IDENTIFICATION AND ISOLATION OF GENES RESPONSIBLE FOR INCREASED SHELF-LIFE IN GUAVA (*Psidium guajava* L.)

Thesis

Submitted to Punjab Agricultural University
In partial fulfilment of the requirements
For the degree of

MASTER OF SCIENCE
in
BIOTECHNOLOGY
(Minor Subject: Plant Breeding and Genetics)

By

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2020

CERTIFICATE I

This is to certify that the thesis entitled, **"Identification and isolation of genes responsible for increased shelf-life in guava (*Psidium guajava* L.)"** submitted for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Namita (L-2017-A-177-M)** under my supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the thesis entitled, **"Identification and isolation of genes responsible for increased shelf-life in guava (*Psidium guajava* L.)"** submitted by **Namita (L-2017-A-177-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Biotechnology** (Minor subject: **Plant Breeding and Genetics**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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ACKNOLEDGEMENTS

"Delight yourself in the lord, and he will give you the desires of your heart" First and foremost I bow my head to **"Almighty God"** for making me a curious being who loves to explore his creation and for giving me the opportunity to write this thesis.

I must offer my gratitude to my major advisor **Dr. Amandeep Mittal**, Fruit Molecular Geneticist, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana who continually and convincingly conveyed a spirit of adventure in regard to research and an excitement in regard to teaching. His patience, motivation, enthusiasm, and immense knowledge helped me in all the time of research and writing of this thesis.

I sincerely acknowledge to the members of my advisory committee *viz.* **Dr. Naresh Kumar Arora**, Horticulturist, College of Horticulture and Forestry, **Dr. Yogesh Vikal**, Senior geneticist, School of Agricultural Biotechnology, **Dr**. **Gurupkar Singh Sidhu,** Assistant Horticulturist, School of Agricultural Biotechnology, **Dr. R.S. Boora**, Horticulturist, Regional Fruit Research Station Bhadurgarh, College of Horticulture and Forestry for their constant support and kind suggestions during the course of investigation. I sincerely thank Director School of Agricultural Biotechnology, **Dr. Parveen Chhuneja** for providing the excellent lab facilities and resources.

No appropriate words could be traced in the present lexicon to express my feelings of indebtness for my lovable parents **Sh. Ravider Kumar** and **Smt. Sonam** and my brothers **Gaurish Kumar, Kartikay, Armaan** and **Ritesh** and my sister **Sudiksha** for their benedictions, affection, remarkable patience, incessant support, infinite love and silent prayers that have brought me upto this stage of career. It was under their watchful eye that I gained so much drive and an ability to tackle challenges head on.

"To hear Complaints with Patience, even when Complaints are Vain, It is one of the Duties of Friendship". I am indebted to my friends **Shaweta Sharma, Ajay Gupta , Lovepreet Kaur, Shiksha Arora, Vasundra Devi, Mamta** and **Sanket ,** who made this arduous journey much more pleasant. Your friendship makes my life a wonderful experience. My dearest comrades and my juniors in the department **Gursimran kaur** were constant sources of laughter, joy, and support.

I fully acknowledge the co-operation rendered by laboratory staff with special thanks to research fellows, my seniors **Sujata Thakur, Manish Jindal** and **Ruchika** who shared their knowledge with me during my research. I expand my thanks to all the field workers and lab attendants **Ranjit Singh** and **Rajinder Singh** for their valuable contribution in completion of my work.

I feel proud to be a part of PAU, Ludhiana where I learnt a lot and spent many unforgettable moments of my life. **Memory and words may fail to acknowledge somebody but the heart cannot**. It is my honest conviction and sincerest prayer that the Almighty God bless you all.

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Place: Ludhiana **Namita**

Title of the Thesis	**:**	Identification and isolation of genes responsible for increased shelf-life in guava (*Psidium guajava* L.)
Name of the student and Admission No.	**:**	Namita L-2017-A-177-M
Major Subject	**:**	Biotechnology
Minor Subject	**:**	Plant Breeding and Genetics
Name and Designation of Major Advisor	**:**	Dr. Amandeep Mittal Fruit Molecular Geneticists
Degree to be Awarded	**:**	M.Sc.
Year of award of degree	**:**	2019
Total pages in thesis	**:**	116 + VITA
Name of the University	**:**	Punjab Agricultural University, Ludhiana – 141 004, Punjab, India

ABSTRACT

Guava is a climacteric fruit and ripening continuous even after harvesting leading to various textural changes. Abscisic acids (ABA) triggers the production of ripening hormone ethylene at fruit maturation stage. Most of the post-harvest changes are attributed to biosynthesis and signaling of ethylene further leading to cell wall metabolic changes imparting softness to fruit. Here, we tested 14 guava cultivars for ripening behavior and measured the physio-chemical attributes and biochemical changes at 3 ripening intervals in 2018-19 winter season crop. White fleshed Allahabad Safeda, Hisar Safeda, Punjab Safeda, Thailand guava, Shweta, Sardar guava and CISH-G5 and pink fleshed Punjab Pink, Lalit, Hisar Surkha, Purple local, 17-16, Punjab Kiran and Arka Kiran cultivars were analysed. Average TSS among genotypes varied from 8-11.5(^{o}brix), Acidity 0.28 - 0.46%, reducing sugar 3.1-8.9%, Vitamin C 97.7 – 121 mg/100g, Firmness 4.66 - 6.73lb in 8 days of shelf life experiment. Highest fruit firmness at 4 days post-harvest was found in Thailand guava (9.8 lb). Our physico-chemical and gene expression analysis for ethylene & ABA biosynthesis, signaling and cell wall degrading enzyme genes reveal that ABA and ethylene biosynthesis is delayed in the highest shelf life cv. Thailand guava. Comparative genome sequence analysis of Thailand and Allahabad Safeda identified the specific nucleotide changes in ethylene biosynthesis gene *SAM3* leading to change in glutamic acid to aspartic acid in an important domain. The genes *ACO5*, *EIL3*, *EIN2*, *ERF6* and *PL* have SNPs in the untranslated and/or the promoter regions that might be impacting the docking of transcriptional machinery and concomitant delay in ripening. SNP based molecular markers in Thailand vs Allahabad Safeda might help in marker assisted selection for transferring superior alleles for delayed ripening in popular guava cultivars.

Keywords: ABA, *ACO5*, *EIL3*, *EIN2*, *ERF6*, guava, *PL*, SNPs

Signature of the major advisor **Signature of the student**

ਖੋਜ ਗ੍ਰੰਥ ਦਾ ਸਿਰਲੇਖ	:	ਅਮਰੂਦ (*ਸੀਡੀਅਮ ਗੁਜ਼ਾਵਾ* ਐਲ.) ਦੀ ਮਿਆਦ ਵਧਾਉਣ ਵਾਲੇ ਜੀਨਾਂ ਦੀ ਪਹਿਚਾਣ ਅਤੇ ਨਿਖੇੜਨ
ਵਿਦਿਆਰਥੀ ਦਾ ਨਾਮ ਅਤੇ ਦਾਖਲਾ ਨੰ.	:	ਨਮੀਤਾ ਐਲ-2017-ਏ-177-ਐਮ
ਪ੍ਰਮੁੱਖ ਵਿਸ਼ਾ	:	ਬਾਈਓਟੈਕਨੋਲਜੀ
ਸਹਿਯੋਗੀ ਵਿਸ਼ਾ	:	ਪਲਾਂਟ ਬ੍ਰੀਡਿੰਗ ਅਤੇ ਜੈਨੇਟਿਕਸ
ਮੁੱਖ ਸਲਾਹਕਾਰ ਦਾ ਨਾਮ ਅਤੇ ਅਹੁਦਾ	:	ਡਾ. ਅਮਨਦੀਪ ਮਿੱਤਲ ਫਰੂਟ ਮੋਲੀਕਿਉਲਰ ਜੈਨੇਟਿਸਿਸਟ
ਡਿਗਰੀ	:	ਐਮ.ਐੱਸ.ਸੀ.
ਡਿਗਰੀ ਮਿਲਣ ਦਾ ਸਾਲ	:	2020
ਖੋਜ ਪੱਤਰ ਵਿੱਚ ਕੁੱਲ ਪੰਨੇ	:	116 + ਵੀਟਾ
ਯੂਨੀਵਰਸਿਟੀ ਦਾ ਨਾਮ	:	ਪੰਜਾਬ ਐਗਰੀਕਲਚਰਲ ਯੂਨੀਵਰਸਿਟੀ, ਲੁਧਿਆਣਾ - 141 004, ਪੰਜਾਬ, ਭਾਰਤ

ਸਾਰ ਅੰਸ਼

ਅਮਰੂਦ ਇੱਕ ਮੌਸਮੀ ਫ਼ਲ ਅਤੇ ਤੁੜਾਈ ਮਗਰੋਂ ਵੀ ਇਹ ਪੱਕਦਾ ਰਹਿੰਦਾ ਹੈ ਜਿਸ ਨਾਲ ਇਸ ਵਿੱਚ ਕਈ ਤਰ੍ਹਾਂ ਦੀਆਂ ਤਬਦੀਲੀਆਂ ਆਉਂਦੀਆਂ ਰਹਿੰਦੀਆਂ ਹਨ। ਐਸਕਾਰਬਿਕ ਐਸਿਡ (ABA) ਫ਼ਲ ਦੇ ਪੱਕਣ ਦੀ ਅਵਸਥਾ ਉਪਰ ਪੱਕਣ ਵਿੱਚ ਸਹਾਈ ਇਥਲਿਨ ਹਾਰਮੋਨ ਦਾ ਉਤਪਾਦਨ ਕਰਦੇ ਹਨ। ਤੁੜਾਈ ਉਪਰੰਤ ਆਉਣ ਵਾਲੀਆਂ ਬਹੁਤ ਸਾਰੀਆਂ ਤਬਦਲੀਆਂ ਜੈਵਿਕ ਸਿੰਥੇਸਿਸ ਲਈ ਜ਼ਿੰਮੇਵਾਰ ਹੁੰਦੀਆਂ ਹਨ ਅਤੇ ਇਥਲਿਨ ਨਾਲ ਕੋਸ਼ਿਕਾ ਝਿੱਲੀ ਦੀਆਂ ਮੈਟਾਬੋਲਿਕ ਤਬਦੀਲੀਆਂ ਕਾਰਨ ਫ਼ਲ ਨਰਮ ਪੈ ਜਾਂਦਾ ਹੈ। ਇਸ ਅਧਿਐਨ ਦੌਰਾਨ, ਪੱਕਣ ਦੇ ਵਤੀਰੇ ਦੇ ਲਿਹਾਜ਼ ਨਾਲ ਅਮਰੂਦ ਦੀਆਂ 14 ਕਿਸਮਾਂ ਦੀ ਜਾਂਚ ਕੀਤੀ ਗਈ ਅਤੇ 2018-19 ਦੀ ਸਰਦ ਰੁੱਤ ਦੀ ਫ਼ਸਲ ਵਿੱਚ ਪੱਕਣ ਦੇ 3 ਅੰਤਰਲਾਂ ਉਪਰ ਫ਼ਲ ਵਿੱਚ ਆਉਣ ਵਾਲੀਆਂ ਭੌਤਿਕ-ਰਸਾਇਣਕ ਗੁਣਾਂ ਅਤੇ ਜੀਵ-ਰਸਾਇਣਕ ਤਬਦੀਲੀਆਂ ਦਰਜ ਕੀਤੀਆਂ ਗਈਆਂ। ਅਮਰੂਦ ਦੀਆਂ ਸਫੇਦ ਗੁੱਦੇ ਵਾਲੀ ਕਿਸਮਾਂ ਇਲਾਹਾਬਾਦ ਸਫੇਦਾ, ਹਿਸਾਰ ਸਫੇਦਾ, ਪੰਜਾਬ ਸਫੇਦਾ, ਥਾਈਲੈਂਡ ਅਮਰੂਦ, ਸ਼ਵੇਤਾ, ਸਰਦਾਰ ਅਮਰੂਦ ਅਤੇ CISH-G5 ਅਤੇ ਗੁਲਾਬੀ ਗੁੱਦੇ ਵਾਲੀਆਂ ਕਿਸਮਾਂ ਪੰਜਾਬ ਪਿੰਕ, ਲਲਿਤ, ਹਿਸਾਰ ਸੁਰਖ, ਪਰਪਲ ਲੋਕਲ, 17-16, ਪੰਜਾਬ ਕਿਰਨ ਅਤੇ ਅਰਕਾ ਕਿਰਨ ਦਾ ਮੁਲਾਂਕਣ ਕੀਤਾ ਗਿਆ। ਮਿਆਦ ਦੇ 8 ਦਿਨਾਂ ਉਪਰ ਕੀਤੇ ਗਏ ਤਜ਼ਰਬੇ ਦੌਰਾਨ ਅਮਰੂਦ ਦੀਆਂ ਕਿਸਮਾਂ ਵਿੱਚ ਔਸਤਨ ਟੀ.ਐਸ.ਐਸ., ਐਸੀਡਿਟੀ, ਰਡਿਊਸਿੰਗ ਸ਼ੂਗਰ, ਵਿਟਾਮਿਨ ਸੀ ਅਤੇ ਠੋਸਤਾ ਕ੍ਰਮਾਵਾਰ 8-11.5(^{o}brix), 0.28-0.46%, 3.1-8.9%, 97.7-121 mg/100g ਅਤੇ 4.66-6.73lb ਸੀ। ਤੁੜਾਈ ਤੋਂ ਚਾਰ ਦਿਨਾਂ ਮਗਰੋਂ ਥਾਈਲੈਂਡ ਗੁਆਵਾ (9.8 lb) ਦੀ ਠੋਸਤਾ ਸਭ ਤੋਂ ਵਧੇਰੇ ਦਰਜ ਕੀਤੀ ਗਈ। ਭੌਤਿਕ-ਰਸਾਇਣਕ ਅਤੇ ਇਥਲਿਨ ਅਤੇ ABA ਜੈਵਿਕ ਸਿੰਥੇਸਿਸ, ਸਿਗਨਲਿੰਗ ਅਤੇ ਕੋਸ਼ਿਕਾ ਝਿੱਲੀ ਨੂੰ ਨੁਕਸਾਨ ਪਹੁੰਚਾਉਣ ਵਾਲੇ ਇੰਜ਼ਾਈਮ ਜੀਨਾਂ ਦੇ ਜੀਨ ਐਕਸਪ੍ਰੇਸ਼ਨ ਮੁਲਾਂਕਣ ਤੋਂ ਪਤਾ ਚੱਲਿਆ ਕਿ ਸਭ ਤੋਂ ਵਧੇਰੇ ਮਿਆਦ ਵਾਲੀ ਕਿਸਮ ਥਾਈਲੈਂਡ ਗੁਆਵਾ ਵਿੱਚ ABA ਅਤੇ ਇਥਲਿਨ ਜੈਵਿਕ ਸਿੰਥੇਸਿਸ ਵਿੱਚ ਦੇਰੀ ਹੋਈ। ਥਈਲੈਂਡ ਅਤੇ ਇਲਾਹਾਵਾਦ ਸਫੇਦਾ ਕਿਸਮਾਂ ਦੇ ਤੁਲਨਾਤਮਕ ਜੀਨੋਮ ਅਨੁਕ੍ਰਮਣ ਮੁਲਾਂਕਣ ਤੋਂ ਇਥਲਿਨ ਜੈਵਿਕ ਸਿੰਥੇਸਿਸ ਜੀਨ *SAM3* ਵਿੱਚ ਵਿਲੱਖਣ ਨਿਉਕਲੀਓਟਾਈਡ ਤਬਦੀਲੀਆਂ ਹੋਈਆਂ ਅਤੇ ਇੱਕ ਮਹੱਤਵਪੂਰਨ ਖੇਤਰ ਵਿੱਚ ਗਲੁਟੇਮਿਕ ਐਸਿਡ ਤਬਦੀਲ ਹੋ ਕਿ ਐਸਪਾਰਟਿਕ ਐਸਿਡ ਬਣਿਆ। ਅਨੁਵਾਦ-ਰਹਿਤ ਅਤੇ/ਜਾਂ ਪ੍ਰੋਮੋਟਰ ਖੇਤਰਾਂ ਵਿੱਚ *ACO5, EIL3, EIN2, ERF6* ਅਤੇ *PL* ਜੀਨ ਵਿੱਚ SNPs ਦੀ ਹੋਂਦ ਦਰਜ ਕੀਤੀ ਗਈ ਜਿਸਨੇ ਟ੍ਰਾਂਸਕ੍ਰਿਪਸ਼ਨਲ ਮਸ਼ੀਨਰੀ ਦੀ ਡੋਕਿੰਗ ਨੂੰ ਪ੍ਰਭਾਵਿਤ ਕੀਤਾ ਅਤੇ ਇਸ ਨਾਲ ਫ਼ਲ ਦੇ ਪੱਕਣ ਵਿੱਚ ਦੇਰੀ ਹੋਈ। ਥਾਈਲੈਂਡ ਬਨਾਮ ਇਲਾਹਾਬਾਦ ਸਫੇਦਾ ਵਿੱਚ SNP ਅਧਾਰਿਤ ਆਣਵਿਕ ਮਾਰਕਰ, ਅਮਰੂਦ ਦੀਆਂ ਪ੍ਰਚਲੱਤ ਕਿਸਮਾਂ ਦੇ ਪੱਕਣ ਵਿੱਚ ਦੇਰੀ ਲਿਆਉਣ ਲਈ ਵਧੀਆ ਐਲਿਲਸ ਦੇ ਸਥਾਂਨਾਤਰਣ ਲਈ ਮਾਰਕਰ ਅਧਾਰਤ ਚੋਣ ਵਿੱਚ ਮਦਦ ਕਰ ਸਕਦੇ ਹਨ।

ਮੁੱਖ ਸ਼ਬਦ: ABA, *ACO5, EIL3, EIN2, ERF6,* ਅਮਰੂਦ, *PL,* SNPs

ਪ੍ਰਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Guava (*Psidium guajava* L.) the "apple of tropics" is an important fruit crop due to its taste and nutritional quality. Guava a native of central and South America belongs to the family *Myrtaceae* possessing ~ 150 species. It was introduced in India in seventeenth century and became an important fruit crop of India after mango, banana and citrus (Table 1). Guava carries wider adaptability to diverse climatic and soil conditions. Although a native to tropical America, it is also cultivated in different tropical and subtropical countries. Guava is mainly valued for its delicious taste, aroma and four times higher vitamin C content than orange fruits (Sato *et al* 2010). It is a good source of pectin, calcium, phosphorus and flavonoids. Owing to its nutrient composition and gelling properties, guava is an important trade commodity of tropical countries (Table 2).

Guava fruits can be of varied shapes ranging from round, ovoid to pear shaped with a size range of 2-5 inches (5-12.5 cm) long. Its thin skin varies in colour from pale green, light-yellow to red. Generally, the fruit gives a strong, musky odour (due to carbonyl compounds) when ripened. The central pulp may be slightly darker in the tone, is juicy and normally filled with very hard, yellowish seeds with TSS varying from 5-14%. Based on the variation in these traits the coloured and non-coloured varieties of guava have been released at national and international level. In India, many guava cultivars are released for commercial production. **Punjab Pink** a hybrid between Portugal x L-49 = F1 x Apple colour bears fruits of medium to large size with attractive golden yellow colour and a TSS of 10.5 to 12.0 %. **Lalit** (chance seedling) raised at CISH, Lucknow has medium sized fruits with attractive yellow colour with a pink blush. **Arka kiran** a hybrid of Kamsari x Purple Local developed in IIHR, Bangalore has 12% TSS. **Hisar Surkha** a hybrid of Apple colour x Banarsi Surkha, has round fruits, saffron yellow skin and TSS of 13.6%. **Purple Local** has a dark red fruit, shape vary from oval to round. **Shweta** a selection from the open pollinated seedlings of Apple colour guava has TSS of 10.5-11.0%, with semi-hard seeds. **Sardar guava (L-49)** is a selection from Allahabad Safeda has fruit with rough surface and ribs on shoulders and carries excellent taste with 10-12 % TSS. **Allahabad Safeda** is round and smooth with pleasant flavour and 10-12% TSS. **Hisar Safeda** a cross between Allahabad Safeda x Seedless developed at CCSHAU has TSS of 13.4%. CISH-G5 is a seedling selection from CISH, Lucknow. CISH-G5 fruits are of apple coloured, medium sized with TSS of about 13.2% (Anonymous 2019).

Table 1.1: Comparison of area and production in five major fruit crops

	India		Punjab	
Fruits	**Area (ha)**	**Production (MT)**	**Area (ha)**	**Production (MT)**
Mango	304111	2737008	6743	107572
Citrus	98566	1330638	47101	1017725
Banana	90483	316689	163	9317
Guava	6938	104077	8205	180775
Grapes	425	8925	420	12105

Table 1.2: Comparison of caloric and nutrient value for 100g edible portion of the three most important fruits of Punjab state *viz*. guava, orange and mango

Parameters	**Guava (100g)**	**Orange (100g)**	**Mango (100g)**
Calories	68 Kcal	49 Kcal	70 Kcal
Protein	2.55 g	0.94 g	0.5 g
Fat	0.95 g	0.3 g	0.27 g
Carbohydrate	14.3 g	11.84 g	17 g
Minerals			
Calcium (Ca)	18 mg	40 mg	10 mg
Iron (Fe)	0.26 mg	0.09 mg	0.13 mg
Potassium (K)	417 mg	179 mg	156 mg
Magnesium (Mg)	22 mg	10 mg	9 mg
Vitamins			
Vitamin C	228 mg	48.5 mg	27.7 mg
Thiamin	0.067 mg	0.087 mg	0.058 mg
Riboflavin	0.04 mg	0.04 mg	0.057 mg
Niacin	1.084 mg	0.274 mg	0.584 mg
Phyto-nutrients			
Lycopene	5404 µg	-	0 µg
Carotene (beta)	374 µg	-	445 µg

***source*: USDA National Nutrient Database for Standard Reference**

In a broad sense, fruits are divided into two categories; climacteric and non-climacteric. Climacteric fruits undergo ripening even after harvesting whereas non-climacteric fruits lacks post-harvest ripening. Guava comes under the category of climacteric fruits as it ripens rapidly after harvesting resulting into change in texture and quality in 3-4 days after harvesting at ambient temperature. Guava fruit comprises high percentage of their fresh weight as water. Consequently, it shows relatively high metabolic activity which continues after harvesting and makes it a highly perishable commodity. Its soft skin leads to high susceptibility to bruising and mechanical injury due to which it cannot be stored for more than a week even during winter season. During peak harvesting time, when there is a surplus of its fruits in the local market, rate of post-harvest loses are also high. To reduce percent losses in guava, it is necessary to evolve technologies for prolonging its keeping quality so that, its transportation to distant markets should become possible. Development of practical solutions to these post-harvest problems requires detailed understanding of biochemistry and molecular biology of fruit ripening process.

Ripening is a genetically programmed and highly coordinated physiological event of organ transformation from unripe to ripe stage. The making of a fruit is a developmental process unique to plants. It involves a complex network of interacting genes and signalling pathways. In fleshy fruits, it involves three distinct stages namely, fruit set, fruit development, and fruit ripening. Of these, ripening has received most attention from geneticists and breeders as fruit ripening is a process that causes the fruits to become more palatable by increasing their sweetness. Also, it makes the fruit less green and soft. Just like any other development process, fruit ripening is also under the control of plant growth regulators (Seymour *et al* 2013). Ethylene is routinely found to be involved in ripening (Lelievre *et al* 1997) and hence is referred to as the "ripening hormone". Ethylene regulates the expression of several genes involved in fruit ripening. Thus, ripening is a physiological process that involves speeding up of metabolic processes by up regulation of genes encoding enzymes involved in ethylene biosynthesis and a cascade of gene activation thereafter (Alexander and Grierson 2002). Other plant hormones such as auxin, abscisic acid (ABA) and brassinosteroids also play major roles in ripening process (McAtee *et al* 2013). In tomato and grapes, it has been found that low level of auxin is required during ripening (Gillaspy *et al* 1993), whereas ABA concentration is low in unripe fruit and it increases during ripening. Study on tomato (Vardhini and Rao 2002) and grapes (Symons *et al* 2006) suggested that brasinosteroid concentration is also increased at onset of ripening. Physiological changes that accompany ripening include increased production of cell wall degrading enzymes such as polygalacturonate (PG), pectin methyl esterase (PME), cellulase and β-galactosidase (Brummell and Harpster 2001). Methyl jasmonate is another plant hormone that initiate fruit

ripening. It was found that application of jasmonate in apple increases the ethylene and ester biosynthesis, red colour anthocyanin and β-carotene content.

Generally, to conduct safe transportation of fruits, certain chemicals are sprayed on fruits for increasing shelf life. Chemicals like 2, 5– norbornadieno, diazocyclopentadiene and silver thiosulphate are used for delaying the ripening process. Importantly, all the three chemicals have toxic effects compared to 1-methylcyclopropene (MCP) and can be used to prevent over-ripening (Fabi *et al* 2007). However high cost and unstable gaseous nature confines its use (Paul *et al* 2010). In banana it was shown that 1-HCP (1- hexylcyclopropene) is more effective than 1-MCP (Kebenei *et al* 2003). Also, 1- HCP effects the rate of respiration, colour, concentration of organic acids and sugar in "Kommeet" tomato fruit (Khan *et al* 2016). Nitric oxide has also been used to increase shelf life of tomato and it was found that tomato fruit when treated with nitric oxide show reduced ethylene biosynthesis, probably due to reduced expression of *LeACO1, LeACOH2 and LeACO4* genes (Eum *et al* 2009). Also, the use of nitric oxide in banana hinders the expression of *MA-ACSI* and *MA-ACO1* gene (Cheng *et al* 2009).

We wished to look at the physico-chemical and morphological traits of guava cultivars commercially grown in Punjab and/or National level during ripening. I measured total soluble sugars, titratable acidity, vitamin C, reducing sugars and firmness during ripening in white fleshed and pink fleshed genotypes variable in keeping quality. White fleshed cultivars included Allahabad Safeda, Punjab Safeda, Thailand guava, Hisar Safeda, Shweta, L-49/Sardar guava pink fleshed were Punjab Pink, Punjab Kiran, Arka Kiran, 17-16, Hisar Surkha, Lalit, Purple Local and Apple coloured CISH-G5. We also wanted to understand the genetic mechanism of guava ripening in slow ripening vs fast ripening cultivars. With this vision we laid out the objectives as below

Objectives:

a) Study the physio-chemical fruit characteristics of selected colored and white fleshed guava genotypes

b) Quantify the expression of genes involved in fruit ripening and maturation in contrasting genotypes

c) Characterization and isolation of genes responsible for increased shelf life

CHAPTER II

REVIEW OF LITERATURE

Plant species have evolved numerous mechanisms for seed dispersal for obtaining successive generations of viable and competitive progeny. Fruits are considered as main part of this mechanism and can be narrowly defined as mature carpels. Fruits can be categorised into dehiscent or dry and non-dehiscent or fleshy fruits. *Arabidopsis thaliana* and pods of legumes are examples of dehiscent fruits. Non-dehiscent fruits such as tomato and melons serve as model for plant development study and are the source of human diet. It makes these crops important for studying molecular basis of development and ripening.

The process of ripening is completely biochemical and physiological changes at the terminal stage of fruit development which makes the organ edible and imparts value to fruit, and make fruit important as agriculture commodity (Giovannoni 2001). Ripening and maturation is a developmental process unique to plants. It involves a highly co-ordinated, genetically programmed, irreversible and metabolically active process involving a series of physiochemical, biochemical and organoleptic changes. These changes leads to modification of cell wall structure and texture, conversion of starch to sugars, increased susceptibility to post harvest pathogens, alteration in pigment biosynthesis and accumulation of flavours and aromatic volatiles which influence appearance, texture, flavor and aroma of fruit so that it can achieve an edible final quality for consumption (Giovannoni 2001, 2004; Bouzayen *et al* 2010; Gapper *et al* 2013; Tohge *et al* 2014).

Two classes of ripening fruits, climacteric and non-climacteric, have been characterised to distinguish fruit on the basis of respiration and ethylene biosynthesis rates. Climacteric fruit, such as tomato, cucurbits, avocado, banana, peaches, plums, and apples are distinguished from non-climacteric fruits, such as strawberry, grape, and citrus, by their increased respiration and ethylene biosynthesis rates during ripening (Lelievre *et al* 1997). Although non-climacteric fruits, such as citrus, may respond to ethylene but ethylene is not required for fruit ripening.

From agricultural perspective, ripening brings both positive and negative attributes to fruits. Ripening imparts desirable flavour, colour and texture at considerable expense of loss in crop and increased fruit pathogen susceptibility. As guava is a climacteric fruit, ripening leads to excessive textural softening. Fully ripe guava fruits bruise easily and are highly delicate which leads to mechanical injuries, high incidence of rotting caused by fungi, shrivelling of the fruit and deformity (Jain *et al* 2003). Ripening depreciates the post-harvest quality of guava and makes its handling, storage, transport and marketing difficult (Kader

2002). Genetically regulated changes in fruit physiology necessitates the use of pesticides, post-harvest fumigants, controlled atmosphere storage and shipping mechanism to minimize losses. So, it is important to know the genetics of ripening and effect of chemicals on gene expression to understand the key control points in global ripening and regulation process such as carotenoids, flavours, vitamins and flavour volatiles accumulation which will allow manipulation of nutrition and quality characteristics associated with ripening (Giovanani 2001).

2.1 Model systems for fruit development and ripening

Tomato is mostly utilized as model plant for understanding the mechanism of ripening owing to its diploid genetics, short generation period (approx. 45-100 days), ease of seed and clonal propagation, well characterized single gene mutants, recombinant inbred lines (Eshed and Zamir 1995), availability of mapping population and comparatively small and well annotated genome sequence (Tomato Genome Consortium 2012). Critical role of ethylene and its molecular basis was determined first using tomato as a model system (Lincoln and Fischer 1988). Also, Ethylene biosynthesis and transduction has been well studied in model plant Arabidopsis. In Arabidopsis the gene encoding the ethylene receptors have been isolated using positional cloning (Chang *et al* 1993).

2.2 Ethylene production and role in climacteric and non-climacteric fruits

Two systems are described in ethylene biosynthesis, System I and System II. System I correspond to low ethylene production in the pre-climacteric period of climacteric fruit and is present throughout the development of non-climacteric fruit. System II refers to "autocatalytic synthesis" which involves auto-stimulated massive ethylene production and is specific to climacteric fruit. Therefore, the major ethylene-related differences which distinguish climacteric and non-climacteric fruit is the presence or absence of autocatalytic ethylene production (McMurchie *et al* 1972; Alexander and Grierson 2002).

The ethylene biosynthetic pathway was given by Yang and Hoffmann in 1984. Methionine is adenylated (addition of AMP) and converted to S-adenosyl-L-methionine (SAM) using methionine adenosyl transferase / SAM synthetase. ACC synthase (ACS) converts SAM into ACC 1-aminocyclopropane-1-carboxylic acid (ACC). ACC oxidase (ACO) converts ACC into ethylene (Sato and Theologis 1989; Hamilton *et al* 1990, 1991) (Fig.2.1.1). *ACO* and *ACS* belongs to multigene family of five and nine members, respectively in tomato, with expressions differentially regulated during fruit development and ripening (Barry *et al* 2000).

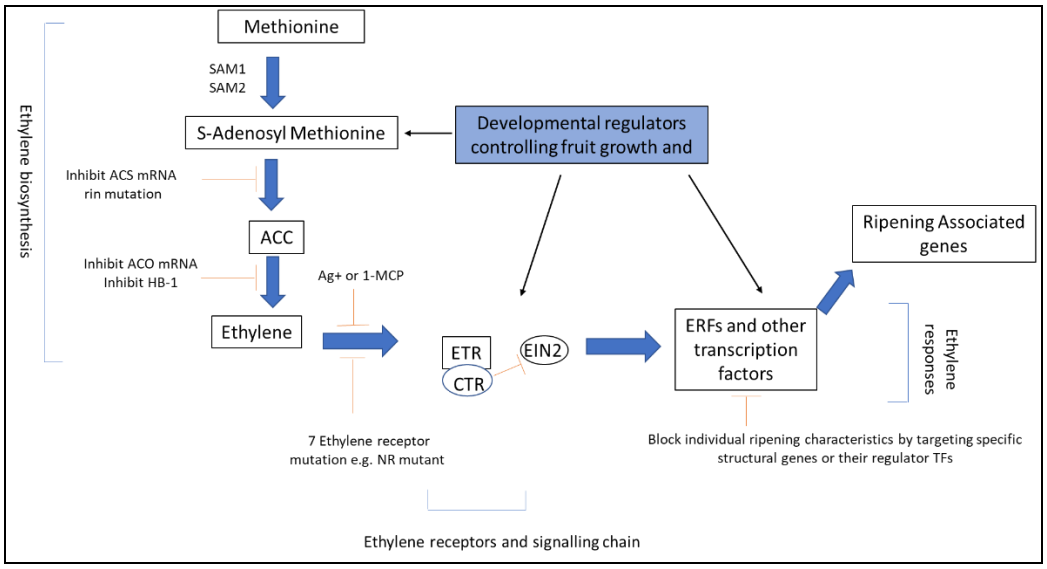

Fig. 2.1: Ethylene Biosynthesis and Signalling (modified from Leng *et al* 2014)

2.2.1 Expression of ethylene biosynthesis genes during ripening

During onset of ripening *LeACO1* and *LeACO4* genes shows upregulation and continue being active throughout ripening whereas *LeACO3* shows only transient activation at the breaker stage of fruit ripening. *Le ACS6* and *LeACS1A* are expressed at the pre-climacteric stage (system 1), while, *LeACS4* and *LeACS1A* are the most active genes during transition to ripening. Subsequently, *LeACS4* shows continue high expression during climacteric phase, whereas *LeACS1A* expression declines. The increase in ethylene production marks the initiation of *LeACS2*, and the inhibition of *Le ACS6* and *LeACS1A* expression.

ACS genes are supposed to play a critical role to switch from pre-climacteric system I to climacteric system II (Barry *et al* 2000). System I is characterized by inhibitory feedback of ethylene in its own biosynthetic pathway, whereas the transition to system II is characterized by autocatalytic production. The importance of ethylene in triggering the ripening of climacteric fruit has been clearly demonstrated by the downregulation of *ACO* and *ACS* genes in transgenic plants using an antisense strategy. The ethylene-suppressed lines showed strongly delayed ripening in tomato (Oeller *et al* 1991; Picton *et al* 1993). Study on other fruits also show same results, such as in melon (Ayub *et al* 1996) and apple (Dandekar *et al* 2004). However, ethylene-independent ripening pathways exist in climacteric fruit, as illustrated in melon fruit, where part of softening, sugar accumulation, and coloration of the flesh occur in ethylene suppressed fruit (Flores *et al* 2001). These results have led to the conclusion that climacteric (ethylene-dependent) and non-climacteric (ethylene-independent) regulation coexists in climacteric fruit (Pech *et al* 2008).

2.2.2 Ethylene perception and signal transduction during fruit ripening

Mutation studied in Arabidopsis have made it possible to reveal ethylene perception and signal transduction like identification of the ethylene-insensitive mutants, *ETR1* (Bleecker *et al* 1988). Ethylene receptors are encoded by a small multigene family of structurally distinct genes but functionally redundant proteins working either as hetero or homo-multimers. In tomato, six ethylene receptor genes have been isolated and found to be expressed in all plant tissues, three of these showing a net increase during ripening, while two express constitutively. Also, it was verified that the tomato Never-ripe (Nr) mutation, which results in impaired ripening, occurs in one of the ethylene receptor genes. Ethylene receptors are rapidly degraded during fruit ripening; however, the transcription rate remains high. The receptor level determines the timing of ripening (Kevany *et al* 2007). Moreover, the suppression of the ethylene receptor *LeETR4* led to an early ripening of tomato fruits (Kevany *et al* 2007). The *CTR1* gene (Constitutive Triple Response) was first isolated from Arabidopsis which is another major component of ethylene signalling lying downstream of the receptor acting as a negative regulator of the ethylene transduction pathway (Kieber *et al* 1993). The tomato *CTR1* gene (*Sl-CTR1*) was first isolated from fruit tissue (Leclercq *et al* 2002), and in spite of being a negative regulator of ethylene responses, its transcripts are up-regulated during fruit ripening, corresponding with the rise in ethylene production. In tomato *CTR* family consist of four genes, each gene exhibiting a specific pattern of expression during ripening and in response to ethylene, with *Sl-CTR1* being the most actively expressed during fruit ripening (Adams-Phillips *et al* 2004). Additional ethylene receptor family member has been identified in tomato, GREEN-RIPE (GR) fruit-specific ethylene insensitive mutation of tomato (Barry and Giovannoni 2006). GR is a homolog of Arabidopsis gene, *REVERSION TO ETHYLENE SENSITIVITY1 (RTE1)* (Resnick *et al* 2006). GR and its allele, *Never-Ripe2 (NR2)* are dominant non-ripening mutants having similar fruit phenotypes to NR. Interestingly, Barry *et al* 2005 found that the fruit-specific ethylene insensitivity of GR fruit is a result of the over-abundance of *GR* expression in a tissue usually not normally found at high levels, which result in dramatic inhibition of fruit ripening even though the fruit still produce normal to elevated levels of ethylene. The exact role of *GR* and *RTE1* remain unclear. However, they influence ethylene receptor- copper interaction due to their role in membrane localization and putative copper-binding activities (Rodriguez *et al* 1999; Chen *et al* 2002).

Positive regulators which mediate later steps of ethylene signalling are *ETHYLENE INSENSITIVE 2 (EIN2)* and *ETHYLENE INSENSITIVE 3 (EIN3)*. In both Tomato and Arabidopsis *CTR* negatively regulates *EIN2* and there is just one *EIN2* gene found, through which all ethylene processes are transmitted. In tomato, *EIN2* expression increases at the

onset of ripening (Wang *et al* 2007). In addition to transcriptional control of *EIN2* mRNA accumulation, *EIN2* is also a target for protein turnover *via* the 26S proteosome. Qiao *et al* (2009) identified two E3 ligases from Arabidopsis designated *EIN2 TARGETING PROTEIN 1 (ETP1)* and *EIN2 TARGETING PROTEIN 2 (ETP2)* that bind to *EIN2* and mediate its degradation.

