCCA | 56.2 |

**Table 3.** Limonin content and sensory profile of studied contrasting citrus genotypes

| Citrus hybrid/parents | Limonin content (ppm) | | Bitterness score | | Group |
|---|---|---|---|---|---|
| | Fresh juice | Stored juice | Fresh juice | Stored juice | |
| SCSH-9-11/12 | 0.32 ± 0.01 | 0.75 ± 0.01 | 1.20 ± 0.03 | 2.43 ± 0.04 | Low limonin |
| SCSH-11-9/13 | 0.00 ± 0.00 | 1.00 ± 0.03 | 1.23 ± 0.04 | 3.10 ± 0.06 | Low limonin |
| SCSH-17-8/14 | 1.23 ± 0.03 | 5.08 ± 0.06 | 1.73 ± 0.03 | 5.00± 0.08 | High limonin |
| White Fleshed Pummelo | 2.55 ± 0.05 | 6.76 ± 0.14 | 1.70 ± 0.04 | 4.93 ± 0.12 | High limonin |
| Sweet orange cv Mosambi | 1.39 ± 0.02 | 4.49 ± 0.08 | 0.27 ± 0.02 | 3.97 ± 0.10 | High limonin |

*Limonin content in the fresh juice and after 24 h storage was measured using LC-MS/MS, and the sensory properties using a hedonic scale

*glucosyltransferase* from *C. sinensis* (cv. Valencia; 502 amino acids) was retrieved from the NCBI protein database. Sequence variants identified in the target genomic region of low- and high-limonin genotypes were translated *in-silico* to their corresponding amino acid sequences. For protein-level mapping, the translated sequences from each genotype were aligned with the NCBI reference protein using MEGA X(Molecular Evolutionary Genetics Analysis) software, employing the MUSCLE algorithm for multiple sequence alignment. Missense and synonymous variants were classified based on the predicted amino acid changes, and the positions of missense substitutions were mapped onto the reference sequence.

## Results

The findings of the present study revealed that the limonin content significantly differed in the fresh and stored juice among the selected interspecific citrus hybrids and parental genotypes. Additionally, sensory profiling of the juice of new hybrids by the 10-member panel determined the bitterness (1–5 scale) and overall acceptability (1–9 hedonic scale). In the fresh juice, limonin content varied from 0 to 2.55 ppm, which was increased to 0.75 to 6.76 ppm after 24 hours of storage of juice at 4°C. Sensory profiling also indicated that the bitterness score in the fruit juice showed a marked increase after storage, whereas the acceptability (hedonic) score for fruit juice decreased for all the studied genotypes. Among the new hybrids and parental genotypes, SCSH-9-11/12 and SCSH-11-9/13had lower delayed bitterness. On the other hand, inter-specific hybrid SCSH-17-8/14, White Pummelo, and Mosambi exhibited a higher magnitude of delayed bitterness.

The molecular study on genetic variations for limonoid-associated delayed bitterness was carried out to understand whether any length variability is present or not in the amplified *Limonoid UDP-glucosyltransferase* gene for the selected primers (nine) across 16 hybrids and two parental citrus genotypes. However, no polymorphism was observed (Fig. 1).

To assess any sequence variations, the amplicon from contrasting genotypes with low and high delayed bitterness, based on limonin content (SCSH-9-11/12 and SCSH-11-9/13, having low limonin content, and SCSH-17-8/14, White Pummelo, and Mosambi, exhibiting high limonoid bitterness), was selected for Sanger sequencing.

The obtained sequences were mapped with the available genomic resources of Limonoid UDP-glucosyltransferase for sweet orange (*C. sinensis* cv. Valencia). Variant annotation was performed using snpEff (version 4.3t; http://pcingola.github.io/SnpEff/), following the conversion of genetic to genomic coordinates via in-house Perl scripts. This pipeline annotated and predicted the potential effects of genetic variants on protein-coding genes. The analysis of variants and their annotation in the genomic region of the reference genome indicated a total of 20 variants for low limonin and 36 variants for high limonin content among the studied genotypes (Fig. 2).

