MOLECULAR AND BIOCHEMICAL ANALYSIS OF MANGO (CV. ALPHONSO) FLAVOR

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Research Guide

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Certified that the work in the Ph.D. thesis entitled **'Molecular and Biochemical Analysis of Mango (cv. Alphonso) Flavor'** submitted by **Mr. Ram Kulkarni** was carried out by the candidate under my supervision. The material obtained from other sources has been duly acknowledged in the thesis.

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I hereby declare that the thesis entitled **'Molecular and Biochemical Analysis of Mango (cv. Alphonso) Flavor'** submitted for Ph.D. degree to the University of Pune has not been submitted by me for a degree at any other university.

Date: Ram Kulkarni

Pune

Dedicated to my parents

and

The researchers who made an outstanding contribution to the field of plant volatiles

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LIST OF ABBREVIATIONS

°C	Degree Celsius
AAT	Alcohol acyl transferase
ADH	Alcohol dehydrogenase
AEDA	Aroma extraction dilution analysis
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
bp, kb	Base pairs, kilo base pair
cDNA	Complementary DNA
CTAB	Cetyltrimethylammonium bromide
DAMD	Directed amplification of minisatellite DNA
DEPC	Diethyl pyrocarbonate
DAH	Days after harvest
DMAPP	Dimethylallyl pyrophosphate
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EAD	Electroantennographic detection
EF1α	Elongation factor 1α
EO	Enone oxidoreductase
EtBr	Ethidium bromide
FID	Flame ionization detector
FPP	Farnesyl pyrophosphate
FPPS	Farnesyl pyrophosphate synthase
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GC-O	Gas chromatography-olfactometry
g	Relative centrifugal force
g, mg, ng, kg	Gram, milligram, microgram, kilogram
GGPP	Geranylgeranyl pyrophosphate
GGPPS	Geranylgeranyl pyrophosphate synthase
GLC	Gas liquid chromatography
GLV	Green leaf volatiles
GPP	Geranyl pyrophosphate
GPPS	Geranyl pyrophosphate stynthase
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA
HMMF	4-hydroxy-5-methyl-2-methylene-3(2H)-furanone
HPL	Hydroperoxide lyase
HPLC	High-performance liquid chromatography
IDS	Isopentenyl diphosphate synthase
IPP	Isopentenyl pyrophosphate

ISSR	Inter simple sequence repeat
ITS	Internal transcribed spacer
L, ml, µl	Liter, milliliter, microliter
LC	Liquid chromatography
LSD	Least significant difference
LOX	Lipoxygenase
LSU	Large subunit
MEP	Methylerythritol phosphate
MS	Mass spectrometer
MVA	Mevalonic acid
mRNA	Messenger ribonucleic acid
MRM	Multiple reaction monitoring
MMT	Million metric tons
NADH	Nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NCL	National Chemical Laboratory
NIST	National Institute of Standards and Technology
ORF	Open reading frame
ppb	Parts per billion
PCA	Principal component analysis
PCR	Polymerase chain reaction
RACE	Rapid amplification of cDNA ends
RAPD	Random Amplified Polymorphic DNA
RT	Room temperature
RNA	Ribionucleic acid
RNase	Ribonuclease
SCoT	Start codon targeted
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPE	Solid phase extraction
SPME	Solid phase microextraction
SSU	Small subunit
TE	Tris-EDTA
TAE	Tris-acetate-EDTA
TOF-MS	Time-of-flight mass spectrometer
TPS	Terpene synthase
UTR	Untranslated region

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THESIS ABSTRACT

Mango is one of the highly popular tropical fruits not only in India, the country of its origin, but throughout the world. India stands the first in the list of mango producing and exporting countries. Out of several cultivars of mango found in India, Alphonso is the most popular one because of its highly attractive flavor. Apart from this, Alphonso possesses many significant attributes such as attractive color, ample sweetness, low fiber containing pulp and long shelf life. Although Alphonso cultivation is widespread in many regions of India with varying extent, the quality of the fruits, especially in terms of taste and flavor is not the same in all of these regions. Because of this, the main cultivation of Alphonso is concentrated in the 700 km long, narrow coastal belt of western India, known as the Konkan region. Even within this region, the fruits show conspicuous variation in the flavor. In order to understand chemical basis for such locality dependent variation in the flavor of Alphonso, ripening fruits of Alphonso from three cultivation localities in India, Dapoli, Deogad and Vengurle were analyzed for the content of volatiles by gas chromatography. This study revealed that Alphonso fruits from Dapoli were rich in terpene volatiles; whereas, those from Deogad contained higher concentration of lactones and furanones than the other localities. Further, biosynthesis of terpenes was studied with respect to the genes encoding geranyl and farnesyl pyrophosphate synthase; whereas, that of furanones was studied with respect to the gene of enone oxidoreductase. The coding sequences of these genes were expressed in *E. coli* and the recombinant enzymes were characterized biochemically. The structural characteristics of the proteins were also predicted. Expression profiling of these genes in the ripening stages of the Alphonso fruits suggested key ripening-related role of these genes.

General Introduction and Review of Literature

GENERAL INTRODUCTION AND REVIEW OF LITERATURE

From the smallest bacteria to the largest animals, from the aquatic organisms to the birds, from the grasses to the huge trees, all the life forms have similar biochemical basis for the operation of fundamental cellular processes. All the organisms produce primary metabolites, sugars, fats, proteins and nucleotides, which are the heart of cellular organization and life-keeping processes. In spite of this similarity, living organisms differ from each other in many noticeable characters which force them to get divided into different groups called Kingdoms. One of the most obvious factors that distinguish plants from the other organisms is their inability to move. Nevertheless, every disadvantage has its advantage; nature has compensated immobility of the plants by gifting them an ability to produce an amazing array of special chemicals called secondary metabolites.

1.1. Secondary metabolites: not really secondary

In contrast to the primary metabolites, which have been studied to an enormous depth for their biosynthesis and whose functions in growth and development of the plants have been well established, secondary metabolites have earlier received less attention of the plant biologists. One of the most important reasons for this apathy was that the secondary metabolites were considered as the chemicals not required for the normal growth and development of the plants; rather they were considered as by-products of the primary metabolism (Zahner, 1979). As a consequence, the earlier studies carried out on the secondary metabolites were very limited in scope; they only addressed the issues such as 'isolation and structure elucidation of new secondary metabolites' (Hartmann, 2007). It is only since about last 50-60 years that the secondary metabolites are also being studied from the functional biology point of view (Chu et al., 2011). The studies carried out to understand the functions of secondary metabolites have indicated that these compounds play important roles in plants' life including but not limited to protection from the herbivores and pathogens, attracting the pollinators and seed dispersing

agents, gaining competitive advantage against other plants (allelopathy), protecting the cells from the oxidative stress, etc (Croteau et al., 2000). As an example, it was shown in *Nicotiana attenuata* that linalool, one of the monoterpenes produced by the plants, decreases the oviposition rates of an insect on *N. attenuata*, thereby protecting it from the future herbivory (Kessler and Baldwin, 2001). Many phenolic compounds, such as catechin, that are secreted by the plants through their roots into the surrounding rhizosphere are known to protect the plants from pathogens and participate in the plant-plant and plant-insect interactions (Bais et al., 2006). Similarly, in *Pseudowintera colorata*, anthocyanins were found to protect the cells from the oxidative effects of the radicals generated from hydrogen peroxide (Gould et al., 2002). In the light of such a diverse contribution of secondary metabolites in the plant life, these chemicals have been suggested to be commonly called as 'natural products' or 'specialized chemicals' instead of secondary or by-product metabolites (Croteau et al., 2000) and have caught great attention of the researchers for their functional studies and commercial utilization.

1.2. Volatile natural products

Out of the numerous natural products that plants produce, about 1% chemicals are released into the surrounding environment by volatilization because of their high vapor pressure which in turn results from small size and the hydrophobic nature of these chemicals. The volatile chemical agents, released from the flowers and fruit or from the vegetative parts such as leaves or roots of the plants, transmit its message to the other organisms. This message can act in several ways on different organisms; it can magnetize the pollinators and seed disseminators towards the flowers and fruits or can warn insect herbivores and pathogens through their toxic properties (Dicke et al., 2003; Kessler and Baldwin, 2001; Xiang et al., 2007). As an evidence, in potato the volatile chemicals produced by the enzyme hydroperoxide lyase were shown to intoxicate the aphids feeding on the plants (Vancanneyt et al., 2001). In some cases the volatile message can be a "cry for help" for the friends, such as the predators of the plant-attacking insect or the neighboring

plants which may keep themselves prepared for the similar herbivore/pathogen attack (Dicke et al., 2003; Kessler and Baldwin, 2001). In the studies demonstrating such functions of volatiles, chemicals released from one plant upon herbivory were shown to induce release of similar defensive chemicals by inter- and intraspecific plants (Baldwin et al., 2006). In certain cases, volatiles form the media of negative interactions between plants through their allelopathic properties. For example, *Salvia leucophylla* releases toxic monoterpenes which retard growth of the surrounding plants, thereby giving competitive advantage to *S. leucophylla* (Romagni et al., 2000). Apart from such ecological roles, volatile compounds also participate in the internal physiological functions of the plant by increasing their thermo-tolerance and by protecting them against oxidative stress (Dudareva et al., 2004). For example, isoprene, one of the few hemiterpenes, was shown to protect common bean leaves from the increased temperatures (Sharkey et al., 2001).

1.3. Flavor: The functional volatiles

Apart from playing many vital roles in the plants' life cycle, volatiles also offer the frugivorous animals an aromatic way to locate their pleasantly flavored food. Animals can perceive the odor by either orthonasal (through the nasal cavity) or retronasal (through the mouth cavity) route (Fig. 1.1). At the molecular level, the sensation of odor is perceived via the interaction of the volatiles with the proteinaceous receptors in the olfactory system of the animals. Because of such molecular interactions, nature of elicited response varies with the varying architecture of the compound. Some of the volatiles such as formic acid are not at all detected by human olfactory system at the concentration as high as 4,50,000 ppb; whereas, some others like maple furanone is detected at the concentration as low as 0.00001 ppb (Kobayashi, 1989). Apart from such quantitative variation, the responses that the volatiles induce are also broad in nature, ranging from the foul smell of putrescine, to the highly pleseant, flowery odor of 2-phenylethanol. Several attempts have been made to understand the structure-function relationship of the odor perception (Zarzo, 2007). Some of these studies have provided hints towards

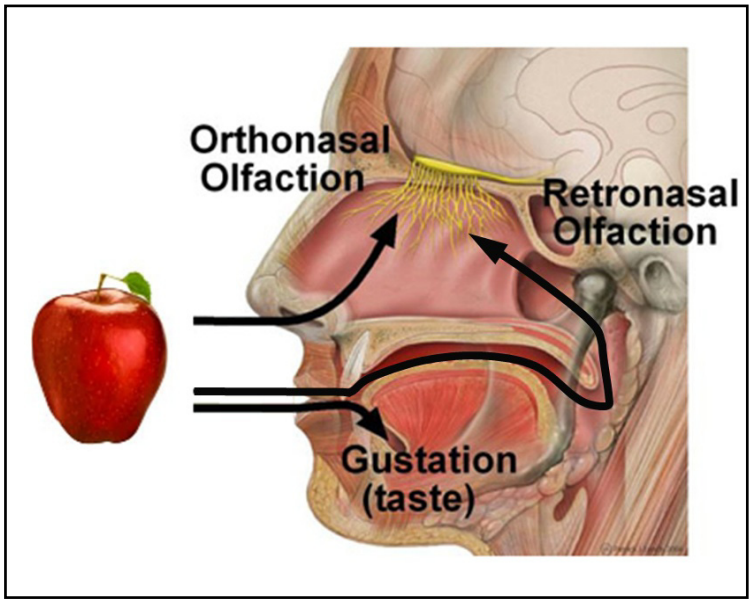


Figure 1.1
Orthonasal and retronasal perception of the odor (reproduced with modifications from Anonymous 2007).

the functional groups, called osmophoric moieties, of the chemical that decide the odor prduced. For example, esters [-O-(C=O)-] usually have a fruity smell, compounds having caramel-like flavor have planar enol-carbonyl substructure in a cyclic dicarbonyl compound, isothiocyanate group (-NS) gives the compounds a mustardy smell, etc (Hodge et al., 1963; Zarzo, 2007). In spite of these generalizations, there are many puzzles about odor perception that are annoying to the researchers. It is not yet clearly understood how certain chemicals having strikingly different structures elicit the same odor response and why few molecules having very similar structure and osmophoric moieties result in entirely different response (Sell, 2006) (Fig. 1.2). The situation is further complicated by the results suggesting that a single chemical does not bind to a specific olfactory receptor, but to an array of receptors resulting in the "mixed" response (Malnic et al., 1999). Because of these reasons, predicting odor of the chemical based on its structure still remains a statistical procedure with only 77% accuracy (Sell, 2006).