The ethylene signalling cascade ends with transcriptional activation of a large family of transcription factors termed *ETHYLENE RESPONSE FACTORs (ERFs).* The *ERFs* are transcriptionally activated by *ERFs* (Klee and Giovannoni 2011) indicating autocatalytic feed forward mechanism of regulation. Lee *et al* (2012) defines that *SlERF6* act as a negative regulator for ethylene and carotenoid biosynthesis in maturing tomato fruit.

2.2.3 Ethylene plays a major role in regulating ripening and softening of climacteric fruit

Study in Charentais melon (Ayub *et al* 1996), revealed the expression of some ripening-related cell wall-associated genes and activities, including those of *PGs (Polyglactouranase*) (Sitrit and Bennett 1998; Hiwasa *et al* 2003, 2004), *expansins* (Rose *et al* 1997), and *EGases (*Endo-1,4-β-D-glucanases) (Lashbrook *et al* 1994). It was found that when the fruit were treated with exogenous ethylene the rapid softening of the pulp occurred coincident with an induction of *PG* gene expression. In 1-MCP-treated (*1-Methylcyclopropene*) fruit however, pulp firmness showed only a marginal decrease during ripening, but insufficient to produce soft, edible fruit. No significant increase in *PG* mRNA levels, and PG activity were observed (Fabi *et al* 2009). It has been found that genetically engineered tomatoes and melons with suppressed ethylene biosynthesis exhibit delayed and reduced fruit softening (Murray *et al* 1993; Picton *et al* 1993; Ayub *et al* 1996; Guis *et al* 1997; Flores *et al* 2001). However, whether melon fruit softening is totally, or partially, dependent on ethylene has not been clearly established, since transgenic melon with reduced expression of the *1-aminocyclopropane-1-carboxylate oxidase (ACO)* gene showed a delayed but significant decrease in flesh firmness during ripening (Ayub *et al* 1996; Guis *et al* 1997; Flores *et al* 2001). The application of a highly potent inhibitor of ethylene perception, 1-methylcyclopropene (1-MCP) (Sisler *et al* 1996) provides an alternative approach to assess the role of ethylene in fruit softening. It was previously demonstrated that treatment of pear fruit with 1-MCP after the onset of ripening restricted softening and prevented the accumulation of *PG* mRNA and endo-PG activity (Hiwasa *et al* 2003).

2.2.4 Transcriptional Regulation of Fruit Ripening

Several well-characterised tomato mutations and their dramatic effects on fruit ripening reveal the transcriptional control of fruit maturation such as study on *ripening-inhibitor (rin), non-ripening (nor) and Colourless non-ripening (Cnr)* mutations helped to

understand transcriptional control. *MADS-box* transcription factor was the first gene to be characterized that is partially deleted in *rin* mutants (Vrebalov *et al* 2002). The RIN-MADS protein binds the consensus *MADS-box* CArG DNA-binding site and interacts with promoters of numerous ripening-related genes (Fujisawa *et al* 2013; Zhong *et al* 2013). Transcriptome studies on normal and *rin/rin* fruit shows that *RIN-MADS* activity contributes to the expression of hundreds of ripening-related genes.

Martel *et al* (2011) used chromatin immunoprecipitation (ChIP) to determine interaction of the *RIN* protein with the promoters of target genes, involved in most ripening-related processes and those are *CNR-SPL* dependent. As studied in other MADS-box proteins, RIN-MADS forms complexes with additional MADS-box proteins (Ito *et al* 2008; Martel *et al* 2011) and has been associated with promoter binding of CArG-box recognition sequences. Numerous putative promoter targets have been identified on this basis (Kumar *et al* 2014; Qin *et al* 2012). Specific interactions of CNR-SPL with ripening gene targets are less clear though *TDR4*, a tomato *FRUITFUL (FUL)* ortholog, is normally found to be ripening-induced and substantively suppressed in fruit of the *Cnr* mutant. Jaakola *et al* (2010) reported that transgenic suppression of *TDR4* (also known as *FUL1*) had little effect on tomato fruit ripening while simultaneous suppression along with the closely related *FUL2* gene resulted in ripening phenotypes (Bemer *et al* 2012). Interestingly, allelic variation at the apple *FUL* locus has also been associated with fruit ripening attributes, specifically softening (Cevik *et al* 2010), indicating as with *RIN* the possibility of evolutionary conservation and translational utility for crop improvement (Manning *et al* 2006).

Using reverse genetic approaches, a number of additional ripening related transcriptional regulators have been identified (Lin *et al* 2008). For an instance HD-zip Homeobox protein has been found to bind with *ACC oxidase (leACO1).* Hamilton *et al* 1990; Picton *et al* 1993 showed that antisense silencing of *LeACO1* in tomato cause reduced ethylene and also proved that *LeHB1* act as a positive regulator of *LeACO1*. Martel *et al* (2011) also suggested the involvement of *RIN, LeHB1 and ACO1*in ripening. Two AGAMOUS-like MADS-box proteins, TOMATO AGAMOUS 1 (TAG1) and TOMATO AGAMOUS-LIKE 1 (TAGL1), physically interact with the RIN-MADS protein and the genetic repression in these genes results in ripening inhibition (Fujisawa 2014, Martel 2011) similar as in *cnr* and *rin* phenotype (Itkin *et al* 2009). TAGL1 is the tomato ortholog of the duplicated and redundant Arabidopsis *SHATTERPROOF 1 (SHP1)* and *SHP2* genes, which are necessary for normal development of valve margins in the silique and subsequent organ shattering and seed release (Liljegren *et al* 2000).

Zhu *et al* (2013) and Kou *et al* (2016) reported that *NAC* gene have modest ripening effects in tomato. *FRUITFULL HOMOLOG-1(FUL1/TDR4)* and *FRUITFULL HOMOLOG-2*

(FUL2/MBP7) (Bemer *et al* 2008), interact with *RIN* (Martel *et al* 2011) and are involved in ripening process. Transgenic tomato plants developed by knocking down the expression of *TAG1* resulted in reduced carotenoids, thin pericarp and yellow fruit color due to decreased expression of *RIN* target *ACS2* and *TAG1* (Karlova *et al* 2014). ERF subfamily contains single *AP2-ERF* domain member which interact with GC regulator DNA sequence (GCC BOX) associated with ethylene-mediated transcription of target gene (Ohmetakag and Shinshi 1995). In tomato fruit pattering, determinacy and early development is regulated by one or more miR156-targeted *SQUAMOUSA* promoter binding protein (SBP/SPL). Fruit formation and fruit yield are affected in tomato by overexpression of *miRl56* (Zhang *et al* 2011). *SlAP2a* and *SlMADS1* were shown to act as negative regulators of fruit ripening (Chung *et al* 2010). RNAi repression of these genes in tomato results in a fruit that overproduce ethylene indicating *SlAP2a* act as a modulator of tomato ripening. The *non-ripening (nor)* mutations were originally identified in tomato and gene controlling fruit (Tigchelaar 1973) traits have been characterized as *NAC* domain family transcription factors (Martel *et al* 2011) and *nor* is proposed to function in transcriptional activation cascade regulating ripening-related processes. *RIN* and *CNR* mutations block ripening process in fruit, due to the inhibition of ethylene production (Manning *et al* 2006). In *RIN* mutants, autocatalytic genes involved in ethylene biosynthesis, *SlACS2* and *SlACS4* are suppressed (Barry *et al* 2005). In apple, *MADS819* gene was found to control fruit ripening characters such as starch degradation and ethylene modulated ripening traits. *Colorless NON-RIPENING MUTATION(CNR)* lead to epigenetic changes and cause increased cytosine methylation in upstream region of promoter of *SQUAMOSA* promoter-binding protein-encoding genes leading to decreased gene expression and inhibition of normal fruit ripening (Manning *et al* 2006).

2.2.5 Major genes and transcriptional factors involved in fruit ripening

ACC2/4 ACC synthase (ACS) is comprised of multigene families whose members have different physiological roles and are regulated by different mechanisms (Mathooko *et al* 2001). *ACC2/4* expression increases during ripening in peach and belongs to System II in ethylene biosynthesis (Wu *et al* 2018; Hayama *et al* 2006). Treatment of apricot with ethylene inhibitors such as 1-MCP resulted in decrease in the expression of *ACC* genes and delayed ripening (Munoz-Robredo *et al* 2012). *ACS4* is the most important gene expressed during ripening (Oeller *et al* 1991; Theologis 1992).

EIN3 is a nuclear localized protein that exhibit DNA binding activity and has been confirmed to target *Ethylene Response Factors (ERFs)* (Solano *et al* 1998). There are three *EILs* in tomato and have been proposed to regulate ethylene responses. It has been confirmed through antisense expressions that *EIL1-3* are the positive regulators of ethylene response.

Also, it has been suggested that differential expression and regulation of ethylene response occurred downstream from *EILs* at *ERF* level (Tieman *et al* 2001). Overexpression of *LeEIL* overcomes the negative regulation of mutant *NR* receptors and activates ethylene signal transduction pathway downstream which results in restoring ripening related genes such as *Polyglalctouranase* and *LeACO1* (Chen *et al* 2004) providing the evidence of role of *EIL* in ripening.

ERF6 is highly expressed in fleshy fruits and has been shown to have strong correlation with carotenoid and ethylene regulation. Expression analysis of *SlERF6* suggested that first the expression increases at mature green stage, reaches its maximum level at post breaker stage and then further decreases thus showing correlation with later fruit development and ripening (Li *et al* 2013). *ERFs* have been found to control some genes which effect ripening-related traits, such as color, firmness, aroma, taste, and postharvest shelf life (Solano *et al* 1998; Ju and Chang 2015). Also, the decreased expression of ERF6 was observed in *RIN* and *NOR* mutant fruits confirming that *SlERF6* is the positive regulator of ethylene responses and plays a primary role in fruit ripening (Li *et al* 2019).

MADS box gene is regarded as master regulator for molecular development and regulation in tomato (Giovanani *et al* 2004; Itkin *et al* 2009). Positional cloning of *RIN* locus, gene repression and mutant complementation reveals the role of *MADS-box* gene in tomato fruit ripening (Vrebalov *et al* 2002). A mutation in *RIN* locus as a ripening inhibitor was first reported half a century ago (Robinson and Tomes1968). *RIN* is regarded as the major locus for ripening as it initiates and accelerates all the ripening associated changes including red pigmentation, fruit softening and ethylene biosynthesis (Verbalov *et al* 2002; Fujisawa *et al* 2013). Mutation in this locus prevents ripening by never turning to red, retain flesh firmness and suppress ethylene biosynthesis (Tigchelaar 1978). CRISPR-Cas induced mutation of *RIN* gene results in low pigmentation and delayed ripening (Ito *et al* 2008).

TAG1 is another *MADS* box protein involved in ripening. RNAi repression of *TAG1* gene in tomato led to inhibition of ripening and reduce pericarp thickness (Vrebalov *et al* 2009). TAG1 repression also cause change in fruit pigmentation. Overexpressing *TAGL1* accumulated more lycopene (Itkin *et al* 2009; Vrebalov *et al* 2009), and reducing *TAGL1* mRNA led to reduced carotenoids, thinner fruit pericarp and decreased ethylene, which inhibited ripening changes significantly and resulted in yellow-orange colored fruits with increased firmness (Vrebalov *et al* 2009;Gimenez *et al* 2016). This suggests that *TAGL1* controls carotenoid synthesis by modulating ethylene synthesis and signalling in combination with *MADS-RIN* (Garceau *et al* 2017). *FUL1* and *FUL2* are functionally redundant in fruit ripening regulation and dual suppression of both genes substantially inhibited ripening by blocking ethylene biosynthesis and decreasing carotenoid accumulation (Wang *et al* 2014).

FUL1/FUL2 homologs function in many biological processes both in cooperation with and independent of *MADS-RIN* (Fujisawa *et al* 2014), and also regulate ethylene-dependent and independent aspects of fruit ripening (Bemer *et al* 2012; Shima *et al* 2014).

TDR4 plays a role in ripening by reducing the lycopene levels by downregulating the expression of *1-deoxy-D-xylulose5 phosphate synthase1(DXS1)*, which affected lipid and cuticle metabolism resulting in fruit wilting and a significant decrease in free glutamic acid by upregulating the expression of the glutamic acid decarboxylase gene (Bemer 2012).

Mutation in the gene encoding a member of *NAC* transcription factor family results in *Nor* mutation which plays an important roles in diverse physiological processes during development, including the stress response, flowering, and senescence (Martel *et al* 2011). *NAC-NOR* mutant exhibits altered ripening than the normal gene such as higher firmness, reduced lycopene accumulation etc (Karlova 2014). Through VIGS induced gene silencing in *NAC* gene confirmed that expression of *SAM1* and *ACO* is regulated by *NOR* gene (Yuan *et al* 2016). Various *NAC* genes involved in ripening are *SlNAC1*and *SlNAC4* (Zhu *et al* 2014).

LeETR3, the gene encoding tomato *NEVER RIPE* affect the ripening in tomato plant (Wilkinson *et al* 1995). Wilkinson *et al* (1995) mapped *LeETR3* to chromosomal locus corresponding to *NEVER RIPE(NR)*. A tomato ripening mutant which is well categorized for its insensitivity to ethylene in a variety of physiological processes including fruit ripening, floral senescence, hypocotyl elongation, epinasty and organ abscission (Lanahan *et al* 1994). Lashbrook *et al* (1998) provide evidence for the regulation of ethylene by *NR* genes, resulting significant increase in ethylene sensitivity observed in ripening fruit.

ETP1 and ETP2 also play a role in ripening as downregulation of *ETP1* and *ETP2* genes resulted in partial ethylene-insensitive phenotypes as compared to wild type phenotypes (Qiao *et al* 2009). ETP1 and ETP2 interacts with *EIN2*. Down-regulation of *EIN2* by a co suppression mechanism or via VIGS strategy resulted in ethylene insensitivity and ripening inhibition associated with reduced expression of ethylene- and ripening-related genes (Fu *et al* 2005; Hu *et al* 2010), suggesting that *LeEIN2* is a positive regulator of ethylene-mediated responses during fruit ripening.

CTR1, acts directly downstream of the ethylene receptors. *CTR* is the Mitogene-activated protein kinase kinase. The *ctr1* loss-of-function mutations result in the constitutive activation of ethylene response in seedlings and adult plants, demonstrating that the encoded protein acts as a negative regulator of ethylene signalling (Lin *et al* 2008; Klee and Giovannoni 2011). So far, four *CTR1* homologs (*SlCTR1*, *SlCTR2*, *SlCTR3*, and *SlCTR4*) have been identified in the tomato (Leclercq *et al* 2002; Adams-Phillips *et al* 2004; Lin *et al* 2008). All tomato *CTRs* show ability to interrelate with one or more ethylene receptors in

yeast two-hybrid systems (Zhong *et al* 2008). The ethylene-responsive *CTR1* (Zegzouti *et al* 1999; Leclercq *et al* 2002) exhibit a ripening-related expression pattern. *SlCTR1* displays a typical ripening-regulated expression, whereas *SlCTR2* shows a steady increase in its expression during ripening and was up-regulated in ripening-impaired mutants *Nr* and *rin* (Lin *et al* 2008a), suggesting its putative role in the ripening process. The suppression of *SlCTR1 via* VIGS strategy was reported to promote tomato fruit ripening, consistent with *CTR* being a negative regulator of climacteric ripening (Fu *et al* 2005).

LeETR4 under the control of CaMV35S promoter exhibited a constitutive ethylene response and were severely affected (Tieman *et al* 1999). When antisense plants were developed, using this receptor with fruit-specific promoter, fruits showed early ripening. Expression analysis confirms that *ACO1* and *ACO4* are the main genes supporting ripening-associated ethylene production (Nakatsuka *et al* 1998). In addition knockouts of *ACO1* in the "galia" melon parental line confirmed that *ACO1* is inhibiting 99% ethylene synthesis (Nunez-Palenius *et al* 2007). ACO expression increased in peach and apricot throughout ripening (Mbeguie-A-Mbeguie *et al* 1999; Wu *et al* 2018).

2.3 Role of ABA in fruit ripening

Role of Abscisic acid was first discovered in plant wilting and stomatal closure (Mittelheuser and Van Steveninck 1969; Wright and Hiron 1969). Later on, ABA was identified to play a crucial role in fruit development and ripening. It was found that ABA accumulation was increased at onset of ripening in both climacteric and non-climacteric fruits (Buesa *et al* 1994). By applying exogenous ABA, production of various ripening related metabolites increased (Cherny and Zeevaart 2000; Ban *et al* 2003; Jeong *et al* 2004; Giribaldi *et al* 2010). ABA deficient mutant exhibits different growth pattern as compared to wild type (Taylor *et al* 2000; Galpaz *et al* 2008). Also, Rodrigo *et al* (2003) suggested that de-greening stage of fruit began later in ABA deficient plant. All these above studies provide an evidence for the role of ABA in fruit development and ripening.

2.3.1 ABA biosynthetic Pathway

ABA is synthesised *de novo* from C_{40} carotenoid. The first step in carotenoid biosynthesis is the formation of phytoene from two molecules of geranylgeranyl diphosphate (GGPP) followed by lycopene formation by four desaturase steps: cyclization at both ends of lycopene molecule give rise to *α-* or *β-carotene* which further undergoes hydroxylation at C3 and C3′ to form the xanthophylls, lutein and zeaxanthin. In plastids ABA biosynthesis is initiated with the hydroxylation and epoxidation of *β-carotene* to produce all trans-xanthophylls, zeaxanthin and violaxanthin. After that violaxanthin gets converted into 9-cisepoxyxanthophylls to yield xanthophylls which is the first C_{15} intermediate of ABA.

Xanthonin further oxidised in two steps to form ABA (Qin and Zeevaart 1999; Taylor *et al* 2005). *NCED* is the rate limiting step in the ABA biosynthesis (Thompson *et al* 2000; Iuchi *et al* 2001;Tung *et al* 2008). The hydroxylation reaction give rise to ABA catabolic pathway I. In this pathway C-8' is at predominant position for hydroxylation reaction among three different methyl groups. In Arabidopsis C-8' is controlled by *CYP707A* gene family (Kushiro *et al* 2004; Saito *et al* 2004). Moreover, ABA homeostasis can be altered by ABA conjugation by cytosolic *UDP glucosyltransferases (GTs)* or release by *β-glucosidases (BGs)*, through intracellular or inter tissue transport processes (Sauter *et al* 2002; Seo and Koshiba 2011).

ABA content in fruits can be determined by studying the dynamic balance between biosynthesis (*NCED genes*), catabolism (*CYP707A genes*), and reactivation (*BG/GT genes*). Changes in dynamic balance results in the alteration in ABA biosynthesis and among these *NCED* is the key biosynthetic enzyme which was first isolated in maize *vp14* mutant (Tan *et al* 1997), also characterized in various climacteric fruits such as in apple (Lara and Vendrell 2000), peach (Alvaro *et al* 2013), tomato (Burbidge *et al* 1999), and melon (Sun *et al* 2013). Rodrigo and Zacarias (2007) and Wheeler *et al* (2009) also characterized this gene in non-climacteric fruits such as orange and grape. Huo *et al* (2013) cloned four *NCED* genes from tomato. ABA level in tomato fruits is regulated by *SlNCED1* at transcriptional level (Zhang *et al* 2009a). It was observed that tomatoes overexpressing *SlNCED1* had more ABA accumulation, drought tolerance and lower transcription (Thompson *et al* 2000). Also, ABA deficient mutant had lower mean fruit weight and showed different development and ripening pattern as compared to the wild type (Galpaz *et al* 2008). In transgenic tomato fruits having silenced *SlNCED1* gene showed reduction in endogenous ABA level and inhibit cell wall degradation which suggests that *NCED* is a key gene in ABA biosynthetic pathway (Sun *et al* 2012).

CYP707A which encodes ABA 8' hydroxylase can also alter dynamic balance of ABA levels (Saito *et al* 2004). As determined in Arabidopsis seedling enhanced expression or loss of function of *CYP707A* gene can effect ABA level and change their sensitivity to exogenous glucose (Zhu *et al* 2011). Reactivation step also plays a major role in altering ABA levels (Price *et al* 2003; Burla *et al* 2013). Role of BGs (Beta galactosidase) in activation of hormones groups including ABA, cytokinin and auxins has been reported in various plants (Brzobohaty *et al* 1993; Lee *et al* 2006). Gene silencing of *FABG3* gene in strawberry shows that *FABG3* gene effect ABA accumulation (Li *et al* 2013) (Fig. 2.2).

2.3.2 ABA signal transduction and transcription regulation

Last decade marks the progress in understanding of ABA signal transduction and receptor molecules. ABA mediated signalling cascade is initiated by perception of ABA through ABA receptors. ABA receptors includes *ABAR/CHLH* (Mg- chelated H subunit)

(Mochizuki *et al* 2001; Shen *et al* 2006; Shang *et al* 2010) and *GPCR* type *G* protein (Liu *et al* 2007). Studies on these receptors suggest that the exact nature and role of these receptors are still unclear (Muller and Hansson 2009; Wu *et al* 2009). Breakthrough step in ABA signalling was with the identification of PYR/PYL/RCAR protein family, the family of type 2C protein phosphatase and subfamily 2 of SNF1- related kinase (Ma *et al* 2009; Melcher *et al* 2009; Gonzalez-Guzman *et al* 2012).

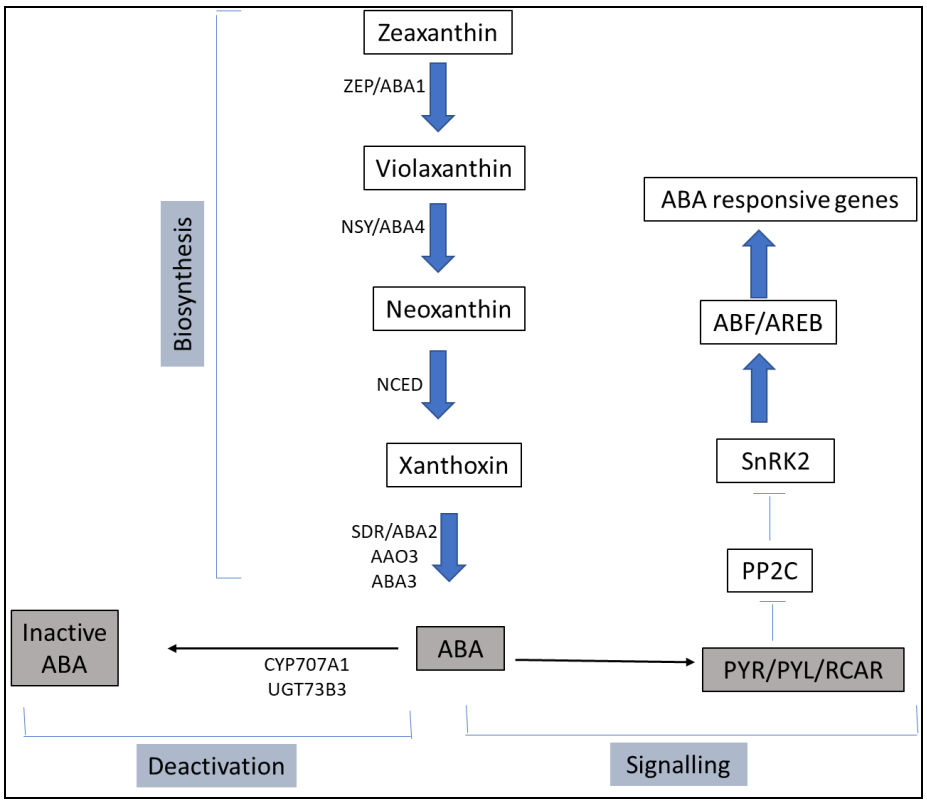

Fig. 2.2: ABA biosynthesis and Signalling (modified from Leng *et al* 2014)

2.3.2.3 ABA signal transduction model

In the absence of ABA, *SnRK2* is inhibited by *PP2Cs* by their physical interaction and phosphatase activities. ABA molecule when binds to ABA receptors *PYR/PYL/RCAR* give rise to structural changes in receptors and enables the interaction of ABA receptors with PP2Cs and hinders the interaction between *PP2Cs* and *SnRK2s*. *SnRK2s* are released from *PP2Cs* and activates their downstream targets including transcription factors such as ABA-responsive element binding proteins (AREBs) and ABA-responsive element binding factors (ABFs) (Klingler *et al* 2010). These transcription factors are the basic leucine zipper family (bZIP)-type DNA-binding domains that binds the ABA-responsive element and have a pivotal role in ABA-dependent gene activation (Choi *et al* 2000; Hattori *et al* 2002; Kang *et al* 2002; Gomez-Porras *et al* 2007). For instance *AREB1*, *AREB2*, *ABF3*, and *ABF4* positively regulate the expression of ABA signalling response genes (Yoshida *et al* 2010). Several TFs which are

important in fruit ripening are also essential for understanding ABA signalling pathway comprising *vvABF2* which is a grape *bzip* transcription factor and overexpression of *vvABF2* in grapes leads to up regulation and modification in ABA responses which results in enhancement and change in phenol synthesis and cell wall softening (Nicolas *et al* 2014).

MYB10 is another TF involved in flavonoid/ /phenylpropanoid pathway during ripening in strawberry fruit. ABA activates the *R2R3-MYB10* in strawberry during ripening. Many early regulated and late regulated genes used for anthocyanin production in ripened fruits are regulated by *MYB10* (Medina-Puche *et al* 2013). Anthocyanin biosynthesis in red coloured cherry was controlled by ABA and PacMYB expression, furthermore ABA might control PacMYB (Shen *et al* 2014). Fruit development can also be controlled by other TFs like *FVCDPK* a calcium dependent protein kinase from wild type strawberry and is expressed during ripening and is upregulated by ABA (Jia *et al* 2016).

2.3.3 Evidences for the role of ABA in fruit ripening

There is evidence to support role of ABA in photosynthate unloading from phloem in developing fruits. For instance, ABA treatment boosted the uptake of sugar into vacuoles in apple fruit flesh (Yamaki and Asakura 1991) and increased the sugar content of developing citrus fruit (Kojima *et al* 1995). Recently, it has been found that the manipulation of endogenous sucrose content alters the expression of *FaNCED* genes and the content of endogenous ABA in strawberry fruit (Jia *et al* 2013). Anthocyanin biosynthetic pathway is regulated by ABA and anthocyanin accumulation induced by ABA results in enhancing fruit colour and activates plant tissue defence against potential damage by prompting biosynthesis of phenols which act as an antioxidant (Lacampagne *et al* 2009). For an instance, in bilberry ABA levels and expression of *VmNCED1* and the *neoxanthin synthase gene (VmNSY)* increased sharply at the onset of ripening, the stage in which expression of the *chalcone synthase (VmCHS)* and *anthocyanidin synthase genes (VmANS)* also increases along with the accumulation of ABA (Karppinen *et al* 2013). Recent studies showed that ABA treatment results in the up regulation of transcriptional regulators of structural genes in the phenylpropanoid and flavonoid pathways, as well as genes considered to be involved in the acylation and transport of anthocyanin into the vacuole (Koyama *et al* 2010; Berli *et al* 2011).

Cell wall have an important role in fruit texture formation. This fruit texture is also regulated by ABA. Alteration in the cell wall results in fruit softening, which is associated with the expression of a number of hydrolase and transglycosylase genes (Huber 1983), together with increases in enzyme activity and mRNA levels of *polygalacturonase (PG)* (Smith and Gross 2000), *pectin methylesterase (PME)* (Tieman *et al* 1992), *endo-β1,4-glucanases (Cels)* (Lashbrook *et al* 1994), and *expansin (Exp1)* (Brummell *et al* 1999;

Cosgrove 2000). Generally, it is believed that fruit softening is induced by ethylene and due to variable effects of ethylene on the activities of hydrolases during ripening (Nishiyama *et al* 2007). However, a direct observation and molecular level evidence of the involvement of ABA in fruit softening has recently been provided (Sun *et al* 2017). In *SlNCED1-RNAi* transgenic fruits, the pectin content was reported to be significantly higher, the shelf life was longer, and the pulp was firmer and more flexible than in control fruits during the ripening stage (Sun *et al* 2013). Also, the role of ABA in regulating these genes rest on the stage of fruit development.

2.3.4 ABA effect on Ethylene biosynthesis and its role in ripening

Fruit ripening is not simply modulated by individual hormone, but is under the regulation by a complicated network of feedback and crosstalk among different phytohormones (Kumar *et al* 2014). Earlier role of ABA was found only in non-climacteric fruits, but latter on role of ABA was determined in climacteric fruits (Zhang *et al* 2009b). Some prior studies indicate the effect of ABA on ripening process, such as in mango and tomato ABA has been found to affect cell wall catabolism to softening (Zaharah *et al* 2013; Sun *et al* 2012). Recent studies confirmed that ABA has been widely acknowledged to regulate ethylene biosynthesis and signalling during fruit ripening. Mou *et al* (2016) reported change in expression of genes involved at various stages of ethylene biosynthesis with respect to ABA. Such as there is decrease in the activity of *SAM* genes in the presence of ABA and the expression is enhanced in nordihydroguaiaretic acid (NDGA) treated tomato fruits. Similarly, *LeACS4, LeACS6* and *LeACO1* are up regulated in *NDGA* treated fruits and *ETR6, ERF3,* and *ERF*4 shows enhanced expression in the presence of ABA.

2.4 Cell- wall metabolism

One of the most noticeable change occurring during ripening is cell wall remoulding which results in change in texture in fleshy fruits making them more palatable (Fabi *et al* 2007). Cell wall metabolism participates in cell wall turgor and fruit texture. The genes involved for these changes are the main targets for the genetic manipulations of fruits (Vicente *et al* 2007; Goulao and Oliveria 2007; Matas *et al* 2009). Pulp softening results in post-harvest deterioration and losses and this softening is due to structure and composition of primary wall of plant cell which is composed of cellulose microfibrils crosslinked with hemicellulose embedded in pectin matrix (Carpita and Gibeaut 1993) which consists of complex domain of both linear and highly branched polysaccharides and having a backbone of galactosyluronic acids (Brummnell 2001). The cell wall loosing, and disintegration is due to enzyme hydrolysis. The dismantling of cell wall take place by the action of pectin degradation enzymes. Several enzymes are identified which are involved in cell wall metabolism among them *polyglactouranase* is thought to be the major enzyme involved in

fruit softening that removes galactourysl residues from pectin (Atkinson *et al* 1998). Fabi *et al* (2009) described the role of PG in fruit softening in papaya. Increase in PG was inversely correlated with decrease in firmness or softening (Fabi *et al* 2009). Mostly genes encoding cell wall modifying enzymes were isolated in tomato (Gapper *et al* 2013). α -L-*arabinofuranosidase* is a cell wall modifying enzyme which play a role in softening by removing arabinosyl and some other residues from pectin (Sozzi *et al* 2002; Itai *et al* 2003). α-1,2 linkages between galacturonosyl and rhamnosyl residues in pectin is hydrolysed by rhamnogalacturonase (Wong 2008). Galactosidase removes galactosyl residues from pectin and xyloglucans (Smith *et al* 2002) and participates in ripening (Smith and Gross 2000). *β-galactosidase* II (*TBG4*-encoded gene product), was shown to increase more than 4-fold during ripening in wild type but not *rin* and *nor* fruit (Carey *et al* 1995). Mannase hydrolysis β-linkage mannan backbone (Rodriguez-Gacio *et al* 2012). Pectate Lyases catalyse the eliminative cleavage of de-esterified pectin (Marin-Rodriguez *et al* 2002) and pectin methyl esterases degrade methyl esterified polyuronides (Wakabayashi *et al* 2003). Tieman *et al* 1992 reported that antisense repression of *PME* in tomato results in pectin degradation (Rose *et al* 1997) Brummell *et al* (1999) reported that expansins interrupt the hydrogen bond between cellulose microfibrils and cross linking glycans. *Expansin* genes are found to be upregulated in tomato and strawberry during ripening (Civello *et al* 1999) and overexpression of *expansins* show increased softening of mature green fruit (Brummell *et al* 1999). All the above-mentioned cell wall proteins associated with tissue and developmental stages often leadsto rapid changes in size and shape of the fruits (Cosgrove 2000).

Recent studies in tomato suggests that cuticle structure and composition is considered to be associated with substantial variation in fruit texture and softening (Saladie *et al* 2007). Cuticle has various function that have significant influence on fruit quality and texture as it helps to maintain fruit skin integrity (Hovav *et al* 2007), restrict transpiration (Kerstiens 1996) and limits microbial infection (Reina-Pinto and Yephremov 2009). The waxy component of cuticle is composed of triterpenes alkanes, esters and alcohols (Gapper *et al* 2013). Vogg *et al* (2004) determined that *Slcerf6* is a tomato *β-ketoacyl Co-A* synthase essential for formation of long chain C31 alkanes and C32 aldehydes. Kosma *et al* (2010) revealed that cuticle undergoes various changes during fruit ripening by exploring cuticle metabolite components of two tomato fruit ripening mutant *rin* and *nor*.

2.4 Integrated role of Abcisic acid, Brassinosteriods and Methyl jasmonate in fruit ripening

ABA is a major plant growth regulator and plays a key role in fruit development and ripening. With increase in ABA concentration ethylene biosynthesis increases in climacteric fruits (Zhang *et al* 2009b). For instance, in ‘Granny Smith’ apples, increased ABA level

results in synthesis of *de novo* ACO protein, causing an increase in endogenous ethylene levels, which subsequently enhances *ACS* expression (Lara and Vendrell 2000). Genes like NCED have a key role in ABA biosynthesis. NCED is cloned in various fruits such as tomato, peaches, grapes (Zhang *et al* 2009a). In tomato it was found that expression of *LeNCED1* occurred before ethylene biosynthesis genes such as *LeACS* and *LeACO* therefore, it was suggested that ABA is regulating expression of ACS and ACO. In peaches and grapes *PpNCED1* and *VvNCED* expresses at high level in the beginning of fruit ripening, triggering the accumulation of ABA (Zhang *et al* 2009a). ABA biosynthesis is inhibited by several compounds such as fluridone, norflorawn, and NDGA. These inhibitors suppress ABA biosynthesis by inhibiting biosynthesis of carotenoids (Han *et al* 2004).

Brasinosteriods (BRs) are the group of steroidal plant hormones involved in various plant growth and development processes such as cell elongation, cell division, vascular differentiation, and abiotic stress tolerance (Clouse 2002). In tomato it was proposed that exogenous application of BRs promotes ripening of tomato pericarp disc due to increased ethylene production (Vardhini and Rao 2002).

Methyl Jasmonates are synthesised from α-linolic acid by several enzymes in octadecanoid pathway, including lipoxygenase (Lox), Allen oxide synthase (AOS) and 12-oxophytodienoate 3 (OPR3). Jasmonates also stimulate ethylene biosynthesis in climacteric fruits (Ziosi *et al* 2009) by inducing the expression of ACS and ACO genes. Recent study on strawberry suggested that jasmonate treatment changes expression of anthocyanin biogenesis genes such as Poly Amino Lyases (PAL1).

2.5 Physiochemical and Biochemical changes during fruit ripening

2.5.1 Firmness

Firmness is one of the major indices for determining fruit maturation. Firmness is mainly due to pectin polymers (Fertonani 2006) which can be bonded to ions, mainly Ca^{2+}, which maintains adjacent chains bonded among themselves (Taiz and Zeiger 2004). Bashir and Abu-Goukh (2003) reported decrease in fruit firmness during ripening of guava. The decline in firmness was about 8-fold from hard mature green to final soft ripen stage. It has been confirmed that Pectate Lyase deficient fruits show inhibition of pectin solubilization and depolymerization (Uluisik *et al* 2016).

2.5.2 Total soluble solids

The total soluble solids (TSS) which generally contain sugars, minerals and acids, give a reliable index to judge the proper stage of maturity. Hegde and Chharia (2004); Tosun *et al* (2008) stated that TSS increased significantly throughout development and ripening in

guava and blackberries. Ingle *et al* (1982) described that there is an increase in TSS during ripening in sapota. Similarly, Bashir and Abu-Goukh (2003); Rodriguez *et al* (1971) reported that TSS increased 1.2 folds during ripening in guava. The TSS concentration was found to be low in immature and mature fruits initially and increased later in ripe stage (Gangwar 1972; Hussain and Shah 1975). Also, studies revealed that in guava *cv.* 'Kampuchea' the TSS level increased from about 5 to 70 ^{o}Brix (Lazan and Ali 1998) whereas in Indian cultivars, 'Allahabad Safeda' and 'Sardar' the increase in TSS was from 10 to 13^{o} Brix until ripe stage (Tandon *et al* 1983; Selvaraj *et al* 1999). The initial increase of TSS observed during ripening is possibly due to conversion of starch and other insoluble carbohydrates into soluble sugars (Singh *et al* 1981) whereas, slight decline at the later stages was due to utilization of soluble solids and sugars in respiratory processes.

2.5.3 Titratable acidity

Titratable acidity (TA) gives a measure of the amount of acid present in a fruit. The organic acids contributing to acidity in guava are mainly citric, malic, glycolic, tartaric and lactic acids (Hui 2006). Organic acids usually decline during ripening as they are respired or converted to sugars. Titratable acidity increased during development but was less in ripe blackberry fruits (Tosun *et al* 2008). However, Damodaran *et al* (2001) have reported that acidity declined throughout the period of ripening in sapota *cv.* Cricket Ball. Similar results were observed by Nag *et al* (2011) in guava. Total acid level in guava fruit, ranged from 0.2 to 1.1% on fresh weight basis (Bashir and Abu-Goukh 2003). Total acidity initially increases with progressive maturity and then decreases at full maturity after climacteric peak.