In the low-limonin genotypes, a total of 20 sequence variants were detected in the target genomic region (Fig. 2). Of these, 12 were missense variants (60%), (i.e., DNA change that results in different amino acids being encoded at a particular position in the resulting enzyme), and eight were synonymous (40%) i.e., DNA change that results in the same amino acid being encoded at a particular position in the resulting enzyme, without altering its primary amino acid sequence. Missense variants were distributed across multiple codon positions, with the highest clustering between positions 2586644 to 2586765 (Table 4). Further, base substitution patterns revealed a predominance of transversions over transitions (14 vs. 4), corresponding to a

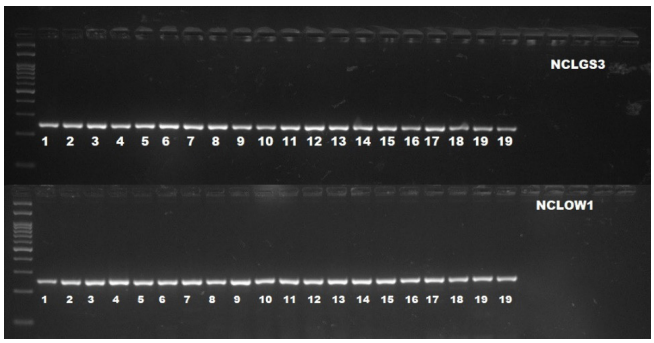


**Fig. 1.** PCR ampliconsusing primers NCLGS3 and NCLOW1 in citrus hybrids and parental genotypes

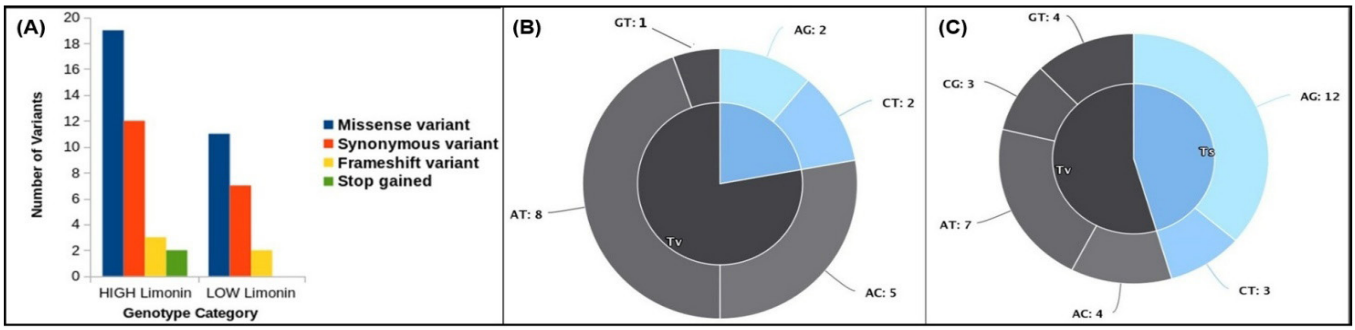


**Fig. 2.** Variant annotation statistics for citrus genotypes with contrasting bitterness properties. (a) Distribution of variant functional categories, including missense, synonymous, frameshift, and stop-gain mutations. (b) Transition and transversion variant counts in low-limonin genotypes and (c) Transition and transversion variant counts in high-limonin genotypes

transition/transversion (Ti/Tv) ratio of 0.29 (Fig. 2). Among transversions, AT substitutions were the most frequent (8 instances), followed by AC (5) and GT (1) changes whereas no CG transversions were detected in this group. Transitions were equally represented by AG (n = 2) and CT (n = 2) substitutions (Fig. 2). Comparative analysis with high-limonin genotypes showed differences in variant composition. High-limonin genotypes carried a greater number of missense changes (19 variants) and exhibited a higher Ti/Tv ratio (0.83), whereas the low-limonin group had a lesser number of missense variants (Table 5).

A total of 20 sequence variants were identified in the low-limonin candidate region. Annotation against the NCBI reference protein (limonoid UDP-glucosyltransferase, *C. sinensis*, 502 amino acids) showed that 12 of these variants were missense (resulting in amino-acid substitutions), whereas the remaining eight were synonymous (no change in the encoded amino acid). The missense variants mapped to the following residue positions of the 502-amino-acid reference sequence: residue 267 (SNV-9), 269 (SNV-8), 272 (SNV-9/2), 273 (SNV-5, SNV-6, SNV-7/2, SNV-8/2), 279 (SNV-4), 293 (SNV-3), 308 (SNV-2) and 324 (SNV-1) (Fig.3). Amino-acid substitutions were predicted by aligning the variant sequences to the reference protein sequence retrieved from NCBI; the specific residue changes for each SNV are provided in Fig. 3.