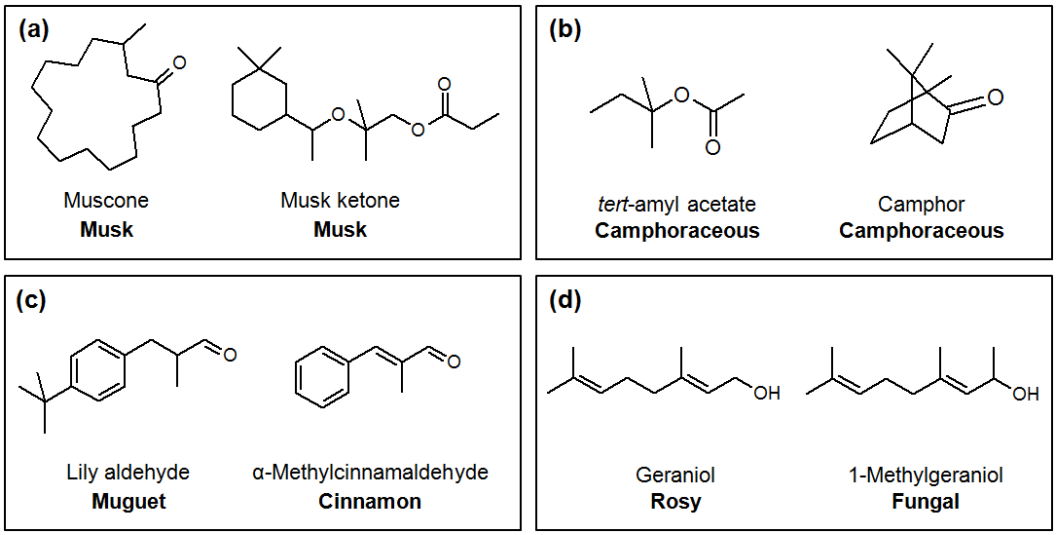


Figure 1.2
The chemicals having similar odor but different structures (a, b) and similar structures but strikingly different smells (c, d)

1.4. Analytical advances: trap them!

In the earlier times, analysis of the volatile compounds was limited mainly to the essential oils from the plants. These essential oils being volatile, the easiest way of separating them from the rest of the components of the plant was distillation. Many variations in the distillation technique were further made in order to increase the efficiency and reduce the time required for preparing the samples. Out of these, 'simultaneous distillation-extraction' became the most popular (reviewed by Chaintreau, 2001). Although, even today, distillation is the most common technique of isolating volatile chemicals from the plants, the high temperature involved is known to induce chemical changes in the components of the volatile blend (Fischer et al., 1988). Another widespread method of isolating the volatiles is a simple extraction using organic solvents. Although this method is not capable of selectively isolating the volatiles, important reasons for its popularity for a long time are its simple procedure; gentle nature and capability of extracting a broad range of volatiles (Linskens and Jackson, 1997) (Fig. 1.3).

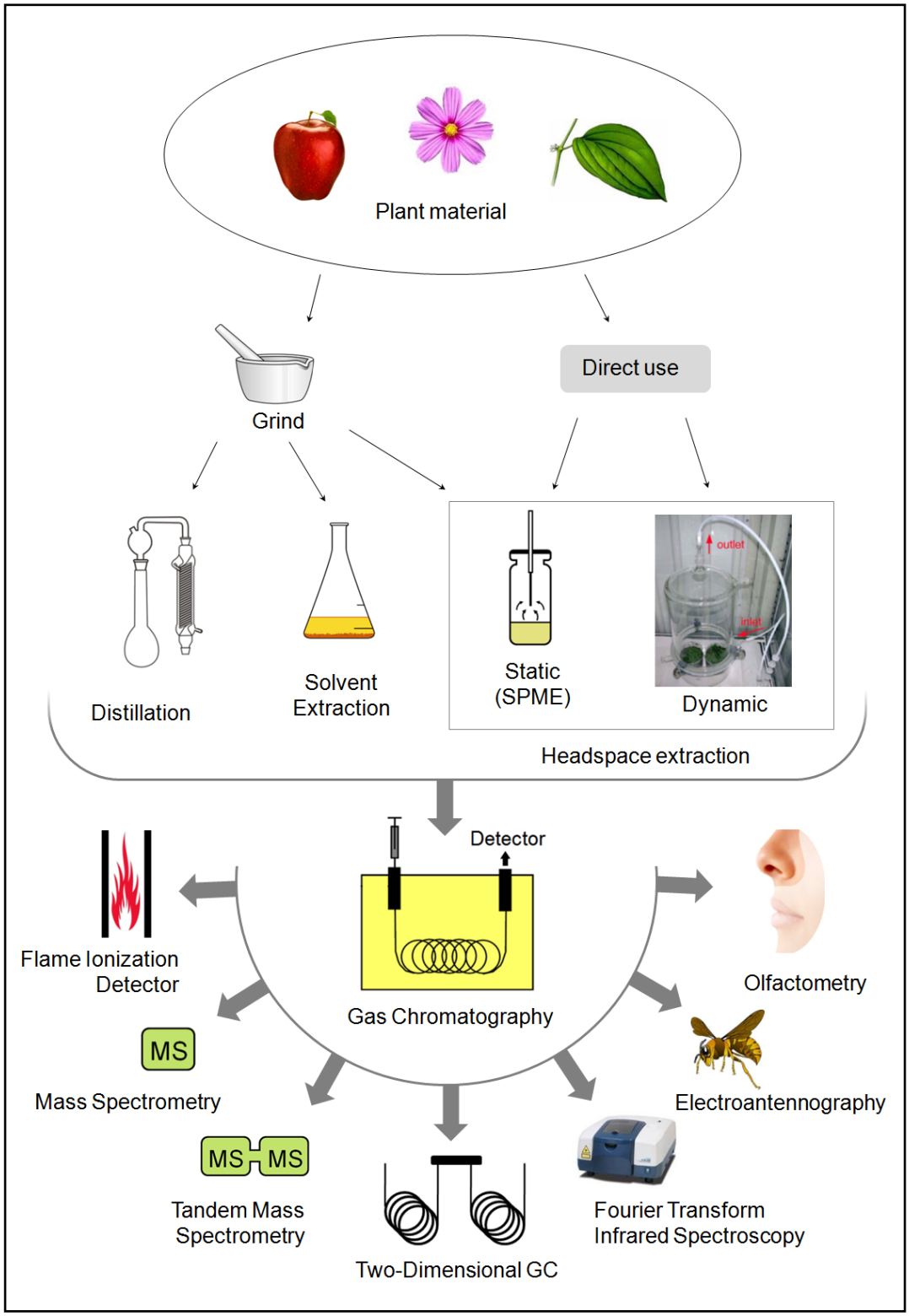


Figure 1.3

General flow-chart of analysis of plant volatiles. Only the most commonly used methods of extraction and chromatographic analysis of volatiles are represented.

Increasing interest in understanding the functional roles of the plant volatile compounds has lead to the development of the advanced techniques of extraction and analysis of these compounds. One such technique is headspace analysis. It involves capture of the volatiles released into the air surrounding the sample (headspace) and is highly selective for isolating the volatiles. In the static headspace analysis, the headspace air is sampled either directly or the volatiles are adsorbed on the adsorbent fiber by solid phase microextraction (SPME). SPME has become a popular method because of its solvent-free nature and highly specific and sensitive detection of volatiles (Vas and Vekey, 2004). In the dynamic headspace sampling, the headspace air is continuously circulated with the help of variable-pressure pumps, through the adsorbent material for a particular period of time. The volatiles adsorbed on the matrix can then be easily eluted from the matrix with organic solvent and used for further analysis (Tholl et al., 2006) (Fig. 1.3).

Analysis of the constituents of the volatile blend obtained by the methods described above, is generally performed by gas chromatography (GC) coupled to the detector such as flame ionization detector (FID) (Tholl et al., 2006) (Fig. 1.3). The identification of the compounds is performed using the mass spectrometer (MS); the spectra obtained are matched with those stored in the libraries of the mass spectra such as NIST or Weily by a software such as MS search. The identification, merely based on the mass spectra is generally not completely reliable and thus, it is complementated by the other methods such as determining and matching the retention index of the test compound with those of the authentic standards. The volatile blend produced by the plants usually being a complex mixture containing multiple isomers of a single compound, more advanced techniques have been established for the exact and sensitive analysis, such as chiral GC (Schurig, 2001), GC x GC (two dimensional GC) (Beens et al., 1998), GC-MS/MS (tandem mass spectrometry) (Granero et al., 2004), GC-TOF-MS (time-of-flight mass spectrometer) (Matisova and Domotorova, 2003), GC-FT-IR (Fourier transform infrared spectroscopy) (Ragunathan et al., 1999), etc. For measuring the contribution of constituent volatiles to the aroma, techniques

such as GC-olfactometry (GC-O) and aroma extraction dilution analysis (AEDA) are also used (Jackson and Linskens, 2002). In these techniques, the sample is serially diluted and analysed by GC. The GC eluent is divided into two parts, one goes to the regular detector; whereas the other is used for measuring the odor intensity by a human nose. Alternatively, to study the insect response to the volatiles, GC- electroantennography (GC-EAD) is used. Here, after separating through GC, the volatiles are passed onto an insect antena which is removed from the insect and connected to the electrodes and the electrical activity in the antenna is then measured (Topazzini et al., 1990) (Fig. 1.3).

1.5. Classification and biosynthesis of plant volatiles

The diversity in the function of plant volatiles originates from the variability in their chemical structures which are mainly represented by terpenoids, furanones, phenylpropanoids and fatty acid derivatives. Even within these classes, a great variability in the sizes and the functional groups of the chemicals is observed.