2.5.4 Sugars

The sugars present in fruits impart sweetness, which influence the taste and flavor. Tosun *et al* (2008) recorded increase in total sugars during the ripening period in blackberries. The increase in sugar with the fruit growth might be due to conversion of certain cell wall material such as pectin and hemicelluloses into reducing substance during ripening (Kumar 2008). Sastry (1965) also reported that increase in activities of sugars during growth of guava fruit appear to be due to the synthesis and conversion of cell wall materials and later on reduction due to utilization of sugar in the process of respiration. Also, the relative combination of non-reducing sugars (sucrose) is rather low in guava when compared to reducing sugars (fructose and glucose). Sugar content shows a constant rise from initial stage of fruit development till ripening (Patel *et al* 2015) Comparable outcomes were obtained by Mitra and Bose (1996); Bulk *et al* (1997); Mercado-silva *et al* (1998); Bashir and Abu-Goukh (2003) in various genotypes of guava. The total sugars generally increased during ripening of

detached fruits and fructose was found as the major component of total sugars followed by glucose and sucrose (Wilson 1980).

2.5.5 Vitamin C

Ascorbate plays a key of role in plant cells. Important properties of ascorbate are its antioxidant capacity and the finalization of oxidative chain reactions resulting in non-oxidative products such as dehydroascorbate (DHA) and 2,3-diketogulonic acid (Davey *et al* 2000). The importance of ascorbate is in scavenging Reactive Oxygen Species (ROS). The change in ascorbate levels during fruit ripening is a trait dependent on the species. A linear increase in ascorbic acid was observed till fruit ripening and a slight decrease thereafter (Patel *et al* 2015). It was reported that ascorbic acid increases very slowly at initial stages of development and increased rapidly and reached to its maximum level at ripe stage (Mitra and Bose 1996). Increase in ascorbic acid content with advancement of fruit maturity and ripening were also reported by Bulk *et al* (1997). They studied the changes in chemical composition of four guava cultivars. Selvaraj *et al* (1999) studied the changes in chemical composition of guava fruits during growth and development at four stages of maturity *viz.*, green mature (GM 150 DAF and 140 DAF), peel colour turning (CT 155 DAF and 145 DAF), yellow hard (YH 160 DAF and 150 DAF) and ripe stage (R165 & 155 DAF) respectively for 'Safeda' and 'Sardar' cultivars. They also confirmed that compositional changes during fruit ontogeny and changes in physico-chemical, biochemical and mineral constituents were associated with ripening. Similar results were observed in tomato (Dumas *et al* 2003; Gautier *et al* 2008; Ioannidi *et al* 2009; Badejo *et al* 2012), grape (Cruz-Rus *et al* 2010) and strawberry (Cruz-Rus *et al* 2011), ascorbate content increases as the fruit ripens. The increase in ascorbic acid is correlated with changes in the activity of enzymes affecting the redox state of the fruit during the breaker stage (Gautier *et al* 2008; Jimenez *et al* 2002). Unlike tomato, grape and strawberry, kiwifruit showed a maximal ascorbate level at the immature green stage due to its high biosynthesis rate, which decreased as it ripened and then remained fairly stable until complete ripening (Zhang *et al* 2018). In peach fruits, ascorbate content gradually decreased during ripening (Imai *et al* 2009).

CHAPTER III

MATERIAL AND METHODS

During fruit ripening, a number of biochemical changes including synthesis and degradation of pigments, conversion of starch to sugars, production of volatiles and hydrolysis of cell wall components and enhanced rate of respiration occur. These changes are associated with the alteration in firmness and texture of a fruit to yield an edible fruit with optimum blend of color, taste, aroma and texture. Study of these physiological and biochemical changes of the fruit are of major concern for understanding metabolic processes such as fruit ripening, softening and in more general the process of senescence.

The present study deals with analysing total soluble solids (TSS), vitamin C, titratable acidity, reducing sugars, density and firmness of the guava fruits from popular cultivars grown in northern India. Visual and taste aspects were also analysed at different time points to determine the shelf life of fruits. After determining the early and delayed ripening cultivars, genetic mechanism behind the ripening control was delved into using the quantitative PCR approach for well-known genes involved in fruit ripening and maturation. Finally, the genes with differential regulation were analysed for sequence variation analysis among the early and late cultivars. Below are the materials and methods used in this study.

The experiments were carried out in the laboratories of the School of Agricultural Biotechnology and department of Fruit science, Punjab Agricultural University, Ludhiana, Punjab.

3.1 Physio-chemical analysis of different guava genotypes

The research was carried out on different white and Pink fleshed genotypes of guava. White-fleshed genotypes were Allahabad safeda, Punjab safeda, Thailand, Shweta, L-49, and Hisar safeda. Pink fleshed genotypes were 17-16, Punjab pink, Punjab kiran, Hisar surkha, G-5, purple local, Lalit and Arka Kiran. The fruits of different genotypes were obtained from Regional Fruit Research Station (RFRS) in Bahadurgarh (Patiala).

3.1.1 Total soluble solids (TSS)

For measuring the TSS of the fruits the pulp of fruits with three replicates from all the genotypes at three different timepoints i.e., 1 days post-harvest (DPH), 4 DPH and 7 DPH was crushed. Juice was extracted from the crushed fruits. TSS of juice was measured using Hand-refractometer (0-32^{o}brix) and it is expressed as per cent soluble solids.

3.1.2 Titratable acidity

For analysing titratable acidity, 2-3 drops of phenolphthalein solution were added into 5 ml of juice. This solution was titrated against 0.1N NaOH solution till the appearance of pink colour. The results are expressed as percentage of citric acid, malic acid and tartaric acid.

Below is the formula used for calculating % titratable acidity using the values from titration curve.

$$Acidity\ (\%) = \frac{\frac{1}{10} Titre\ vol\ (ml) \times Noramlity\ of\ alkali \times q.wt.of\ acid \times volume\ made\ (ml) \times 100}{Vol.of\ aliquot\ (ml) \times volume\ of\ sample\ (ml) \times 1000}$$

3.1.3 Vitamin C

Measuring the vitamin C content of fruits involves two steps, first is standardization of indophenol solution and second is actual estimation of vitamin C from fruits.

3.1.3.1 Standardization of indophenol solution

Two ml of ascorbic acid with 5 ml of reagent 1 (7.5 g Metaphosphoric acid and 20 ml of acetic-acid in 250 ml distilled water) was titrated against indophenol dye until rose pink colour appears for 5 seconds. The titre volume is considered as the dye factor and is later used for vitamin C calculation.

3.1.3.2 Estimation of Vitamin C from juice

For estimations, 2ml of juice with 5ml of reagent 1 was titrated against indophenol dye. Noted the volume of dye used. It is expressed as mg/100ml of juice.

$$Vitamin\ C\ (mg\ per\ 100\ \ ml\ juice) = \frac{Dye\ factor \times vol.of\ the\ dye\ used \times 100}{Vol.of\ the\ juice\ taken}$$

3.1.4 Reducing Sugars

For estimating reducing sugars, 2ml of saturated lead acetate was added into 5ml of juice and kept for half an hour at room temperature. Then the solution was filtered using Whatman's filter No.1 and 5ml of potassium oxalate was added to the filtrate. The solution was again filtered through Whatman's filter No.1 and 100 ml volume was made using water and the solution was transferred to a burette. Separately, in a 25 ml conical flask 2.5ml of each Fehling solution A and B was taken and heated till it starts boiling. Then the heated Fehling's solution was titrated against the filtrate from step 1 until the redbrick colour appears. Then 3-4 drops of methylene blue indicator were added and titration was continued again until the appearance of brick red colour. Volume of filtrate used was noted.

$$Reducing\ sugars = \frac{factor \times volume\ made \times 100}{Weight\ of\ sample \times 100}$$

.1.5 Firmness

Flesh firmness was measured using Penetrometer. Fruit skin was removed with stainless steel peeler and tip was forced into the fruit with uniform speed and reading was noted to the nearest 0.5 lb or 0.25 kg.

3.1.6 Visual and sensory aspects

Fruit color, appearance, taste and aroma were the visual and sensory aspects taken for this study. All the characteristics for different genotypes in triplicates were noted down at three different timepoints (1, 4 and 7 DPH).

3.2 Gene expression analysis of ripening and maturation involved genes in fruits

Genes related to ripening were identified by studying model plants such as tomato, peach and apple (explained in results and discussion).

3.2.1 Selection of genes and Primer Designing

Gene specific primers were designed using PerlPrimer and Primer3. Two types of primers were designed from i) exonic region ii) exon-intron span junction.

3.3 RNA extraction quantification and cDNA synthesis

RNA was extracted from fruit samples of selected genotypes collected in liquid nitrogen at 3 different time points (2, 4 and 8 DPH) and was stored in -80ºC until processed. For RNA extraction, tissue samples were crushed in liquid nitrogen to fine powder. 10µl Mercapto-ethanol was added per ml of lysis solution and mixed briefly.600 µl of this mixture was added in the powder, mixed vigorously on a vorte x shaker for 20 seconds and placed at room temperature for 5 minutes. Then the mixture was centrifuged for 3 minutes at 13000 rpm at 4°C. The supernatant was transferred to blue filteration column and centrifuged at maximum speed for 1 min. 750 µl of binding solution was added into clarified lysate and mixed thoroughly by pipetting in and out for at least 10 times. 700 µl of this mixture was transferred into binding column and centrifuged at maximum speed for 1 minute. It was repeated twice. 300 µl of wash solution 1 was added into binding column and centrifuged for 1min at maximum speed. Then for removing DNA during RNA purification additional treatment of on-column DNAse was given after binding of RNA to column. Briefly, Ten µl of DNase1 with 70 µl of DNase digestion buffer was added and incubated for 20 minutes at room temperature. I pipetted 500 µl of washing solution 1 into binding column and centrifuged for 1 min at max speed. This step was repeated twice. After wash centrifugation the column was dried by centrifuging for 1 min to remove remaining flow through. The column was then transferred to a new collection tube and 35 µl of elution buffer was added on the centre of binding matrix. Incubated the column for 5 min at room temperature before centrifugation for 1 min at max. speed. Finally, additional 25 µl of elute was added and incubated on ice for 5 min, centrifuged and the final purified RNA was stored at -20 ºC.

3.3.1 RNA Quantification

RNA was quantified on 1.2% agarose formaldehyde denaturing MOPS gel.

3.3.1.1 10X MOPS buffer preparation

For 1000 ml of 10X RNA gel buffer 41.84g of MOPS was added to a beaker along with 10.88 g of Sodium acetate in 500 ml of DEPC water. While constantly stirring the

solution 20 ml of 0.5 M EDTA was added and pH was adjusted to 7 using NaOH pellets. Final volume was made to 1000 ml with DEPC treated water Table 3.1.

Table 3.1: 10X MOPS buffer preparation

Reagent	Final Concentration	Amount
MOPS	0.2M	41.84g
Sodium Acetate	80mM	10.88g
EDTA	10mM	20ml of 0.5M
Adjusted pH to 7 with NaOH Pellets and DEPC treated water added to final volume of 1000 ml.		

3.3.1.2 1X RNA gel Buffer

For 500 ml of 1X RNA gel buffer 50ml of 10X RNA gel buffer was added to 450 ml of DEPC water.

3.3.1.3 1.2% denaturing agarose gel

For 200 ml of gel preparation 2.4g of agarose in 105ml of DEPC water was boiled for 40 seconds intervals until agarose was completely dissolved. Solution was allowed to cool down to 65°C under fume hood and 12 ml of 10X RNA gel buffer was added with 3ml of formaldehyde into the gel solution (Table 3.2). Gel was casted and allowed to solidify for 45 minutes. 1X RNA gel buffer was poured into gel apparatus so as to cover the gel and comb was removed. The gel was pre-run for 10 minutes at 70-100 V for equilibration with buffer.

Table 3.2: 1.2% denaturing agarose gel

Components	Final concentration	Amount
Agarose	1.2%	1.2g
*MOPS Buffer	1 X	10ml of 10X
*Formaldehyde	1%	3ml of 37%(w/v)
DEPC Water	Added to 100ml	

*Formaldehyde and 10 X MOPS buffer should be added after boiling the agarose gel.

3.3.1.4 RNA Sample Preparation

One volume of 5X loading buffer was added to 4 volumes of RNA sample and mixed well. The mixture was then incubated at 65°C for 5-10 min for breaking the secondary structure of RNA. After denaturation samples were chilled on ice for 5 minutes, spinned briefly, loaded onto equilibrated gel and run for 1hr 30 min at 70-80 V (5 V/cm).

3.3.2 cDNA synthesis:

cDNA was synthesised using Maxima first strand cDNA synthesis kit. The kit uses Maxima Reverse Transcriptase (RT), an advanced enzyme derived by *in vitro* evolution of M-MuLV RT. The enzyme features high thermostability, robustness and increased cDNA synthesis rate compared to wild type M-MuLV RT at high temperature like 55 °C. Table 3.3 shows the components for cDNA synthesis kit

Table 3.3: cDNA synthesis reaction set up

5X buffer	4µl
Maxima enzyme mixture	1 1µl
Template	1 - 5 µg
Nuclease free water to 20 µl reaction volume	

Maxima Enzyme Mix contains Maxima Reverse Transcriptase and Thermo Scientific RiboLock RNase Inhibitor. The recombinant RiboLock™ RNase Inhibitor effectively protects RNA template from degradation by RNases A, B and C at temperatures up to 55°C. 5X Reaction mix contains the remaining reaction components: reaction buffer, dNTPs, oligo (dT)18 and random hexamer primers. Nuclease-free water provided with the kit was used to make-up the reaction volume.

All the components were added to sterile RNAase free tube, mixed gently and centrifuged. The reaction mixture was then transferred to PCR tubes and incubated in a thermocycler at 25 ºC for 10 minutes, followed by 50 ºC for 30 minutes and finally 5 minutes at 85 ºC. The synthesized cDNA was diluted to 100 µl and was stored at -20 ºC until qPCR analysis.

3.4 Quantitative PCR analysis

Real time quantitative PCR is a very powerful approach for relative and absolute quantification of gene expression. This technique combines the approach of polymerase chain reaction and fluorescence signal detection. The output obtained from RT-qPCR is in the form critical threshold (CT) or quantitation cycle (CQ). This CT/CQ is the cycle of amplification during PCR, when a minimum threshold fluorescence signal has been detected. The range of CT value lies between the number of amplification cycles specified during the PCR run. Generally higher the CT value means lower the gene expression or vice-versa. RT-qPCR technique is commonly utilized for copy number estimation and to study the fold change in expression of a gene among treatments. For relative quantification, it requires a reference gene (a housekeeping gene) with equal expression among all developmental stages and tissues

for normalization. In a relative quantification experiment we must set four reactions; control sample with housekeeping gene (HC), control sample with target gene (TC), experimental sample with housekeeping gene (HE), experimental sample with target gene (TE). We perform the reactions in triplicates. After averaging, we obtain 4 CT values and then we have to perform following calculations,

$$\Delta\text{CT (control)} = TC - HC$$

$$\Delta CT\ (experimental) = TE - HE$$

$$\Delta\Delta CT = \Delta CT\ (experimental) - \Delta CT(control)$$

$$Fold\ change = 2^{-\Delta\Delta CT}$$

The ΔCT is normalized value and ΔΔCT is actual difference of gene expression between the two samples at control and experimental conditions. A negative value indicates higher gene expression while a positive value indicates lower gene expression in experimental conditions as compared to control conditions. It is very powerful measure as we can observe the difference as lower as 0.5-fold (Livak *et al* 2001).

In my experiments, I used histone 3 as an internal control for normalization. The treatment samples were cDNA from fruit tissues collected at 2 DPH, 4 DPH and 8 DPH and the control samples were cDNA from Allahabad Safeda fruit tissues collected at same time points.

The reaction was set up using the reaction mixture as given in Table 3.4

Table 3.4: qRT-PCR reaction set up

Component	**Volume (15 µl/well)**
Template	1 µl (out of 100 µl)
Power-up™ Sybr® green	5.5µl
Primer F/R (5 µM)	1.25 µl
Nuclease free water	7.25 µl
Total	15 µl

3.4.1 Reverse Transcriptase – quantitative PCR (qRT-PCR) setup

qRT-PCR was preincubated at 50°C for 2 minutes, followed by denaturation at 95°C for 3 minutes, then run for 40 cycles with denaturation at 95°C for 15 seconds followed by primer annealing and extension at 60°C for 1 minute. Primer specificity was tested using melt curve analysis by melting the final PCR product at 95°C for 15 seconds and then cooling down slowly to 60°C for reannealing of products. Multiple peaks in this analysis indicates multiple PCR products and non-specificity of the primer. Contrarily, a single peak indicates that the product accumulated is specific. The reaction was carried out in Roche LightCycler®

96 instrument and data was analyzed using LightCycler® 96 1.1 software and Micro Soft Excel.

3.5 Gene sequence variation detection among differentially expressed genes

Genome sequencing of Allahabad Safeda and genome re-sequencing of Punjab Safeda and Thailand guava was carried out under the aegis of "Whole genome and transcriptome based SSR and SNP markers development in guava (*Psidium guajava* L.) for linkage mapping and trait association". Transcriptome assembly of Allahabd Safeda has already been done separately in an in-house project lead by Dr. Amandeeep Mittal. Bioinformatics tools were used to identify the haplotypic variations among Allahabad safeda, Punjab Safeda and Thailand for all the differentially regulated genes. The steps and computer programs used for the purpose are:

1. The genomes of three guava cultivars *viz.* Allahabad Safeda, Punjab Safeda and Thailand were aligned to each other using bowtie program. This gives the output in .bam format.
2. The aligned.bam file was uploaded to Integrative genomic viewer (IGV) (Robinson *et al* 2011).
3. The genomic and transcriptomic sequences of the genes from Allahabad Safeda were used as query for BLAT search tool integrated in IGV itself.
4. The corresponding regions in Punjab Safeda and Thailand were analysed for single nucleotide polymorphisms (SNPs) or insertion-deletions (InDels).
5. The gene sequence with SNPs or INDELs in Punjab Safeda or Thailand were fetched from IGV individually for Punjab Safeda and Thailand.
6. The FGENESH programme (http://www.softberry.com/) was used to convert the gene sequences into coding sequences (cds).
7. The converted coding sequences from Punjab safeda and Thailand were aligned to coding sequences from Allahabad Safeda using pairwise alignment tool Clustal O.
8. SNPs and indels were detected in the coding regions.
9. Proteins sequences were generated from the cds sequences of genes using ExPASy (https://web.expasy.org/translate/) and FGENESH tools.
10. Protein sequences were aligned using Clustal O to analyse the variations at protein level. Also, the protein sequences were BLAST back using NCBI BLAST tool to confirm their molecular functioning.
11. The proteins with variations were modelled using MODELLER (Webb *et al* 2014) to analyse the effect of SNP/indel on the structure of proteins.

CHAPTER IV

RESULTS AND DISCUSSION

During fruit ripening, a number of biochemical changes including synthesis and degradation of pigments, conversion of starch to sugars, production of volatiles and hydrolysis of cell wall components and enhanced rate of respiration occur. These changes are associated with the alteration in firmness and texture of a fruit to yield an edible fruit with optimum blend of color, taste, aroma and texture. Study of these physiological and biochemical changes of the fruit are of major concern for understanding metabolic processes such as fruit ripening, softening and in more general the process of senescence.

The present study deals with analysing total soluble solids (TSS), vitamin C, titratable acidity, reducing sugars, density and firmness of the guava fruits from popular cultivars grown in northern India. Visual and taste aspects were also analysed at different time points to determine the shelf life of fruits. After determining the early and delayed ripening cultivars, genetic mechanism behind the ripening control was delved into using the quantitative PCR approach for well-known genes involved in fruit ripening and maturation. Finally, the genes with differential regulation were analysed for sequence variation among the early and late cultivars.

4.1 Physiochemical characteristics of selected guava genotypes

4.1.1 Total soluble solids (TSS)

TSS was observed in three data points *viz*. 1-day post harvesting (1 DPH), 4 DPH, and 7 DPH. Data was collected for three different seasons *viz*. November, January and February. TSS (^{o}brix) of fruits for 1 DPH varied in the range of 6.5 to 11 for November, 6.6 to 9.8 for January, and 6.5 to 10.55 in February averaged out for all the genotypes together (Fig. 4.1). In general, TSS of fruits harvested in November was non- significantly less than the fruits of January and February. So, we focused our analysis on fruits collected in November only. TSS observed from fruits 1 DPH was higher than fruits 7 DPH (Fig. 4.1) except Thailand guava. Our data shows that the TSS of fruits show an increasing trend during ripening but decreases sharply at over ripe stage. It was observed that TSS of pink-fleshed varieties was higher as compared to the white-fleshed varieties. Among the pink-fleshed varieties Punjab Kiran has the highest TSS of 12.7 and Arka Kiran has lowest TSS value of 9.83. Where as in white-fleshed varieties Punjab Safeda has highest TSS value of 10.9 and Thailand had lowest TSS value of 6.8 (Fig.4.2). The Increase in TSS during ripening might be due to depolymerization of polysaccharides and conversion of fruit starch to sugars (Patel *et al* 2015). However slight decline at the later stages could be attributed to the utilization of soluble solids in respiratory processes. Highest decline in TSS was observed in Lalit (Fig. 4.2). The genotypes Thailand and Hisar Safeda did not follow the same trend and an increase in TSS was observed at later stages during ripening in these (Fig.4.2). These observations are in agreement with those reported earlier in sapota and guava fruit (Pawar *et al* 2011).

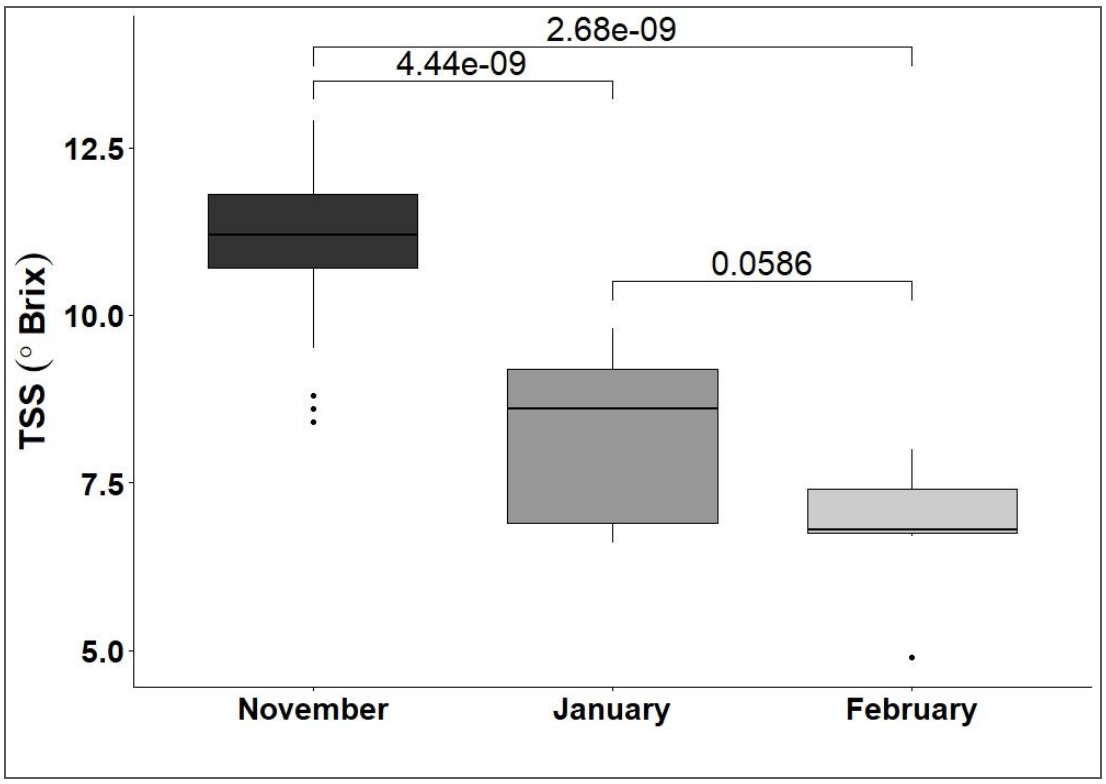

Fig. 4.1 Mean Total Soluble Solids measured from fruits collected in November, January and February averaged for 1-, 4- and 7-days post-harvest. Tukey's test p-values are mentioned among the groups.

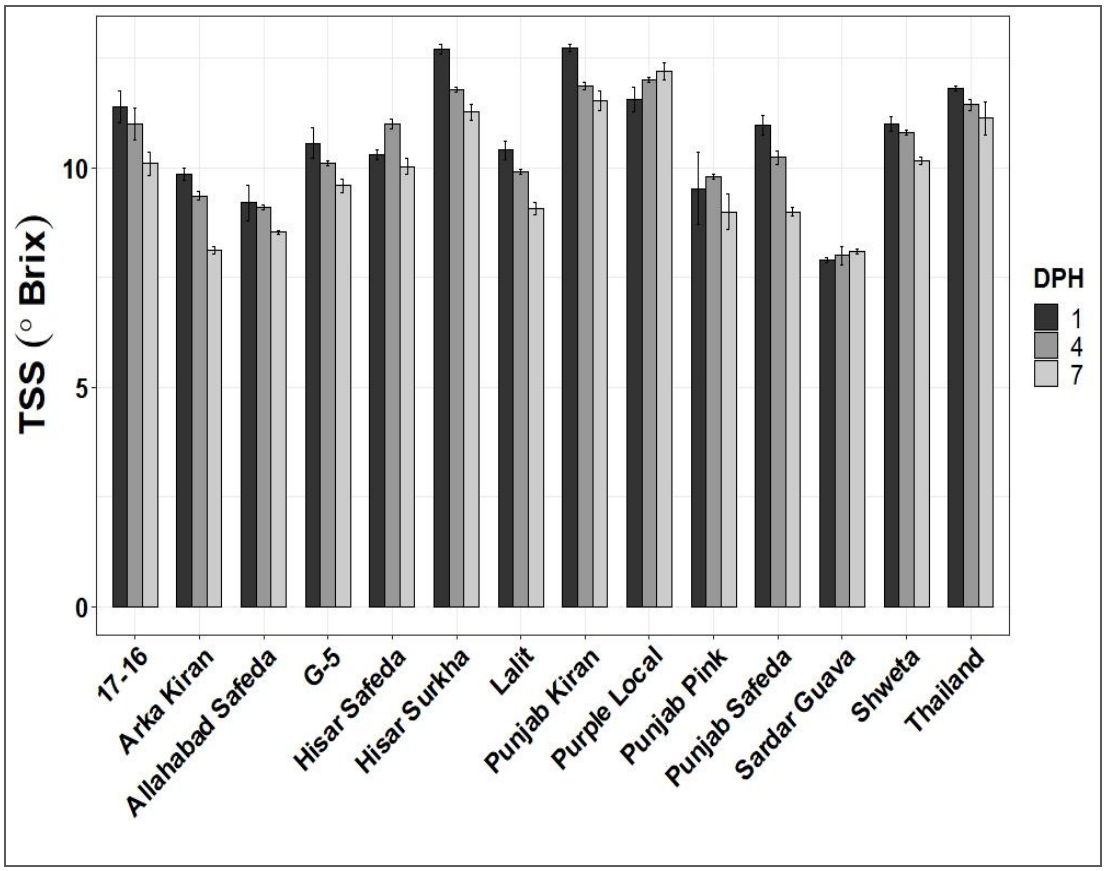

Fig. 4.2: TSS of selected guava genotypes measured after 1 DPH, 4 DPH and 7 DPH in November 2018.

4.1.2 Titratable acidity (TA)

Titratable Acidity was observed in three data points *viz.* 1 DPH, 4 DPH and 7 DPH in November, January and February. Percent TA measured from fruits 1 DPH varied in the range of 0.2 to 0.62 for different cultivars in November, 0.19 to 0.77 in January and 0.26 to 0.83 in February. TA of fruits in November was lower than January and February fruits (Fig. 4.3). We focussed our analysis on fruits collected in November hereafter. Our data shows that the TA decreases during ripening as fruits become sweeter. The values drop to very low at 7 DPH except for 4 cultivars; Hisar safeda, Hisar surkha, Thailand and Lalit. Among pink fleshed cultivars Arka Kiran had highest TA value of 0.56 measured 1 DPH while Hisar Surkha had lowest acidity value of 0.24 measured 1 DPH. Whereas, in case of white-fleshed varieties Sardar guava had highest acidity value of 0.56 measured 1 DPH and Thailand had lowest acidity value of 0.34 measured 1 DPH (Fig. 4.4). TA generally corresponds to the concentration of organic acids present in the fruit, which is an important parameter in maintaining the quality of fruits (Shirzadeh and Kazemi 2011). The concentration of these acids increased before ripening from immature green (IG) to mature green (MG) apple and finally decreased during ripening (Shirzadeh and Kazemi 2011). The increase in TA from IG to MG apple may be due to the formation and accumulation of organic acids during maturation. It could also be due to high concentration of undissociated organic acids stored in the vacuole.

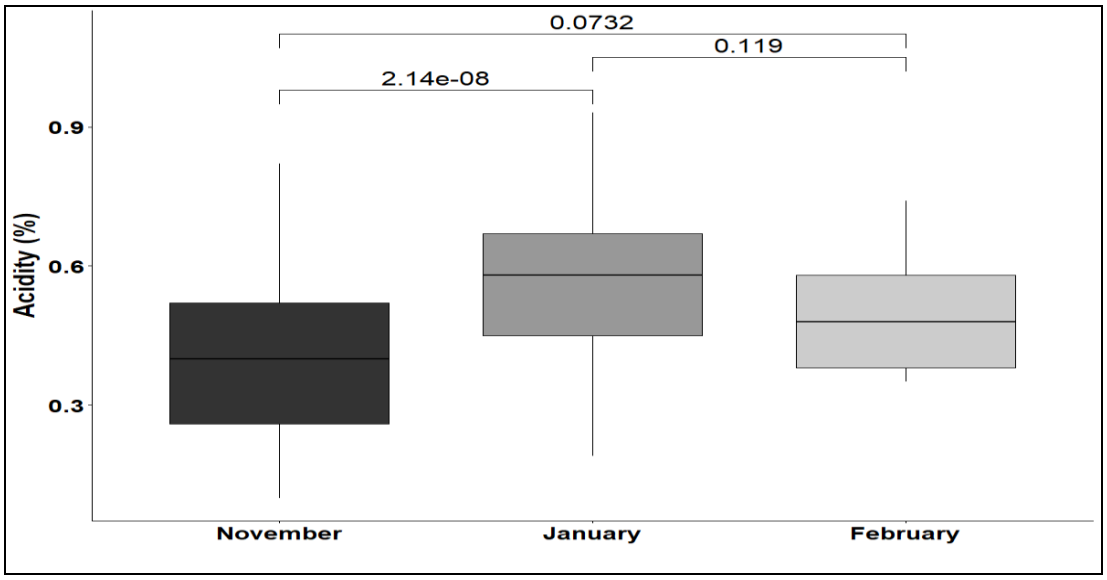

Fig. 4.3: Mean Titratable acidity measured from fruits collected in November, January and February averaged for 1, 4 and 7 days post-harvest. Tukey's test p-values are mentioned among the groups.

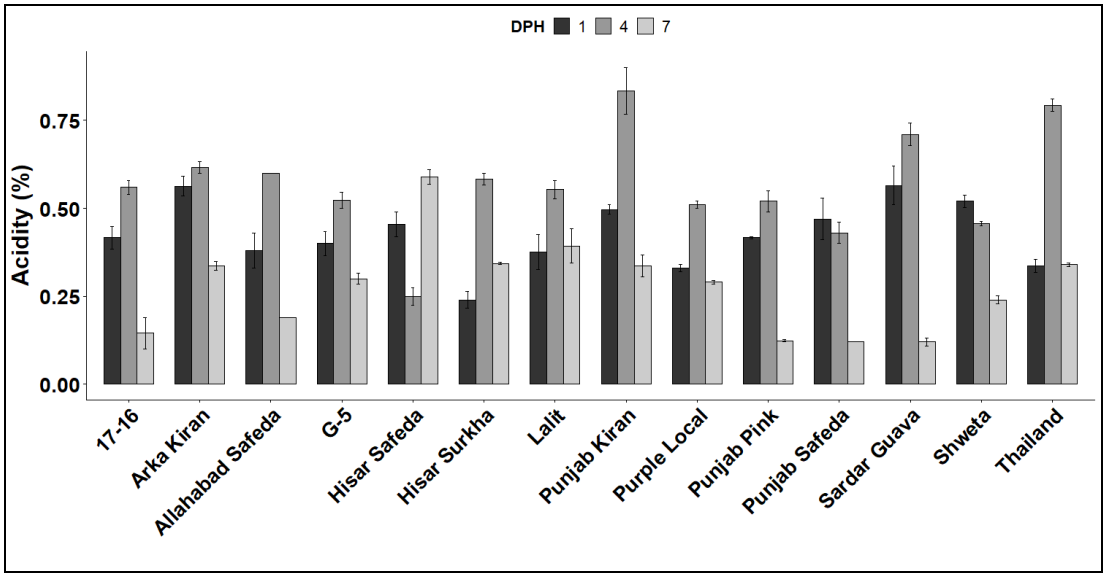

Fig. 4.4: Acidity of guava genotypes measured on 1,4 and 7 DPH in November 2018.

As the ripening progressed, these organic acids declined due to their utilization in respiration process and conversion into sugar thus, leading to decrease in TA (Tosun *et al* 2008). TA of genotype Sardar guava declined highest during ripening. While the TA of genotypes Hisar Safeda, Hisar Surkha and Thailand increased during ripening and did not follow the pattern as of other genotypes (Fig. 4.4). These results are in accordance with the earlier reported results on guava (Selvaraj *et al* 1999; Hedge and Chharia 2004). Decrease in acidity during ripening has also been reported in other fruits such as sapota (Damodaran *et al* 2001) and ber (Kannan and Susheela 2003). Damodaran *et al* (2001) and Nag *et al* (2011) have also reported that acidity declined throughout the period of ripening in sapota and guava, respectively.

4.1.3 Vitamin C

Vitamin C was also measured in three data points *viz*. 1 DPH, 4 DPH and 7 DPH in November, January and February 2018-19. Vitamin C (mg/100g) content of different genotypes measured at 1 DPH varied from 89 to 122 in November, 87.8 to 116.5 in January and 104.5 to 165.65 in February. In general, vitamin C content of fruits collected in February was found to be higher than the fruits of November and January (Fig. 4.5). We focussed our analysis on fruits collected in November hereafter. Among the pink fleshed cultivars, Hisar Surkha had highest vitamin C content of 117.5 mg/100g while Purple Local had the lowest vitamin C content of 93.6. Among the white-fleshed cultivars, Allahabad Safeda had highest Vitamin C content of 105.33 while Thailand was found to had lowest Vitamin C content of 91.33 measured 1 DPH (Fig. 4.6). Vitamin C content did not follow a clear pattern during ripening but increased at over ripe stage. L-Ascorbic acid (vitamin C) is an antioxidant and

imparts important nutritional benefits to the consumers. Guava is one of the richest source of vitamin C. The content of vitamin C in the guava fruits showed continuous increase throughout fruit development and ripening. Only the genotype Punjab Pink had a minor decline in vitamin C at over ripe stage, except that all other genotypes had increasing trend of vitamin C content (Fig. 4.6). We have observed only the increasing trends in vitamin C as we were considering the fruits till 7 DPH only. Many articles reported the decrease in vitamin C content at over ripe stage which should be beyond 7 DPH as we have not seen any decline till 7 DPH. Rajkumar *et al* (2016) reported increase in vitamin C content of guava fruit cv. Allahabad Safeda from immature to ripe stage and then a sharp decline at over ripe stage. Mondal *et al* (2004) observed increase in tomato fruit vitamin C content up to turning stage

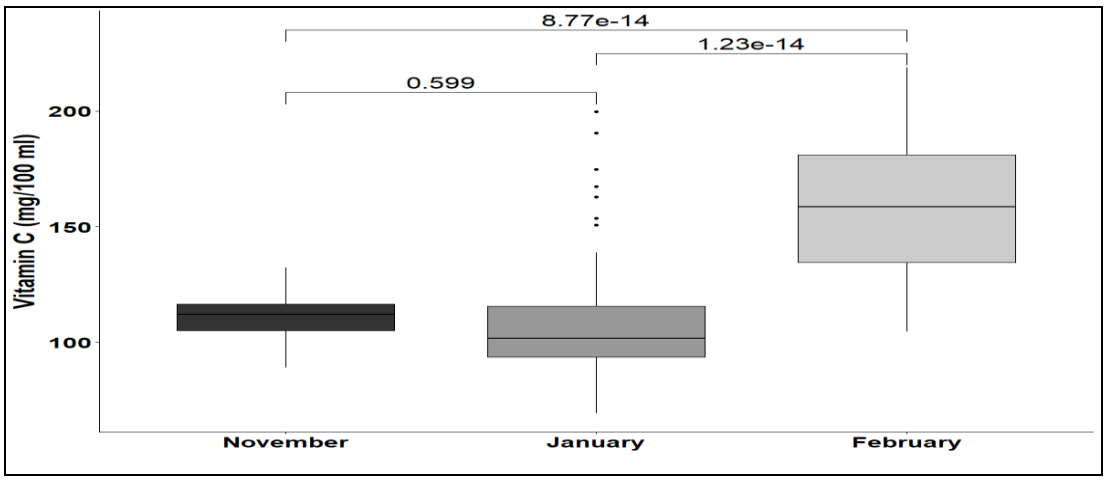

Fig. 4.5: Mean vitamin C measured from fruits collected in November, January and February averaged for 1, 4 and 7days post-harvest. Tukey's test p-values are mentioned among the groups.