## Discussion

The delayed bitterness remains a major challenge in citrus juice processing, which is primarily attributed to limonin, an extremely bitter compound from the limonoid family. Previous studies have reported the predominant role of genetic factors underlying the biosynthesis and degradation/glucosylation, which play crucial roles in determining their accumulation (Baldwin et al. 2010; Saini et al. 2019). Limonoid content is typically highest during the early stages of citrus fruit development and subsequently declines due to metabolic conversion, dilution during fruit enlargement, and glycosylation of limonoid aglycones.

**Table 4.** Single-nucleotide variations (SNVs) were observed for low limonin in the studied citrus genotype

| S. No. | Position | Reference | Alteration | Functional category of variant |
|---|---|---|---|---|
| 1 | 2586599 | T | G | Missense |
| 2 | 2586612 | A | T | Synonymous |
| 3 | 2586644 | C | A | Missense |
| 4 | 2586647 | C | A | Missense |
| 5 | 2586648 | T | A | Synonymous |
| 6 | 2586650 | T | A | Synonymous |
| 7 | 2586686 | C | TA | Synonymous |
| 8 | 2586687 | A | T | Synonymous |
| 9 | 2586688 | A | T | Synonymous |
| 10 | 2586689 | A | T | Synonymous |
| 11 | 2586693 | C | T | Missense |
| 12 | 2586734 | A | G | Missense |
| 13 | 2586741 | A | G | Missense |
| 14 | 2586752 | T | A | Missense |
| 15 | 2586753 | C | A | Missense |
| 16 | 2586756 | C | A | Missense |
| 17 | 2586761 | A | T | Synonymous |
| 18 | 2586763 | T | C | Missense |
| 19 | 2586765 | C | A | Missense |
| 20 | 2586771 | A | C | Missense |

Increased activity of *limonoid UDP-glucosyltransferase* promotes the conversion of bitter limonoid aglycones into glucosides, reducing their levels (Wang et al. 2016). While the remaining aglycone form (LARL) present in fresh fruit converts slowly into the extremely bitter form after juice extraction, it leads to the development of bitterness in

**Table 5.** Single-nucleotide variations (SNVs) observed for high limonin in the citrus genotype

| S. No. | Position | Reference | Alteration | Functional category of variant |
|---|---|---|---|---|
| 1 | 2586612 | A | G | Missense |
| 2 | 2586615 | T | C | Frameshift |
| 3 | 2586622 | A | T | Missense |
| 4 | 2586632 | G | A | Missense |
| 5 | 2586641 | T | G | Missense |
| 6 | 2586644 | C | A | Frameshift |
| 7 | 2586646 | A | T | Stop gain |
| 8 | 2586652 | T | A | Synonymous |
| 9 | 2586658 | C | A | Synonymous |
| 10 | 2586663 | G | A | Missense |
| 11 | 2586667 | G | A | Synonymous |
| 12 | 2586679 | T | C | Stop gained |
| 13 | 2586688 | A | C | Synonymous |
| 14 | 2586689 | A | T | Synonymous |
| 15 | 2586693 | C | T | Missense |
| 16 | 2586713 | G | C | Missense |
| 17 | 2586727 | A | T | Missense |
| 18 | 2586729 | C | T | Missense |
| 19 | 2586737 | C | A | Missense |
| 20 | 2586741 | A | G | Missense |
| 21 | 2586751 | G | A | Synonymous |
| 22 | 2586752 | T | A | Missense |
| 23 | 2586790 | T | G | Frameshift |
| 24 | 2586802 | A | C | Missense |
| 25 | 2586804 | G | A | Missense |
| 26 | 2586959 | A | G | Missense |
| 27 | 2586960 | G | C | Synonymous |
| 28 | 2587105 | G | A | Synonymous |
| 29 | 2587130 | A | G | Synonymous |
| 30 | 2587165 | T | G | Synonymous |
| 31 | 2587185 | T | A | Missense |
| 32 | 2587195 | T | G | Synonymous |
| 33 | 2587200 | T | G | Missense |
| 34 | 2587204 | G | C | Synonymous |
| 35 | 2587212 | G | A | Missense |
| 36 | 2587237 | G | A | Missense |

stored juice.