1.5.1. Terpenoids

Terpenoids, also known as isoprenoids, form the largest class of plant volatiles. The chemicals represented by this class can be as small as isoprene having the molecular weight of 62 and as large as rubber having the molecular weight greater than 1,00,000 (McGarvey and Croteau, 1995). The volatile terpenes are commonly known or identified by a typical aroma imparted to the plants. For example, carvone, a monoterpene ketone is responsible for an unique aroma of caraway; whereas, the distinctive aroma of citrus fruits is because of the presence of monoterpene aldehyde, citral. The other non-flavor but popular members of this class include the chemicals such as taxol, an anticancer diterpene derivative from *Taxus* species (Croteau et al., 2006) and artemisinin, an antimalerial sesquiterpenoid from *Artemisia annua* (Klayman, 1985). Terpenes are considered as the polymers of C5 units, based on which, they are classified as hemiterpenes (C5) monoterpenes (C10), sesquiterpenes

(C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), etc. Out of these classes, only hemiterpenes, monoterpenes and sesquiterpenes are volatile because of their small sizes (Bouvier et al., 2005). Biosynthesis of all of these terpenoids proceeds in three stages, generation of C5 building blocks, condensation of the C5 units into isopentenyl pyrophosphates and actual synthesis of terpenes (Fig. 1.4) (Bouvier et al., 2005).

1.5.1.1. *Generation of building blocks*

All the terpenes are derived from the two C5 units, dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). These two building blocks of the terpenes are derived by two pathways in the plants: cytosolic mevalonic acid (MVA) pathway and plastidic methylerythritol phosphate (MEP) pathway. The cytosolic MVA pathway provides the precursors for the production of sesquiterpenes, triterpenes and polyterpenes. It starts with the condensation of three molecules of acetyl-CoA with each other, giving rise to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA is an important intermediate of the MVA pathway; its reduction into mevalonic acid by the enzyme HMG-CoA reductase has shown to be the regulatory step of isoprenoid biosynthesis in animals as well as in plants (Chappell et al., 1995). Mevalonic acid is finally transformed by a series of reactions into IPP which gets converted by a reversible reaction into DMAPP. Recently discovered MEP pathway provides the precursors for the production of monoterpenes, diterpenes and tetraterpenes in the plastids (Lichtenthaler, 1999). It starts with the condensation of glyceraldehyde-3-phosphate and pyruvate. Since the organelles of the plant cells are presumed to have originated through prokaryotic endosymbiosis, the genes of plastid-located MEP pathway show high similarity to the respective bacterial genes (Bohlmann et al., 2000).

1.5.1.2. *Prenyltransferases: The central regulators*

An incredibly structurally diverse set of terpenes is synthesized starting from the fused products of IPP and DMAPP. Although head-to-tail is the most

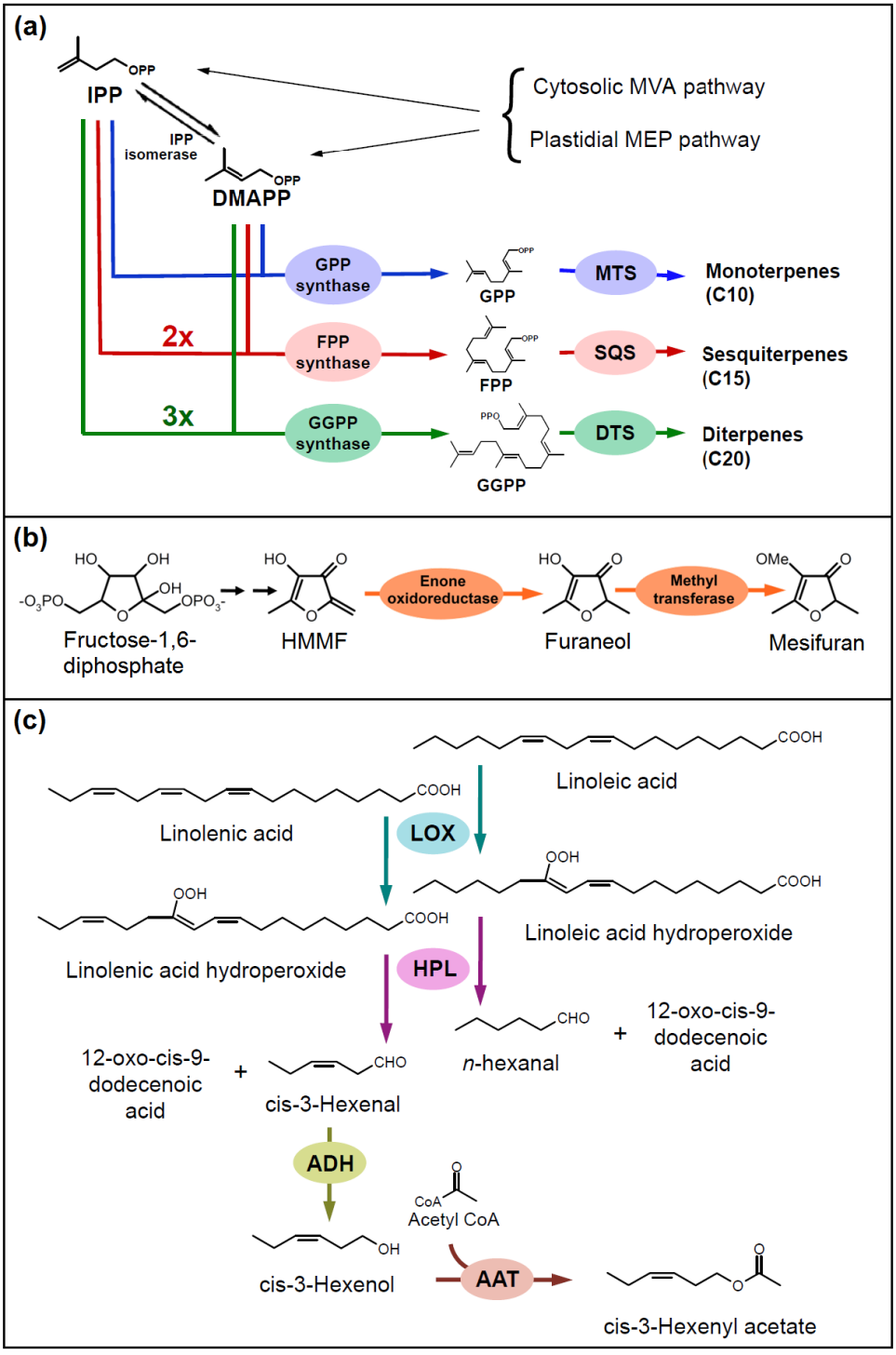


Figure 1.4

Biosynthetic pathway of a few classes of flavor compounds, terpenes (a), furanones (b) and the fatty acid derivatives (c)

MVA: mevalonic acid, MEP: methylerythritol 4-phosphate, IPP: isopentenyl pyrophosphate, DMAPP: dimethylallyl pyrophosphate, GPP: geranyl pyrophosphate, FPP: farnesyl pyrophosphate, GGPP: geranylgeranyl pyrophosphate, MTS: monoterpene synthase, SQS: sesquiterpene synthase, DTS: diterpene synthase, HMMF: 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone, LOX: lipoxygenase, HPL: hydroperoxide lyase, ADH: alcohol dehydrogenase, AAT: alcohol acyl transferase

common fashion in which these building blocks unite, other ways of their condensation such as head-to-head and head-to-middle are also observed. Fusion of one molecule of each of IPP and DMAPP yields geranyl pyrophosphate (GPP). Sequential additions of two IPP units to DMAPP results in the production of farnesyl pyrophosphate (FPP); whereas, after union of three IPP molecules with DMAPP, geranylgeranyl pyrophosphate (GGPP) is formed. The respective enzymes, GPP synthase, FPP synthase and GGPP synthase are collectively known as isopentenyl diphosphate synthases (IDSs) or short-chain prenyltransferases (Vandermoten et al., 2009). IDSs are the important branch-point enzymes in the terpene biosynthesis; since DMAPP and IPP are the precursors of all the types of terpenes, the extent of IDS activity is likely to decide the DMAPP and IPP pool diverted towards the biosynthesis of respective terpenes. The reactions catalyzed by IDSs proceed by the carbocationic mechanism. The divalent metal ions such as Mg^{2+} or Mn^{2+}, required as a cofactor for the activity of prenyltransferases, promote the ionization of the allylic substrate (for example, DMAPP), yielding a carbocation which is attacked by IPP, resulting in the formation of isopentenyl diphosphate longer by 5 carbons (Vandermoten et al., 2009).

In contrast to FPPS and GGPPS, which have been studied in greater details in several plant species, biosynthesis of terpenes is less clearly understood with respect to GPPS. It was earlier thought that plants might not have a special enzyme catalyzing the synthesis of GPP, rather small amounts of GPP produced by FPPS and GGPPS might be utilized in the biosynthesis of monoterpenes (Croteau, 1987). This was later proved to be untrue by purifying the first enzyme from the cell culture of *Lithospermum erythrorhizon* that solely synthesized GPP (Heide, 1988). This was followed by purification of similar GPPS enzymes from various other plants such as *Salvia officinalis* (Croteau and Purkett, 1989), *Pelargonium roseum* (Suga and Endo, 1991), *Vitis vinifera* (Clastre et al., 1993) and *Abies grandis* (Tholl et al., 2001). The isolation of GPPS enzyme in mint was paralleled by the discovery of the first gene for the corresponding enzyme (Burke et al., 1999). Surprisingly, this enzyme turned out to be heterodimeric and it was found to be formed by a large and a

small subunit (LSU and SSU). Genes encoding similar heterodimeric GPPS were also later characterized from *Antirrhinum majus* (Tholl et al., 2004) and *Humulus lupulus* (Wang and Dixon, 2009). LSU from all of these three plants share high sequence similarity (> 65%) with each other and with the plant GGPPS. On the other hand, enzymatically, these three LSU were shown to be much different from each other. Mint LSU was inactive on itself (Burke et al., 1999); *Antirrhinum majus* LSU was active as a GGPPS (Tholl et al., 2004); whereas, *Humulus lupulus* LSU produced GPP, FPP as well as GGPP (Wang and Dixon, 2009). In contrast to LSU, SSU have much lower sequence identity (20-30%) with the plant GPPS and GGPPS and they are shown to be inactive by themselves. The other kind of plant GPPS is represented by homodimeric enzymes which further have two subtypes (Schmidt and Gershenzon, 2008). Out of these two types known from the plants, one is found only in conifers. The genes of this class have been characterized from *Abies grandis* (Burke and Croteau, 2002) and *Picea abies* (Schmidt and Gershenzon, 2008). These genes also show high sequence similarity to the conifer GGPPS. The genes of the other class of homodimeric GPPS have been characterized from four plants, Arabidopsis (Bouvier et al., 2000), *Picea abies*, *Quercus robur* (Schmidt and Gershenzon, 2008) and tomato (van Schie et al., 2007) and they show quite low sequence identity (30%) with the members of the first class (Schmidt and Gershenzon, 2008).