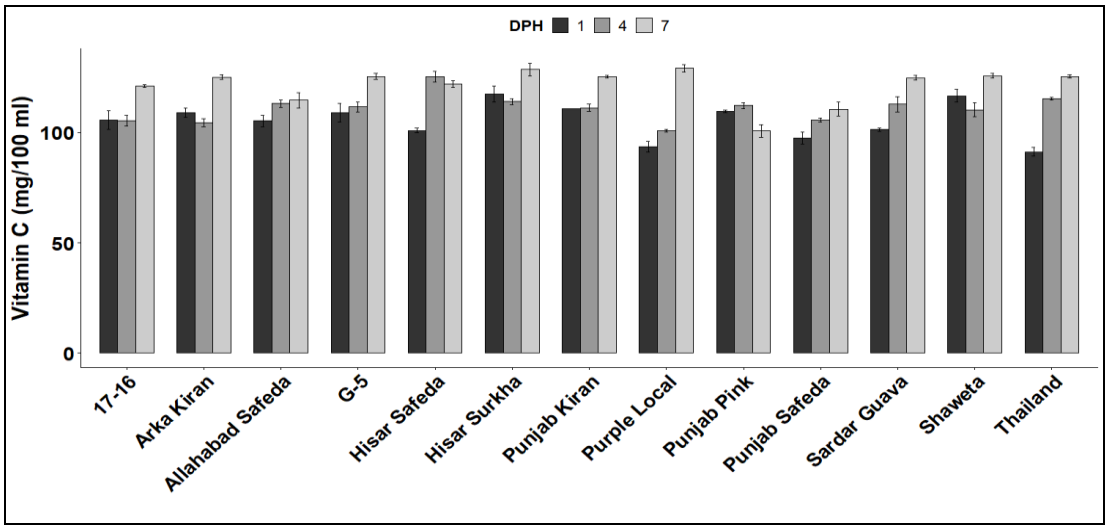

Fig. 4.6: Vitamin C of guava genotypes measured on 1, 4 and 7 DPH in November 2018.

followed by decline at ripe stage. Vitamin C content was reported to increase during the ripening of banana (Mustaffa *et al* 1998) and jujube (Bal and Josan 1980). While a continuous decline has been observed in sapota (Lakshminarayan and Subramanyam 1966) and cherry (Bulk *et al* 1997; Kadioglu and Yavru 1998). In guava fruits also, vitamin C content has been reported to show a S-shaped increase during ripening (Dhillon *et al* 1987). These observations are also in agreement with those of Zheng *et al* (2007b) in peach and Zheng *et al* (2007a) in mango. Bulk *et al* (1997) reported that vitamin C content increased slowly during initial growing period and significantly decreased during maturation and ripening in different guava cultivars. Vitamin C in fruits of guava *cvs.* L-49 and Banarsi Surkha increased up to MG stage and then decreased dramatically at over-ripe stage (Jain *et al* 2003). Vitamin C content of ber fruits increased during ripening on tree as well in storage (Kannan and Susheela 2003). Nunes *et al* (2006) compared the vitamin C content during ripening of strawberries in storage as well as in field and reported that the total vitamin C content of fruits increased during ripening and development irrespective of the ripening conditions.

4.1.4 Reducing sugars

Amount of reducing sugars in fruits of November was lower than January and February (Fig. 4.7). The value of reducing sugars was found to be in the range of 4.76 % to 8.85 % in January, 5.13 % to 6.87 % in February fruits measured at 1 DPH. The highest value of 8.85 % reducing sugars was found for Hisar surkha and Thailand in January fruits. The lowest value of 4.76 % reducing sugars in January was found for Allahabad safeda. In general, our data shows increase in reducing sugars as ripening proceeds. The reducing sugars of fruits collected from January and February was found to be following similar pattern and range. Reducing sugars involve glucose, fructose and galactose. As depicted in Fig. 4.8, our results show an increase in reducing sugars during fruit ripening in various genotypes of guava. The increase was continuous and slow from 1 DPH to 4 DPH, while rapid from 4 DPH to 7 DPH. Our results are supported by the increase in monosaccharides content during ripening reported in ber (Baviskar *et al* 1995; Goel and Siddiqui 1999; Malhotra 2010), and blackberries (Tosun *et al* 2008).

Increase in reducing sugars is due to conversion of starch and sucrose into glucose (Wills *et al* 1981). Mowlah and Itoo (1982) showed that glucose, fructose and sucrose were the main sugars in the white- and pink-fleshed guavas. The level of fructose increased during guava fruit ripening as reported by Lee *et al* 2010. Jain *et al* (2003) also found that reducing sugars increased during guava fruit ripening. Similar results were also reported in mango fruits (Abu-Goukh and Abu-Sarra 1993).

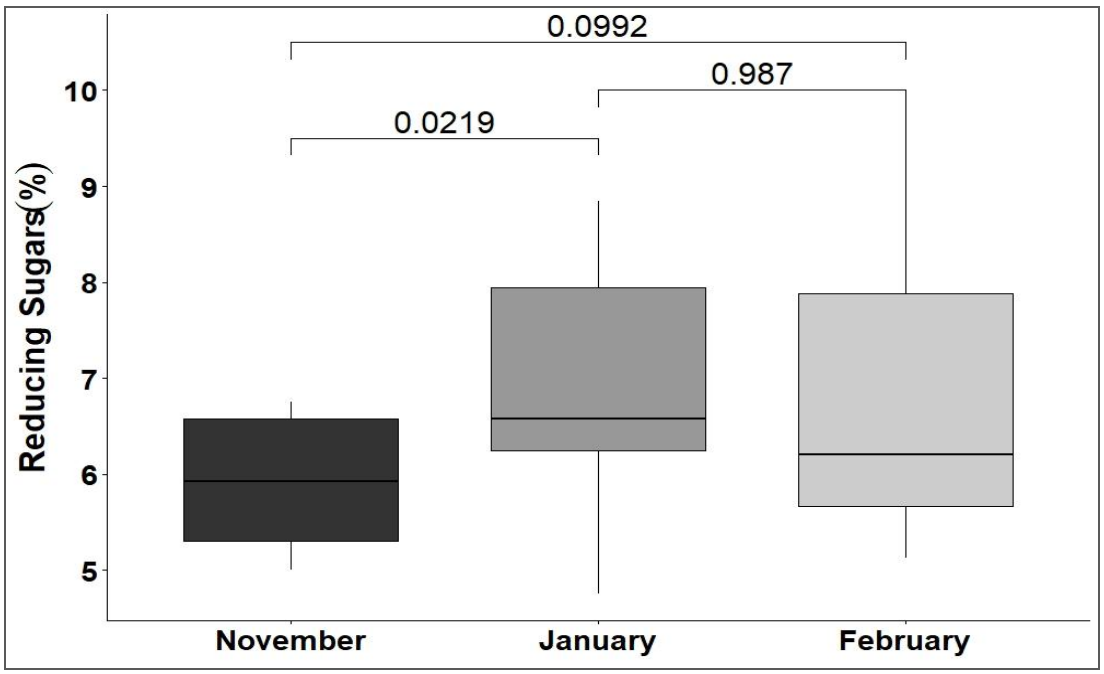

Fig. 4.7: Mean Reducing Sugars measured from November, January and February fruits. Tukey's test p-values are mentioned among the groups.

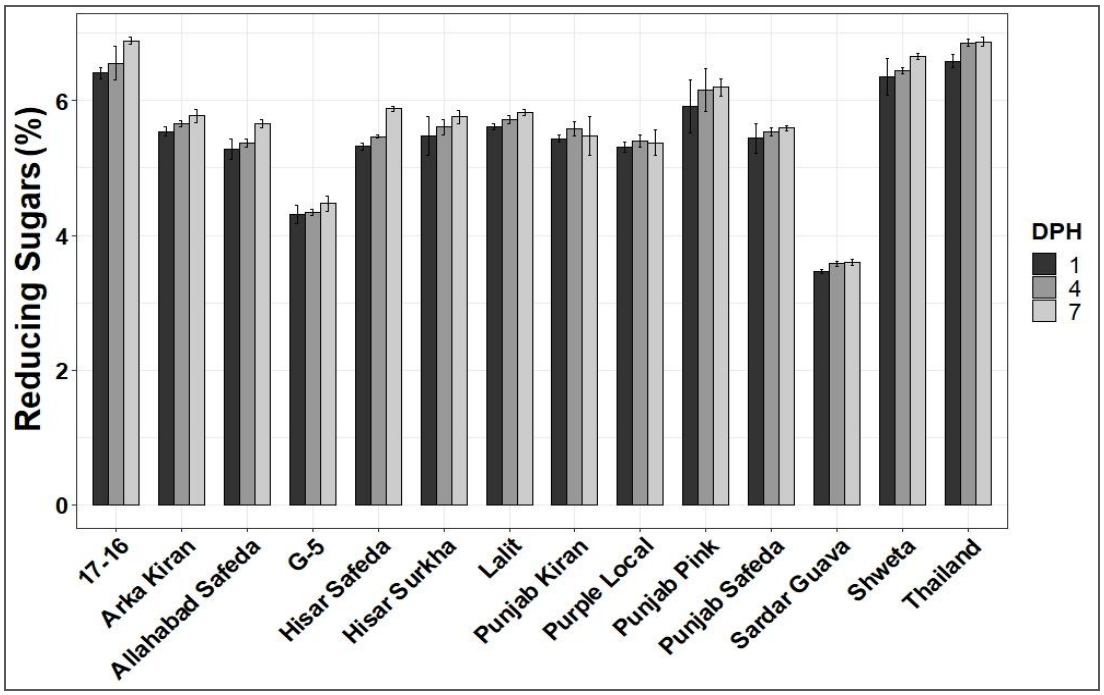

Fig. 4.8: Reducing Sugars guava genotypes measured 1, 4 and 7 DPH in February 2019.

4.1.5 Fruit firmness

Fruit firmness was measured in November and January 2018-19. Firmness was measured at 1 DPH and 4 DPH. The values of firmness calculated at 1 DPH lie in the range of 1.6 to 15.07 (lb) for the fruits collected in November and 4 to 16 for fruits collected in January. In general, fruits firmness of November fruits was more than in January (Fig. 4.9). Among the pink fleshed genotypes firmness was higher in Hisar Surkha (6.5 lb) and lower in Punjab Pink (2 lb). In case of white fleshed fruits Punjab Safeda was found to have the

highest firmness value of 15.07 and L-49 had lowest firmness value of 5.6 (Fig. 4.10). Firmness is an important physical parameter for determining the fruit ripening and quality. Firmness of fruit decreases during ripening process (Bashir and Abu-Goukh 2003).

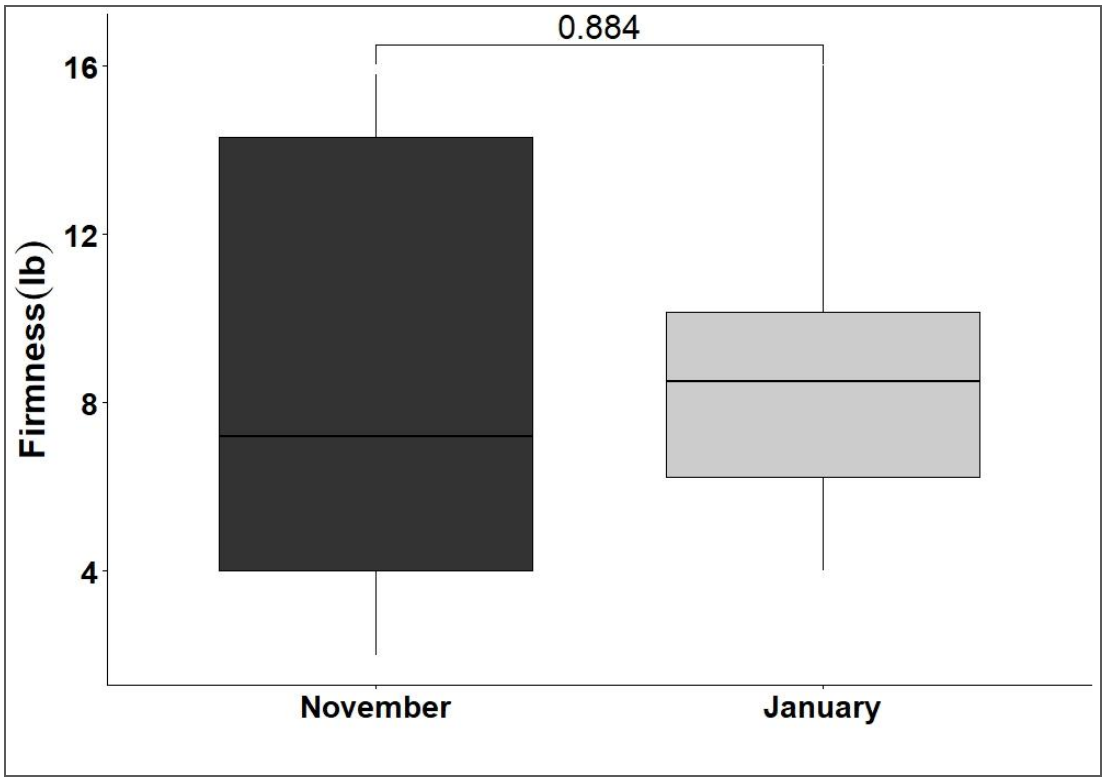

Fig. 4.9: Mean fruit firmness recorded in November and January. Tukey's test p-values are mentioned among the groups.

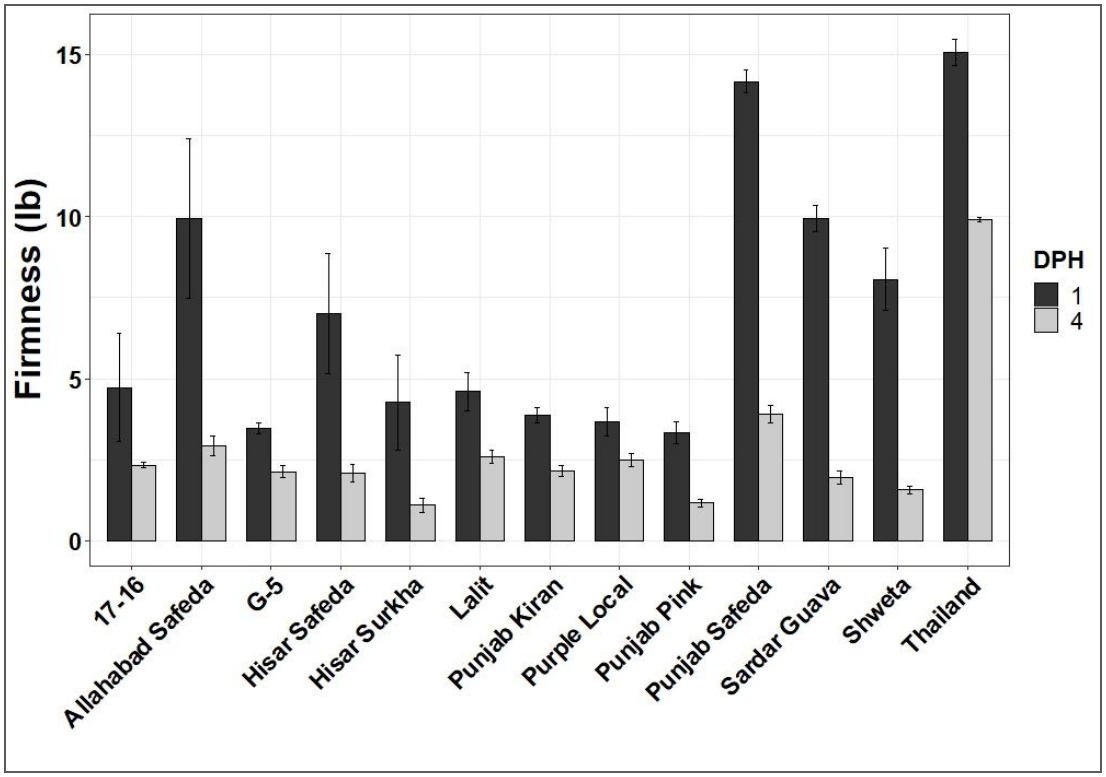

Fig 4.10: Firmness of guava genotypes calculated 1 and 4 DPH in November 2018.

Our results also show decrease in fruit firmness during ripening. Punjab Safeda and Thailand were found to be having highest firmness among all the fourteen genotypes (Fig. 4.10). Firmness is contributed mainly by cell wall constituents and in guava higher firmness is due to presence of pectic substances (Ahmed and Labavitch 1980). Due to the activity of pectin degrading enzymes, the softening of fruits is exhibited by degradative changes in the pectic substances (Huber 1983). Decrease in firmness is probably due to change in cell wall polysaccharides caused by hydrolytic enzyme activity (Rose and Bennett 1999; Payasi *et al* 2009). Similar drops in guava fruit firmness have been reported by Rodriguez *et al* (1971). Abu-Goukh and Abu-Sarra (1993) observed a rapid decrease in flesh firmness during ripening of three mango cultivars. Similar patterns of changes were reported for banana (Abu-Goukh *et al* 1995), pear (Luton and Holland 1986), apple, peach, apricot (Salunkhe and Wu 1973) and date (Barrevelled 1993).

4.1.6 Quality of fruits picked in November was higher than January and February fruits

As previous studies confirmed that winter season guava is better than rainy season guava (Rajkumar *et al* 2016). In the winter season we picked guava at three time points *viz.* November, January and February. In present study we found that November fruits were better than the fruits of January and February as shown in Fig.4.11 and 4.12 in respect of TSS and TA. These comparisons are drawn on the basis of characteristics of 1 DPH fruits. These fruits are ready to eat and most valued from consumer point of view. TSS of November fruits was higher as compared to January and February (Tukey's test p-value 0.111 and 0.00453 for January-November and February- November respectively, Fig. 4.11). Acidity was found to be higher in February and January than November (Tukey's test- p value 0.0294 and 0.04 for February-November and January- November respectively, Fig.4.12). Decline in quality might be due to lower temperature during fruit developmental stage of January and February picked fruits.

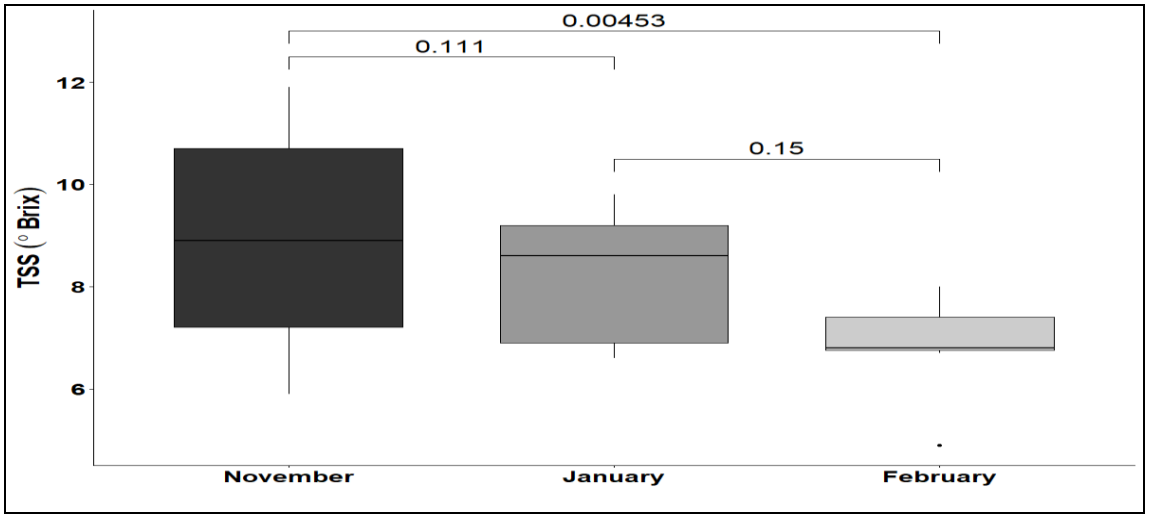

Fig. 4.11: Boxplot of TSS measured from fruits after 1 DPH collected in November, January and February. The values on the above are p-values for Tukey's test for multiple comparisons among the three months.

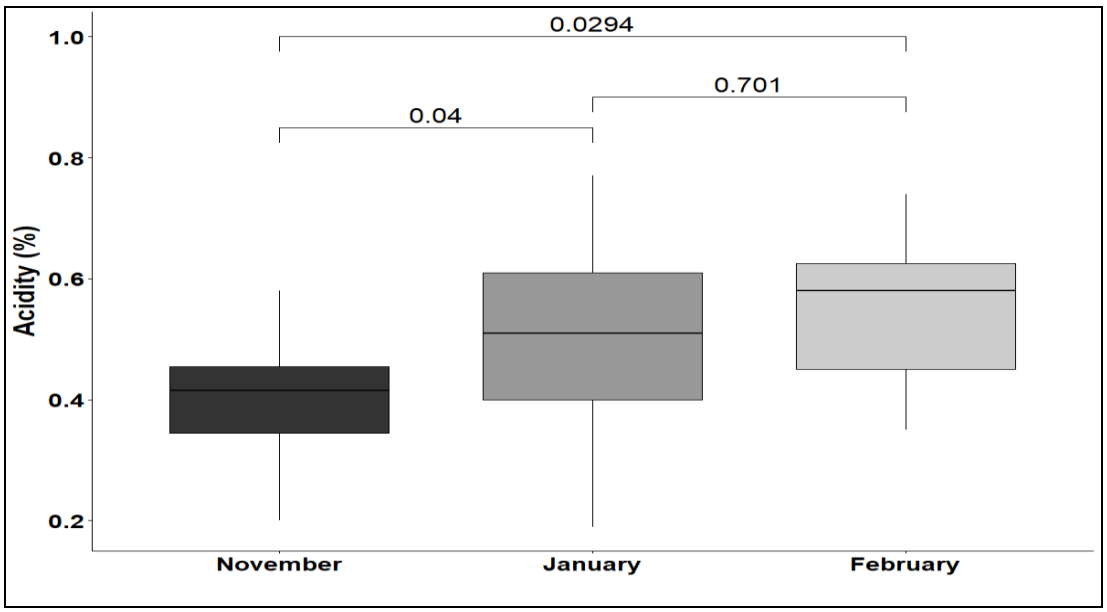

Fig. 4.12: Boxplot of acidity measured from fruits after 1 DPH collected in November, January and February. The values on the above are p-values for Tukey's test for multiple comparisons among the three months.

4.1.7 Deterioration of taste and fruit appearance is associated with ripening

Taste and appearance are the most important characteristics of a fruit and determine the market value of fruits. The present study investigated changes in taste and appearance of guava fruit during their ripening. The fruits were stored in room temperature in a closed room at relatively low humidity. On day 1 after harvesting, all the genotypes were green to pale yellowish in colour and good in taste. Which shows they were ripe and ready to eat when picked from trees. Fruits were again analysed for taste and appearance on day 4. Most of the genotypes turned dark yellowish to brown in color (Fig.4.13) and their taste was also deteriorated. Which shows most of the genotypes had a shelf life of 3-4 days at room temperature, but some genotypes such as Punjab Safeda, Thailand and Arka Kiran were better in taste and appearance. Purple local had a good taste as well, but its appearance was not good. Similarly, on day 7 we found that all the genotypes except Punjab Safeda, Thailand, Hisar Safeda, and Purple Local had bad taste, rotten appearance and were emitting foul smell (Table 4.1). This result indicates that these four genotypes have better shelf life and keeping quality than others at room temperature.

Major factor associated with the post-harvest deterioration of fruit quality is the rate of softening. Excessive softening results in shorter shelf life during storage, transportation and distribution, and increased wastage. Several genes potentially involved in degradation, rearrangement and structural manipulation of cell wall have been isolated, and most of these have been studied in the tomato. Up to 40% reduction of tomato fruit softening has been achieved by down-regulating the *TBG4* a β-galactosidase gene (Smith *et al* 2002). The first ever commercial transgenic crop product was Flavr-Savr tomato. In this the gene encoding a

cell wall degrading enzyme polygalacturonase was knocked down using RNAi technology and it stayed green for longer time than the wild type tomatoes (Bruening and Lyons 2000). More recently, another class of cell wall-degrading enzyme pectate lyases, appears to have more important role in ripening in tomato than previously known (Uluisik and Seymour 2019). In strawberry, a non-climacteric fruit, suppression of the pectate lyase mRNA resulted in significantly firmer fruits (Jimenez-Bermudez *et al* 2002).

Skin colour is widely used as a visual maturity index in many fruits. The change in skin colour from deep green to yellowish green is attributed to the disappearance of chlorophyll and is considered as a criterion in judging harvest maturity in guava (Kumar and Hoda 1974). Hence it is the best maturity index in guava as it could be monitored non-destructively during fruit ripening and storage (Mercado-Silva *et al* 1998; Asrey *et al* 2008).

Table 4.1: Changes in colour and taste of genotypes during ripening

S. No.	Genotype	1 DPH	4 DPH	7 DPH
1	17-16	Green, hard, sweet taste	Yellowish, soft, bad taste	Yellowish- brown, bad taste same as Arka Kiran
2	Arka Kiran	yellow	Yellowish, soft	Stinky in taste
3	Allahabad Safeda	Green, hard	Yellowish-green colour, soft	Smelly, not good in taste
4	G-5	Green, hard	Green- pale yellow, hard	-
5	Hisar Safeda	Green, hard	Pale yellow, soft	Better in taste than Allahabad Safeda
6	Hisar Surkha	Green, hard	Pale yellow, semi-hard	Bad taste
7	Lalit	Green, hard	Yellow-green, soft	Bad taste
8	Punjab Kiran	Green, hard	Yellow, soft	Bad taste
9	Purple Local	Good in appearance, purple green, hard	Shrunken, soft	Not good in appearance, smells good and better than all coloured flesh cultivars
10	Punjab Pink	Yellow	Yellow, soft	Bad taste
11	Punjab Safeda	Green, hard	Yellow colour, good taste, hard	Better in taste than Allahabad Safeda and Hisar Safeda
12	L-49	Green, hard	Yellow-green, soft	Bad taste
13	Shweta	Green, hard	Yellowish-green soft	Bad taste
14	Thailand	Green, very hard	Green colour, hard	Hard and crispy

1 DPH	4 DPH	7 DPH
		
		
		
		
		
		
		

Fig. 4.13: Visual characterization of guava genotypes at 1, 4 and 7 DPH in November 2018.

4.2 Expression quantification of genes involved in fruit maturation and ripening among contrasting genotypes and genetic mechanism of ripening in guava fruit

Based on the visual characteristics, firmness and TSS value the genotypes Allahabad Safeda, Punjab-pink and Lalit were considered to be in category of early ripening as they had higher TSS value and were less firm and deteriorated very early. Whereas the genotypes Punjab Safeda, Hisar Safeda and Thailand guava were considered as late ripening as they had higher firmness and they deteriorate very late. Thailand, Punjab Safeda and Hisar Safeda genotypes were taken into study for dissecting the genetic basis of delayed ripening. qPCR analysis was carried out on these cultivars with 29 different primers for different genes known to be involved in ripening in model organisms such as tomato. Basically, the genes we were looking at were involved mainly in ABA biosynthesis, ABA signalling, ethylene biosynthesis, ethylene signalling and cell wall degradation pathways. Our focus was to find out the genes as well as the major pathway responsible for delayed ripening. Genetic mechanism of ripening control in guava is not well understood thus we took this opportunity to produce some new information which could be translated to other tree species especially whose genome is yet unavailable.

4.2.1 Identification of genes involved in ripening from different fruit species

Fruit ripening is widely studied in tomato and other climacteric fruits such as peach and apple. More than 100 genes have been identified to be involved in ripening and maturation in fruits based on mutant screens / functional studies. Most of these genes are directly or indirectly related to ethylene or ABA- biosynthesis and/or signalling and cell wall degrading enzymes. Table 4.2 lists 99 genes identified during search for finding genes related to ripening.

Table 4.2: Genes Related to Ripening

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
1	Ethylene biosynthesis	(Holdsworth *et al* 1987)			
2	1-Aminocyclopropane-1-Carboxylic Acid Synthase (ACS4/ACC4)	(Lincoln *et al* 1993)	476	289/442 (65%)	273/422(65%)
3	1-Aminocyclopropane-1-Carboxylic Acid Synthase (ACS2/ACC2)	(Rottmann *et al* 1991)			
4	Ethylene Insensitive like 3 (EIL1)	(Chen *et al* 2004)			
5	Ethylene Insensitive like (EIL2)				
6	Ethylene Insensitive like (EIL3)		601	363/620(59%)	363/620(59%)
7	Ethylene Response factor (ERF6)	(Lee *et al* 2012)	254	116/248 (46%)	118/269(44%)
8	Ethylene Insensitive EIN2	(Alonso *et al* 1999)	1294	625/1291(48%)	652/1270(51%)
9	Constitutive Triple Response CTR1-like protein kinase	(Adams-Phillips *et al* 2004)	488	218/316 (69%)	247/405(61%)
10	14-3-3a protein	(Shi & Zhang 2014)			
11	MADS-box protein 1	(Dong *et al* 2014)			
12	MADS-RIN	(Vrebalov *et al* 2002)	242	173/254(68%)	147/248(59%)
13	Tomato Agamous like TAG1	(Pnueli *et al* 1994)	248	125/157(79%)	162/252(64%)
14	MADS-box 2	(Pimentel *et al* 2010)			

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
15	Agamous-like MADS-box protein AGL9	(Li *et al* 2019)			
16	Tomato FRUITFULL Homologs TDR4/Ful-like MADS-box	(Jaakola *et al* 2010)			
17	CmGal1 (beta-galactosidase)	(Nishiyama *et al* 2007)			
18	CmGal3 (beta-galactosidase)				
19	Polygalacturonase (PG)	(Fabi *et al* 2009)	397	170/323(53%)	242/366(66%)
20	Polygalacturonate 3	(Fabi *et al* 2014)	444	139/391 (35%)	240/448(54%)
21	Endoglucanase	(Nishiyama *et al* 2007)			
22	Endotransglucosylase/hydrolase 1 (XTH1)				
23	Expansin (EXP1)	(Nishiyama *et al* 2007)			
24	Cellulase	(Seymour *et al* 2011)	496	295/498(59%)	412/495(83%)
25	Tomato fruit ripening specific mRNA	(Giovannoni *et al* 2017)			
26	*Arabidopsis* pseudo-response regulator 2 gene APRR2-like protein	(Pan *et al* 2013)			
27	Inositol monophosphatase 3	(Imai *et al* 2009)			
28	Aldo-keto reductase	(Agius *et al* 2003)			
29	Lipoxygenase 1	(Zhang *et al* 2006)			

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
30	Lipoxygenase 3	(Zhang *et al* 2006)			
31	Lipoxygenase 4				
32	Lipoxygenase 5				
33	Lipoxygenase 6				
34	Phenylalanine ammonia-lyase (PAL)	(Sparvoli *et al* 1994)			
35	9-Cis-epoxycaretenoid dioxygenase 1 (NCED1)	(Zhang *et al* 2009a)	246	190/246(77%)	189/246(77%)
36	9-Cis-epoxycaretenoid dioxygenase 2 (NCED2)		247	201/247(81%)	201/247(81%)
37	ABA 8'-hydroxylase (CYP707A1)	(Li *et al* 2012)	324	209/337(62%)	228/322(79)
38	Beta-glucosidase 3	(Jia *et al* 2013)			
39	Basic Leucine Zipper Domain bZIP	(Davies & Robinson 2000)			
40	Cysteine protease 4 (Cp4)				
41	MYB2	(Jaakola *et al* 2010)	115	77/140(55%)	228/322(79%)
42	Expansin (Exp2)	(Civello *et al* 1999)			
43	Auxin (AUX/IAA)	(Yuan *et al* 2016)			
44	Polygalacturonase (PG-2a)	(Bird *et al* 1988)			
45	NAC transcription factor Non-Ripening	(Gao *et al* 2018)	355	204/453(42%)	197/395(50%)

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
	(NAC-NOR)				
46	Ethylene inducible 4(E4)	(Cordes *et al* 1989)			
47	Ethylene inducible 8 (E8)	(Deikman & Fischer 1988)			
48	Tomato FRUITFULL Homologs (TDR4)	(Busi *et al* 2003)	245	183/279(66%)	159/255(62%)
49	Ethylene Receptor (ETR3)	(Lashbrook *et al* 1998)	635	429/648(66%)	432/588(73%)
50	Phytoene synthase 1(Psy1)	(Luo *et al* 2013)			
51	EIN2 targeting protein ETP1 (F box)	(Qiao *et al* 2009)	415	86/349(25%)	86/349(25%)
52	EIN2 targeting protein 2 ETP2 (F box)	(Qiao *et al* 2009)	388	72/257(28%)	72/257(28%)
53	Ethylene Receptors (ETR4)	(Tieman & Klee 1999)	761	481/749(64%)	479/740(65%)
54	Never-ripe	(Wilkinson *et al* 1995)			
55	1-Aminocyclopropane-1-Carboxylate Synthase (ACS2)	(Rottmann *et al* 1991)			
56	1-Aminocyclopropane-1-Carboxylate Synthase (ACS3)	(Rottmann *et al* 1991)			
57	1-Aminocyclopropane-1-Carboxylate Synthase (ACS5)	(Yuan *et al* 2016)			
58	1-Aminocyclopropane-1-Carboxylate Synthase 7 (ACS7)	(Tomato Genome Consortium 2012)			

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
59	Ripening Regulated gene (DDTFR8)	(Gao *et al* 2018)			
60	1-Aminocyclopropane-1-Carboxylate Oxidase (ACO5)	(Alexander & Grierson 2002)	301	199/330(60%)	198/299(66%)
61	Non-Ripening (NOR)	(Gao *et al* 2019)			
62	1-Aminocyclopropane-1-Carboxylate Oxidase (ACO6)	(Yuan *et al* 2016)	319	103/113(91%)	259/316(82%)
63	1-Aminocyclopropane-1-Carboxylate Oxidase (ACS8)				
64	1-Aminocyclopropane-1-Carboxylate Oxidase (ACS4)				
65	1-Aminocyclopropane-1-Carboxylate Synthase 7-like				
66	1-Aminocyclopropane-1-Carboxylate Oxidase 4				
67	1-Aminocyclopropane-1-Carboxylate Syntahse 1A (ACS1A)				
68	1-Aminocyclopropane-1-Carboxylate Synthase-like				
69	Linoleate 9S-lipoxygenase B	(Ferrie *et al* 1994)			
70	Triacylglycerol Lipase 2	(Aoki *et al* 2010)			

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
71	Lipoxygenase (loxC)	(Shen *et al* 2014)			
72	Lipoxygenase (TomloxE)				
73	Linoleate 9S-lipoxygenase	(Ferrie *et al* 1994)			
74	3-ketoacyl-CoA synthase 6	(Leide *et al* 2007)	496	425/496(86%)	425/496(86%)
75	Cytochrome P450	(Yuan *et al* 2016)			
76	Chalcone--flavonone isomerase 3				
77	Glucosyltransferase 2	(Griesser *et al* 2008)			
78	UDP-glycosyltransferase 75C1	(Sun *et al* 2017)	470	283/470(60%)	283/470(60%)
79	UDP-glycosyltransferase 76E1		475	165/339(49%)	187/360(52%)
80	S-adenosyl Methionine 1 (SAM1)	(Yuan *et al* 2016)	393	371/392(95%)	373/392(95%)
81	1-Aminocyclopropane-1-Carboxylate Oxidase -like (E8)				
82	1-Aminocyclopropane-1-Carboxylate Oxidase 1 (ACO1)				
83	S-Adenosyl Methionine 1 (SAM3)		390	365/388(94%)	365/388(94%)
84	ACO3 homolog isoform 1		363	108/259(42%)	184/369(50%)
85	Cellulose Synthase 3		1083	463/954(48%)	957/1083(88%)

Sr. No	Gene Name	Reference	Query Length	Identity	
				Translated Guava Genome	Translated Guava Transcriptome
86	Polygalacturonate PG2	(Bird *et al* 1988)			
87	Pectate Lyase (PL)	(Yang *et al* 2017)	403	311/404(76%)	332/381(87%)
88	Cellulose synthase 2	(Yuan *et al* 2016)			
89	Phytoene synthase (Psy1)	(Luo *et al* 2013)			
90	15-cis-zeta-carotene	(Chen *et al* 2010)			
91	Acid invertase	(Sato *et al* 1993)			
92	Endoglucanase 2	(Yuan *et al* 2016)			
93	Endoglucanase 25-like				
94	Alpha-galactosidase 3				
95	Mannan endo-14-beta-mannosidase	(Wang *et al* 2009)	399	123/390(32%)	219/373(59%)
96	Glyceraldehyde-3-phosphate	(Yuan *et al* 2016)			
97	Glucose-6-phosphate 1-dehydrogenase				
98	Beta-glucosidase 42				
99	Beta-galactosidase 4		724	130/216(60%)	528/696(76%)

4.2.2 Identification of genes in guava genome and transcriptome

The protein sequences of these 99 genes were retrieved from NCBI. These protein sequences were used as query for tBLASTn against guava genome and transcriptome in–house data available in our lab (Mittal *et al* unpublished). Out of 99 protein sequences 32 aligned with guava genome and transcriptome. From the protein sequence of these 32 genes, the genic sequence from both genome and transcriptome data was fetched using samtools program. To confirm the retrieval of correct sequences, NCBI BLAST was again performed on these genes. All the genes did show hits on similar type of genes (predicted and validated).

4.2.3 qPCR primer designing

All the 32 genes were used for designing qPCR primers. Primer3 and PerlPrimer were used for designing the primers. Table (4.3) shows the primers designed and used in this study.

Table 4.3: Forward and reverse primers for the transcripts/ genes with their contig number and product size in guava transcriptome assembly.