Biochemical analysis of fruit juice in our study revealed a marked increase in limonoid concentration after 24 hours of storage. Notably, in the commercially cultivated variety *Mosambi*, the fresh juice contained relatively low levels of limonin (1.39 mg/L), but its concentration increased approximately fourfold within 24 hours in stored juice. These findings are in agreement with the previous research studies in citrus fruits and highlight the issue of limonin-associated delayed bitterness as a major constraint in citrus fruit processing (Kita et al. 2000). Raithore et al. (2016) previously reported that the juice of citrus hybrids and their parental genotypes developed delayed bitterness within four hours of storage, which was correlated with an increase in limonin concentration.

Furthermore, among the newly developed inter-specific hybrids (Pummelo × Sweet orange cv Mosambi), SCSH-9-11/12, and SCSH-11-9/13 had the limonin content below the threshold bitterness value reported in the previous studies, and these newly developed hybrids were found suitable for juice processing and storage due to the lower limonin-associated bitterness. The sensory evaluation also confirmed the biochemical results of limonin content in the fruit juice analyzed through LC-MS analysis. Our results pertaining to the limonin content and corresponding sensory analysis were in line with previous findings on different citrus species (Guadagni et al. 1973; Zaare-Nhandi et al. 2008; Kore and Chakraborthy 2015; Zhang et al. 2020; Huang et al. 2021; Liu et al. 2012).

Molecular analysis of the observed variations in limonoid-associated delayed bitterness among the new citrus hybrids was carried out using the sequence information of the *Limonoid UDP-glucosyltransferase* gene (LOC102630581; Gene ID: 102630581). The expression of the *Limonoid UDP-glucosyltransferase* gene and its enzyme activity has been identified as a major debittering factor in citrus fruit juice, as the enzyme catalyzes the transfer of glucose to the limonoid moiety, resulting in the formation of stable limonoid glucosides (Karim and Hashinaga 2001; Huang et al. 2021). Gel electrophoresis results could not decipher polymorphism based on the length of the amplified product for the 16 citrus hybrids and their parental genotypes studied. Therefore, five genotypes from two groups (low-limonin and high-limonin) were selected for Sanger sequencing of the PCR-amplified products to observe sequence variations. The nucleotide sequences obtained for new interspecific citrus hybrids were submitted to the NCBI Genomic Sequences Repository (OR465050, OR474192, OR474193, OR518553, OR518554, and OR518555).

Further analysis of variants and their annotation in the genomic region of the reference genome revealed that low-limonin genotypes exhibited a total of 12 missense variants (i.e., single or multiple base changes in DNA that