Farnesyl pyrophosphate synthase (FPPS) provides direct precursor for sesquiterpene volatiles and also for the higher terpenes such as dolichols, sterols and ubiquinones (Grunler et al., 1994). FPPS is one of the highly studied prenyltransferases in both, animals and plants, where it has shown to be a regulatory enzyme of the terpenoid biosynthesis. Avian FPPS was the first enzyme of the terpenoid biosynthetic pathway whose X-ray crystal structure was determined (Tarshis et al., 1994). The gene encoding FPPS has been isolated and characterized from several plants such as Lupin (Attucci et al., 1995), *Parthenium* (Pan et al., 1996), rice (Sanmiya et al., 1997), tomato (Gaffe et al., 2000; Sallaud et al., 2009) *Ginkgo* (Wang et al., 2004), *Centella* (Kim et al., 2005), *Taxus* (Liao et al., 2006), Norway spruce (Schmidt and

Gershenzon, 2007), wintersweet (Xiang et al., 2010) and ginseng (Kim et al., 2010). Nevertheless, the most significant evidences on importance of FPPS in the terpenoid biosynthesis have come from the study of this gene in *Arabidopsis* (Closa et al., 2010; Cunillera et al., 1996; Cunillera et al., 1997, 2000; Delourme et al., 1994; Manzano et al., 2006; Masferrer et al., 2002) and *Artemisia* (Banyai et al., 2010; Chen et al., 2000; Han et al., 2006; Hemmerlin et al., 2003; Matsushita et al., 1996; Zhao et al., 2003).

1.5.1.3. *Terpene synthases: Let me finish the job*

The isopentenyl diphosphates produced by IDSs are the direct precursors of the terpenes and their conversion in terpenes is brought about by the action of enzymes known as terpene synthases. Since many terpene synthases carry out cyclization reactions leading to the synthesis of cyclic terpenes, these enzymes are also known as terpene cyclases. Because the substrates of prenyltransferases and terpene synthases share common structural feature such as the presence of pyrophosphate moiety attached to the hydrophobic tail, the reactions catalyzed by these enzymes are also similar in some aspects such as the presence of aspartate-rich substrate binding motifs in the enzymes and the electrophilic reaction mechanism. Based on sequence similarity of terpenes synthases with each other as well as on the basis of their functions, seven subfamilies of terpene synthases have been identified in plants, *viz*. TPS-a to d, TPS-e/f, TPS-g and TPS-h (Table 1.1) (Chen et al., 2011). The most peculiar feature of terpene synthases is their ability to synthesize more than one product. For example, a sesquiterpene synthase from grand fir synthesizes the mixture of 52 sesquiterpenes (Steele et al., 1998).

1.5.2. Furanones

Furanones are responsible for the caramel-like flavor of many fruits including strawberry, pineapple, raspberry, grapes, tomato, kiwi and mango (Schwab and Roscher, 1997). Out of the several furanones, furaneol, mesifuran, norfuraneol and homofuraneol are the most important flvorants (Schwab et al., 2008). In addition to having the sweet and pleasant odor, these compounds are characterized by their low odor detection threshold;

Table 1.1

Classification of terpene synthases based on their amino acid sequences and functions

Subfamily	Functional class/es	Occurrence
TPS-a	Sesquiterpene synthases	Angiosperms
TPS-b	Monoterpene synthases Isoprene synthases	Angiosperms
TPS-c	Copalyl synthase/kaurene synthase	Land plants
TPS-d	Monoterpene synthases Sesquiterpene synthases	Gymnosperms
TPS-e/f	Kaurene synthases Other diterpene synthases Monoterpene synthases Sesquiterpene synthases	Vascular plants
TPS-g	Monoterpene synthases Sesquiterpene synthases Diterpene synthases	Angiosperm
TPS-h	Putative bifunctional diterpene synthases	*Selaginella moellendorffii*

suggesting that their odor could be detected at a very low concentration (Schwab and Roscher, 1997).

In spite of the crucial involvement of furanones in determining the flavor of fruits, the studies done on understanding the biogenesis of furanones are scarce. The enzymes and the corresponding genes involved in the biosynthesis of furanones are known merely from two plants, strawberry and tomato. Earlier studies on strawberry showed that out of several radiolabeled substrates fed to the ripening strawberry fruits, fructose-1,6-diphosphate had the highest rate of incorporation into furaneol (Roscher et al., 1998). This, along with the other studies (Schwab, 1998; Wein et al., 2001) confirmed

fructose-1,6-diphosphate as a natural precursor of furanones in the plants. Further studies carried out to understand the biosynthesis of furaneol in plants indicated that fructose-1,6-diphosphate is first converted by an unknown enzyme into an unstable intermediate 4-hydroxy-5-methyl-2-methylene-3(2H)-furanone (HMMF) (Fig. 1.4). The furaneol forming enzymes, enone oxidoreductase, highly similar to the NAD(P)H:quinone oxidoreductase, then reduces the α, β-unsaturated bond of HMMF, resulting in the formation of furaneol (Raab et al., 2006). The presence of HMMF has also been detected in the fruits such as pineapple and raspberry suggesting that the biosynthetic pathways of furanones might be similar in different plants (Klein et al., 2007). Apart from this plant-located pathway, biosynthesis of furaneol in strawberries is also suggested to occur in collaboration with the enzymes from the epiphytic bacteria, *Methylobacterium extorquens,* which is found in association with strawberry (Koutsompogeras et al., 2007; Verginer et al., 2010; Zabetakis, 1997; Zabetakis et al., 2006).

1.5.3. Fatty acid derived compounds

Fatty acids contribute to the volatile compounds produced by the plants by getting converted into the compounds such as aldehydes, alcohols and lactones. The C6 aldehydes, alcohols and their esters, also known as green leaf volatiles (GLVs) are responsible for the typical aroma of the freshly cut leaves. The biosynthesis of these GLVs starts with the well-studied lipoxygenase (LOX) pathway (Fig. 1.4). The enzyme lipoxygenase catalyzes hydroperoxidation of the linolenic acid resulting in the formation of linolenic acid hydroperoxide, which by the action of hydroperoxide lyase (HPL) is converted into (Z)-3-hexenal (Matsui, 2006). Similar catalysis of linoleic acid by LOX and HPL results in the formation of *n*-hexanal. The aldehydes produced in this way are used as the substrates by alcohol dehydrogenase which catalyzes reduction of the aldehydes into corresponding alcohols. Apart from their own role in the aroma produced by the plants, alcohols also contribute to plant volatiles by getting converted into esters by the action of alcohol acyl transferase (AAT) (Beekwilder et al., 2004). Although aldehydes,

alcohols and esters, all three are the products of the LOX pathway and all three contribute to the aroma component of the plants, esters are better known for imparting typical fruity aroma to many fruits including strawberry and banana (Beekwilder et al., 2004); whereas, C6 aldehydes and alcohols are more known and studied for their role in the plant-insect interactions (Matsui, 2006).

Another class of important flavor compounds which are derived from the fatty acids are lactones. These cyclic esters can either contain a five-membered (γ-lactones) or a six-membered (δ-lactones) heterocyclic ring. Lactones are responsible for the fruity, coconut-like, buttery, creamy, sweet or nutty flavor of the fruits. In spite of vital contribution of lactones to the flavor of variety of fruits (Basear and Demirci, 2007), neither the enzymes nor the genes involved in the biosynthesis of lactones have been studied. A few studies have indicated that biosynthesis of lactones might be a collaborative product of metabolism of fatty acids by the peroxidation mediated by LOX and β-oxidation (Albrecht et al., 1992; Schottler and Boland, 1996).

1.5.4. Phenylpropanoids and benzenoids

Phenylpropanoids form another major class of flavor compounds. Phenylpropanoids are primarily derived from phenylalanine and these compounds contribute to the aroma and scents of many plant species (Schwab et al., 2008). Phenylpropanoids enjoy the status of the first class of the plant natural products for which the enzymes involved in the biosynthesis (phenylalanine ammonia-lyase) were isolated (Koukol and Conn, 1961) as well as for which the cDNAs encoding the biosynthetic enzymes were cloned and functionally characterized (Edwards et al., 1985; Kuhn et al., 1984; Ryder et al., 1984). The first committed step in the formation of phenylpropanoids is the conversion of phenylalanine to *trans*-cinnamic acid, catalyzed by phenylalanine ammonia lyase. In the next step, a variety of aldehydes and alcohols are formed from *trans*-cinnamic acid by a series of hydroxylation and methylation reactions (Schwab et al., 2008).

Benzenoids are derived from pheylpropanoids by shortening of the three-carbon chain attached to the phenyl ring of the phenylpropanoids by two carbons. The exact mechanism by which this reaction is brought is not clearly understood and several pathways for this conversion, including CoA-dependent β-oxidation, CoA-independent β-oxidation and the combination of these two reactions, have been proposed (Dudareva et al., 2006).

Apart from these well-known types of the plant volatiles, other pathways also contribute to the odorants produced by the plants. One such type of compounds is apocarotenoids which are derived by the degradation of tetraterpenic carotenoids and include the aroma compounds such as β-ionone (Schwab et al., 2008). In addition to the C6 GLVs produced by the oxylipin pathway, aldehydes and alcohols produced by the degradation of branched chain and aromatic amino acids also contribute to the aroma blend produced by the plants (Schwab et al., 2008).

Although most of the volatiles produced by the above-mentioned pathways are themselves volatile, in some cases, they are further modified by other enzymes via the reactions such as oxidation, reduction, methylation, isomerization, hydration and esterification, which mainly aid in increasing the volatility of the compounds (Dudareva et al., 2004). In some cases, volatiles are also modified by glycosylation which helps in enhancing water solubility and decreasing self-toxicity of the volatiles and thereby assist in the management of the cellular distribution and transport of these compounds (Seigler, 1998).

1.6. Biotechnological implications

Since ancient age, human beings have been using the odorous substances for enjoyment and for the medicinal reasons. As a consequence, in the today's world, flavor and fragrance have developed into one of the most important types of the chemical industries with the total market estimated to be US$18 billion (Guentert, 2007). Due to the high price or the lack of the natural materials, the components used in making the flavors and fragrances are chemically synthesized, mostly from the petroleum raw material. As the

petroleum resource is getting limited and the chemical synthesis procedures are hazardous in terms of the practices such as the use of the heavy metal catalysts, alternative sources of the raw material are being searched (Schwab et al., 2008). Since knowing the way nature (plants) makes the volatile chemicals can give a tool for their *in planta* overproduction or semi-natural production, the scientific community is getting more interested in dissecting the metabolic pathways of the volatiles in plants. This knowledge is also important in terms of the applications such as improving the aroma of the fruits or the flowers.