S. No.	Genes	Forward Primer	Reverse Primer	Amplicon Size (bp)
1	ACC2_27698_c0_seq1	CGAGATGAAGCTGTGGAGGGTG	CTGATCCTCTTAAGCGCCACTTCC	149
2	EIL3_20875_c0_seq6	CGCCTCAAGAGCATCCTGTCTC	CCTGGAAGAAATGGGCTTTTGC	171
3	ERF6_27838_c0_seq1	TGAGCCAAAGGATGAGGCCG	GCATCCAGAGATCAGCCGAAGAC	145
4	EIN2_21623_c0_seq9	ACCGACTTCAGGGAATCATTGAGC	GACCATTTCGAGGAGCATTGCCG	196
5	CTR1_24039_c0_seq30	GGATGCTGCATCTTGTCTTGTCCG	AAGACGTGGAAACCGTAGTGGTG	150
6	MADS RIN_29074_c0_seq1	CATGCTAGGGCTGCTACAGAACTC	TTCGCTAAGAGGAGGAATGGGCTG	128
7	TAG_113510_c0_seq5	GCTTGAGAAGACAATGAGCGCGAC	GCGGATTGAGAACTCGACGAACAG	123
8	PG_27664_c1_seq1	CTCCCAATGCTGATGCCATGTCC	GATGCAAGGTGTGAAGGTGTCGG	197
9	PG3_31033_c0_seq1	CAGCAGTTCAAGTGACGAACGTGG	AAGAAGCTTCAGAGCTCTCAACCG	164
10	Cellulase_14564_c0_seq2	CGAACACCCTAACGGCTCTGTC	CCTTCTCCGACCACAACTGCTG	197
11	NCED1_13093_c0_seq1	GGTGGGCGATCATTGTGGAGTC	AAGTCCATCGGCGAGCTCCA	258
12	NCED2_5330_c0_seq1	ATGCACGACTTCGCCATCAC	CTTGTCGAGGATCCCAAAGCG	140
13	CYPO707A1_23262_c0_seq13	TGAGACTCTCACCGACGAGCA	CGGCGGATAGCATTCTGCTCTG	158
14	MYB_11843_c0_seq1	TGCCGAGAAGGGAATGCAATCTG	GTGAAGGCCATTGGAGATCCCTC	161
15	NAC/ NOR_26107_c0_seq5	GAATTCCCTGAGGCTGGATGACTG	TCGTCAACGAAGGAGGGATGG	130
16	TDR4_26906_c1_seq1	CCGAAACCAATGGGAACTGGGC	GCTGGTGCTCCAAATTCTGAAGC	140
17	ETR3_13476_c0_seq4	TAGCTGATCAGGTTGCAGTTGCTC	CTTCTCTTCTGGCGCGATCAAGTG	113

S. No.	Genes	Forward Primer	Reverse Primer	Amplicon Size (bp)
18	ETP1_26434_c0_seq10	TCGAGAGCGGAGTGGTTCAAGT	TGGAATCTCTCCTCCGATGCAG	100
19	ETP2_25331_c0_seq19	GAATTGGGGAGCGGAGATCAGT	TCGGAGAGAAGAAGCCATGTCG	136
20	ETR4_13692_c1_seq3	GGCTAGTCCAGTTGATGCACGG	TGACATGGACTCTCGGAGCTGG	106
21	ACO5_14429_c0_seq3	CCCCGTGGTTCTTAATCTGCAA	GCAGGAGCAAAACACTGGCACT	165
22	ACO6_13593_c0_seq2	GTCGCCTAAGTTGATGACGATGGA	GTACCAAGGTGAGCAACTACCCTC	190
23	3 Keto acyl _30003_c0_seq1	CGACGAAGAGGACGAGGAAGG	CAAGCTCAAGTACGTGAAGCTGG	193
24	SAM1_21278_c0_seq2	CCGAAGACCATGACCATGTTGG	TGTGTGACCAGATCTCCGATGC	109
25	SAM3_23457_c0_seq2	TTTCAGGCCGGGAATGATCTCT	CTGGGTCCTCACGTCCAAAATG	104
26	ACO3_10259_c0_seq1	ACACAGATAGCGCCTTCCTCACAG	TGGTCCTACGTTCTTTGCCAACAC	196
27	Cellulose synthase_23950_c1_seq18	TGGACAACTCTTCTCATCCCTCC	GGCGTCCCATCAGACCTTTGAG	183
28	PL_27913_c0_seq1	GCCTGCATCTCGGCATTCTCTG	TCACCACGACAAGGTCATGCTG	124
29	Mannose_27726_c0_seq1	AGGAAAGAGGGACAGCTACTTCGG	GGCTCTGCTCCAATATGACTTCGT	154
30	Beta glac_26708_c0_seq112	CGCGTAAAGTCCTGCTTGCTGT	GAAGGCCAAAGATGGAGGCTTG	145
31	UDP 75_26774_c0_seq5	TCGGAAATGAAGCGGGCCAC	ACCTTCGCCACCAGCATCTC	139
32	UDP76_19306_c0_seq7	AAGGGAACGGACAGATCGCCA	AGGGCTTTACAATCTTGCAAGGGA	200

4.2.4 RNA extraction and Normalization:

RNA was extracted from fruit tissue at 2,4 and 8 DPH and quantified on 1.2% agarose gel. After quantification RNA was normalized to 1 µg (Fig. 4.14).

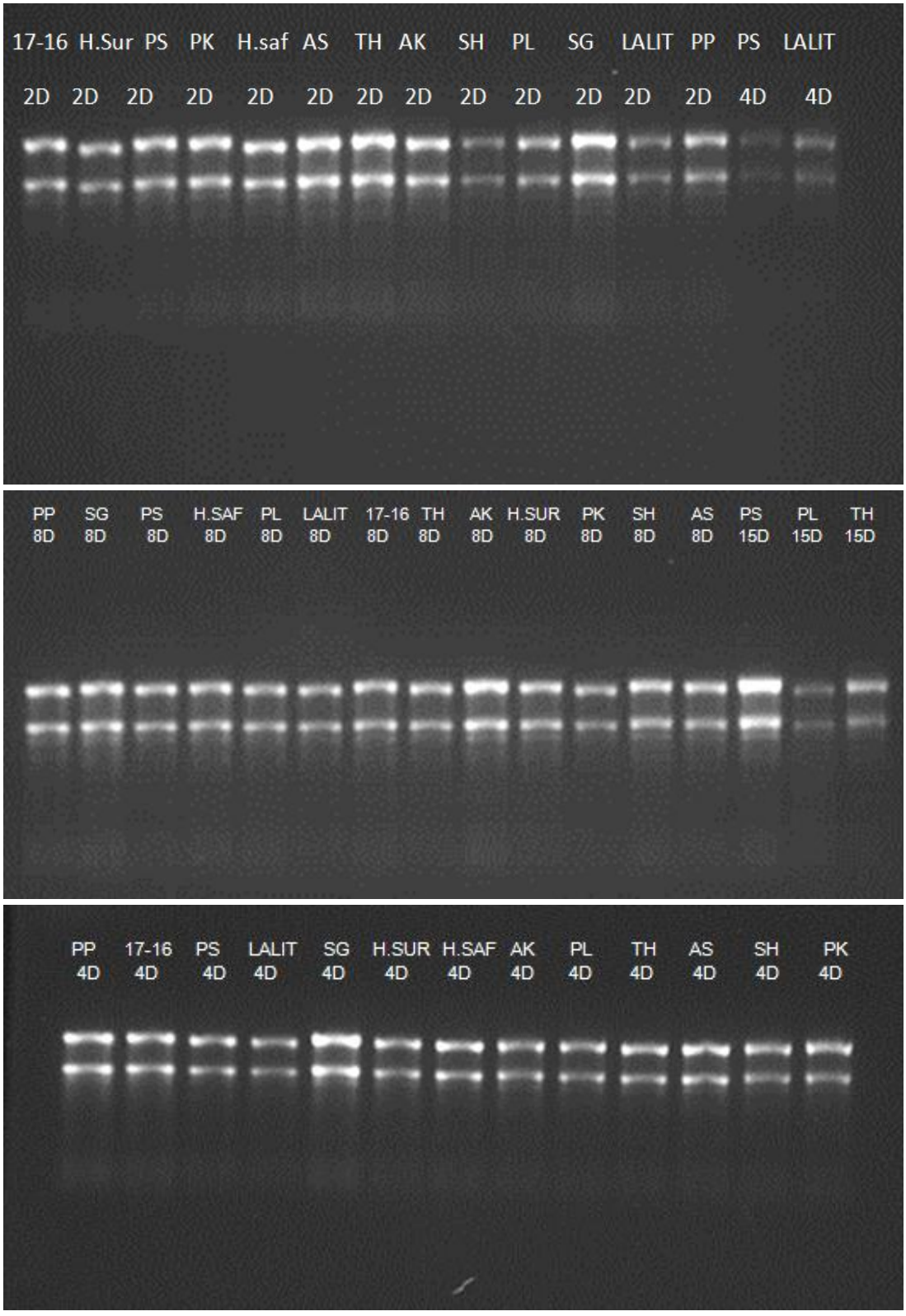

Fig. 4.14: Normalized RNA for 2, 4, 8 and 15 days post-harvest fruits

4.2.5 qPCR primers validation

Primers amplification was validated on cDNA pooled from Allahabad Safeda fruits (2D, 4D and 8D) (Fig. 4.15) without reverse transcriptase control (-RT control). Out of the 32 primers, primer for cellulase synthase didn't show amplification, while primers for ETP1 and PL showed amplification from -RT control (Fig. 4.15). However, the CT for +RT for ETP1 was 22.26 compared to 36.59 for -RT control. Likewise CT for +RT for PL was 21.53 compared to 35.13 for -RT control. All of the genes did have specific PCR product as indicated by their melting curve analysis (Fig. 4.16). PG3 did show CT 33.13 so was not used for gene expression analysis. Cellulose synthase gene role in fruit maturation was not compelling so was not used for expression analysis. Thus, in our study, we used 29 primers for quantifying the gene expression.

Fig 4.15: **Heat map of all the primers validated in Allahabad Safeda cDNA. Green colour indicates low amplification and red colour indicates higher amplification. Rows 1-5 of the figure are primers amplified using Allahabad Safeda cDNA, while rows 7-11 of the figure are primers amplified using Allahabad Safeda RNA (without reverse transcriptase treatment)**

Fig. 4.16: Melt curve peaks for 29 genes. Single peaks show specific primers with single PCR product.

4.2.6 Gene expression analysis for candidate genes among genotypes at different ripening intervals

qRT-PCR analysis of all the 29 genes was carried out on the selected 7 genotypes of guava at 3 different ripening intervals *viz.* 2 DPH, 4 DPH and 8 DPH. The 7 genotypes taken into consideration are selected based on their ripening behaviour. The 7 genotypes selected were Allahabad Safeda (AS), Punjab Safeda (PS), Thailand (TH), Shweta (SH), 17-16, Punjab Pink (PP), and Lalit. Among these cultivars Thailand and Punjab Safeda are the delayed ripening cultivars based on visual inspection and lowest reduction in TSS or rather constant increase in TSS during ripening. For an instance TSS of Thailand increased to 7.4 on 9^{th} day and reached upto 9.2 on 15^{th} day. Also, in Punjab Safeda TSS was 9.5 on 9^{th} day when all other genotypes except Thailand succumbed to complete deterioration. Allahabad Safeda was the earliest ripening cultivar. So, we utilized Allahabad Safeda as the baseline for calculating the fold change, while Thailand and Punjab Safeda as cultivars of focus for pathway analysis. From the qPCR results we obtained the threshold/quantitation cycle (CT/CQ) value. This CQ value was utilized for calculating the fold change among different genotypes. For our analysis we used histone 3 as the housekeeping control to normalize gene expression. To analyse the general trend of gene expression among the different cultivars during ripening we calculated fold change using 2 DPH cDNA as baseline for all the genotypes. For analysis of more specific gene expression we calculated fold change using Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline for other cultivars at the same time point.

4.2.6.1 Candidate gene expression analysis of Ethylene biosynthesis pathway

Ethylene biosynthesis involves three major steps starting from methionine as precursor and are catalyzed by three enzymes *viz.* S-adenosyl Methionine, 1-Aminocyclopropane-1-Carboxylic acid Synthase and 1-Aminocyclopropane 1-Carboxylic acid Oxidase 6.

4.2.6.1.1 S-adenosyl Methionine 1 (*SAM1*)

SAM1 derives the first step in biosynthesis of ethylene by converting (adenylating; addition of AMP) methionine into S-adenosyl methionine. Fig. 4.17a shows that the expression of *SAM1* was lower for all the genotypes at 4 DPH and 8 DPH except for Punjab Safeda compared to 2 DPH in all the cultivars, which shows higher expression at 8 DPH. Fig. 4.17b shows, lower expression of SAM1 in all the genotypes at 2 DPH and 4 DPH and increased expression at 8 DPH compared to Allahabad Safeda. Punjab Safeda showed highest expression of SAM1 at 8 DPH among all genotypes.

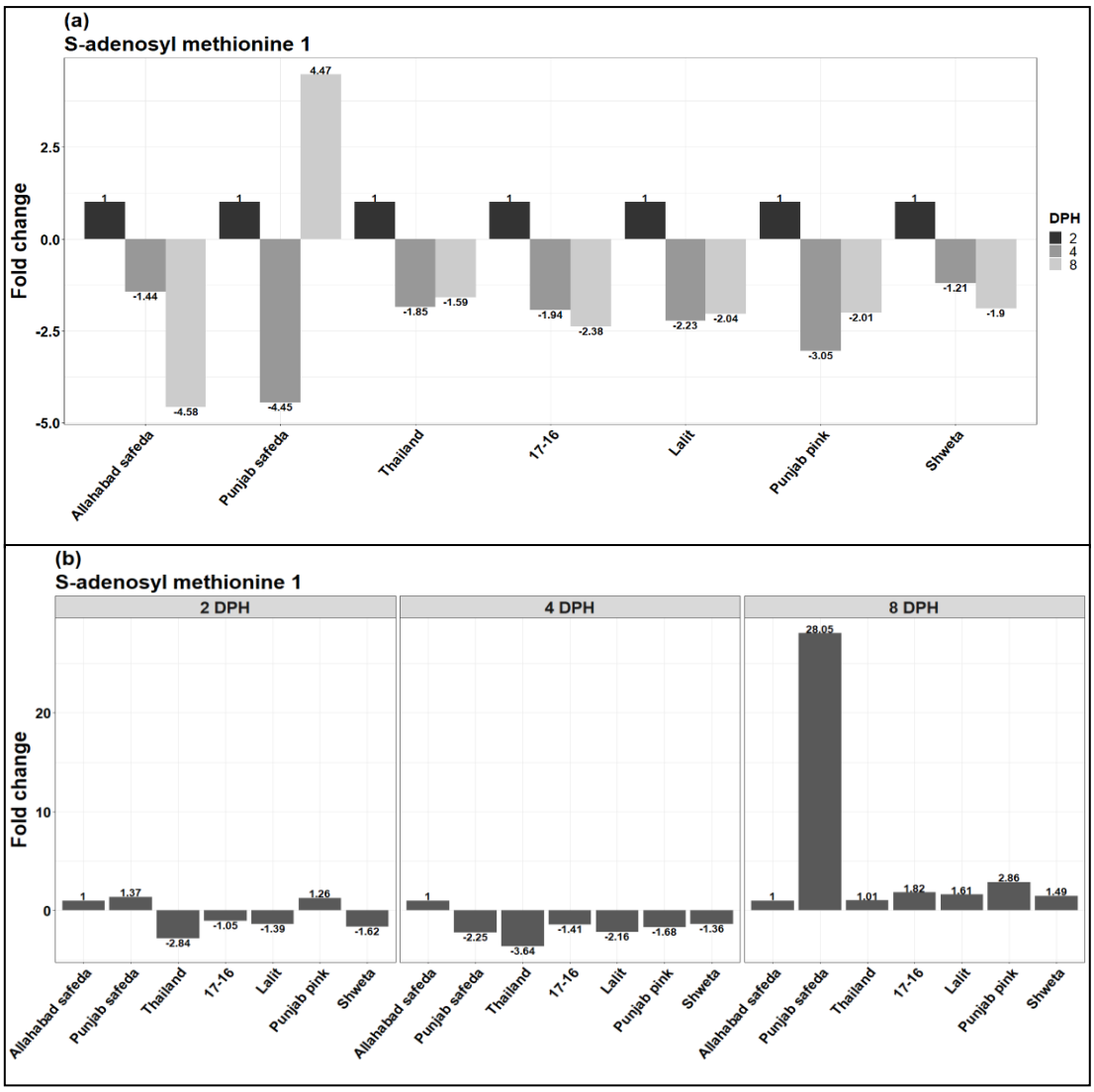

Fig. 4.17: Fold change of *SAM1* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.1.2 S-adenosyl Methionine 3 (*SAM3*)

SAM1 and SAM3 are redundantly present in different genomes. In our experiments, both the genes showed near similar expression pattern. Results of SAM3 expression are shown in figure Fig. 4.18 a & b. SAM1 and SAM3 expression in Thailand and Punjab Safeda cultivars implies that ethylene biosynthesis is normal in both the genotypes.

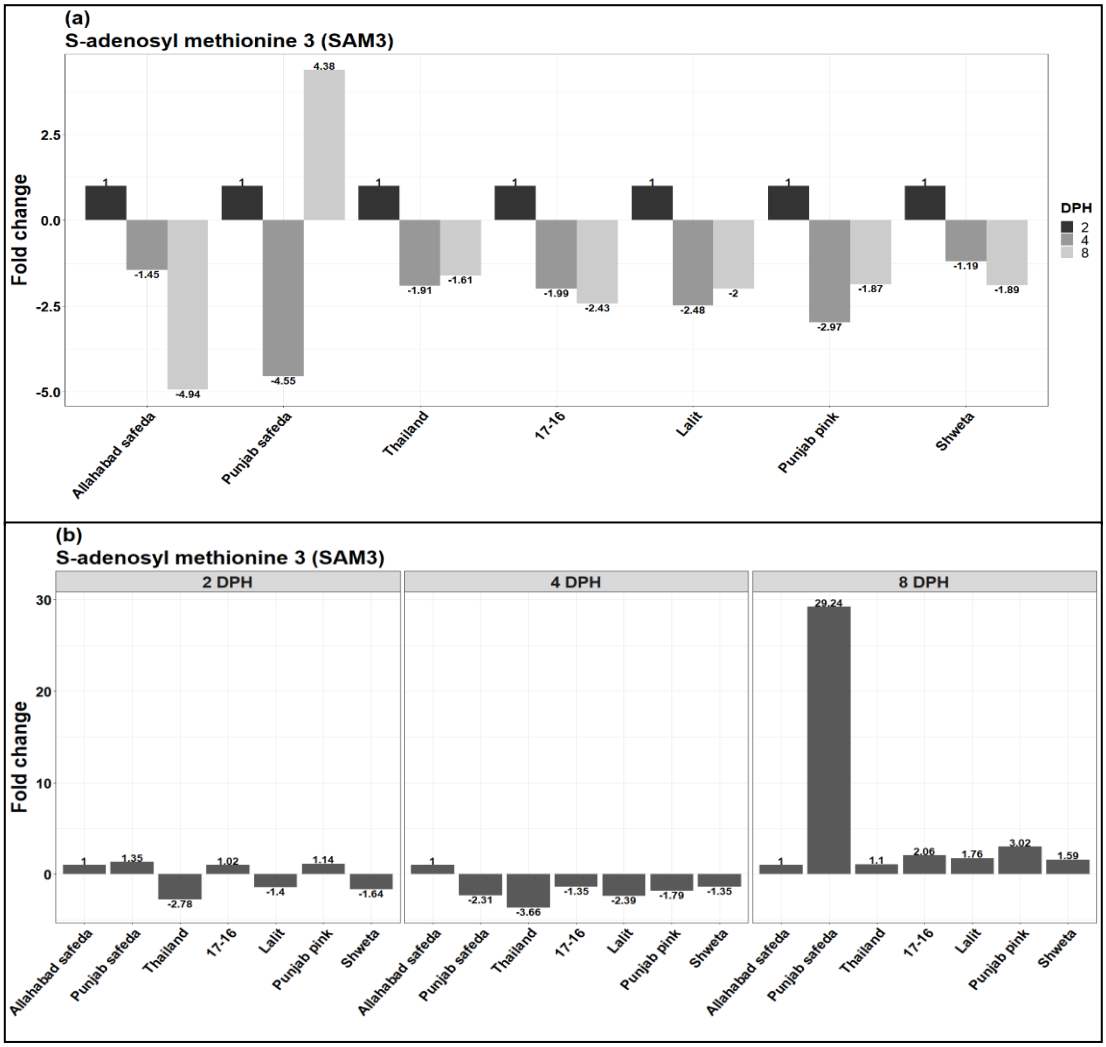

Fig. 4.18: Fold change of *SAM3* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.1.3 1-Aminocyclopropane-1-Carboxylic acid Synthase (ACS2/ ACC2)

ACC2/ ACS2 converts SAM into ACC and was found to be showing relatively higher expression in Punjab Safeda at 8 DPH compared to 2 DPH. (Fig.4.19 a). Thailand shows lower expression of ACC2 in early stage of ripening i.e 2DPH (Fig. 4.19 b). This result indicates the delayed biosynthesis of ethylene in Thailand Guava.

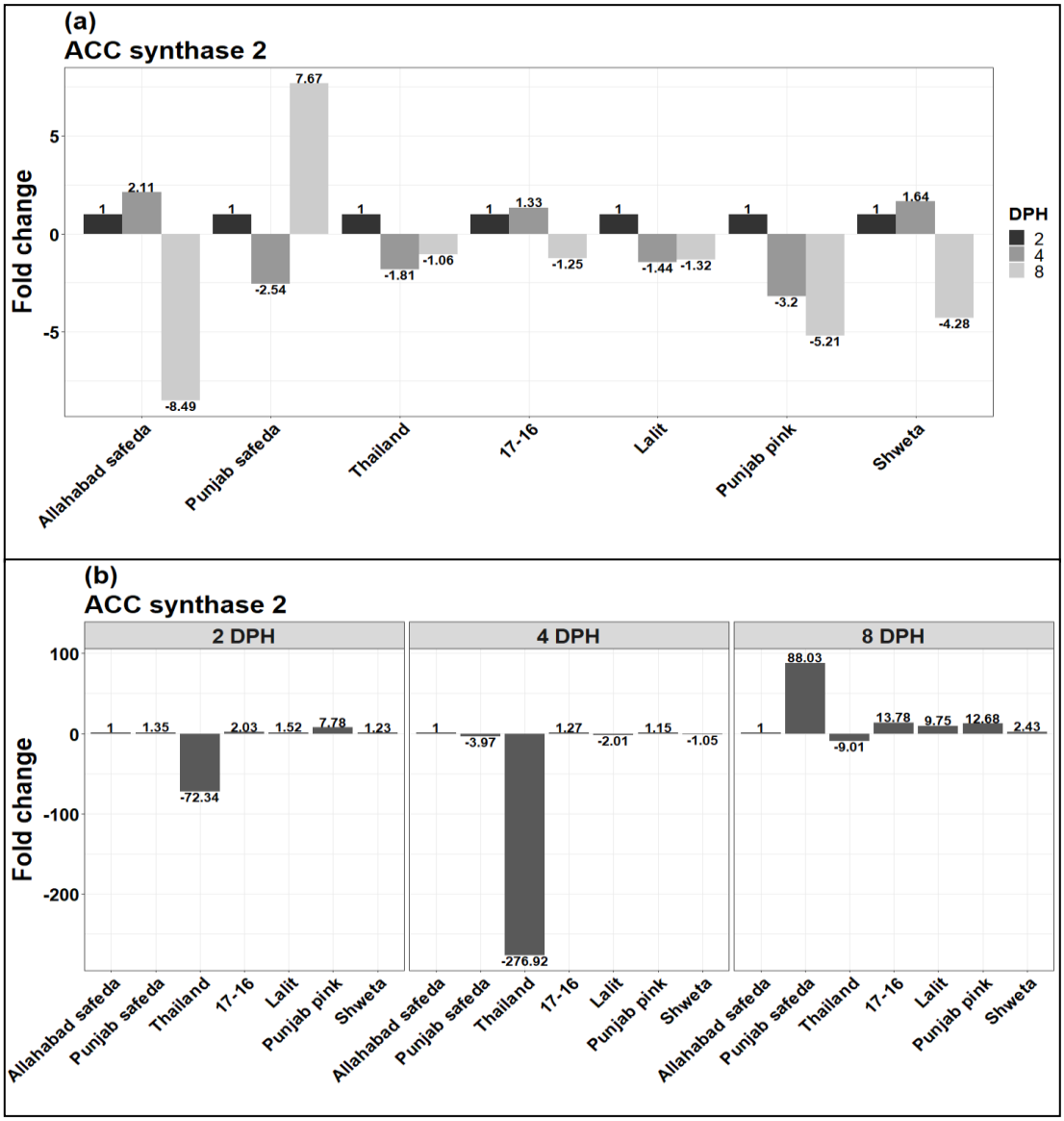

Fig. 4.19: Fold change of *ACC2* gene among different cultivars with (a) 2 DPH as baseline for all cultivars, (b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.1.4 1-Aminocyclopropane 1-Carboxylic acid Oxidase 3 (ACO3)

ACO converts ACC into ethylene in ethylene biosynthesis pathway. *ACO3* expression changes are similar to that of *ACC2 /ACS2* (Fig. 4.20 a & b).

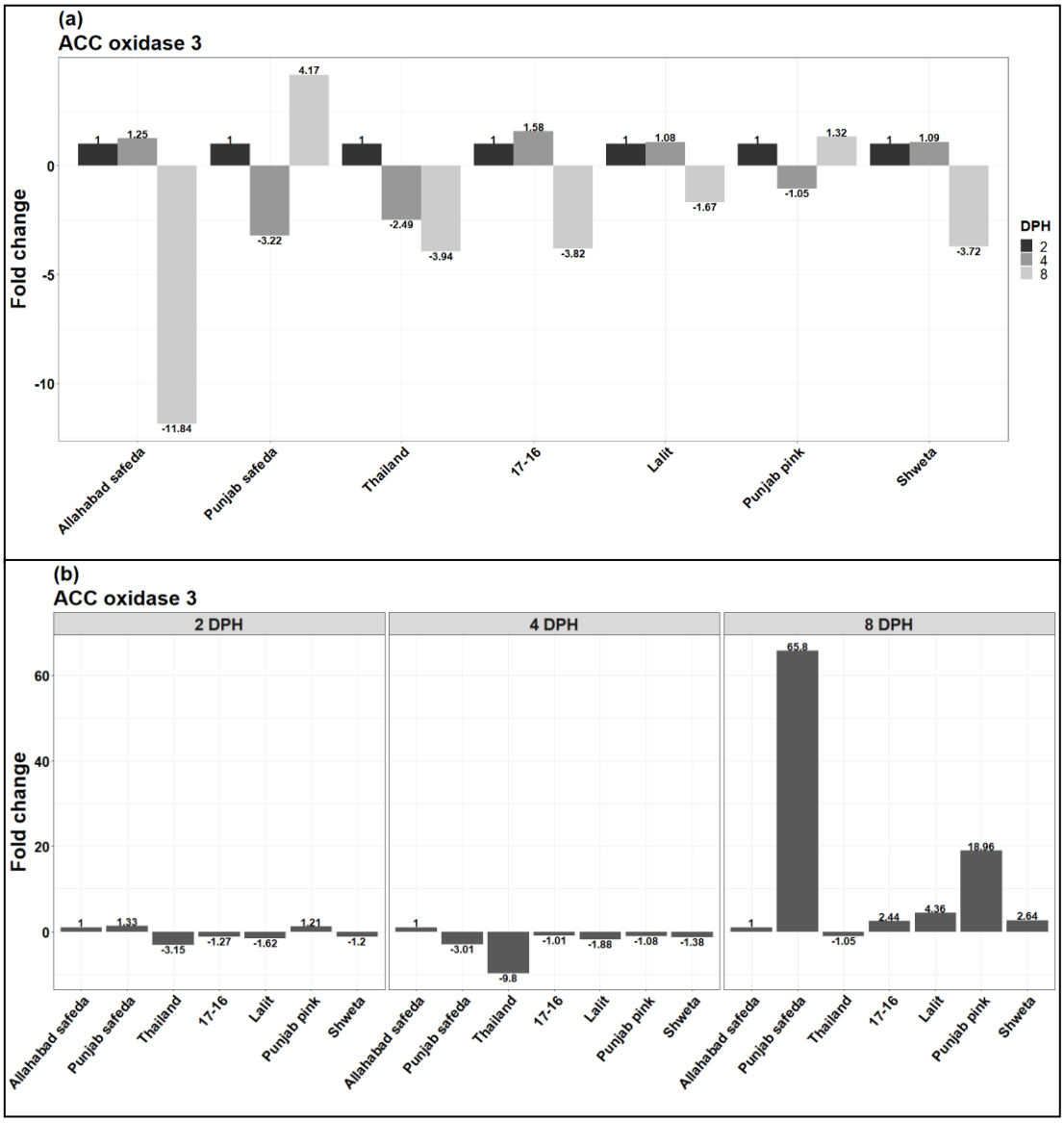

Fig. 4.20: Fold change of *ACO3* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.1.5 1-Aminocyclopropane 1-Carboxylic acid Oxidase 5 (ACO5)

ACO 3 and ACO 5 are the homologous genes and *ACO5* exhibits similar expression changes as that of *ACO 3* (Fig.4.21 a & b).

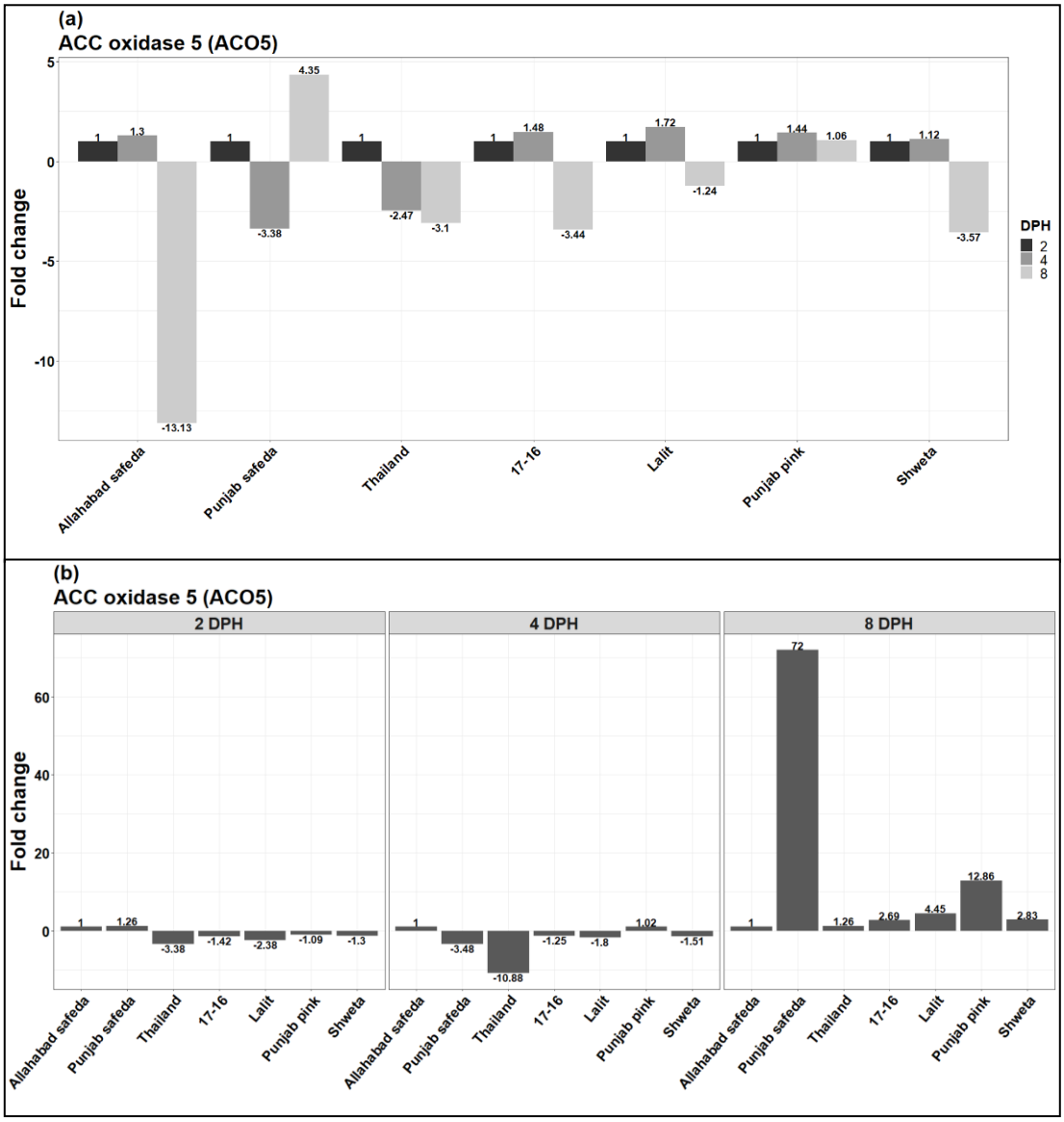

Fig 4.21: Fold change of *ACO5* gene among different cultivars with a) 2 DPH as baseline for all cultivars b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.1.6 1-Aminocyclopropane 1-Carboxylic acid Oxidase 6 (ACO6)

ACO6 shows similar expression changes as that of *ACO3* and *ACO5* showing redundant function of the gene family in guava fruit ripening (Fig. 4.22 a & b).

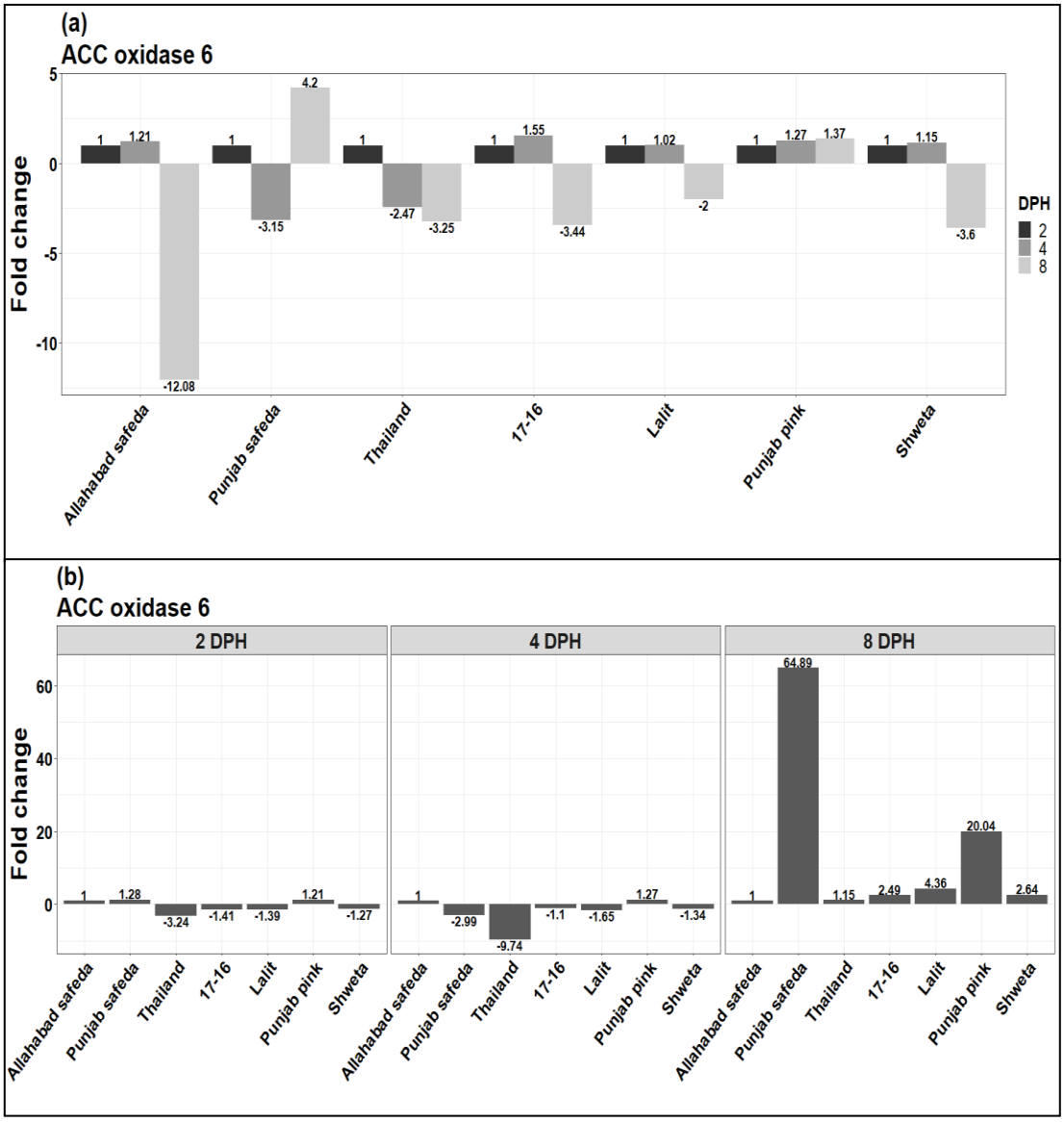

Fig. 4.22: Fold change of *ACO6* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as reference.

4.2.6.2 Candidate gene expression analysis of Ethylene Signaling pathway

Ethylene signaling starts with the receptor ETR3/4. As ETR binds to ethylene, CTR1 enhances the activity of ethylene insensitive genes and TFs.

4.2.6.2.1 Ethylene Receptor 3 (*ETR3*)

ETR3 perceive ethylene and transduce signals to downstream genes to activate ethylene signaling pathway. *ETR3* transcript show decreased expression in Thailand (4 DPH), 17-16 (4 DPH), Punjab pink (4 DPH) and Lalit (8 DPH) (Fig.4.23 a). After comparing the genotypes with specific time point of Allahabad Safeda (2, 4 and 8 DPH), we found decreased expression in Punjab Safeda (2 DPH), Thailand (2 DPH), Punjab pink (4 DPH) and Lalit (2 and 8 DPH), while increased expression at all other time points except 2 DPH (Fig.4.23 b).