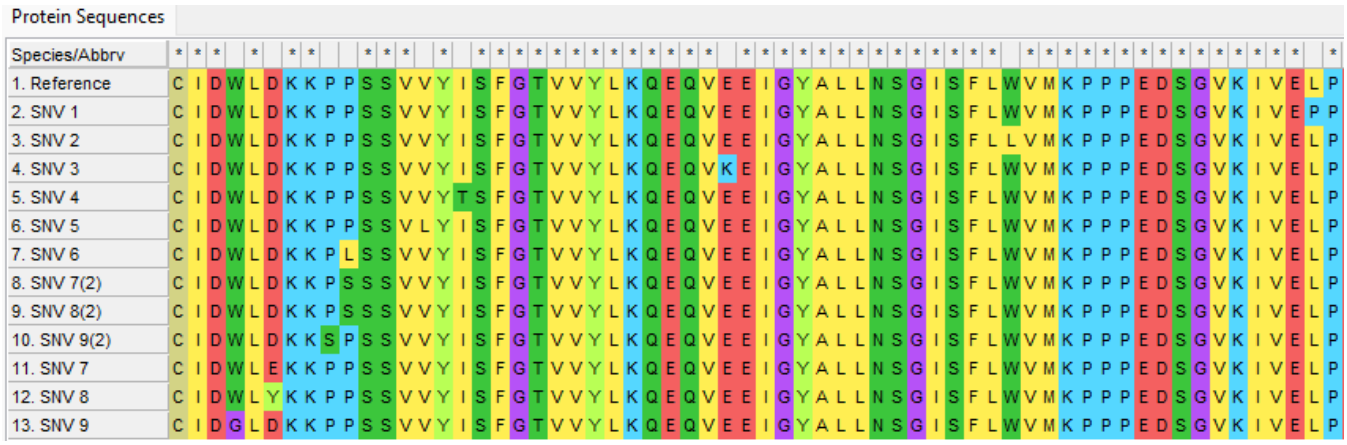


**Fig. 3.** Mapping of missense variants associated with low limonin content to the Citrus sinensis limonoid UDP-glucosyltransferase reference protein (502 amino acids; NCBI accession no. ACD14145.1). Positions of variants are indicated along the linear protein schematic, with the remaining eight synonymous variants excluded from the diagram as they do not alter the encoded amino acid

alter the amino acid sequence without affecting protein length). These included four transitions (2 AG and 2 CT) and 14 transversions (8 AT, 5 AC, and 1 GT), resulting in a transition-to-transversion (Ts/Tv) ratio of 0.29. In comparison, high-limonin genotypes contained 19 missense variants, comprising 15 transitions (12 A↔G and 3 C↔T) and 18 transversions (7 AT, 4 AC, 4 GT, and 3 CG), resulting in a Ts/Tv ratio of 0.83. A transition changes a purine nucleotide into another purine or changes a pyrimidine nucleotide into another pyrimidine. All other mutations substituting a purine for a pyrimidine, or vice versa, are called transversions. Transversions are much more likely to change the encoded amino acid. These results of SNVs (single nucleotide variations) for the *Limonoid UDP-glucosyltransferase* gene in different citrus genotypes suggest that the changes in nucleotide sequences for the enzyme-encoding gene might result in an alteration of gene expression and/or changes in amino acid sequences, leading to a slight alteration of proteins. Previous studies have also reported the nucleotide variations among the *Citrus* species for the *Limonoid UDP-glucosyltransferase* gene and associated variations in limonin content (Kita et al. 2000; Zaare-Nhandi et al. 2008; Huang et al. 2021).

The identification of 12 missense variants within the limonoid UDP-glucosyltransferase gene in low-limonin genotypes suggests potential functional alterations in the encoded enzyme. Functionally, the missense variants in low-limonin genotypes may alter amino acid properties (e.g., charge, polarity, hydrophobicity), potentially affecting limonoid UDP-glucosyltransferase structure and activity, and consequently modulating limonin biosynthesis. The synonymous variants, although not altering protein sequence, may still influence gene expression through codon usage bias or mRNA stability. Further, the results also indicated that variants clustering around residues 267-279 may represent functionally significant domains, potentially affecting the active site or nearby structural motifs critical for enzyme activity. While synonymous variants are unlikely to impact protein function directly, their presence may still reflect genetic linkage with functionally important loci. The results also indicated a larger number of frameshift variants corresponding to the high limonin content in citrus juice. These results are in agreement with the previous findings because the lower expression of the limonoid UDP-glucosyltransferase leads to higher bitterness in fruit juice (Kita et al. 2000; Zaare-Nhandi et al. 2008). These findings also highlight the importance of targeted functional validation of these candidate variants through enzymatic assays or protein modelling to confirm their role in limonin biosynthesis and accumulation.

Overall, integration of positional mapping, substitution type distribution, and functional categorization revealed the nucleotide and amino acid variations underlying low-limonin-associated bitterness among the novel hybrid genotypes and parents, including commercial variety Mosambi. The results of the present study deciphered the putative genomic basis for low bitterness in novel interspecific hybrids developed for processing with low bitterness. The observed SNVs could be potentially used for bitterness-specific markers and validation in vast citrus genetic diversity to harness the potential of marker-assisted selection, which is crucial for breeding perennial fruit crops, as the long juvenile phase requires a 7-9 year wait for observing the bitterness of fruit juice.