Generating the knowledge about the enzymes and the genes encoding them, involved in the biosynthesis of flavorants is one of the first most important steps in knowing the means by which the plants produce volatile compounds. Last two decades have witnessed a huge progress in understanding the genes in the biosynthesis of the flavorants in the plants. One of the earliest examples is provided by the isolation of an acetyl-CoA: benzylalcohol acetyltransferase involved in the production of volatile esters in *Clarkia breweri* flowers (Dudareva et al., 1998). Being rich in the flavorants, strawberry has turned out to be a model system for studying the biosynthesis of volatiles. Many studies have been carried out to understand role and regulation of the flavor biosynthetic genes in strawberry. One of the classic examples that has demonstrated the integration of biochemical and molecular biological techniques for this purpose was the isolation and characterization of a strawberry alcohol acyltransferase (SAAT) gene from strawberry which was further shown to be involved in the biosynthesis of esters, which are the principal flavorants of strawberry fruits (Aharoni et al., 2000). Another prototypic study on the terpenic flavorants in strawberry demonstrated that the flavor difference between cultivated and wild species of strawberry is associated with only a few nucleotide differences in the terpene synthase genes in these species (Aharoni et al., 2004). In contrast to numerous reports now available on the genes in the biosynthesis of the volatiles from many plants (reviwed by Dudareva et al., 2006; Pichersky and Gershenzon, 2002; Schwab et al., 2008), not much is known about the exact mechanism by

which biogenesis of volatiles is regulated. The first transcription factor directly involved in the regulation of volatiles in *Petunia hybrida* flowers, "*ODORANT1*", has been discovered very recently (Verdonk et al., 2005). After this only two transcription factors, *EOBII* and *PhMYB4,* which regulate the production of benzenoid and phenylpropanoid volatiles in *Petunia hybrida*, have been identified till date (Colquhoun et al., 2011; Spitzer-Rimon et al., 2010).

As the next step in studying biogenesis of plant volatiles, many attempts have been made to understand the effect of transgenic over-expression or suppression of the genes on the levels of volatiles. Since the aim of these studies is to have the enhanced fragrance or flavor for the human pleasure, such experiments are considered practically successful only if the change is detectable via olfactory perception by humans, irrespective of detection of the alteration by the analytical techniques such as GC (Dudareva and Pichersky, 2008). Although there are few cases of olfactory detectable enhancement of the volatiles, for example, in the leaves and the flowers of tobacco upon transgenic expression of citrus terpene synthase genes (El Tamer et al., 2003; Lucker et al., 2004), many other experiments were either unsuccessful or they brought about a negligible change in the volatile content. Several reasons including an unavailability of the substrate of the introduced gene or further metabolism of the desired volatile into another non-volatile product can be accounted for this (Dudareva and Pichersky, 2008). One of such examples is the expression of *Clarkia breweri* linalool synthase in tomato, where, although there was the accumulation of linalool in the transgenic tomato fruits, the levels were not sufficient to change the overall flavor perception by humans (Dudareva and Pichersky, 2008; Lewinsohn et al., 2001). Introduction of basil (*Ocimum basilicum*) geraniol synthase in tomato exemplifies one of the most successful experiments that resulted in the perceptible change in the flavor which was because of the large accumulation of geraniol. In this case, about 80% test panelists indicated that the transgenic plants had the stronger aroma than the control plants (Davidovich-Rikanati et al., 2007). Apart from these reports on tomato fruits, studies on the other

plant systems also provide the paradigmic cases of alteration in plant volatiles. For example, modification of vegetative volatiles has been achieved in mint with respect to enhancing the production of the compounds favored by humans (menthol) and down-regulating the accumulation of unfavored chemicals (menthofuran) (Mahmoud and Croteau, 2001, 2003; Mahmoud et al., 2004).

As the pathways leading to the biosynthesis of many flavor volatiles involve multiple steps, it is very important to understand the rate-limiting step of the pathway to be able to modify the production of the final product. Rather, in such a case it is difficult to achieve the change in the volatile content by modifying just a single step of the long pathway. Since regulatory-points of many of these pathways are not clearly understood, most of the studies carried out for the alteration of volatiles have relied on the modification of the final step in the biosynthetic pathway (Dudareva and Pichersky, 2006). Analyzing the genes encoding all of the enzymes involved in the metabolic pathway and understanding their regulatory mechanisms in relation to the metabolic flux should help in the upcoming time for the emergence of the commercial successful example of the plant with the improved fragrance and flavor.

1.7. Mango: the king of fruits

Mango is one of the highly popular tropical fruits not only in India, the country of its origin, but throughout the world. India is also a centre of diversity of mangoes and is blessed with thousands of cultivars of this national fruit, out of which about 50 are commercially cultivated (Salvi and Gunjate, 1988). India produces about 13.6 MMT mangoes and contributes the highest (39.1%) to the world production of 34.5 MMT per year. India also stands first in the list of mango exporting countries by contributing 23% to the total world export of 1.2 MMT (http://faostat.fao.org/site/291/default.aspx) (Fig. 1.5). Within India, mango covers the highest (35%) of the total fruit cultivation area; whereas, in terms of production, mango contributes 22% to the total fruit yield of the country, closely following banana

(http://agricoop.nic.in/hort/hortrevo5.htm). In the light of these figures, mango production certainly plays a significant role in the development of the agro-rural regions of India. Out of the several cultivars from India, Alphonso is the most important in terms of the trade (about 60% of the total mango export) and the popularity, which can mainly be attributed to its highly attractive flavor. Apart from this, Alphonso possesses many significant attributes such as attractive color, ample sweetness, low fiber containing pulp and long shelf life.

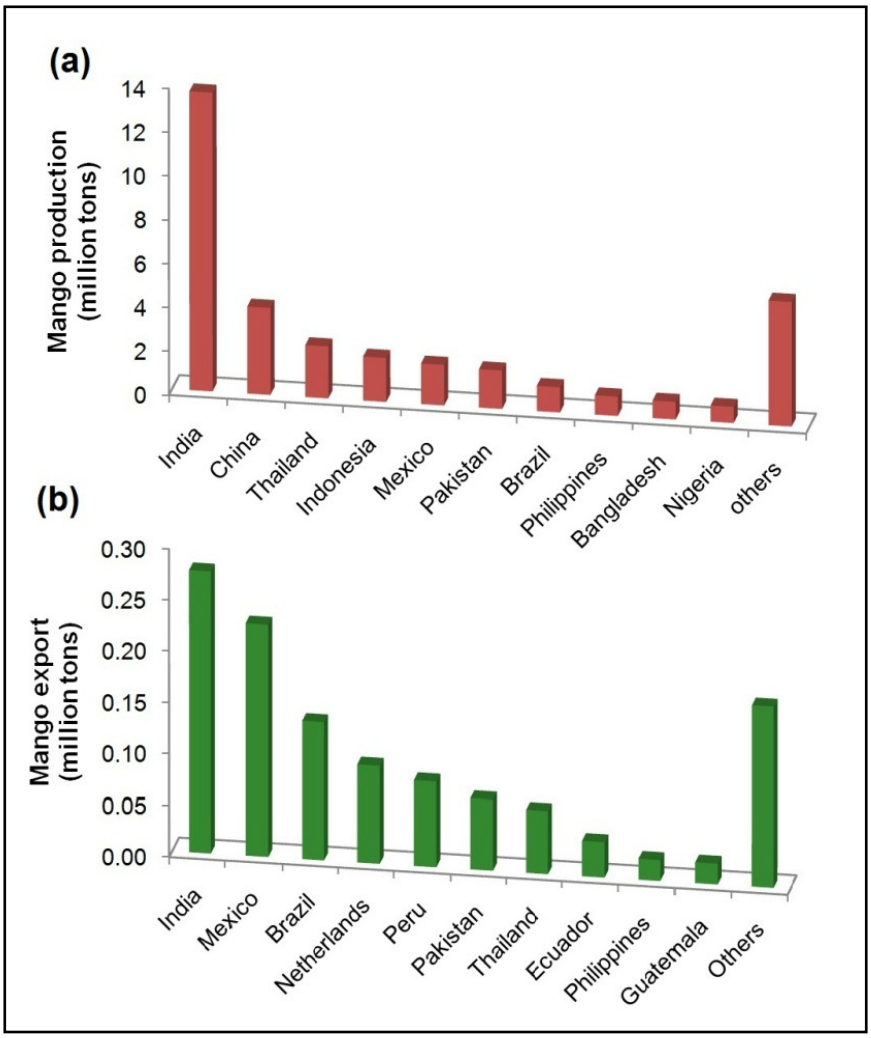


Figure 1.5
Production (a) and export (b) of mangoes by a few top-most countries in the year 2008 (http://faostat.fao.org/site/291/default.aspx).

1.7.1. Mango food products

The largest proportion of mangoes produced in India is consumed for the fresh-table use. Since mango is a seasonal crop and the fruits are highly perishable, the pulp of fruits is industrially processed and preserved in the form of products such as canned pulp, slices, bars, purees, nectars, etc. Mango fruits are also used in making several processed products such as jams, jellies and marmalades. Homogenized pulp of mangoes finds applications in the preparation of many beverages, ice-creams and smoothies. In India, traditionally, the products such as pickles and Aamras (thick semisolid juice made from the fresh pulp) are made from the ripe mango fruits. Similarly, raw fruits are also processed for making pickles, tarts, chutneys, curries as well as for the fresh table use in the form of salads (Bawa, 2007; Tharanathan et al., 2006).

1.7.2. Alphonso cultivation: a challenging task

In spite of being the most popular cultivar of mango in India, Alphonso cultivation is arduous to the farmers because of several reasons. Although Alphonso cultivation is widespread in many regions of India with varying extent, the quality of the fruits, especially in terms of taste and flavor is not the same in all of these regions. Because of this, the main cultivation of Alphonso is concentrated in the 700 km long, narrow coastal belt of western India, known as the Konkan region. Even within this region, the fruits show conspicuous variation in the flavor. Throughout Konkan, the growing practice and post-harvest treatment to the mangoes is the same, wherein, after harvesting the fruits are packed in hay and are immediately transported to a common export centre. After this, it takes about 15 days after harvest for complete ripening. Despite having such consistent cultivation and harvesting practice, the flavor of the mangoes differs between the localities of cultivation. Because of this; Alphonso fruits from a specific region (Deogad) of Konkan are preferred for fresh table use as well as for processed food products, resulting in high market value for the fruits from this region.

Another important problem associated with the Alphonso is the occurrence of spongy tissue, a devastating physiological disorder related to fruit-ripening. No external symptoms of the spongy tissue are observed and only after cutting the tissue, the disorder could be detected in the form of mesocarp tissue near the seed remaining white and unripe. Although several biochemical, nutritional, ecological and physiological reasons have been hypothesized for the occurrence of the spongy tissue, the exact cause of the disorder, which causes loss of 30% of the crop, still remains enigma to the researchers (Janave and Sharma, 2008 and reference therein). Similar to these problems, alternate bearing of the fruits is another cause of the reduction in the yield of Alphonso mango.

1.7.3. Pioneering phytochemical studies

In addition to benefiting the producers economically, mangoes offer high nutrition values to the consumers. Mango fruits are rich in the basic nutrients such as sugars, vitamins, proteins and minerals as well as they contain other health-benefiting components including antioxidants such as carotenoids and prebiotic dietary fibers. Nevertheless, the most important property for which mangoes are liked is their highly attractive flavor. No doubt many studies have till now been carried out to understand chemistry of such pleasant flavor. Being the center of the highest production, earlier studies were restricted to India and being the most favorite, these studies were carried out on the cultivar Alphonso. The earliest analysis of the mango flavor dates back to 1973 and was carried out by the pioneering scientists A. S. Gholap and C. Bandyopadhyay. In this study, the aromatic principles of Alphonso mango were for the first time isolated, concentrated (Bandyopadhyay and Gholap, 1973a) and were further analyzed by GLC (Angelini et al., 1973). This study along with the work on the aroma of canned Alphonso mango (Hunter et al., 1974) indicated that the flavor of mango fruits is represented by the compounds such as hydrocarbons, esters, alcohols, carbonyls and lactones. Aroma of the raw mangoes was also first analyzed by Gholap and Bandyopadhyay (Gholap and Bandyopadhyay, 1977) from the cultivar

Alphonso. This study suggested that the aroma of the latex of the raw fruits, which is common among several mango cultivars, is represented by the monoterpenic compounds (*Z*)-ocimene and β-myrcene. Idstein and Schreier (Idstein and Schreier, 1985) carried out the detailed analysis of the volatiles of the ripe fruits of Alphonso mango suggesting the high diversity of the flavor chemicals in these fruits.