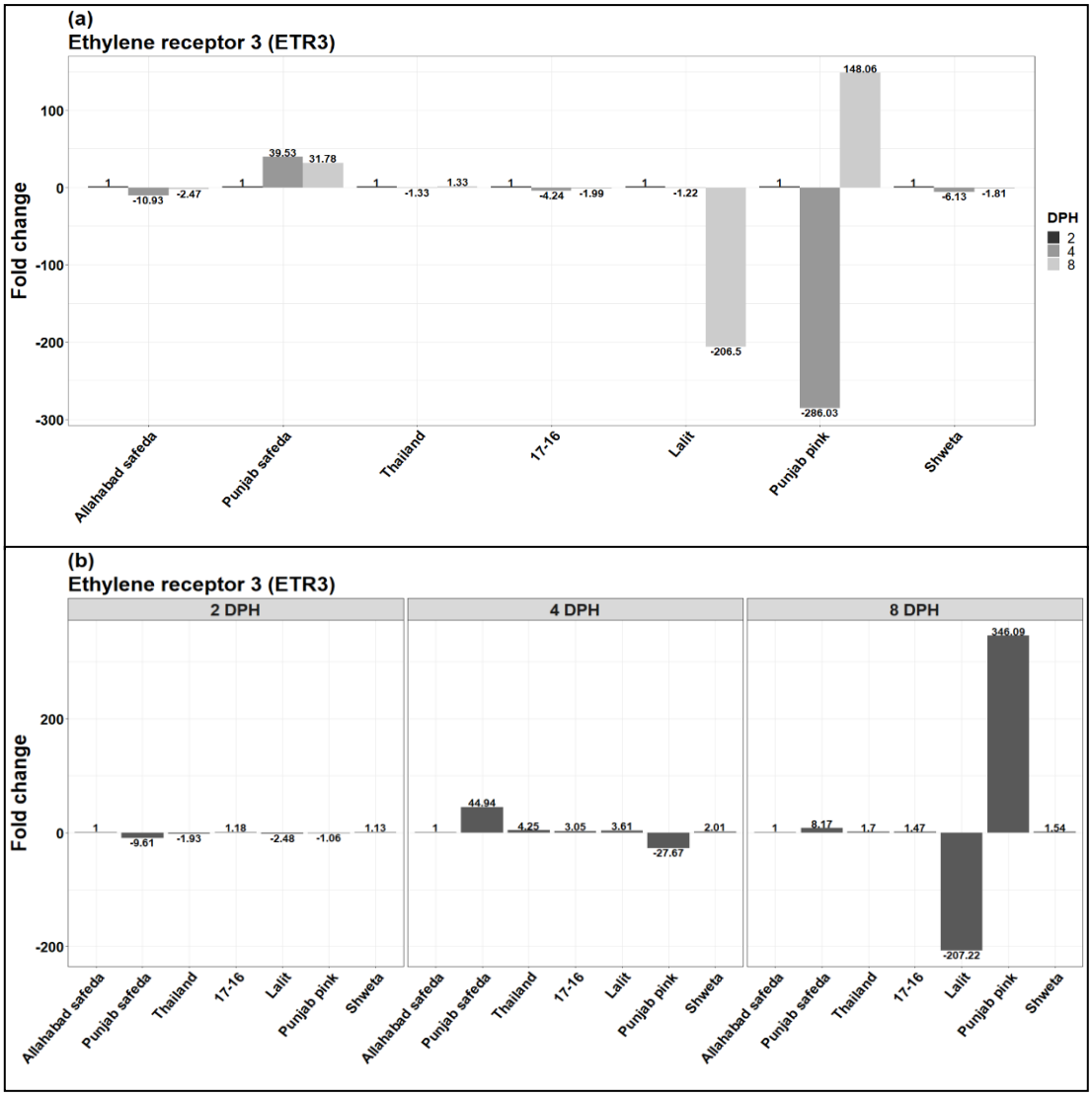

Fig. 4.23: Fold change of *ETR3* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.2 Ethylene Receptor 4 (*ETR4*)

ETR4 also perceive ethylene and transduce signals to downstream genes to activate ethylene signaling pathway. Fig. 4.24 a shows lower expression of *ETR4* in Thailand, 17-16 and Lalit at 4 DPH and 8 DPH. When compared specifically to Allahabad Safeda with the same time scale as that of other genotypes as baseline, the results are little different. Thailand continued to show lower expression in 2 and 4 DPH fruits. But the expression level increased in 8 DPH fruits of all genotypes as compared to Allahabad Safeda 8 DPH (Fig. 4.24 b).

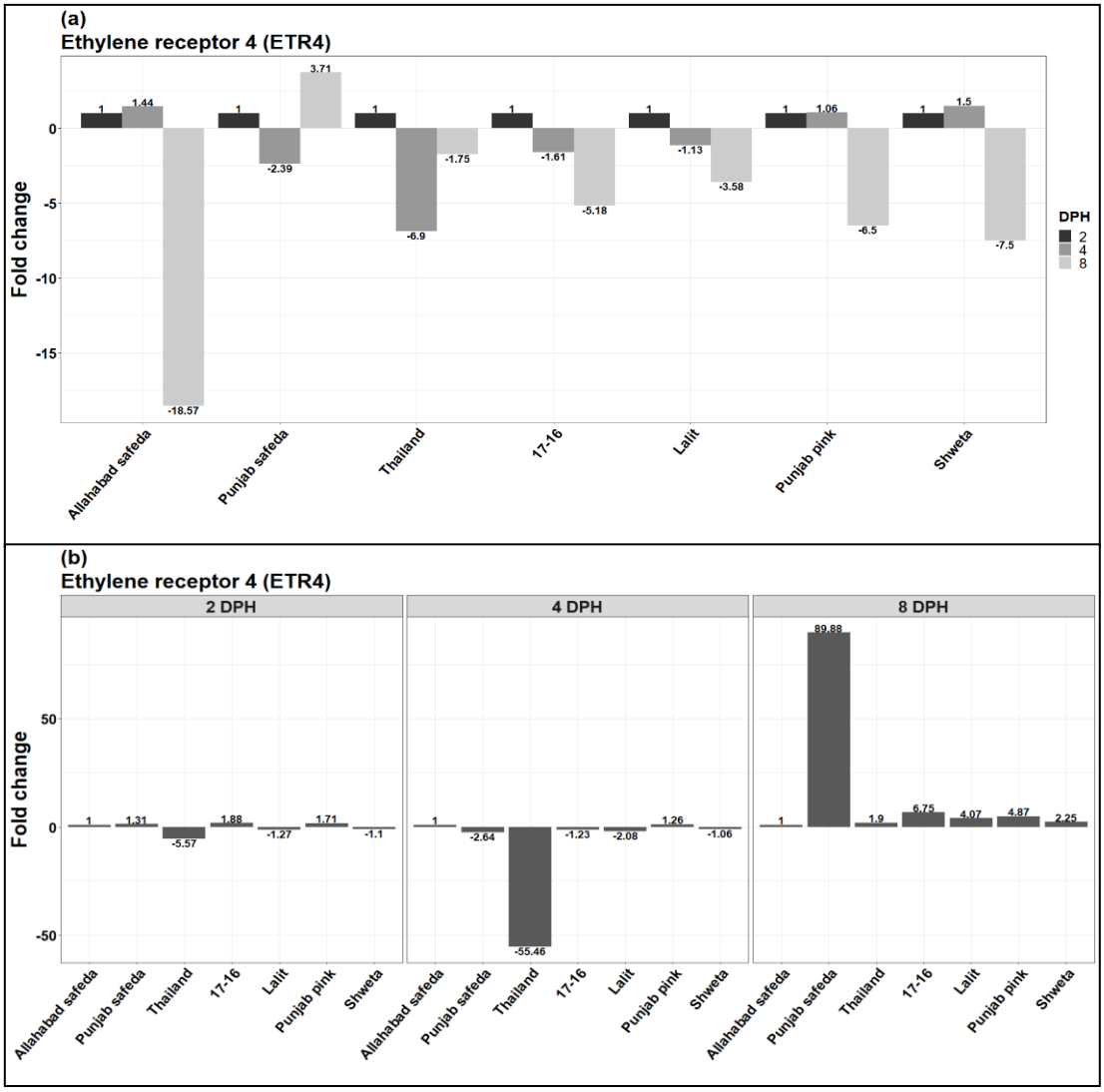

Fig. 4.24: Fold change of *ETR4* gene among different cultivars with a) 2 DPH as baseline for all cultivars b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.3 Ethylene Insensitive 2 (EIN2)

EIN2 is central ethylene signaling player located in the transmembrane of endoplasmic reticulum. Upon ethylene perception, EIN2 is transported and localized into nucleus where it activates the cascade of ethylene signaling. Our results show that the expression of EIN2 is lower in all the genotypes except Lalit, and at all the time points compared to 2 DPH of Lalit as baseline (Fig. 4.25 a). While specific comparisons with Allahabad Safeda with specific time points as baseline show that the expression of EIN2 is upregulated at 8 DPH for all genotypes and Punjab Safeda shows highest expression among all cultivars.(Fig. 4.25 b).

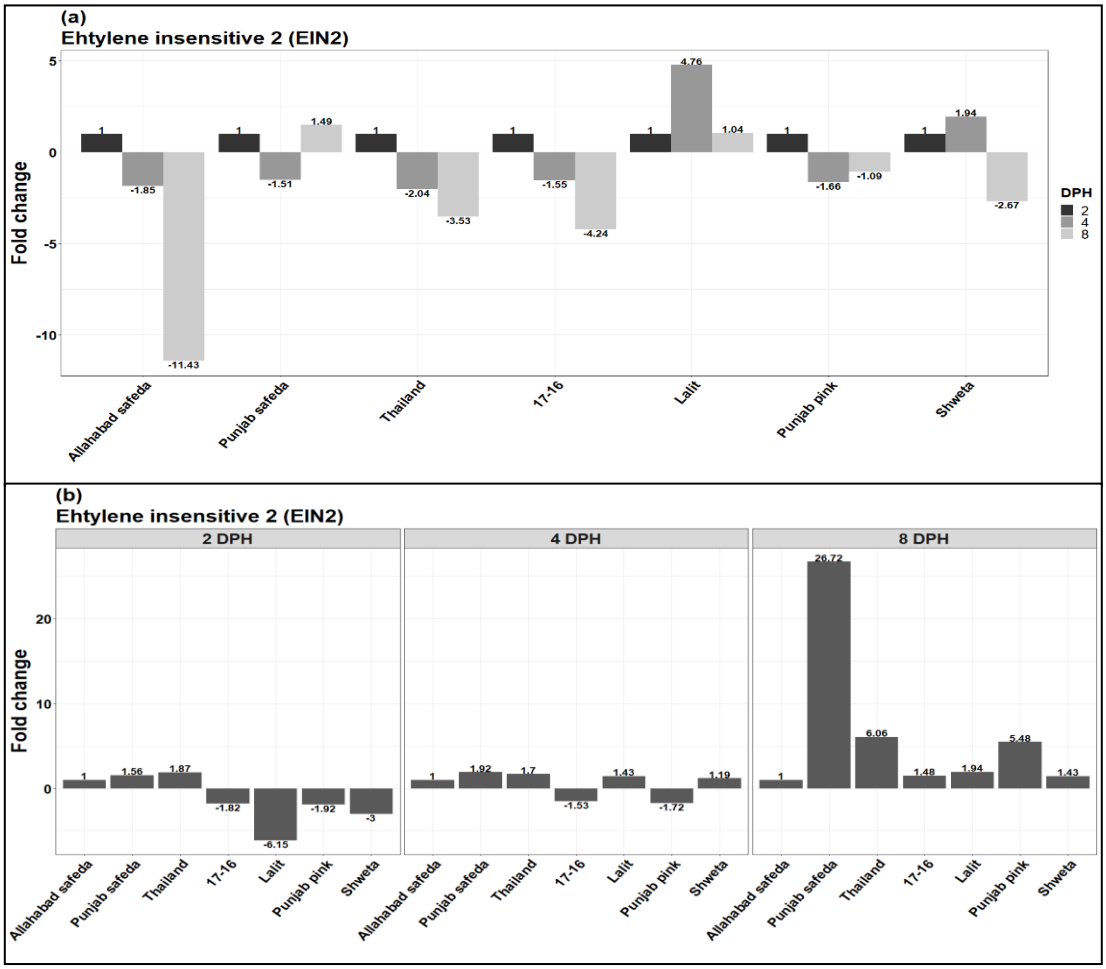

Fig. 4.25: Fold change of *EIN2 gene* among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.4 Ethylene Insensitive like 3 (*EIL3*)

EIL3 is TF activated as a response of ethylene perception. In general, when compared to 2D as a constant, *EIL3* shows lower expression among all the genotypes, but on 8 DPH Punjab Pink shows relatively higher expression. Similar pattern was followed when fold change of 2 DPH, 4 DPH and 8 DPH of different cultivars were calculated using 2 DPH, 4 DPH and 8 DPH of Allahabad Safeda respectively (Fig.4.26).

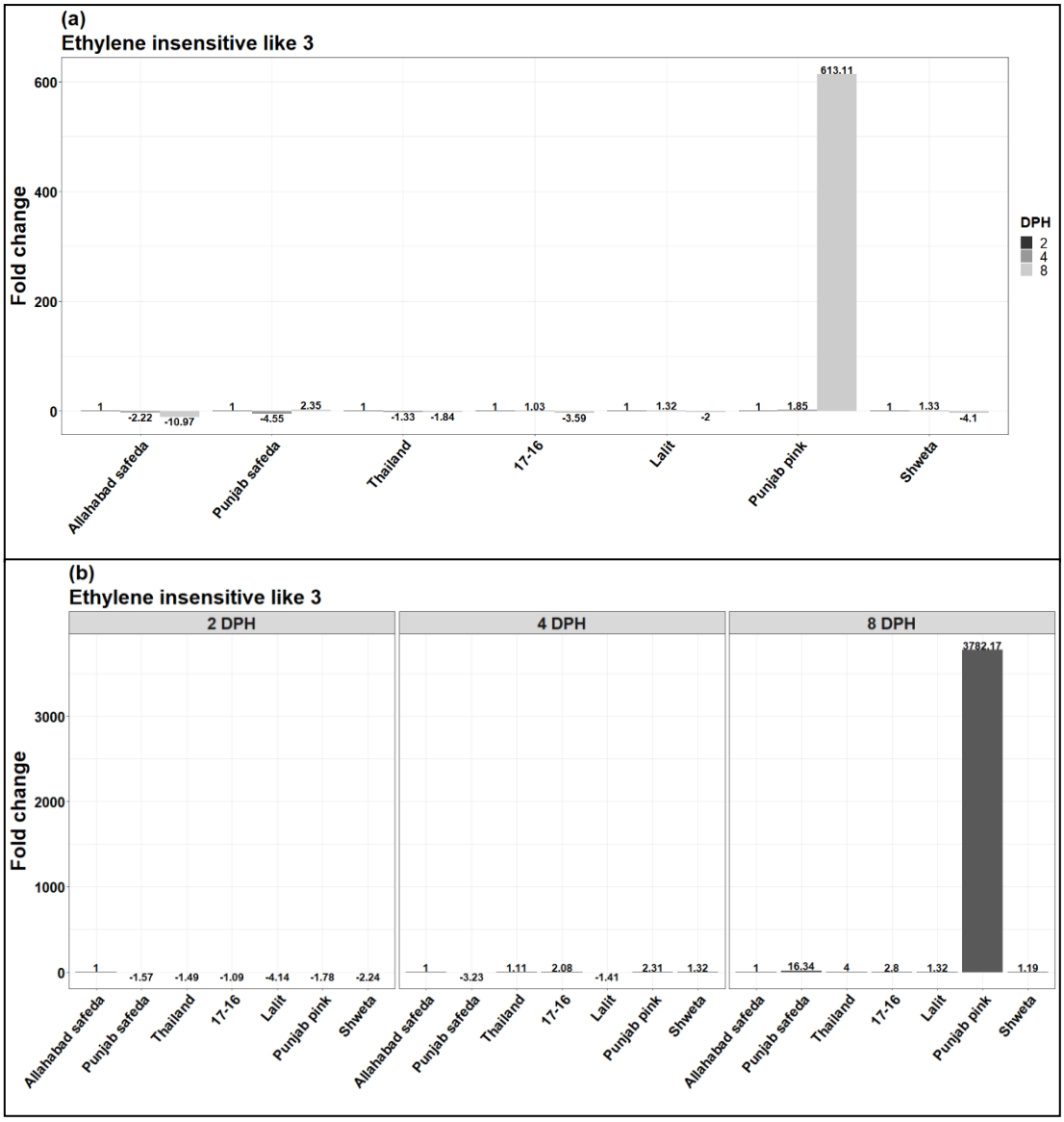

Fig. 4.26: Fold change of *EIL3* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.5 EIN2 targeting protein 1 (ETP1)

ETP1 proteins are involved in binding to the key ethylene responsive protein, EIN2, for its proteasomal degradation. Thus, this gene is positively correlated to ripening. In our experiments, ETP1 was found to be upregulated in 8 DPH Punjab Safeda, Lalit and Punjab Pink while down regulated in 17-16, Shweta, Allahabad Safeda and Thailand at all time points (Fig.4.27 a). When compared to 2, 4 and 8 DPH of Allahabad safeda, Lalit and Shweta shows lower expression (Fig. 4.27 b).

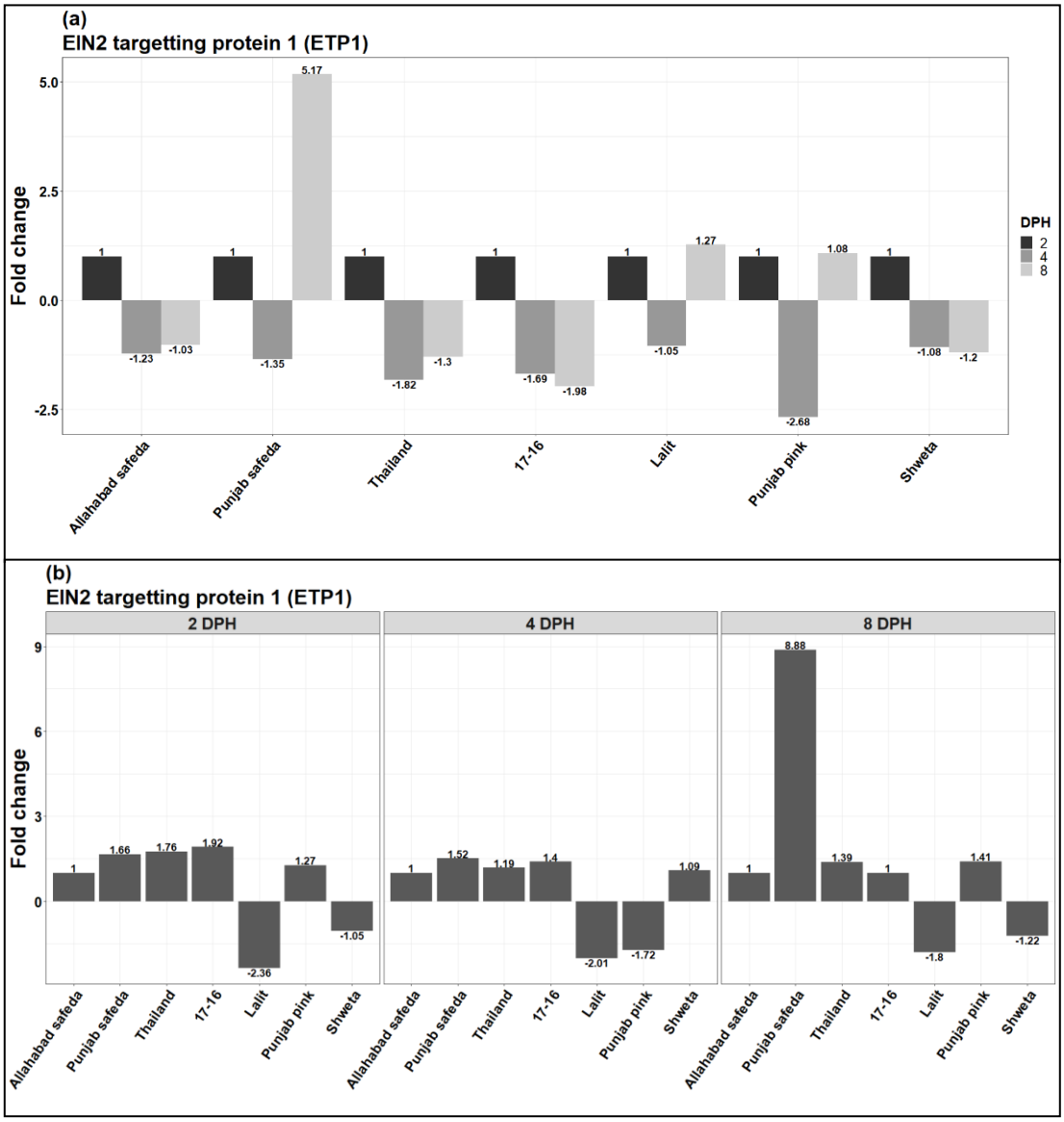

Fig. 4.27: Fold change of *ETP1* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.6 EIN2 targeting protein 2 (ETP2)

ETP1 and ETP2 show a redundant function in tomato and *Arabidopsis*. In our experiments, we found a similar pattern of gene expression among the genotypes for both *ETP1* and *ETP2* (Fig. 4.28 a & b).

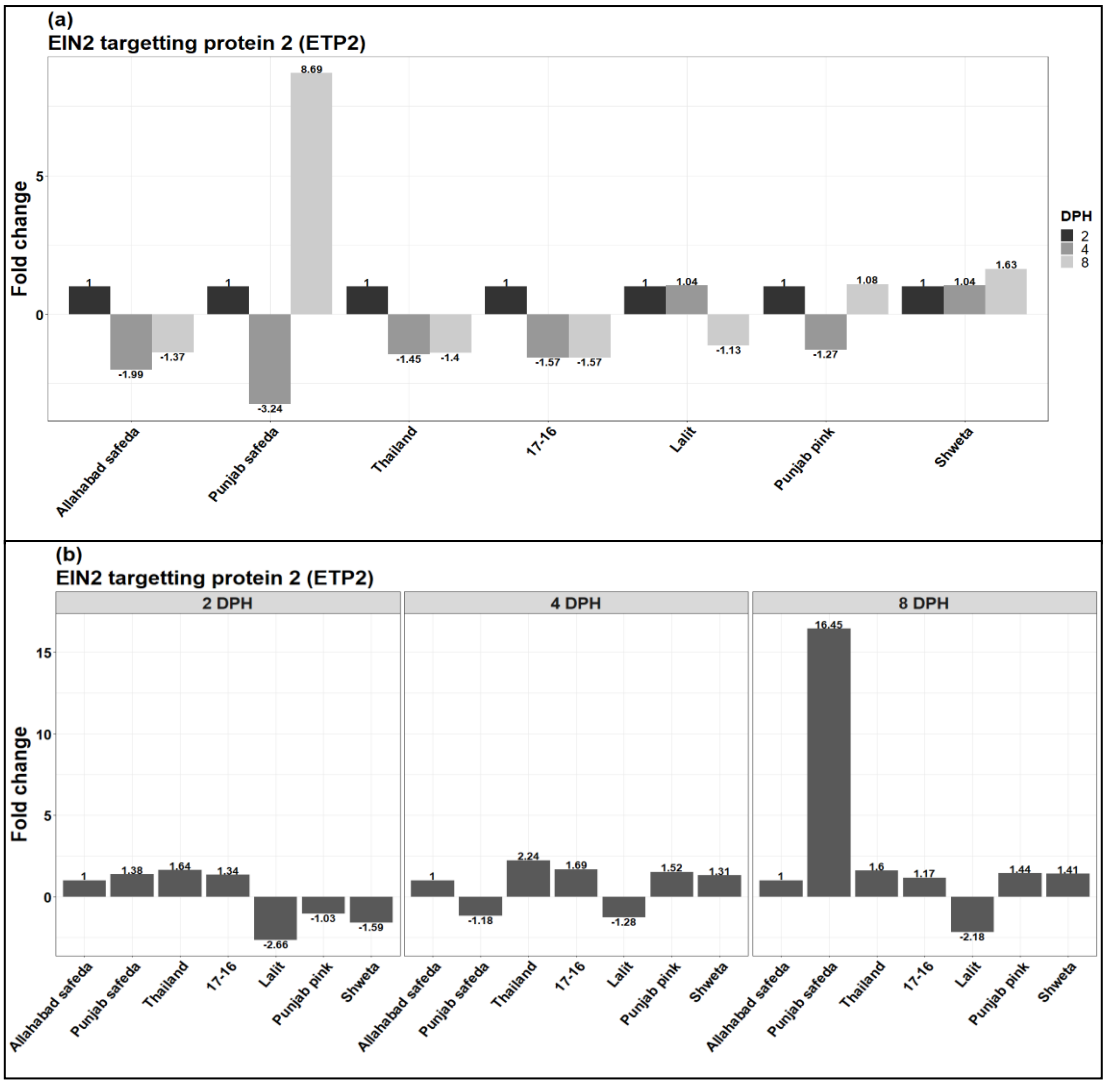

Fig. 4.28: Fold change of *ETP2* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.7 Constitutive triple response 1 (CTR1)

CTR1 is a Raf-protein kinase which binds and phosphorylates ethylene signaling key protein EIN2. In the absence of ethylene, CTR1 prevents the nuclear localization of C-terminus EIN2, thus inhibit ethylene signaling. In our expression results, CTR1 was found to be upregulated in Punjab Safeda at 4 and 8 DPH compared to 2 DPH of Punjab Safeda. In other genotypes there was not much difference except Punjab Pink and Lalit which showed different results in all three time points. Lalit had a lower CTR1 expression at 8 DPH (Fig. 4.29 a). Similar pattern was seen when fold change was calculated using Allahabad Safeda 2, 4 and 8 DPH as baseline for respective time points (Fig. 4.29 b).

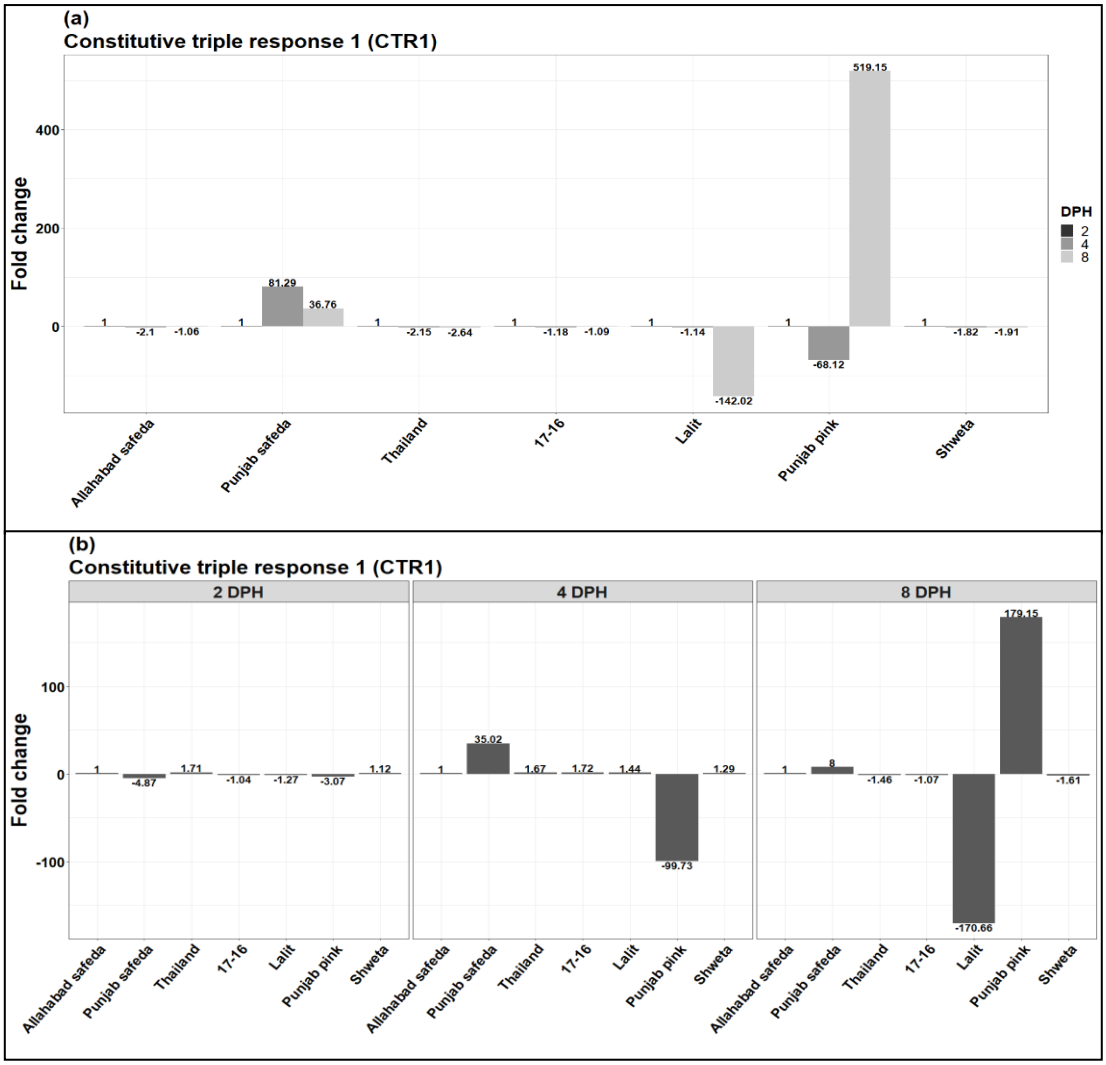

Fig. 4.29: Fold change of *CTR1* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.8 Ethylene response factor 6 (ERF6)

ERF6 is the TF of ethylene signaling and regulate the expression of many genes. Compared to 2 DPH, all the genotypes show decreased expression at all the 3 time points with varying fold change values (Fig. 4.30 a). Comparisons among the specific timepoint shows, increased expression in Thailand, Punjab pink and Punjab Safeda at 8 DPH, while all other genotypes and timepoints show decreased expression of ERF6 (Fig. 4.30 b).

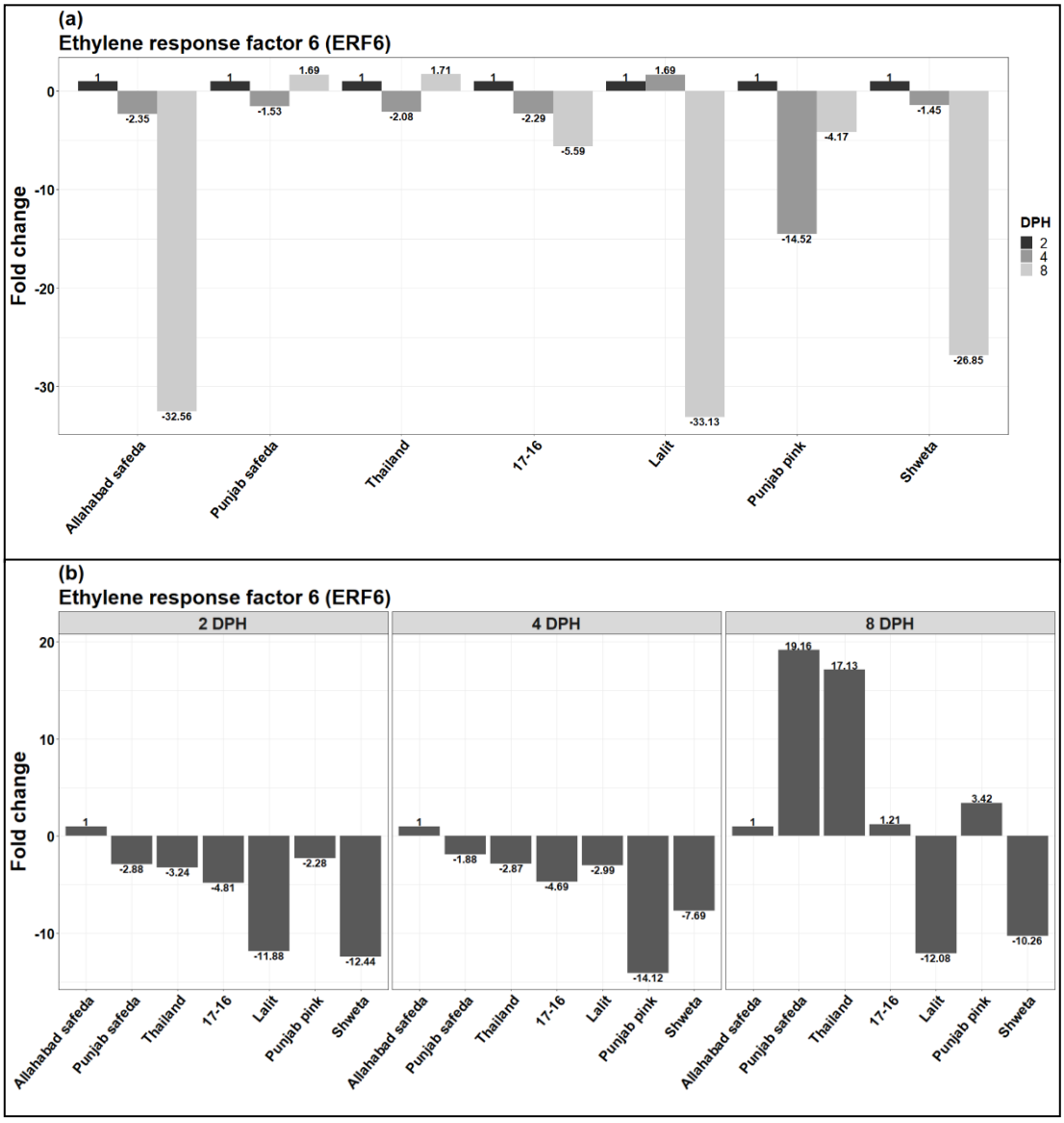

Fig. 4.30: Fold change of *ERF6* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.9 MADS box transcription factor located in Ripening inhibitor locus (MADS-RIN)

MADS transcription factor family is a diverse family involved in fruit ripening, floral transitioning and many other biologically important events. MADS-RIN is the MADS box transcription factor located in the ripening inhibitor locus in tomato genome. In our experiments, expression of this gene was upregulated in Punjab Safeda and Punjab Pink at 8 DPH compared to 2 DPH (Fig. 4.31 a). Similar pattern was seen in expression with little variation in fold change, when different time points were compared with Allahabad Safeda 2, 4 and 8 DPH as baseline respectively (Fig. 4.31 b).

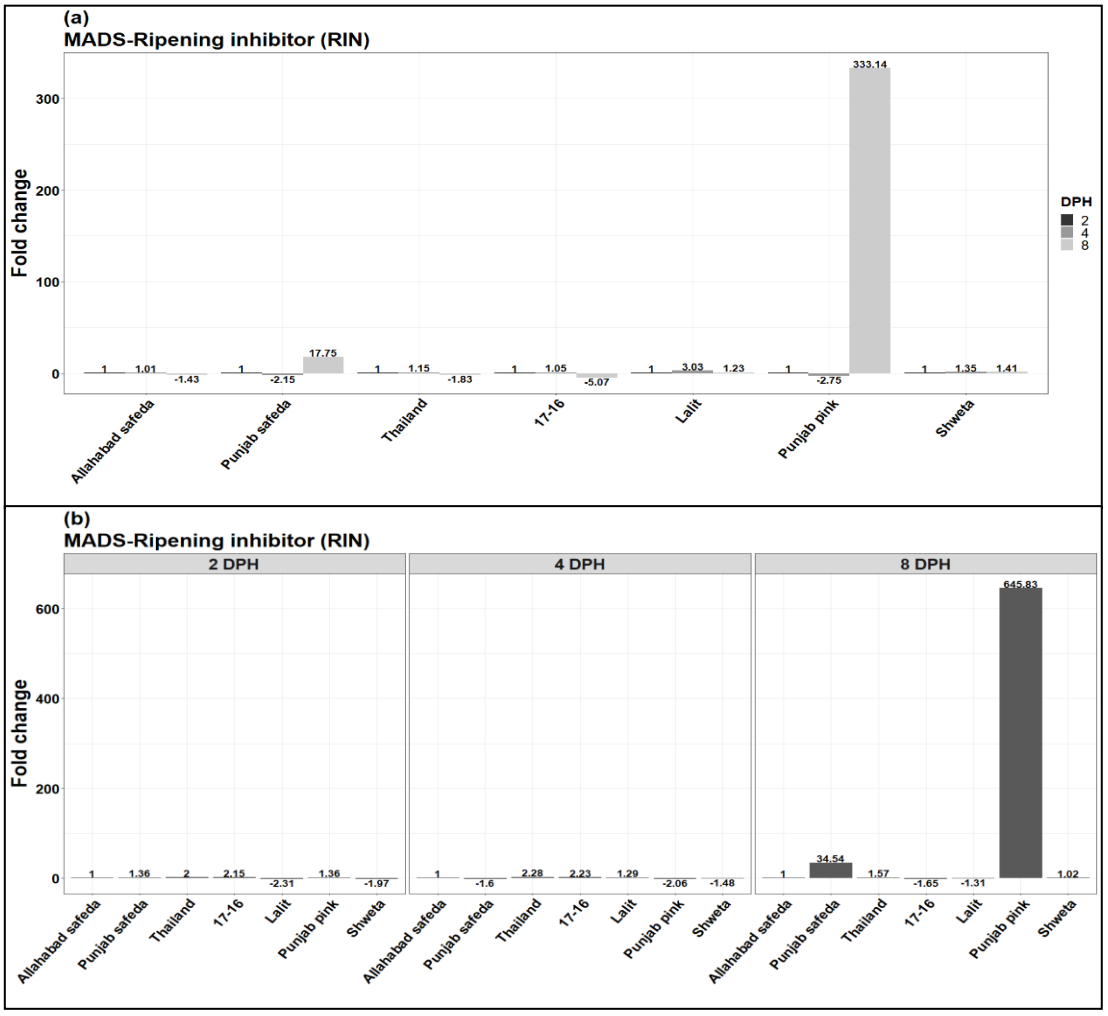

Fig. 4.31: Fold change of *MADS-RIN* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.10 Tomato FRUITFULL Homologs (*TDR4/FUL1*)

TDR4/FUL1 is a MADS-box TF. It acts as a negative regulator of shelf life and positive regulator of ripening. When compared to 2 DPH, TDR4 shows not much difference in expression among the genotypes. 17-16 (2 DPH and 4 DPH) show lower expression. Punjab Pink (2 DPH and 8 DPH) show lower expression. Lalit (8 DPH) show lower expression (Fig. 4.32 a). When compared to 2, 4 and 8 DPH of Allahabad Safeda, all genotypes had increased expression of this gene at 8 DPH (Fig. 4.32 b).