## Authors' contribution

Conceptualization of research (RMS, AKD, NSL, SS); Designing of the experiments (NSL, NS, RMS, AKD, AMS); Contribution of experimental materials (RMS, AKD, NSL); Execution of

field/lab experiments and data collection (NS, MS, NSL, AMS); Analysis of data and interpretation (NSL, SS, BC, AK, OPA, AMS, JS); Preparation of the manuscript (NS, MS, JS, NSL).

## Acknowledgments

The senior author acknowledge the CSIR for Junior Research Fellow/Senior Research Fellow during the doctoral degree programme. The authors sincerely thank the Director and Joint Director (E), ICAR-Indian Agricultural Research Institute, New Delhi, for their invaluable guidance and support throughout this research.

## References

Anonymous. 2023. Area and production of horticultural crops for the year 2021-22. Ministry of Agriculture & Farmers Welfare, Govt of India, New Delhi.https://agriwelfare.gov.in/en/StatHortEst

Anticona M., Fayos M.C., Esteve M.J., Frigola A., Blesa J. and Lopez-Malo D. 2022. Differentiation of juice of mandarin-like hybrids based on physicochemical characteristics, bioactive compounds, and antioxidant capacity. Eur. Food Res. Technol., **248**(9): 2253-2262.

Baldwin E., Plotto A., Manthey J., McCollum G., Bai J., Irey M., Cameron R. and Luzio, G. 2010. Effect of liberibacter infection (huanglongbing disease) of citrus on orange fruit physiology and fruit/fruit juice quality: Chemical and physical analyses. J. Agric. Food Chem., **58**: 1247-1262.

Chandler B.V. and Kefford J.F. 1966. The chemical assay of limonin, the bitter principle of oranges. J. Sci. Food Agric., **17**: 193-197.

De Mori G. and Cipriani G. 2023. Marker-assisted selection in breeding for fruit trait improvement: A review. Int. J. Mol. Sci., **24**(10): 8984.

Deterre S.C., McCollum G., Leclair C., Manthey J.A., Bai J., Baldwin E.A., Raithore S., Stover E. and Plotto A. 2021. Effect of *Poncirus trifoliata* on the chemical composition of fruits in pedigrees of citrus scion hybrids. Sci. Hortic., **277**: 109816.

Doyle J. and Doyle J. 1987. A rapid procedure for DNA purification from small quantities of fresh leaf tissue. Phytochem. Bull., **19**: 11-15.

FAO. 2020. Citrus Fruit, Fresh and Processed: Statistical Bulletin 2020. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy, 2020.

Fong C.H., Hasegawa S., Herman Z. and Ou P. 1989. Limonoid glucosides in commercial citrus juices. J. Food Sci., **54**(6): 1505-1506.

Guadagni D. G., Maier V.P. and Turnbaugh J.G. 1973. Effect of some citrus juice constituents on taste thresholds for limonin and naringin bitterness. J. Sci. Food Agric., **24**(10): 1277-1288.

Gupta A.K. Dhua S., Kumar V., Naik B., Magwaza L.S., Ncama K. and Mishra P. 2023. Current and emerging applications in detection and removal of bitter compounds in citrus fruit juice: A critical review. Food Biosci., 102995.

Huang S., Dong T., Xiong B., Qiu X., Sun G., Liao L. and Wang Z. 2021. Variation in the content and composition of limonoids in fruits of four pummelo varieties during fruit development: The natural debittering process in pummelo fruits. J. Food Compos. Anal., **100**: 103928.

Karim M.R. and Hashinaga F. 2001. Screening and some properties of limonoid glucosyltransferase from selected citrus varieties. Pak. J. Biol. Sci., **4**: 486-483.

Kita M., Hirata Y., Moriguchi T., Endo-Inagaki T., Matsumoto R., Hasegawa S., Suhayda C.G. and Omura M. 2000. Molecular cloning and characterization of a novel gene encoding limonoid UDP-glucosyltransferase in citrus. FEBS Lett., **469**: 173-178.

Kore V.T. and Chakraborty I. 2015. Efficacy of various techniques on biochemical characteristics and bitterness of pummelo juice. J. Food Sci. Technol., **52**: 6073-6077.

Kruger M.J., Davies N., Myburgh K.H. and Lecour S. 2014. Proanthocyanidins, anthocyanins and cardiovascular diseases. Food Res. Int., **59**: 41-52.