1.7.4. Mango volatiles: recent advances

After this, numerous studies have been conducted for the analysis of volatiles of various Venezuelan (Macleod and Detroconis, 1982), Sri Lankan (Macleod and Pieris, 1984), Floridian (Macleod and Snyder, 1985), Australian (Bartley, 1988), Brazilian (Andrade et al., 2000), Thai (Tamura et al., 2001), Cuban (Pino et al., 2005) and Colombian (Quijano et al., 2007) cultivars of mango. These studies have indicated that the mango cultivars are highly diverse in qualitative and quantitative nature of the volatile flavorants. Apart from these merely technical reports of the volatile constitutes of mangoes, more recent studies have focused on the correlation of various biological factors such as pathogenic diseases (Li et al., 2009; Moalemiyan et al., 2006), ripening (Lalel et al., 2003a; Lebrun et al., 2008), maturity stage at harvest (Lalel et al., 2003b), as well as those of physical parameters such as disease control and storage methods (Dea et al., 2010; Lalel and Singh, 2004, 2006; Lalel et al., 2004; Nair et al., 2003), osmotic dehydration (Torres et al., 2007), edible coatings (Dang et al., 2008) and methyl jasmonate treatment (Lalel et al., 2003c), with the mango volatiles. Few studies have also reported the role of mango volatiles in attracting the insects (Cruz-Lopez et al., 2001; De Jesus et al., 2004; Garcia-Ramirez et al., 2004; Hernandez-Sanchez et al., 2001).

1.7.5. Molecular studies in mango

Mango being popular all over the world, many basic studies have been performed in mango. Genetic diversity in the global pool of mango germplasm has been assessed using a variety of molecular markers such as AFLP, DAMD, ISSR, ITS, RAPD and SCoT (Bally et al., 1996; Chunwongse et al., 2000; Eiadthong et al., 1999; Karihaloo et al., 2003;

Kumar et al., 2001; Lopez-Valenzuela et al., 1997; Luo et al., 2010; Ramessur and Ranghoo-Sanmukhiya, 2011; Ravishankar et al., 2000; Ravishankar et al., 2004; Schnell et al., 1995; Srivastava et al., 2005). Out of these, RAPD markers were shown to be able to distinguish monoembryonic and polyembryonic mango cultivars (Lopez-Valenzuela et al., 1997; Karihaloo et al., 2003; Ravishankar et al., 2004). In our laboratory, genetic diversity in the pool of 70 mango cultivars was analyzed by ISSR markers which suggested clear differentiation between Indian and non-India cultivars (Pandit et al., 2007). A numerous studies have been carried out to understand biochemical basis for a vast transformation in the taste, color and texture brought about during mango ripening. Many enzymes such as carbohydrases (Yashoda et al., 2007) polygalacturonase (Chaimanee et al., 2000; Prasanna et al., 2006) and β-galactosidase (Prasanna et al., 2005) have been studied with respect to their role in mango ripening. Similarly, changes in the fatty acids (Bandyopadhyay and Gholap, 1973b), cell wall constituents (Yashoda et al., 2006) and polysaccharides (Prasanna et al., 2004; Yashoda et al., 2005) have been monitored during the process of mango ripening.

Molecular biological studies have given very useful insights in the biology of ripening of mango fruits. Changes in the mRNA and protein content (Lopezgomez and Gomezlim, 1993) along with substantial increase in the total RNA was observed during mango ripening (Chaimanee et al., 1999). Five ripening-related cDNAs were isolated from mango; however, none of these were directly involved in the events associated with ripening (Saiprasad et al., 2004). Later, numerous genes related to the structural changes occurring during mango ripening, such as expansin (Sane et al., 2005), pectate lyases (Chourasia et al., 2006), endo-beta-1,4-glucanase (Chourasia et al., 2008), along with few others such as alcohol dehydrogenase (Singh et al., 2010) have been isolated and their ripening-related expression was shown in the mango fruits.

Surprisingly, in contrast to such a huge interest of the researchers on the several aspects of mangoes, no studies have been conducted to understand the biosynthesis of volatile flavorants in the mangoes.

1.8. Genesis of thesis and its organization

To get biological insights in mango, an economically and socially important crop, and to be able to address the problems associated with the Alphonso, the studies regarding the analysis of flavor of mango fruits, which is one of the most important attributes of this fruit were initiated at the Plant Biochemistry and Molecular Biology Group at NCL. As the first step, diversity in the volatiles of various mango cultivars was analyzed. This study indicated that most of the Indian mango cultivars are rich in terpenes, especially monoterpenes, as well as they contained other compounds like sesquiterpenes, aldehydes, alcohols, lactones and furanones (Pandit et al., 2009a). Alphonso was distinguished from the other cultivars by the presence of high number and concentration of lactones and furanones. Detailed studies on Alphonso in terms of analysis of volatiles through developing and ripening stages, underlined the dominance of mono- and sesquiterpene volatiles throughout the stages and the *de novo* appearance of lactones and furanones in the ripening fruits (Pandit et al., 2009b). The expression of several flavor- and ripening related genes were further profiled in the developing and ripening fruits of Alphonso suggesting the ripening-related pattern of expression of many genes (Pandit et al., 2010).

Having this background knowledge about the mango flavor, I initiated my doctoral work with the main intention of studying the chemical and molecular nature of the geographic variation observed in the flavor of Alphonso and analyzing the flavor biogenesis, with respect to the genes involved in terpene and furanone biosynthetic pathway in Alphonso.

This thesis has been organized in the following manner

Chapter 1: Introduction and review of literature

Chapter 2: Materials and methods

Chapter 3: Results

Chapter 4: Discussion

Summary and future directions

Bibliography

Materials and Methods

2.1. Plant material

Mature raw fruits of Alphonso mango were collected from the orchards of Konkan Krishi Vidyapeeth at Dapoli (N17°45' E73°11') and Deogad (N16°31' E73°20') and from a private orchard at Vengurle (N15°51' E73°39') (Figure 2.1). For each of the three localities, four plants were selected and from each of these plants at least 20 fruits were used for the analysis to justify the statistical validity of the analysis. These randomly chosen plants from the orchard were distantly located (at least 30 m away) from each other and the fruits were harvested from the branches at the periphery and at the height range of 2-5 m to have the fruits exposed to the similar intensity of light. This practice was followed for all the plants from all the three localities. After harvesting, fruits were put in the hay, carried to the laboratory and allowed to ripe at ambient temperature (25-30 °C). Conventionally, ripening of Alphonso is determined based on the period in terms of days after harvesting, change in skin color (green to yellow-orange), smell (raw leafy to sweet fruity) and

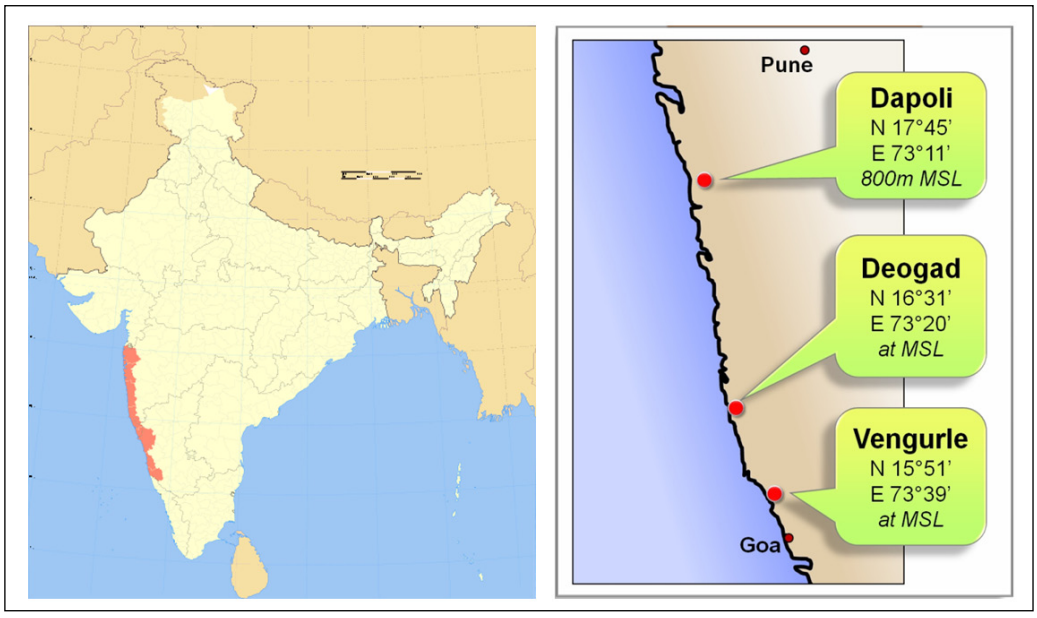


Figure 2.1

Map of India showing the geographic location of Konkan in the orange color (left) and the geographic location of three sampling localities in India, Dapoli, Deogad and Vengurle (right)

softness to touch. According to these indices the fruits of 15 days after harvest (15 DAH) are considered completely ripe (Bandyopadhyay and Gholap, 1973b). Moreover, previous volatile analysis of ripening stages of Alphonso from one location, Deogad, performed in our laboratory has indicated 15 DAH as a completely ripe stage (Pandit et al., 2009b). To span this whole period of ripening, at the interval of every five days, fruits were peeled, pulp was immediately frozen in the liquid nitrogen and stored at -80 °C until use. Thus, the experimental tissues of four ripening stages: 0, 5, 10 and 15 DAH (days after harvest) were obtained from each of the three localities

2.2. Extraction of volatiles

Extraction of the volatiles was carried out by the method described earlier (Kollner et al., 2004) with some modifications. With the help of liquid nitrogen, 2 g fruit mesocarp was ground into fine powder and was extracted for 1 hr in 10 ml dichloromethane containing 40 µg nonyl acetate as an internal standard. The supernatant was decanted, centrifuged at 10,000 *g* for 10 min at RT for removing the remaining tissue debris and dehydrated with sodium sulphate. After carefully concentrating to 1 ml in the rotary evaporator, the extracts were stored at -20 °C overnight to precipitate the fats which were removed the next day by centrifugation at 16000 *g* for 10 min at 4 °C. The extracts were finally concentrated in the similar way to the volume of 50 µl.