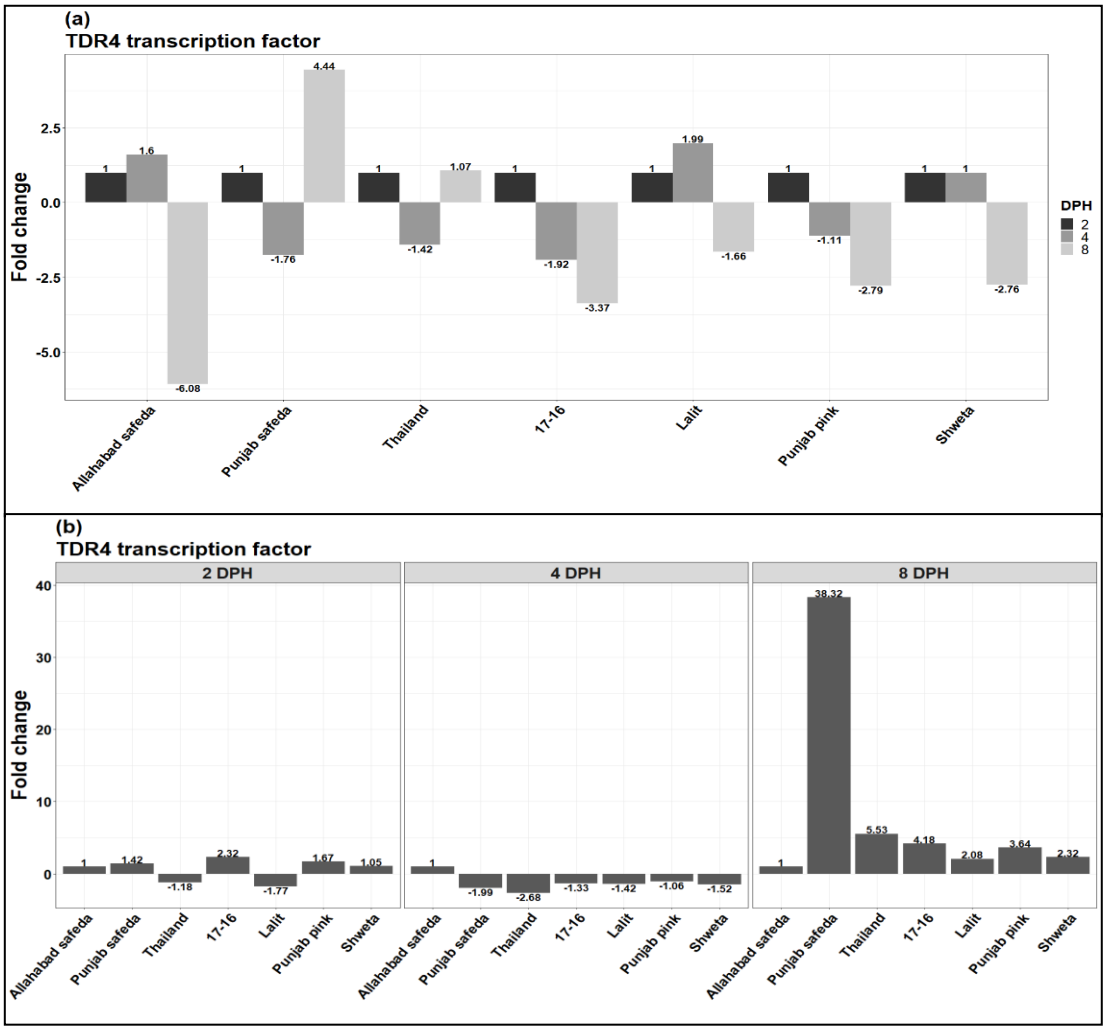

Fig. 4.32: Fold change of *TDR4* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.11 Tomato Agamous 1 (TAG1)

TAG1 is MADS-box protein involved in fruit development and ripening. Mainly this protein is negative regulator of ripening in tomato. In our experiments, we found TAG1 expression was lower in Punjab Pink 4 DPH compared to 2 DPH (Fig. 4.33 a). When the specific timepoints were compared with Allahabad Safeda, we found the expression of TAG1 is upregulated in Punjab Pink 8 DPH, while downregulated or unaffected in other genotypes (Fig.4.33 b).

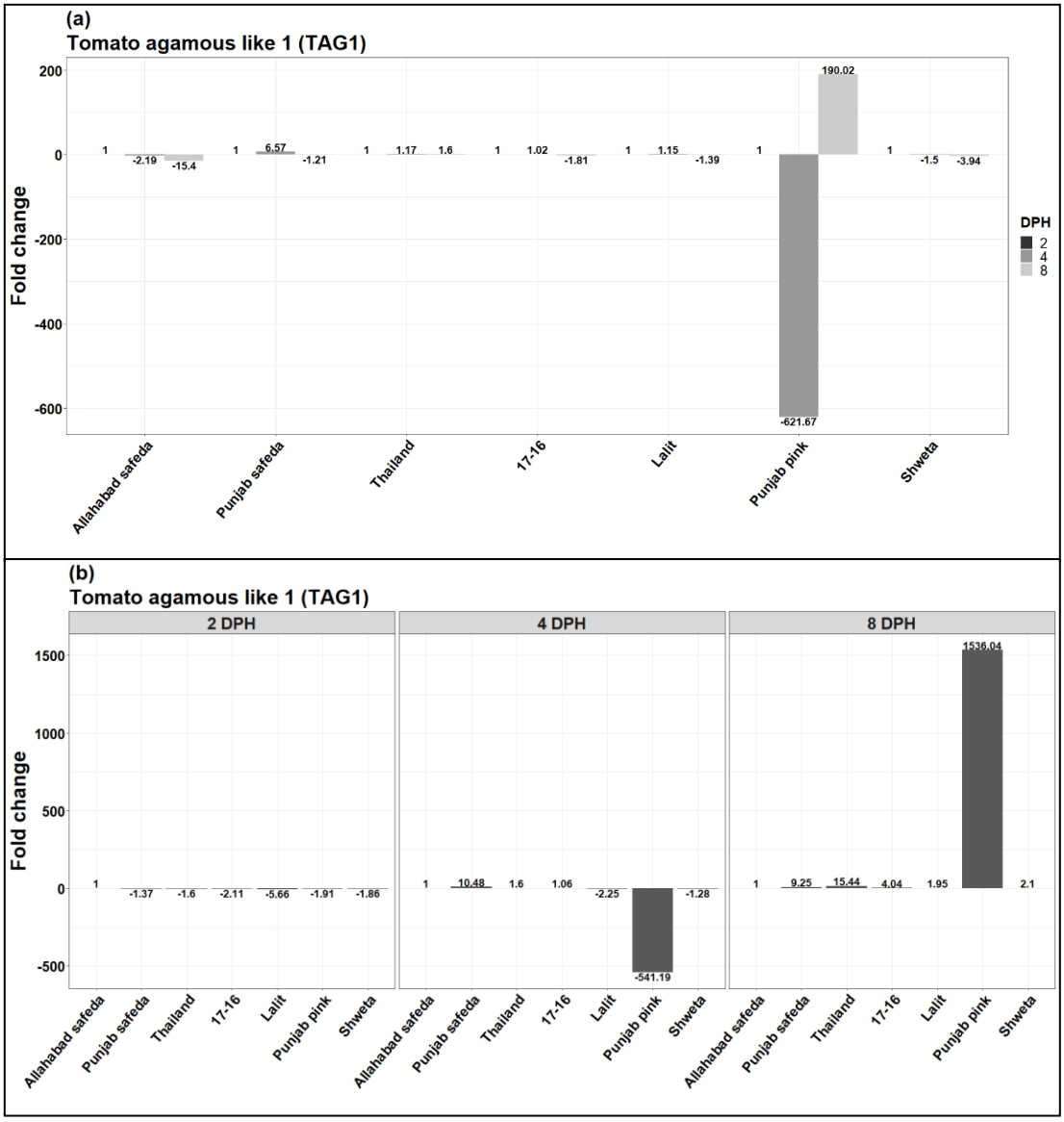

Fig. 4.33: Fold change of *TAG1* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.12 NAC transcription factor Non-Ripening (NAC-NOR)

NAC TFs are important regulators of ripening. The famous tomato Non-ripening mutation (nor) was a mutation in one of the NAC TF. In our results, we found that this gene was slightly downregulated in Thailand and 17-16 as compared to 2 DPH (Fig. 4.34 a). When we used the specific timepoints from Allahabad Safeda as baseline, we found similar results with little varied fold change values. In Punjab Safeda, the gene is upregulated at 8 DPH (Fig. 4.34 b).

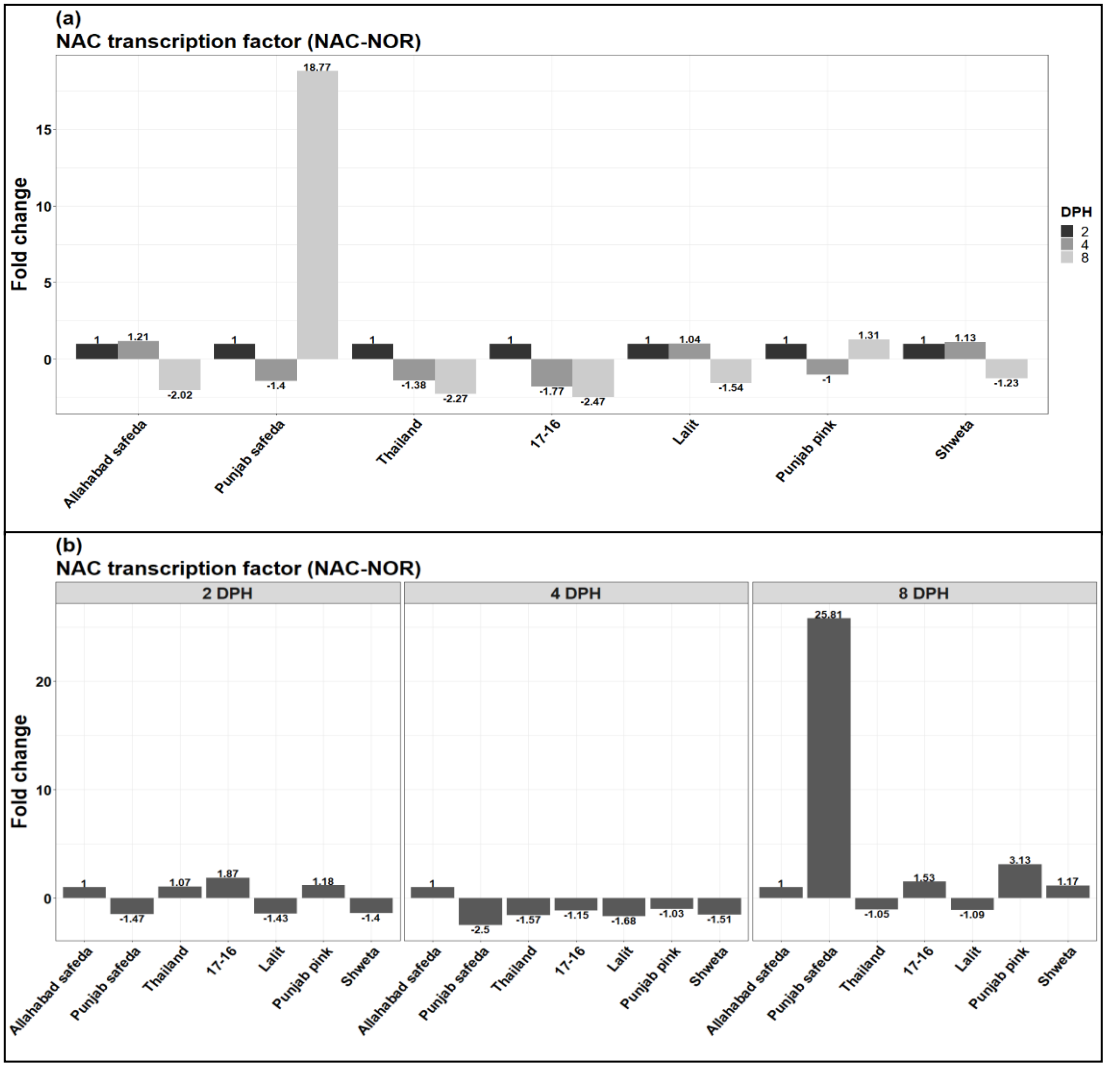

Fig. 4.34: Fold change of *NAC-NOR* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.2.13 MYB transcription factor

MYB TFs are involved in anthocyanin accumulation in fruits. Anthocyanin content is positively related to ripening as during ripening anthocyanin amount is increased. We found higher expression in Punjab Safeda, Lalit and Thailand, while lower expression in 17-16 compared to 2 DPH (Fig.4.35a).

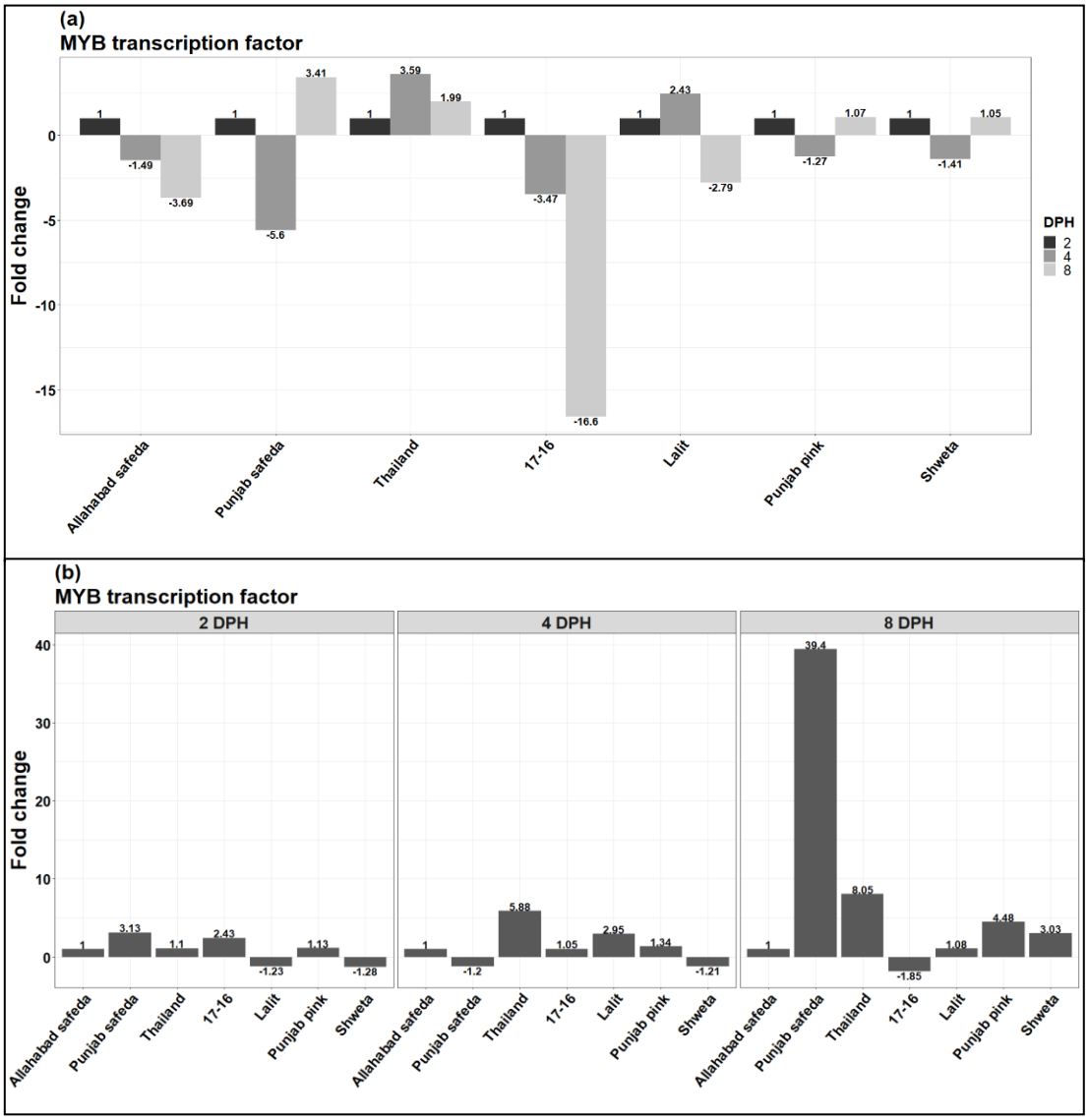

Fig. 4.35: Fold change of *MYB* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

In the search for identification of pathway responsible for delayed ripening we found most of the ethylene signalling pathway genes are downregulated in Thailand. Ethylene signalling is well understood for its role in fruit ripening. The major element of ethylene signalling is ethylene itself. It's a gaseous hormone which binds to receptors and produce

downstream signals. Ethylene signalling starts with conversion of methionine into S-adenosyl methionine (SAM), which is the first step in ethylene biosynthesis. Two enzymes *SAM1* and *SAM2* (S-adenosyl methionine transferase) are known to be involved in this conversion (Yuan *et al* 2016). In Thailand, both of these genes have lower expression than that of Allahabad Safeda after 2 and 4 DPH, at 8 DPH the expression is similar. Punjab Safeda had lower expression at 4 DPH. Thus, in Thailand the initiation of ethylene biosynthesis is slower. Next in ethylene signalling, SAM converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC oxidase (ACO). In Thailand three homolog genes *ACO3*, *ACO5*, and *ACO6* show lower expression at 2, 4 and 8 DPH than the Allahabad Safeda. In Punjab Safeda all three genes are downregulated till 4 DPH. This step is considered as the key rate limiting step in ethylene biosynthesis and lower expression indicates lower ethylene production (Wu *et al* 2018). Similarly, the next step of ethylene biosynthesis is conversion of ACC into ethylene by the enzyme ACC synthase (ACS). The gene *ACS2* also showed lower expression at all three days in Thailand and till 4th day in Punjab Safeda. All these led to the conclusion that in Thailand and Punjab Safeda ethylene is produced at lower rate than other genotypes and in Thailand it is even lower than Punjab Safeda. But what about the downstream genes in ethylene pathway? We investigated some of the receptors and transcription factors for their differential expression. We found that the ethylene receptor ETR4 which transduce the downstream signals and the transcription factor TDR4 both are downregulated in Thailand. This completes ethylene signalling pathway and we can conclude that the delayed ripening in Thailand is due to downregulation of most of the genes from ethylene biosynthetic pathway.

4.2.6.3 Candidate gene expression analysis of ABA biosynthesis pathway

In ABA biosynthesis 9-cis-epoxycaretenoid dioxygenase 1 (*NCED1*) and *NCED2* act as rate key limiting genes and Uridine diphosphate glycosyltransferase (*UDP*) and Cytochrome P450 CYP707A are the key enzymes in ABA catabolism.

4.2.6.3.1 9-Cis-epoxycaretenoid dioxygenase 1 (*NCED1*)

NCED1 is a key rate limiting step in ABA biosynthesis which converts neoxanthin to xanthoxin in ABA signaling pathway. It gets activated during early stages of fruit development and is expressed at lower rates. Compared to the 2 DPH of all cultivars, Punjab pink had relatively higher gene expression. While other genotypes had relative lower gene expression (Fig. 4.36 a). Similar results were found with 2, 4 and 8 DPH of Allahabad Safeda used as baseline for specific time points (Fig. 4.36 b).

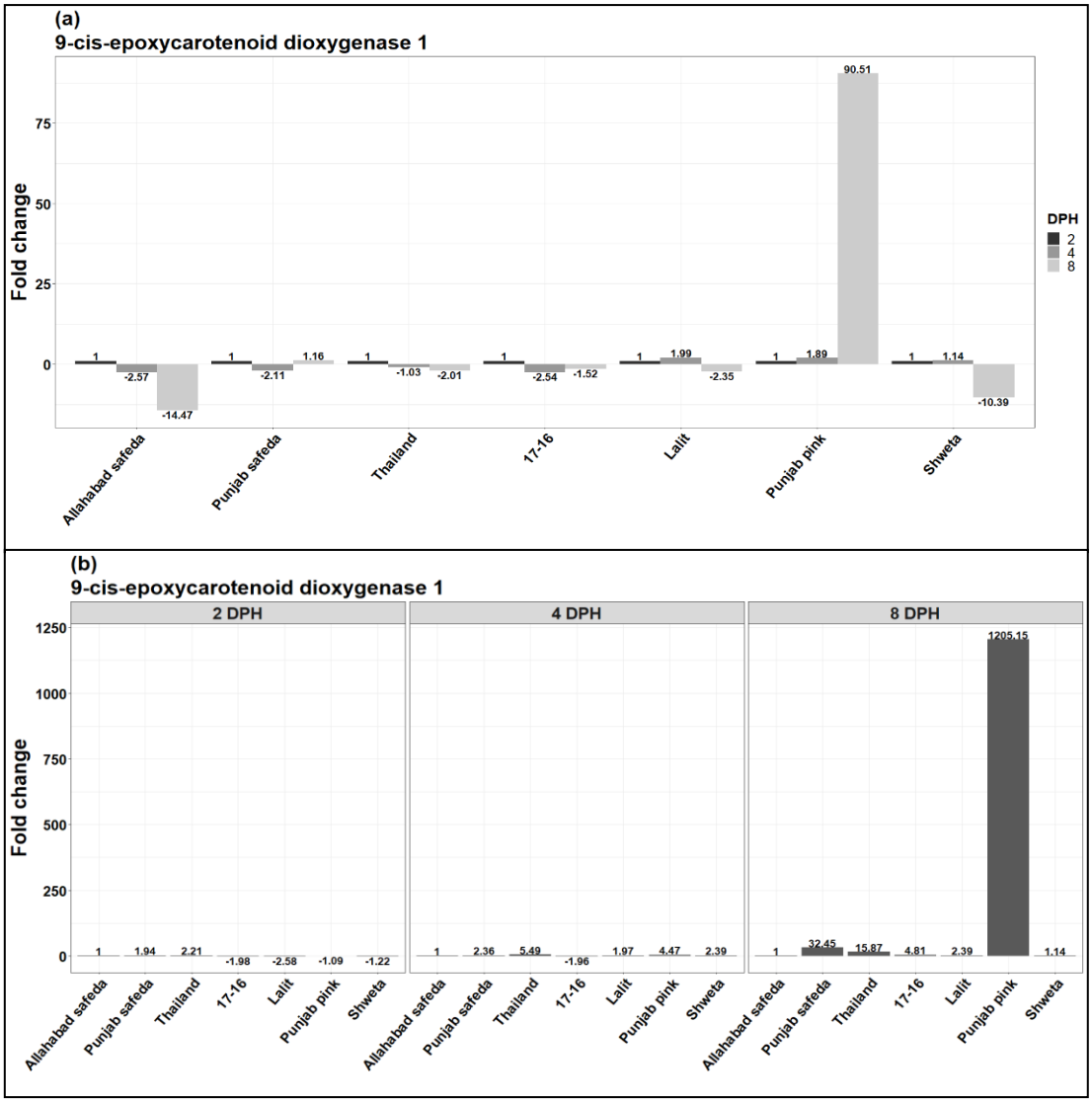

Fig. 4.36: Fold change of *NCED1* gene among different cultivars with a) 2 DPH as baseline for all cultivars b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.3.2 9-cis-epoxycaretenoid dioxygenase 2 (*NCED2*)

Like *NCED1*, *NCED2* also participate in conversion of neoxanthin to xanthoxin in ABA signaling pathway. It gets activated during later stages of fruit development and is expressed at higher rates. *NCED2* shows relative lower expression in all the genotypes compared to 2 DPH as baseline (Fig. 4.37 a). With 2, 4 and 8 DPH as baseline from Allahabad safeda, we found the expression of all the genotypes at 8 DPH was higher (Fig. 4.37 b).

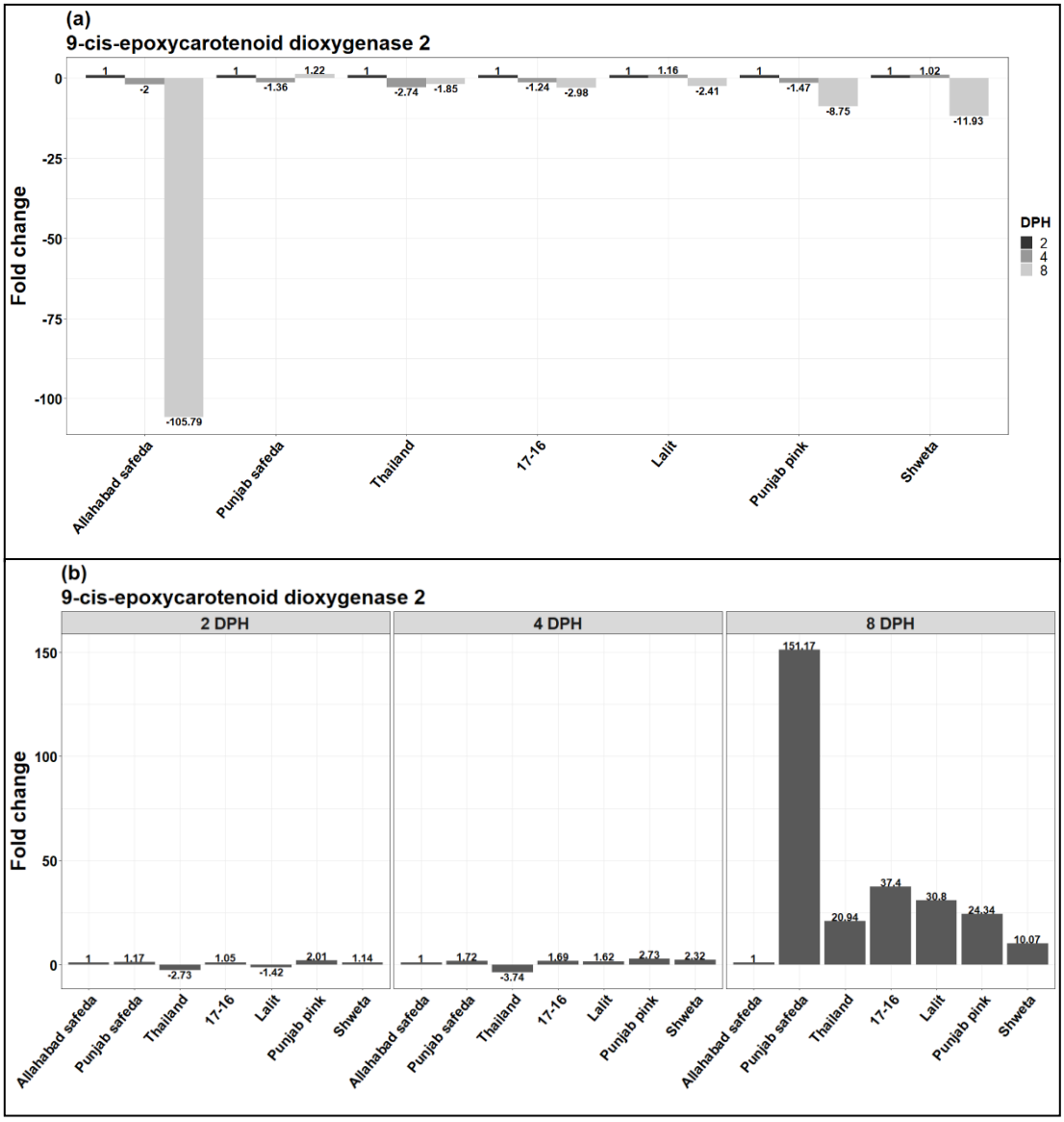

Fig. 4.37: Fold change of *NCED2* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.3.3 Uridine diphosphate glycosyltransferase 75 (*UDP75*)

UDP75 involved in glycosylation and degradation/deactivation of abscisic acid after the synthesis of ABA. The expression is very high in all the genotypes compared to 2 DPH (Fig.4.38 a). The expression was higher for Punjab Safeda and Thailand 4 DPH when compared with 2, 4 and 8 DPH as baseline from Allahabad Safeda (Fig.4.38 b).

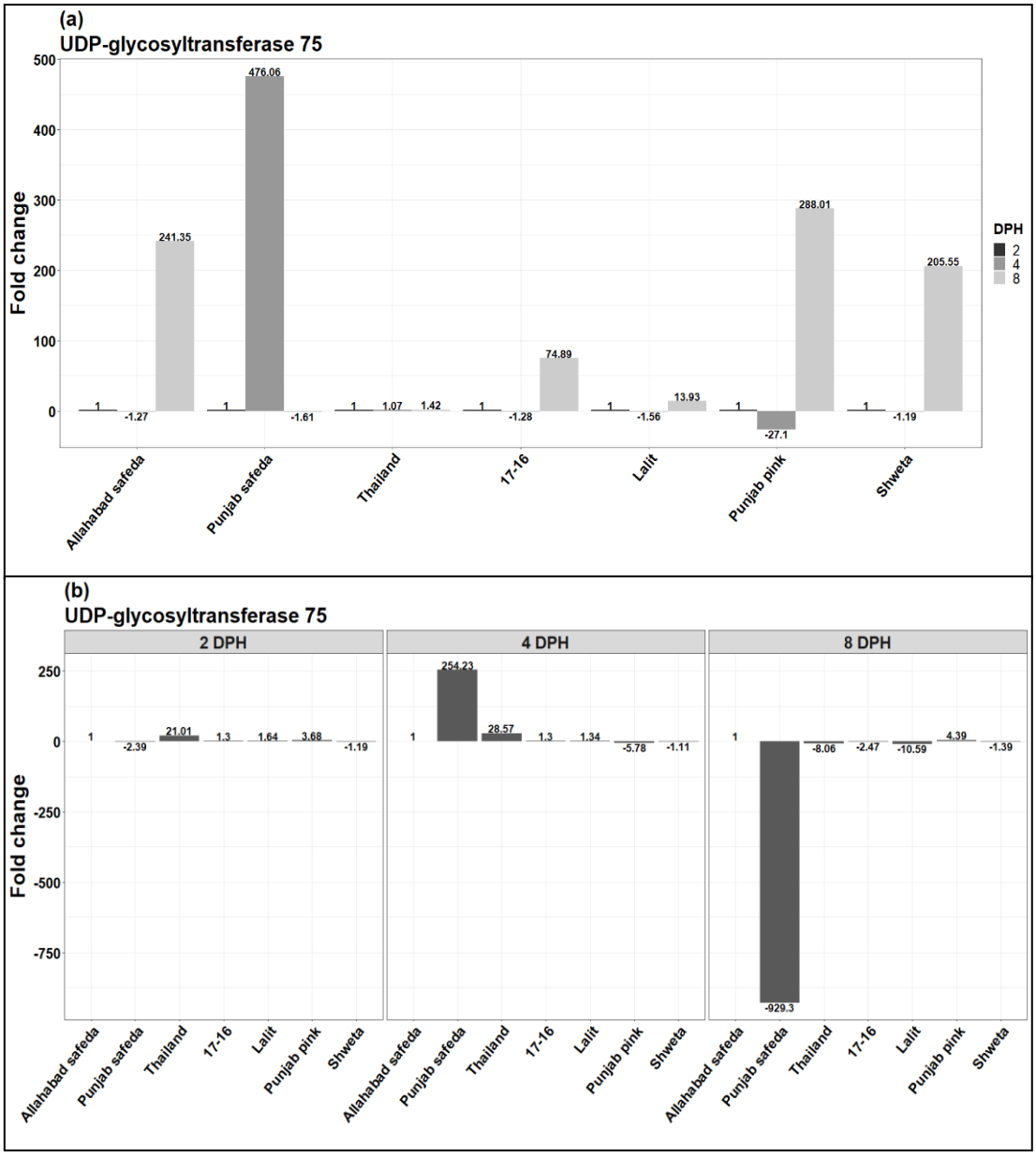

Fig. 4.38: Fold change of *UDP75* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.3.4 Uridine diphosphate glycosyltransferase 76 (*UDP76*)

UDP75 has similar function as that of *UDP76*. Compared to 2 DPH, *UDP76* showed lower expression in all the genotypes except Punjab Safeda and Thailand (Fig. 4.39 a). Specific comparisons show higher expression in Punjab Safeda and Thailand at 8 DPH (Fig. 4.39 b).

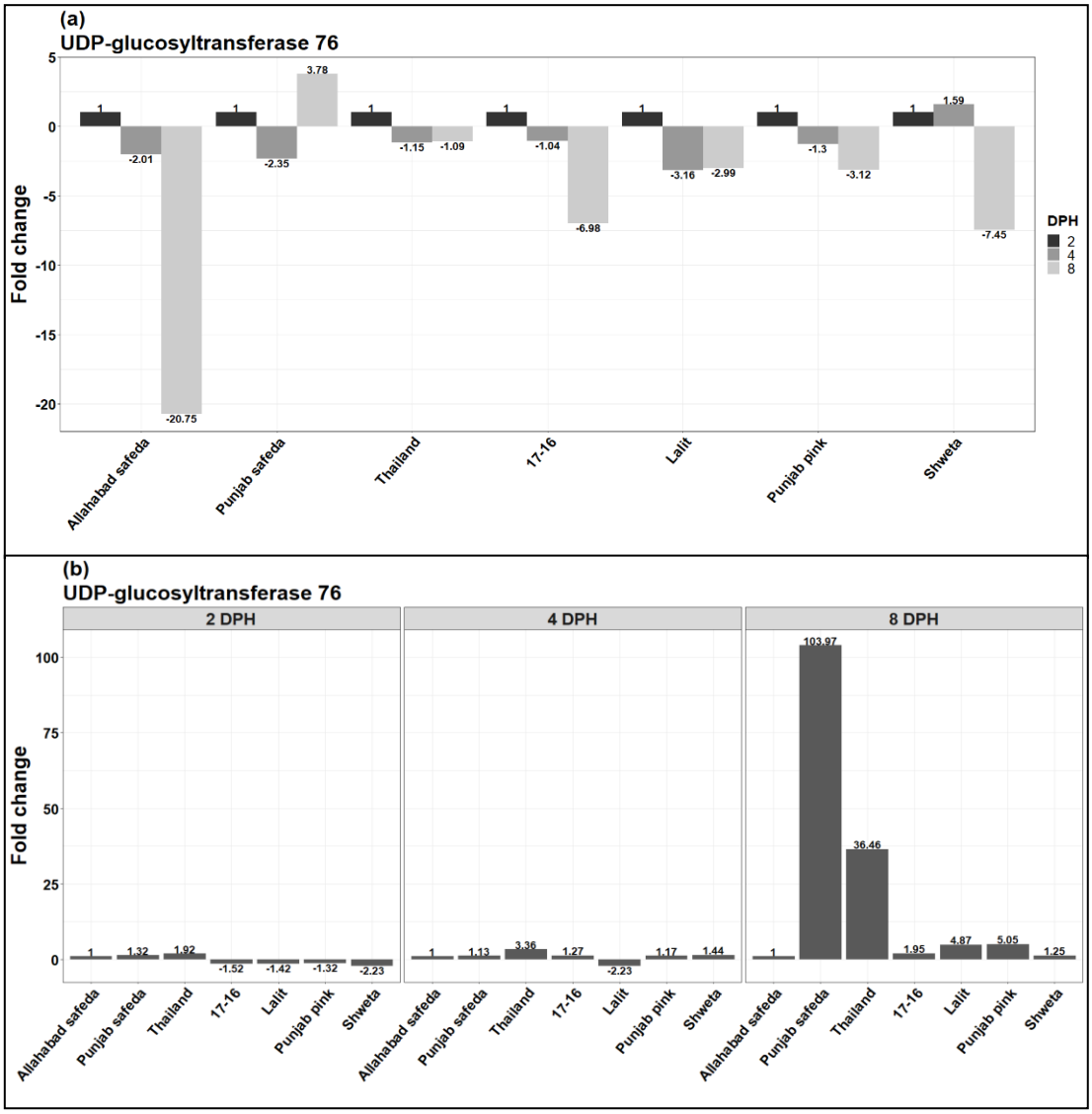

Fig. 4.39: Fold change of *UDP76* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

4.2.6.3.5 Cytochrome P450 *CYP707A*

CYP707A encodes ABA 8'-hydroxylases and is a key enzyme in ABA catabolism. Considering 2 DPH as baseline, all the genotypes had higher expression at 8 DPH except Punjab Safeda (Fig. 4.40 a). Specific comparisons show that Punjab Safeda had higher expression at 4 DPH, while Thailand had lower expression at 8 DPH (Fig. 4.40 b).

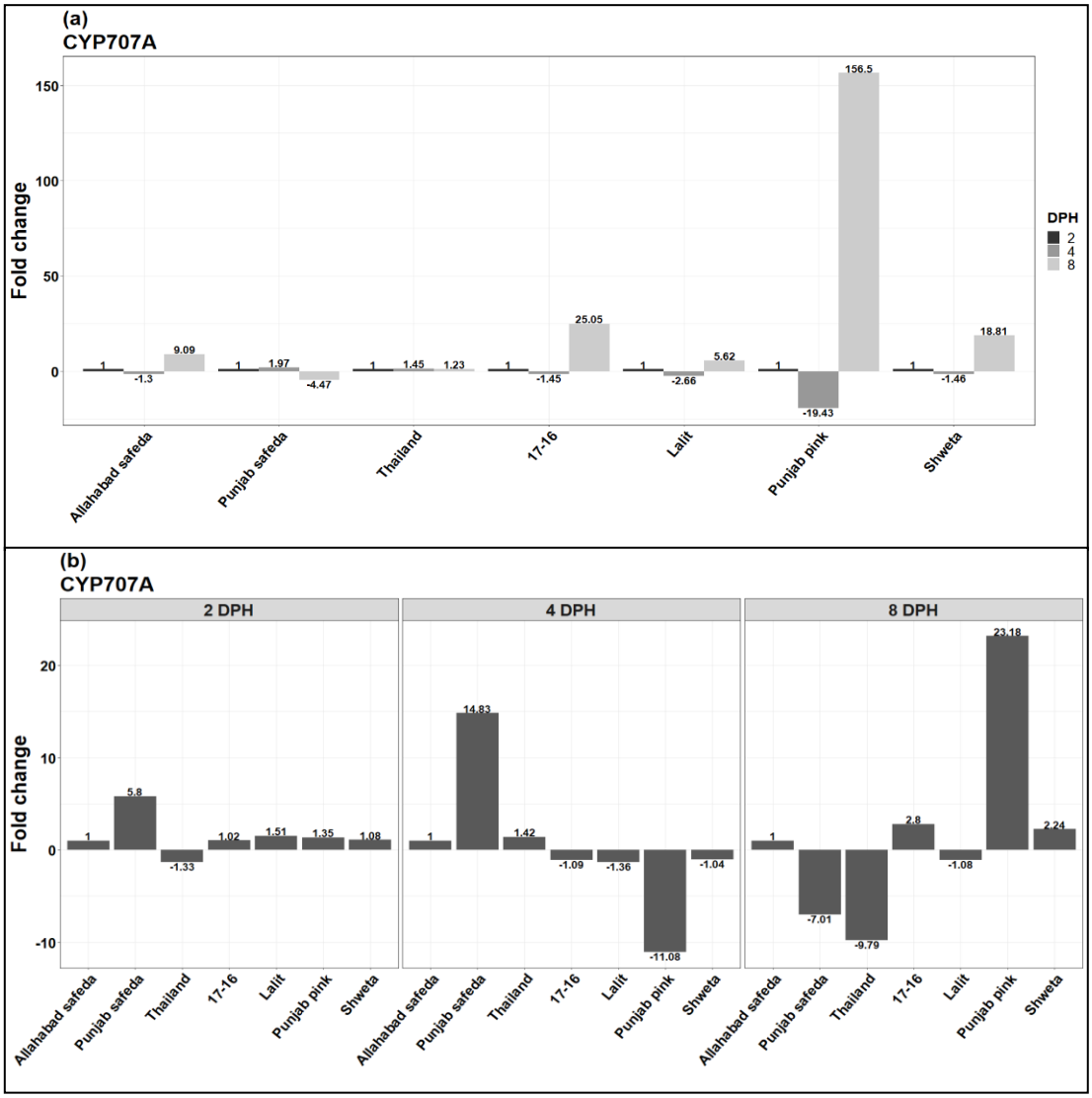

Fig. 4.40: Fold change of *CYP707A* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.4 Candidate gene expression analysis of Cell-Wall Metabolism pathway

4.2.6.4.1 Polygalactouranase (*PG*)

PG is involved in cell wall degradation by hydrolyzing the alpha-1,4 glycosidic bonds between galacturonic acid residues. It results into softening and sweetening of fruits during ripening. In Thailand the expression was lower in all the two days after harvesting compared to 2 DPH (Fig. 4.38 a). Punjab pink shows very high expression at 8 DPH (Fig 4.41 a and b).