Lamo K., Bhat D.J. Kour K. and Solanki S.P.S. 2017. Mutation studies in fruit crops: A review. Int. J. Curr. Microbiol. Appl. Sci., **6**(12): 3620-3633.

Liu Y., Heying E. and Tanumihardjo S.A. 2012. History, global distribution, and nutritional importance of citrus fruits. Compr. Rev. Food Sci. Food Saf., **11**(6): 530-545.

Maier V.P., Hasegawa S. and Hera E. 1969. Limonin D-ring-lactone hydrolase. A new enzyme from citrus seeds. Phytochemistry, **8**: 405-407.

Nishad J., Singh S.P., Singh S., Saha S., Dubey A.K., Varghese E. and Kaur C. 2018. Bioactive compounds and antioxidant activity of selected Indian pummelo (*Citrus grandis* L. Osbeck) germplasm. Sci. Hortic., **233**: 446-454.

Pardo H., Abiola Owoyemi O.B., Goldenberg L., Yaniv Y., Doron-Faigenboim A., Carmi N. and Porat R. 2021. Sensory analysis of a new citrus juice made from 'Aliza' fruit: A new pomelo x mandarin hybrid. J. Food Sci. Nutr. Res., **4**(1): 1-11.

Peterson J.J., Beecher G.R., Bhagwat S.A., Dwyer J.T., Gebhardt S.E., Haytowitz D.B. and Holden J.M. 2006. Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. J. Food Compos. Anal., **19**: S74-S80.

Puri M., MarwahaS.S., Kothari R.M. and Kennedy J.F. 1996. Biochemical basis of bitterness in citrus fruit juices and biotech approaches for debittering. Crit. Rev. Biotechnol., **16**: 145-155.

Raghavan S. and Gurunathan J. 2021. Citrus species - a golden treasure box of metabolites that is beneficial against disorders. J. Herb. Med., **28**: 100438.

Raithore S., DeaS., McCollum G., Manthey J.A., Bai J., Leclair C., Hijaz F., Narciso J.A., Baldwin E.A. and Plotto A. 2016. Development of delayed bitterness and effect of harvest date in stored juice from two complex citrus hybrids. J. Sci. Food Agric., **96**(2): 422-429.

Rouseff R.L. 1982. Nomilin, a new bitter component in grapefruit juice. J. Agric. Food Chem., **30**: 504-507.

Saini M.K., Capalash N., Kaur C. and SinghS.P. 2019. Comprehensive metabolic profiling to decipher the influence of rootstocks on fruit juice metabolome of Kinnow (*C. nobilis* × *C. deliciosa*). Sci. Hortic., **257**: 108673.

Salonia F., Ciacciulli A., Poles L., Pappalardo H.D., La Malfa S. and Licciardello C. 2020. New plant breeding techniques in citrus

for the improvement of important agronomic traits: A review. Front. Plant Sci., **11**: 1234.

Singh N., Sharma R.M., Dubey A.K., Awasthi O.P., Saha S., Bharadwaj C., Sharma V.K., Sevanthi A.M., Kumar A. and Deepak. 2023. Citrus improvement for enhanced mineral nutrients in fruit juice through interspecific hybridization. J. Food Compos. Anal., **119**: 105259.

Wang F., Yu X., Liu X., Shen W., Zhu S. and Zhao X. 2016. Temporal and spatial variations on accumulation of nomilin and limonin in pummelos. Plant Physiol. Biochem., **106**: 23-29.

Wu G.A., Terol J., Ibanez V., López-García A., Pérez-Román E., Borreda C., Domingo C., Tadeo F.R., Carbonell-Caballero J., Alonso R. and Curk F. 2018. Genomics of the origin and evolution of citrus. Nature, **554**(7692): 311-316.

Zaare-Nahandi F., Hosseinkhani S., Zamani Z., Asadi-Abkenar A. and Omidbaigi R. 2008. Delay expression of limonoid UDP-glucosyltransferase makes delayed bitterness in citrus. Biochem. Biophys. Res. Commun., **371**(1): 59-62.

Zhang P., Liu X., Yu X., Wang F., Long J., Shen W., Jiang D. and Zhao X. 2020. The MYB transcription factor CiMYB42 regulates limonoids biosynthesis in citrus. BMC Plant Biol., **20**: 1-12.