2.3. GC-MS and GC-FID analysis

Gas chromatographic analysis was carried out on Clarus 500 (Perkin Elmer, USA) instrument having Rtx-5MS (Restek, USA) capillary column (30 m x 0.32 mm i.d. x 0.25 µm film thickness). Column temperatures were programmed from 40 °C for 5 min, raised to 180 °C at 5 °C min^{-1} followed by increase till 280 °C at the rate of 20 °C min^{-1} and held isothermal for 5 min. Injector and detector temperatures were 200 and 250 °C, respectively. Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. Mass spectra were obtained using Clarus 500 (Perkin Elmer) gas chromatograph–mass

spectrometer at 70 eV with a scan time of 0.2 s for m/z 30–300 under the GC conditions same as those applied for GC–FID analyses. Using a series of n-paraffins (C5–C22), retention indices were determined for all the peaks. Compounds were identified by comparing acquired mass spectra with those of authentic external standards and with the help of Wiley registry of mass spectral data (8th edition). Identification was confirmed by comparison of the obtained retention indices with those of the authentic standards and with those reported in the literature. Quantitation was carried out by internal standard method, where concentrations of different volatiles were normalized with that of nonyl acetate. For each stage of the ripening minimum of two fruits of each of the four trees from each of the three localities were used for two independent extractions while each extract was independently analyzed twice on the GC. Both ANOVA and Principal Component Analysis were carried out using Systat statistical software (version 11, USA). Fisher's LSD test was carried out ($p \leq 0.05$) by ANOVA to compare quantity of each compound and each class of compounds with respect to various stages and cultivation localities.

2.4. RNA isolation and cDNA synthesis

RNA was isolated by the method described previously (Asif et al., 2000; Pandit et al. 2007) with some modifications. Frozen tissue of Alphonso mango of various ripening stages from the above-mentioned three localities was crushed to a fine powder using mortar and pestle in liquid nitrogen. This powder was then homogenized to slurry with preheated (65 °C) extraction buffer containing 100 mM Tris-Cl (pH 8.2), 1.5 M NaCl, 30 mM EDTA (pH 8.0) and 2% CTAB, (10 ml extraction buffer g^{-1} of tissue); 20 µl of 2-mercaptoethanol per ml of buffer (~0.2%) was added just before use. The homogenate was incubated at 65 °C for 20 min, with intermittent but thorough vortexing. After the slurry has been cooled to room temperature (RT), an equal volume of chloroform: iso-amyl alcohol (24:2) was added followed by vigorous shaking with intermittent venting of the tube to form an emulsion. Samples were centrifuged at 12000 *g* for 10 min at RT. The aqueous

phase was collected, to which, prechilled 10 M LiCl was added to a final concentration of 3 M. The RNA was allowed to precipitate at -20 °C for 30 min. The pellet was recovered by centrifugation at 12000 *g* for 10 min at 4 °C; whereas, the supernatant was preserved for the recovery of genomic DNA. The RNA pellet was dissolved in 10 ml diethyl pyrocarbonate (DEPC)-treated water, then extracted once with water-saturated phenol (pH 5.2 to 5.5), followed by extraction with chloroform: iso-amyl alcohol (24:2). To the aqueous phase, 3 M sodium acetate (pH 5.4) was added, to a final concentration of 0.3 M, followed by a range of 0.6 to an equal volume of prechilled isopropanol. RNA was allowed to precipitate at -70 °C for 20 min. RNA was recovered by centrifugation at 12000 *g* for 10 min at 4 °C. The pellet was suspended in 1 ml of 70% EtOH and again centrifuged at 12000 *g* for 10 min at RT. The vacuum-dried pellet was dissolved in an appropriate volume of DEPC-treated water (100 µl g^{-1} starting material). All the reagents were treated with DEPC and were autoclaved (15 psi, 121 °C for 20 min). The exception was Tris-Cl, which was prepared in DEPC-treated water and autoclaved. The entire procedure was conducted under RNase-free conditions.

2.5. Isolation of genomic DNA

To the preserved supernatant from the RNA isolation procedure, 0.1 volume of 3 M sodium acetate (pH 5.4) and an equal volume of prechilled isopropanol were added. DNA was precipitated for 1 h at -20 °C before being recovered by centrifugation at 12000 *g* for 10 min at 4 °C. The pellet was suspended in 1 ml of 70% EtOH, and then centrifuged at 12000 *g* for 10 min at RT. The vacuum-dried pellet was dissolved in an appropriate volume of sterile MilliQ- grade water, i.e., 1 ml g^{-1} starting material. DNA was treated with RNase A for 30 min at 37 °C (15 µg ml^{-1} DNA solution). RNase A was removed via extraction with an equal volume of Tris-saturated phenol (pH 7.8 to 8.0) and centrifugation at 12000 *g* for 10 min at RT. The aqueous phase was re-extracted with equal volumes of chloroform: iso-amyl alcohol (24:2) to remove the traces of phenol by the centrifuging at 12000 *g* for 10 min at RT.

To the aqueous phase, 0.1 volume of 3 M sodium acetate (pH 5.4) was added, followed by 0.6 to an equal volume of prechilled isopropanol. DNA was allowed to precipitate for 1 h at -20 °C and then recovered by centrifugation at 12000 *g* for 10 min at 4 °C. The pellet was suspended in 1 ml of 70% EtOH (RT) and centrifuged at 12000 *g* at RT for 10 min. After vacuum-drying, the pellet was dissolved in an appropriate volume (100 µl g^{-1} starting material) of Tris-EDTA buffer (pH 8.0). DNA was quantified spectrophotometrically at wavelength 260 nm, and the purity was determined by ratios of optical densities at 260/230 nm and 260/280 nm. DNA quality was also assessed by electrophoresing on 1% EtBr-stained agarose gel as described earlier (Sambrook and Russell, 2001).

2.6. Quality assessment of the nucleic acids

RNA and genomic DNA were quantified spectrophotometrically at 260 nm, and their purity was determined by ratios of 260/230 nm and 260/280 nm. RNA quality was assessed by electrophoresing on 1% non-denaturing agarose gel in 0.5x Tris-acetate EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001), using a 1kb ladder (Promega) as the size marker. Genomic DNA was examined by loading on 0.8% agarose gel in 0.5x Tris-acetate EDTA (TAE) (Sambrook and Russell, 2001) containing ethidium bromide (EtBr).

2.7. Isolation of full-length coding sequences of the flavor genes

Based on the conserved regions in the nucleotide sequences of orthologous geranyl pyrophosphate synthase (GPPS), farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase (GGPPS) and enone oxidoreductase (EO) reported in the NCBI database, degenerate primers were designed (Table 2.1). These primers were used for the amplification over the cDNA prepared from ripe fruits of Alphonso mango. The gene specific primers designed (Table 2.2) based on the sequence of the fragments obtained were used for amplification of the ends of the cDNA by rapid amplification of cDNA ends (RACE) using a RACE kit (Clontech, USA). Based on the

Table 2.1

Degenerate primers used for amplifying the fragment of the flavor genes from Alphonso mango (GPPS: geranyl pyrophosphate synthase, FPPS: farnesyl pyrophosphate synthase, GGPPS: geranylgeranyl pyrophosphate synthase, EO: enone oxidoreductase)

Primer	Gene	Orientation	Sequence (5' to 3')
G-D-F1	*GPPS*	Forward	TCTTGTTACNGGTGAAACCATG
G-D-R1	*GPPS*	Reverse	TYAYTTTKTTCTTGTRATGACGC
F-D-F1	*FPPS*	Forward	TKGAYTACAAYGTVCCHGGAG
F-D-F2	*FPPS*	Forward	CYCTYGGYTGGTGYATTGAATGG
F-D-R1	*FPPS*	Reverse	YTAYTTYTGCCTCTTRTADATYTT
GG-D-F1	*GGPPS*[a]	Forward	TSGARATGATHCACACYATGTC
GG-D-R1	*GGPPS*[a]	Reverse	TANGGAATRTAATTMGCYARAGC
GG-D-R2	*GGPPS*[a]	Reverse	TTYCCWGCVGTTTTCCCCARTTC
E-D-F1	*EO*	Forward	GTKGTKGCTGCWKCYVTTAAYC
E-D-F2	*EO*	Forward	AARGMYAYYGAYTCTCCYYTRC
E-D-F3	*EO*	Forward	GGVWSWTTRGCWGARTAYACHGC
E-D-F4	*EO*	Forward	GTTYTRRRWGGHGCTGGKGGWGTTGG
E-D-R1	*EO*	Reverse	GRATSGGRTAYAYRACYACYTTYCC
E-D-R2	*EO*	Reverse	GCYYTHTCHSKYTSYCCWAYTGC
E-D-R3	*EO*	Reverse	RGTRGCTGCTAYYTTDGAWGCACC

N: A/C/G/T; Y: C/T; R: A/G; W: A/T; S: C/G; M: A/C; K: G/T; B: C/G/T; D: A/G/T; H: A/C/T; V: A/C/G

[a] Later characterized as *GPPS*

alignments of the 5' and 3' RACE fragments with the respective sequences reported from the other plants, primers corresponding to the terminal regions of the mRNA were designed (Table 2.3) and were used for the PCR with

Table 2.2

Gene specific primers used to obtain ends of the cDNAs of the flavor genes from Alphonso mango by RACE. (GPPS: geranyl pyrophosphate synthase, FPPS: farnesyl pyrophosphate synthase, GGPPS: geranylgeranyl pyrophosphate synthase, EO: enone oxidoreductase)

Primer	Gene	Orientation	Sequence (5' to 3')
G-R-F	*GPPS*	Forward	AGATGACGTTCTTGATTTCACGGGC
G-R-R	*GPPS*	Reverse	CTTTGAGTTAGATCTAAAAGTGCCCG
F-R-F	*FPPS*	Forward	AGTATTCATTGCCACTTCATTGCCAG
F-R-R	*FPPS*	Reverse	ACTTTCATACTCTGCAAACGCACCC
GG-R-F	*GGPPS*[a]	Forward	ACGACCTTCGTCGGGGAAAACCG
GG-R-R	*GGPPS*[a]	Reverse	GACCCTCAATGCCAATCGATTTCGC
E-R-F1	*EO*	Forward	CGAAGACAGGGCAAGTTCAAGGC
E-R-F2	*EO*	Reverse	GATTCTCCCCTCCCGACTGTTCC
E-R-R1	*EO*	Forward	GGTGTTGGAAGCTTGGTGATTCAG
E-R-R2	*EO*	Reverse	GGGTTCTCTGCTGGTAAATCTATTCT

[a] Later characterized as *GPPS*

mango cDNA as a template. The full-length open reading frames thus obtained for GPPS, GGPPS, FPPS and EO were named as *MiGPPS* (*Mangifera indica* GPPS), *MiFPPS*, *MiGGPPS* (later, named as *MiGPPS2*) and *MiEO*, respectively. The genomic fragment of *MiFPPS* was isolated by carrying out PCR using above-mentioned terminal primers over the Alphonso genomic DNA. Each of the above-mentioned step of PCR was performed in 20 µl reaction volume with 1× buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.6U *Pfu* DNA Polymerase (Promega), 0.5 µM each of forward and reverse primers and suitable quantity of template (1 µl of cDNA or 30 ng genomic DNA). The

Table 2.3

Terminal primers used for amplifying the complete open reading frame of the flavor genes from Alphonso mango