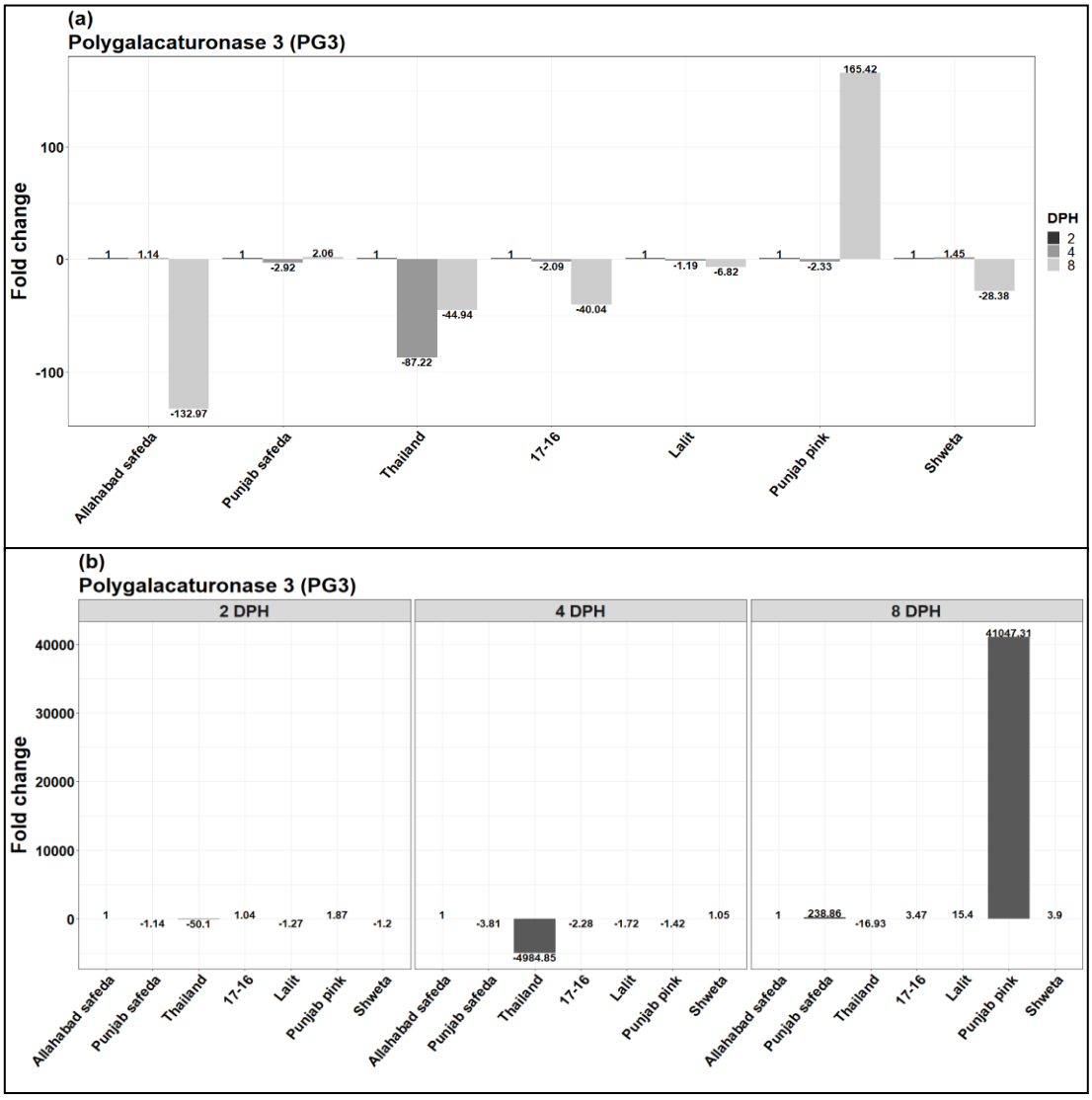

Fig. 4.41: Fold change of *PG31* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.4.2 Pectate lyase (PL)

Pectate lyase is a cell wall degrading enzyme. It is involved in eliminative cleavage of (1→4)-α-D-galacturonan to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends. All the genotypes had lower expression of PL compared to 2 DPH (Fig. 4.42 a). Expression was found to be higher for all the genotypes at 8 DPH when compared to Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline(Fig.4.42 b).

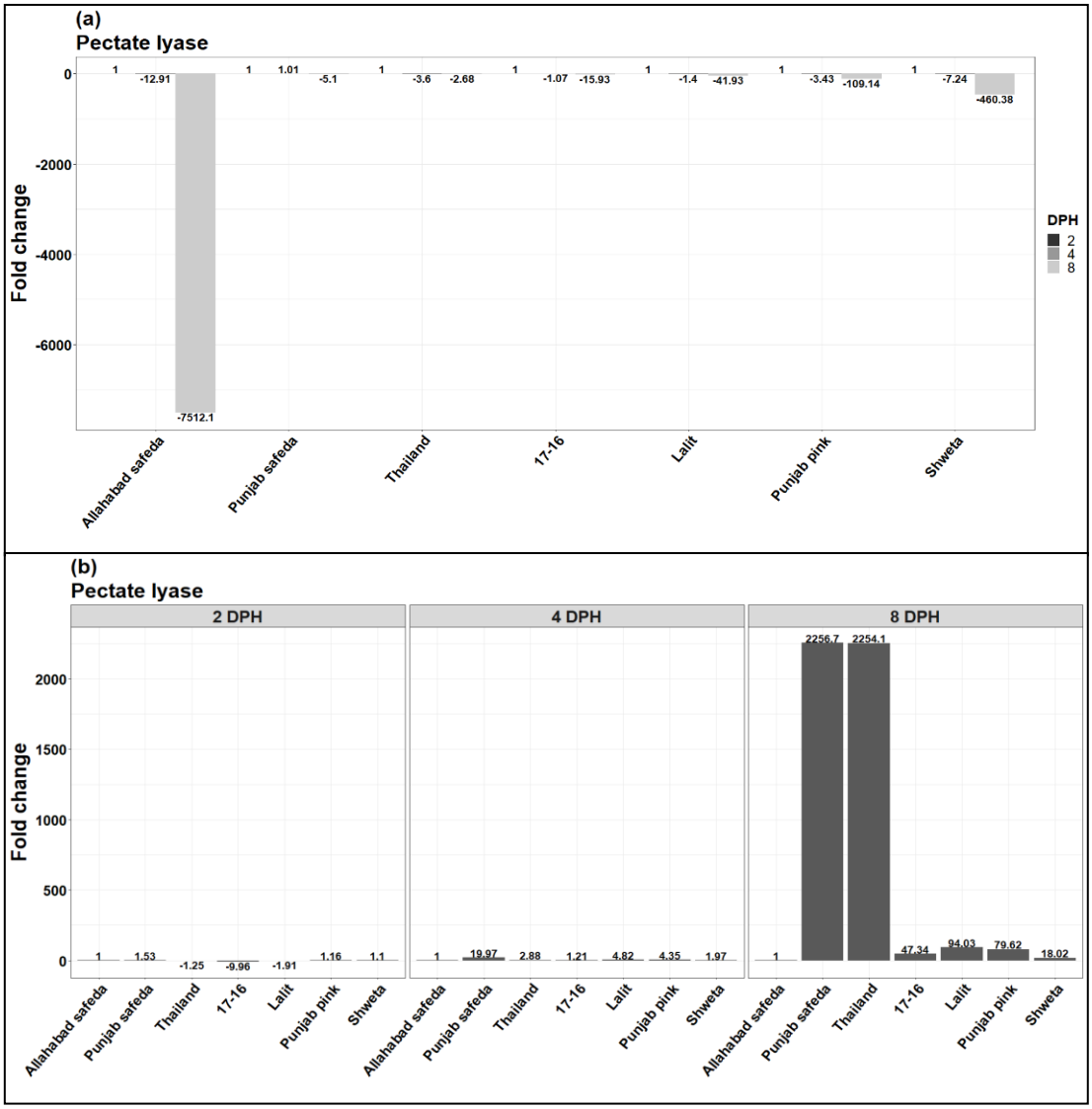

Fig. 4.42: Fold change of *PL* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.5 Candidate gene expression analysis of Sugar metabolism pathway

4.2.6.5.1 *beta-galactosidase*

It converts galactosides into monosaccharide thus increasing sweetness of fruits. Shweta, Punjab Pink and 17-16 all days had lower expression among all genotypes with respect to 2 DPH as a baseline (Fig. 4.43 a). Specific comparison showed higher expression in 8DPH Thailand and Punjab Safeda (Fig. 4.43 b).

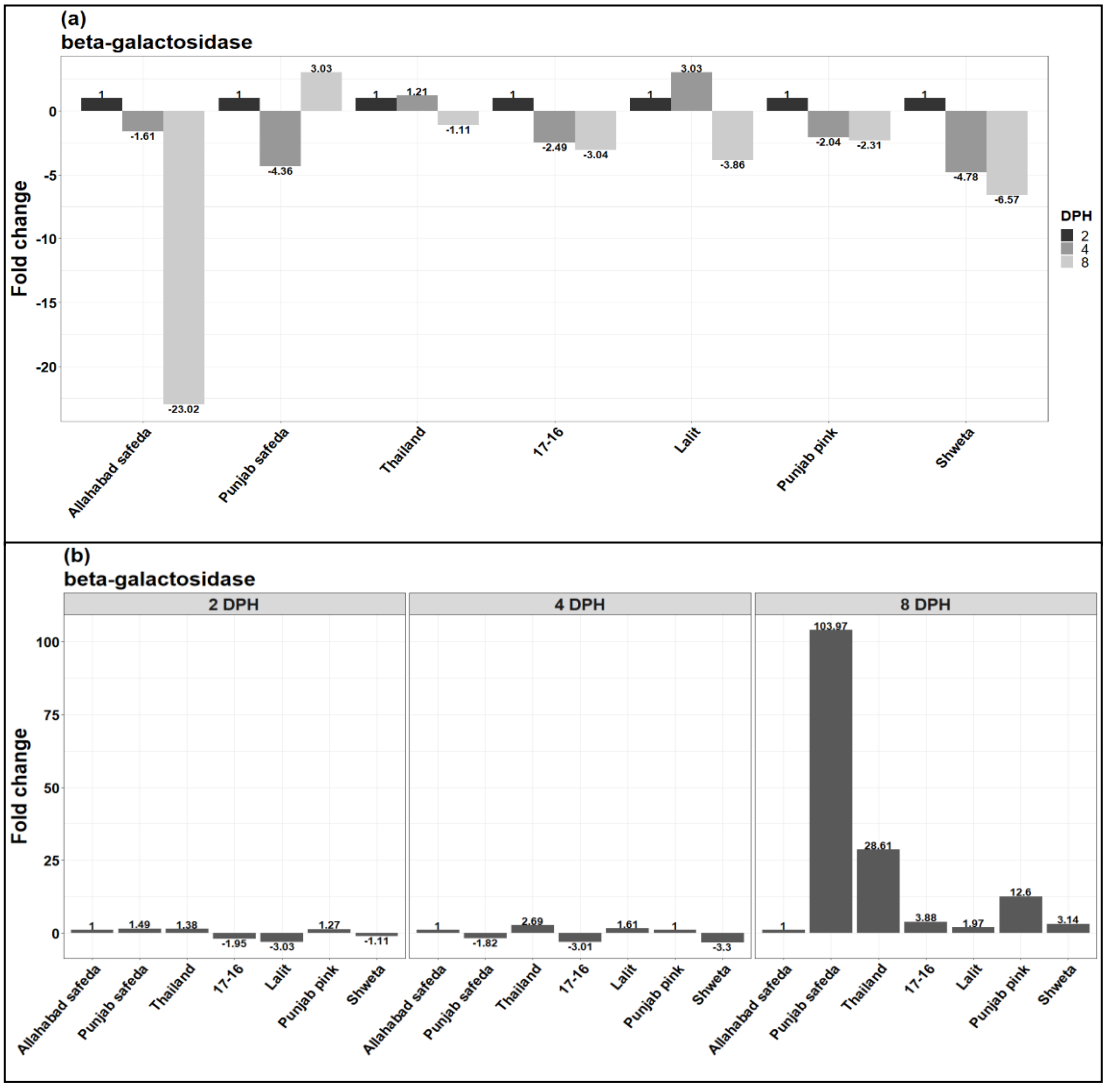

Fig. 4.43: Fold change of *beta-galactosidase* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.5.2 Mannase

Mannase is a simple sugar found in fruits. Thailand had lower expression of mannase among all the genotypes, while Lalit, Shweta and Punjab Safeda show higher expression compared to 2 DPH as a baseline (Fig. 4.44 a and b).

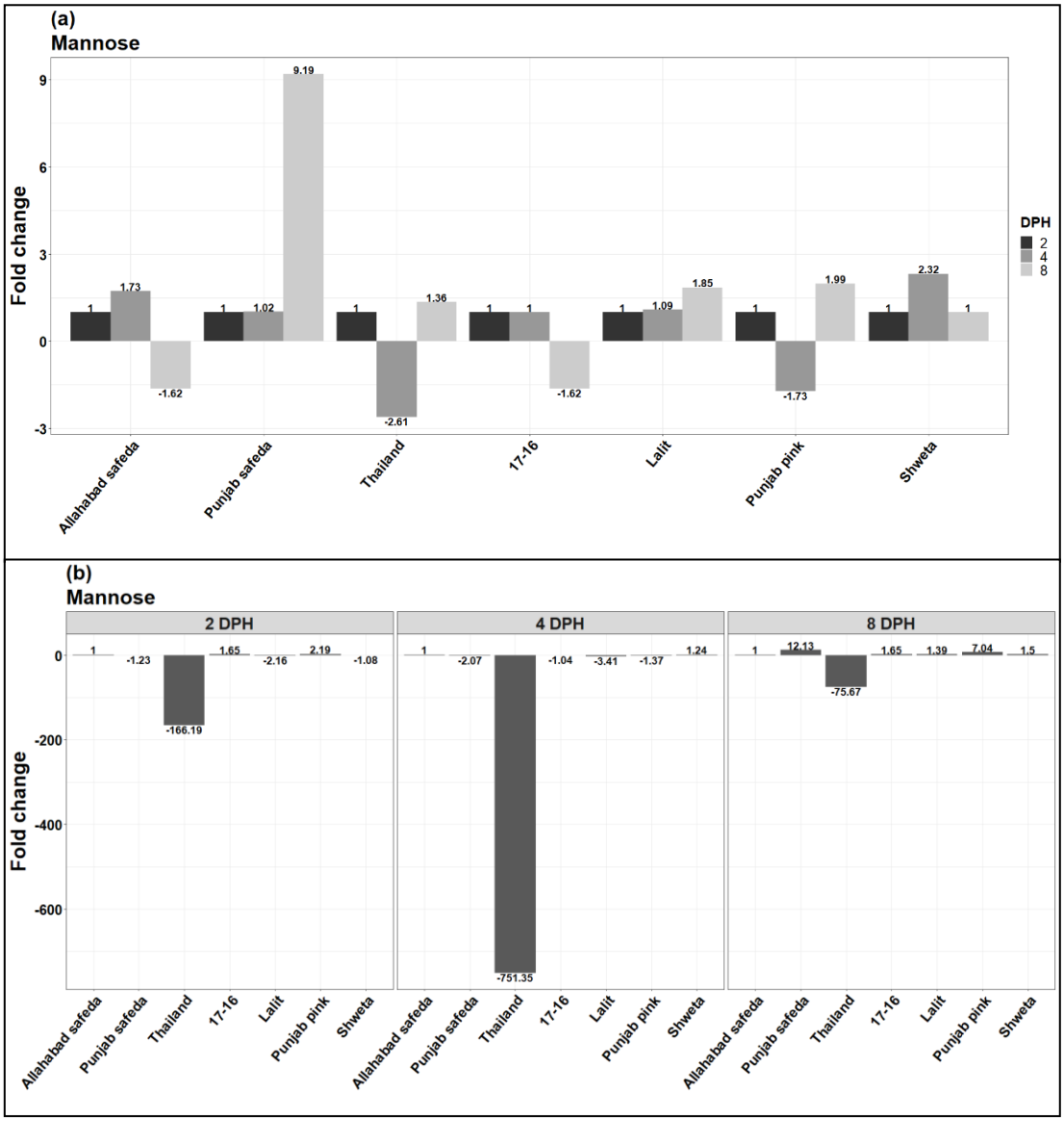

Fig. 4.44: Fold change of *mannose* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline

4.2.6.5.3 3-keto-acyl

3-keto-acyl is involved in cuticular wax and suberin biosynthesis in fruits. All genotypes had lower expression for 2 DPH as baseline (Fig. 4.45 a). Punjab Safeda had higher expression followed by 17-16 and Thailand, compared to Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline (Fig..4.45 b).

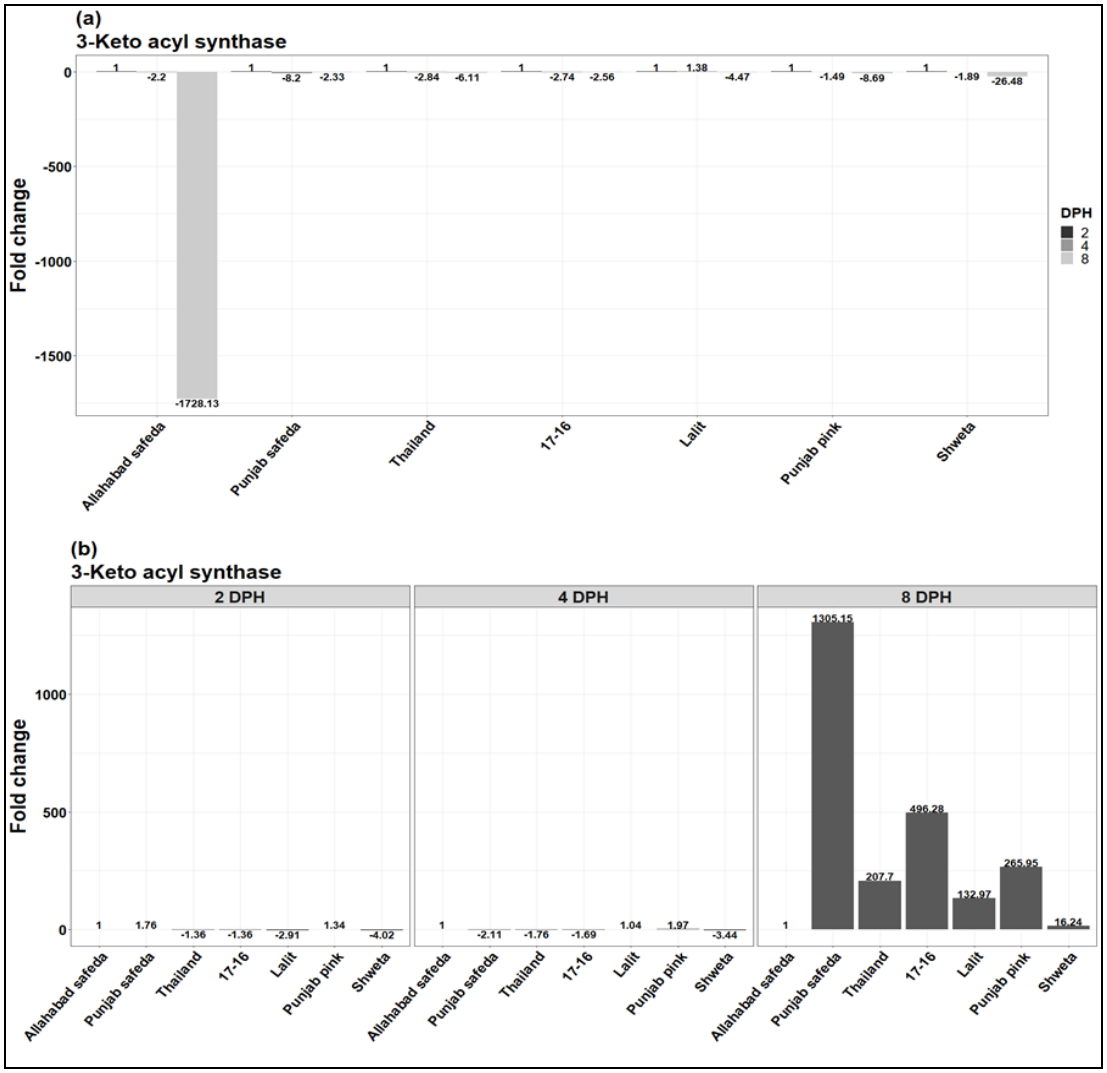

Fig. 4.45: Fold change of *3-keto-acyl* gene among different cultivars with a) 2 DPH as baseline for all cultivars, b) Allahabad Safeda 2 DPH, 4 DPH and 8 DPH as baseline.

It is known that presence or absence of ABA effects ethylene biosynthesis. Higher content of ABA in fruits lead to initiate ethylene biosynthesis (Zhang *et al* 2009b; Mou *et al* 2016). In ABA biosynthesis pathway *NCED1* is a key rate limiting step (Mou *et al* 2016). Among our cultivars we found that in Punjab Safeda and Thailand the expression of *NCED1* was higher at 8 DPH while the expression at 2 and 4 DPH was lower. This explains what we concluded in the last section. In Thailand and Punjab Safeda ABA biosynthesis is triggered

later than other cultivars. This leads to the initiation of ethylene biosynthesis at later stages and thus delays the ripening. Along with delayed synthesis of ABA there is upregulation of UDP75 and UDP76 genes during the early days that is 2 and 4 DPH the expression still remains higher at 8 DPH but not to a great extent. These enzymes are known to degrade ABA thus the cells of Thailand have little to no ABA to initiate ethylene biosynthesis till 8 DPH. Thus, ABA modulates the synthesis of ethylene to delay ripening process in Thailand and Punjab Safeda. But what modulates ABA is not identified in this study. Other than these two major pathway genes, the major gene (Polygalacturonase 3) for cell wall degradation was delayed in these two cultivars. This lower expression could be due to delayed ethylene biosynthesis (Tucker *et al* 2017). Polygalacturonase 3 (PG3) had lower expression in Thailand and Punjab Safeda. The expression was lower at early stages of ripening and was higher at later stage after harvesting.

In the conclusion, we can say that the ethylene signalling and ABA signalling both converge in Thailand and Punjab Safeda to delay the ripening process.

4.3 Multiple Sequence Alignment and Variant Prediction among Allahabad Safeda, Punjab Safeda and Thailand for ripening related Genes

The genomes of three cultivars were aligned to each other and the alignment file was uploaded to IGV. The gene sequences from Allahabad Safeda were used as a query and corresponding regions were fetched from Punjab Safeda and Thailand. The coding sequences and the protein sequences were generated for all the selected 32 genes in Allahabad Safeda, Punjab Safeda and Thailand using FGENESH programme and were aligned using Clustal O. Coding sequence alignment could be achieved for 10/32 genes. Changes in SAM3 and CYP707A involved in Ethylene and ABA Signalling were found in Thailand.

4.3.1 *CYP707A*

In Thailand, there is a G >A substitution at position 23 and G>C substitution at position 44. Protein sequences were also analysed for CYP707A. The protein sequences were searched in PDB to confirm the function of the protein and also for selection of homologues reference protein structure. Protein sequence was modelled using MODELLER for Allahabad Safeda, Punjab Safeda and Thailand. Protein model comparison shows that in Thailand, SNP at position 44, leads to the change in amino acid from histidine to glutamine Fig:4.46

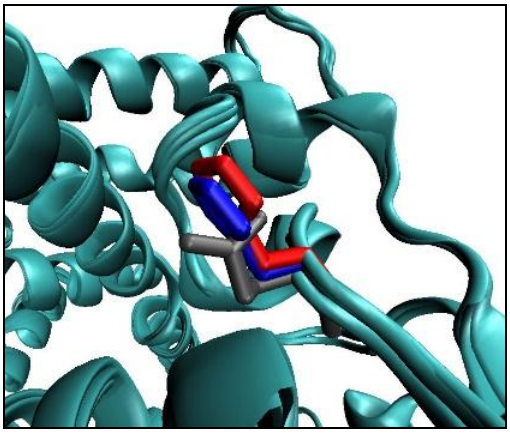

Fig. 4.46: Predicted protein model/structure for *CYP707A*. Cyan area represents identical protein structural regions among three genotypes. Grey area represents glutamine in Thailand, blue and red area represents histidine in Allahabad Safeda and Punjab Safeda respectively.

4.3.2 S-adenosyl Methionine 3 (*SAM3*)

Three SNPs were identified in *SAM3* at position 226, 242 and 250. There is substitution of C >A, G >T and C >T at positions 226, 242 and 250 respectively. Protein sequences were analysed and function was confirmed using PDB. Protein sequences were modelled in Modeller. Comparison shows that SNP at position 226 leads to the change in amino acid from glutamate to aspartic acid in Thailand Fig:4.47

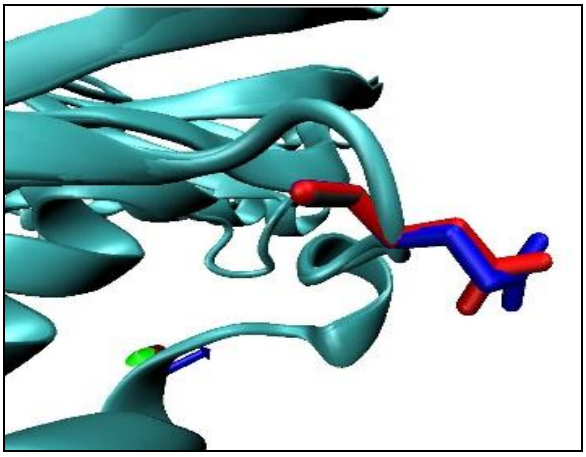

Fig. 4.47: Predicted protein model/structure for *SAM3*. Green area represents identical protein structural regions among three genotypes. Grey area represents aspartic acid in Thailand, blue and red area represents glutamic acid in Allahabad Safeda and Punjab Safeda respectively.

4.3.3 Promoter Analyses of genes for Haplotype Detection:

We observed differential gene expression among various genes through qPCR. Differences in gene expression might be directly related to sequence of its promotor. Mutations/variations in promotor region leads to either upregulation or downregulation of a gene. To test whether the changes in gene expression among the three cultivars is due to promoter sequence variation, we aligned the 500 bp upstream and downstream regions of

selected genes using Clustal O. Seven genes showed variation in promoter regions among three cultivars (Table:4.4)

Table 4.4: Promoter Analyses of Genes

Sr. No.	Position	Allahabad Safeda	Thailand	Punjab Safeda
1	ACO5_scaffold16827_5621-7851			
	413	T	A	T
2	Beta_galactosidase_scaffold 3931:6894-8054			
	28	G	C	G
	86	A	G	A
	166	C	T	C
	249	A	C	A
	262	T	C	T
	362	G	A	G
	500	T	C	T
3	CYP707A scaffold25040:35539-37420			
	34	T	C	T
4	EIL3 C2383712:22712-25556			
	240	G	G	C
5	EIN2 scaffold27768:1-6819			
	21	A	G	A
	145	A	T	A
	186	G	A	G
	238	G	T	G
	405	T	C	T
	473	T	-	T
6	ERF6 scaffold21953:9397-11519			
	184	C	G	C
7	PL scaffold28043:31082-33461			
	47	G	A	G
	93	T	A	T
	153	G	A	G
	191	T	C	T
	212	T	G	T
	475	T	C	T
	493	A	G	A

In a promoter there are some conserved regions known as motifs and the strength of the promoter depends on these motifs as they are required for binding of RNA polymerase to the promoter sequence. Thus, any variation or mutation in these motifs can lead to altered expression of that particular gene. To test whether the differential expression among the seven genes is due to mutation in motifs, we predicted the motifs in the promoters of these genes using online tool MEME suite. We found that there is no change in the motif region for all 10 genes. Fig:4.44 shows an example of motif identification as in ACO6 of Allahabad Safeda.

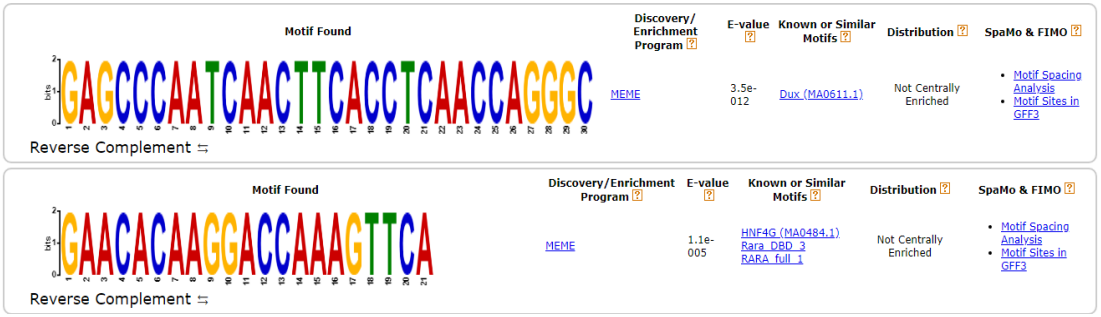

Fig 4.48: Motif identification in ACO6 using MEME Suite

4.3.4 Structural variation analysis and protein modelling gives insight into different ripening behaviour of three cultivars

Structural variation analysis in the candidate genes can lead to increased understanding of gene function and foster the involvement of gene in ripening regulation, if there are variations among cultivars. In our lab, genomic DNA of guava cultivars Allahabad Safeda, Punjab Safeda and Thailand has been sequenced and assembled. We took the advantage of this and analysed all the genes for any structural variations among the three cultivars. In our analysis, we concluded that the gene SAM3 has variations in the coding region. While genes ACO5, beta-galactosidase, EIL3, EIN2, ERF6, and PL have variations in the promoter region. The gene CYP707A has variation in both the coding and promoter region. In SAM3, there is a substitution of aspartic acid with glutamic acid in Thailand. Both the amino acids are of identical nature (acidic) are it is less likely possible that this substitution will result in any kind of structural/functional variations. We did not find any variation in the regulatory sequences for SAM3. Thus, the differential expression among the three cultivars remains unexplained. SAM3 is a very important enzyme in ethylene signalling and initiates the biosynthesis of ethylene. Whether this change in amino acid is contributing to delayed ripening in Thailand needs investigation through improved gene prediction. CYP707A is another protein with a change in amino acid due to substitution in coding region of its gene. In this amino acid substitution, a glutamine has been substituted by histidine in Thailand. Both the amino acids are of different nature (glutamine is polar uncharged while

histidine is positively charged). This substitution can affect the protein structure and hence function. Also, there is a SNP in the regulatory region of this gene. T<C substitution upstream of start codon could be the reason for differential regulation of this gene in Thailand. But both the above inferences need further investigation. Genes beta-galactosidase, EIN2 and PL harbour SNPs in the regulatory regions in Thailand. Although these SNPs are not located in the functional motifs (predicted using MEME suite), but still there could be some role of these SNPs in differential regulation among the genotypes. There are 6 SNPs in EIN2 promoter region, while 7 SNPs each in beta-galactosidase and PL promoter regions. Thus, the effect of these SNPs on ripening regulation needs further investigation. ACO5, EIL3 and ERF6 harbour single SNP each in the 5'-regulatory regions. These SNPs are not located in the functional motifs, but their role in differential regulation and ripening mediation can be validated only with functional assays. Genetic markers for Thailand genotype can be developed using this analysis for marker assisted selection in a breeding program.

CHAPTER V

SUMMARY

Guava is a climacteric fruit and ripening continuous even after harvesting leading to various textural changes. Skin colour changes from green to yellow and fruits start emitting musky odour. Total soluble solids (TSS), titratable acidity (TA), reducing sugars, vitamin C, firmness and density gets altered during storage. Most of the changes are attributed to biosynthesis and signaling of ripening hormone ethylene. Abscisic acids (ABA) triggers the production of ethylene at fruit maturation stage. Ethylene signaling leads to cell wall metabolic changes imparting softness to fruit. Pink fleshed guava cultivars ripen faster than white fleshed cultivars. Finding sequence variation in ethylene production and signaling genes in the slow ripening cultivars has a potential for utilization in molecular breeding strategies.

Here, we tested 14 guava cultivars for ripening behaviour and measured the physio-chemical attributes and biochemical changes at 3 ripening intervals in 2018-19 winter season crop. White fleshed cultivars Allahabad Safeda, Hisar Safeda, Punjab Safeda, Thailand guava, Shweta, Sardar guava and CISH-G5 and pink fleshed Punjab Pink, Lalit, Hisar Surkha, Purple local, 17-16, Punjab Kiran and Arka Kiran were analysed for physio-chemical characteristics. On an average TSS in the season among genotypes varied from 8-11.5(^{o}brix), TA 0.28 - 0.46%, reducing sugar 3.1-8.9%, Vitamin C 97.7 – 121mg/100g, Firmness 4.66 - 6.73 lb in regular intervals of 8 days of shelf life experiment. TSS of fruits increased during ripening and at overripe stage it started decreasing. Highest TSS was found in Lalit 10.37 (°Brix) and Punjab Safeda 11 (°Brix) in November 2018 at 2 DPH. Acidity decreased during ripening and highest TA was found in Allahabad Safeda (0.84%) and Punjab Kiran (0.51%) at 2 DPH. Vitamin C and reducing sugars were found to be increasing during the process of ripening. Highest Vitamin C was recorded in Shweta (127 mg/100g pulp) and Hisar Surkha (132 mg/100 g pulp). Highest values of reducing sugars were recorded in Shweta (6.58%) and in Punjab Kiran (7.32%). As expected, fruit flesh firmness was found to be decreasing during ripening. Highest firmness at 4 DPH was recorded in Thailand (15 lb) and Lalit (2.5 lb).

Genomic resources in guava are in their infancy. Our lab has generated the first *de novo* transcriptome and genome assembly in Allahabad Safeda. Researching the literature, We dissected out all the genes important for fruit maturation and ripening in climacteric and non-climacteric fruits and pulled the protein sequences from NCBI. Alignment of 99 such genes in (Local blast set up in desktop) searched 32 genes involved in ethylene and ABA biosynthesis and signaling in addition to cell wall degradation enzyme genes and transcription factors controlling all these pathways. Primers were designed to query expression changes in

coloured vs non-coloured cultivars variable in physio-chemical characters. RNA extracted from 7 selected genotypes at 3 ripening intervals as 2 days post-harvest (2 DPH), 4 DPH, and 8 DPH was normalized, converted to cDNA and qRT-PCR was performed for selected candidate genes in all these pathways. We found that ethylene signaling is normal in Thailand guava as the expression of ethylene signaling genes ETRs, CTR, EIN, ETP were comparable to Allahabad Safeda, however ethylene biosynthesis is delayed as major ethylene biosynthesis genes *SAM1, SAM3, ACS/ACC2, ACO* had lower expression than the reference Allahabad Safeda. Lower expression of ABA biosynthesis key gene *NCED* in Thailand at 2DPH and 4DPH compared to 8DPH indicates that the ABA biosynthesis and concomitantly ethylene biosynthesis are delayed. Expression of transcription factors *TDR4/FUL1, MYB, MADS-RIN, ERF6, TAG1* upregulated during the ABA and ethylene biosynthesis in Thailand guava at 8 DPH further supports the fruit undergoing delayed ripening. Major cell wall degrading enzyme encoding gene *Polygalacturonase* expression was comparatively low in Thailand guava at all the ripening stages.

Our physico-chemical and gene expression analysis identified that Thailand guava has very slow ripening behavior owing to delayed ethylene and ABA biosynthesis leading to reduced expression of cell wall degrading enzyme gene. Re-sequenceing of Thailand guava genome sequence and comparative analysis to Allahabad Safeda identified the specific nucleotide changes in *SAM3* gene that leads to change in amino acid sequence and protein in an important domain. The genes *ACO5, EIL3, EIN2, ERF6* and *PL* have SNPs in the untranslated and/or the promoter regions that might be impacting the docking of transcriptional machinery and concomitant delay in ripening. Generating molecular markers based on genetic variation in Thailand vs Allahabad Safeda might help in marker assisted selection for transferring superior alleles for delayed ripening in popular guava cultivars.

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