Primer	Gene	Orientation	Sequence (5' to 3')
G-T-F	*MiGPPS*	Forward	ATGCTTGTTGCTAACAGGCTG
G-T-R	*MiGPPS*	Reverse	TCATTTATTTCTTGTGATGACTCTTTGAG
F-T-F	*MiFPPS*	Forward	ATGAGTGATTTGAAGTCCAAGTTCG
F-T-R	*MiFPPS*	Reverse	CTACTTCTGCCTCTTATATATCTTGG
GG-T-F	*MiGGPPS*[a]	Forward	ATGTTCGATTTCAAGTCTTATATGC
GG-T-R	*MiGGPPS*[a]	Reverse	ATTTTGCCTATAGGCAATATAATTAGAC
E-T-F	*MiEO*	Forward	ATGAAAGCGTGGGTGTATGGAG
E-T-R	*MiEO*	Reverse	TTAAGGAATTGGGTATATAACCACC

[a] Later characterized as *GPPS*

reaction conditions were as follows – initial denaturation of 94 °C for 5 min, 35 cycles of 94 ºC for 1 min, annealing temperature (dependent on the primer pair) for 1 min, extention at 72 ºC for 2 min and final extention for 5 min at 72 ºC. The fragments obtained were resolved on 1-1.5% agarose gel in 0.5× Tris-acetate EDTA (TAE) containing ethidium bromide (EtBr) (Sambrook and Russell, 2001) with a 1kb ladder (Promega) as the size marker. The fragments of the expected size were eluted from the agarose gel using GenElute™ Gel Extraction Kit (Sigma Chemical Co., USA) and ligated in pGEM-T Easy vector (Promega). The ligation reactions were transformed in *E. coli* cells (Top10, Invitrogen, USA) and the white colonies on the LB agar plates containing 100 μg ml^{-1} carbenicillin, 1 mM IPTG and 50 μg ml^{-1} X-gal were considered as recombinants. Presence of the insert in the colonies was identified by colony PCR (Sambrook and Russell, 2001). Sequencing of the positive clones was carried out with the help of Megabase 1000 DNA sequencer (Amersham Biosciences, USA) using DYEnamic™ ET Dye Terminator Kit (GE Healthcare, USA). The sequences were aligned and analysed for the presence of uninterrupted reading frame in the MEGA software (Version 4.1) (Tamura et al., 2007).

2.8. Phylogenetic analysis of *MiGPPS* (*MiGPPS1*), *MiFPPS* and *MiGGPPS* (*MiGPPS2*)

Amino acid sequences of the orthologous characterized genes showing the highest identity with the sequences from mango were obtained from the NCBI database and used for constructing a neighbour joining tree using the MEGA software (Version 4.1) (Tamura et al., 2007). The percent bootstrap values were obtained from 1000 replicates.

2.9. Expression cloning and recombinant expression in *E. coli*

Full length sequence of *Mangifera indica* GPPS (*MiGPPS1*), *MiFPPS*, *MiGGPPS* (*MiGPPS2*) and *MiEO* were amplified using Expand High Fidelity PCR System (La Roche, Basel, Switzerland) with the terminal primers (Table 2.3). cDNA prepared from the ripe fruit was used as the template and the resulting fragments for *MiGPPS* (*MiGPPS1*), *MiFPPS* and *MiGGPPS* (*MiGPPS2*) were cloned in the pEXP5-CT/TOPO expression vector (Invitrogen); whereas, that of *MiEO* was cloned in the pCRT7-NT/TOPO expression vector (Invitrogen). Ligation reaction was transformed in the *E. coli* cells (Top10F', Invitrogen) and the transformants were selected on the LB-agar medium containing 100 μg ml^{-1} carbenicillin. The correct orientation of insert was confirmed by carrying out a PCR using forward T7 promoter primer and reverse gene specific primer, as well as by sequencing. The recombinant plasmids of *MiGPPS* (*MiGPPS1*), *MiFPPS*, *MiGGPPS* (*MiGPPS2*) and *MiEO* were transformed in Rosetta(DE3)pLysS (Novagen, USA), BL21(DE3)pLysS (Invitrogen), BL21(DE3)Star (Invitrogen) and BL21(DE3) (Invitrogen) cells, respectively, for recombinant expression. LB medium containing 1 mM sorbitol and 2.5 mM betaine was used for the expression of *MiGPPS* (*MiGPPS1*) and *MiGGPPS* (*MiGPPS2*); whereas, LB media was used for expressing *MiFPPS* and *MiEO*. Starter culture (5 ml) grown for 48 hr at 18 °C was used as inoculum for the expression in 100 ml media with the Overnight Express Autoinduction System 1 (Novagen, USA). Cultures were grown for 24 hr at 18 °C and the pellet obtained after centrifugation was suspended in the buffer containing 25 mM MOPSO (pH 7.2), 10 mM $MgCl_2$

and 10% (v/v) glycerol for *MiGPPS* (*MiGPPS1*), *MiFPPS* and *MiGGPPS* (*MiGPPS2*); whereas, in the same buffer without $MgCl_2$ for *MiEO*. The cells were lysed by sonication and the $(his)_6$-tagged recombinant proteins were purified by passing the cleared lysate through Ni-NTA resin (Qiagen, Germany) following the manufacturer's instructions. Elution was carried out with the buffer containing 250 mM imidazole, 25 mM MOPSO (pH 7.2), 10 mM $MgCl_2$ and 10% (v/v) glycerol for *MiGPPS* (*MiGPPS1*), *MiFPPS* and *MiGGPPS* (*MiGPPS2*); whereas with the same buffer without $MgCl_2$ for *MiEO*. Both crude lysate and the purified protein were checked for the presence and size determination of the recombinant protein by SDS-PAGE (Sambrook and Russell, 2001).

2.10. Assay for the enzymatic activity

2.10.1. Isopentenyl diphosphate synthase

In vitro assays for determining the activity of *MiGPPS* (*MiGPPS1*), *MiFPPS* and *MiGGPPS* (*MiGPPS2*) were carried out in the final volume of 200 µl containing about 0.5 µg of the purified protein, 25 mM MOPSO (pH 7.0), 10% (v/v) glycerol, 2 mM DTT, 10 mM $MgCl_2$ and 67 µM of each DMAPP and IPP (Echelon Biosciences, USA). For the optimum pH determination of the recombinant enzyme, assays were performed in 25 mM MOPSO (pH 6 and 6.5), 25 mM HEPES (pH 7 and 7.5) or 25 mM TRIS (pH 8, 8.5 and 9) containing the other required components as mentioned above. The assays for determining the optimum Mg^{2+} concentration were performed at varied concentration of $MgCl_2$ along with the other required components as mentioned above. After overnight incubation, the assay reactions were deproteinized by washing with equal volume of chloroform and directly used for the LC-MS/MS analysis.

Analysis of isoprenoid pyrophosphates was performed on an Agilent 1200 HPLC system (Agilent Technologies, USA) coupled to an API 3200 triple quadrupole mass spectrometer (Applied Biosystems, USA). The column used was an Agilent ZORBAX Extended C-18; 1.8 µm, 50 x 4.6 mm (Agilent Technologies). Mobile phase consisted of 5 mM ammonium bicarbonate in

water as solvent A and acetonitrile as solvent B, flow rate was set at 0.8 ml min^{-1} and column temperature at 20 °C. Separation was achieved using a gradient starting at 0% B increasing to 10% B in 2 min, 64% B in 12 min and 100% B in 2 min keeping it at 100% B for 1 min followed by a change to 0% B in 1 min and keeping it there for 5 min before the next injection. Injection volume for samples and standards was 10 µl. Mass spectrometer was used in negative electrospray ionization mode. Optimal settings were determined using standards purchased from Sigma-Aldrich. Ion source gas 1 and 2 were set at 60 and 70 psi having a temperature of 700 °C, curtain gas was set at 30 psi and collision gas at 7 psi. Ion spray voltage was maintained at -4200 V. Monitored MRM transitions were m/z 312.9/79 for GPP, m/z 380.9/79 for FPP and m/z 449/79 for GGPP. Data analysis was performed using Analyst Software 1.5 Build 3385 (Applied Biosystems).

2.10.2. Enone oxidoreductase

Purified protein was incubated overnight at 30 °C with 60 mg fructose-1,6-diphosphate and 3 mg NADH in 1 ml buffer containing 25 mM MOPSO and 10% glycerol (pH 7). The products formed were purified by solid phase extraction (SPE) using the DSC-18 columns having the capacity of 3 ml (Sigma, USA). The SPE column was first equilibirated with acetonitrile, followed by the assay buffer. After passing the incubation mixture, the products were eluted from the coulmn with the help of dichloromethane and were analyzed by GC-MS. The product separation was carried out on the GsBP-5MS column having the dimensions of 30 m x 0.32 mm i.d. x 0.25 µm film thickness (General Separation Technologies, USA). Oven temperatures were programmed from 40 °C for 5 min, raised to 220 °C at 10 °C min^{-1} and held isothermal for 5 min. Injector and detector temperatures were 150 and 250 °C, respectively. Helium was used as carrier gas at a flow rate 1 ml min^{-1}. Mass spectra were obtained using Clarus 500 (Perkin Elmer) gas chromatograph–mass spectrometer at 70 eV with a scan time of 0.2 s. To enhance the selectivity of the detection, only the ion of m/z 128 of furaneol was monitored. In the separate analysis total ion chromatograph was also

recorded and was used for examining the spectra of the furaneol formed in the test assays.

2.11. Complementation assay for *MiGGPPS* (*MiGPPS2*)

The functional GGPPS from *Picea abies* (PaIDS5) (Schmidt and Gershenzon, 2007) was used as a positive control. The plasmid carrying carotenoide cluster genes from *Erwinia uredovora* except GGPPS (pACCARΔcrtE) (Sandmann et al., 1993), was a kind gift from Prof. G. Sandmann, University of Frankfurt, Germany. Plasmids in the combination of PaIDS5 + pACCARΔcrtE, *MiGGPPS* (*MiGPPS2*) + pACCARΔcrtE, and pACCARΔcrtE alone, were transformed in BL21(DE3)Star cells (Invitrogen). The transformants were selected on LB agar medium containing 50 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} carbenicillin and for the transformation of PaIDS5 + pACCARΔcrtE and *MiGGPPS* (*MiGPPS2*) + pACCARΔcrtE; and on LB agar medium containing only 50 μg ml^{-1} chloramphenicol for the transformation of pACCARΔcrtE alone. After growing at 28 °C, the colonies obtained for all the three transformation experiments were sub-cultured on LB agar medium containing 50 μg ml^{-1} chloramphenicol and were allowed to grow for 2 days at 28 °C.

2.12. Homology modeling

Three-dimensional structures of *MiFPPS* and *MiGGPPS* (*MiGPPS2*) were determined on CPHmodels 3.0 server (Nielsen et al., 2010). Avian FPPS (PDB ID: 1FPS) (Tarshis et al., 1994) which shows 50% sequence identity with *MiFPPS* and Mint GPPS (PDB ID: 3KRF) (Chang et al., 2010) which shows 78% sequence identity with *MiGGPPS* (*MiGPPS2*) were used as templates. Ramchandran plot assessment of the structure was carried out on RAMPAGE server (Lovell et al., 2003). Further quality parameters of the generated model were assessed on a web-based program, ProSA (Sippl, 1993; Wiederstein and Sippl, 2007). The final structure was visualized in the program UCSF Chimera, production version 1.5.

Table 2.4

Primers used for the real-time quantitative PCR of the Alphonso flavor genes

Primer	Gene	Orientation	Sequence (5' to 3')
G-Q-F	*MiGPPS*	Forward	AGGCTGCGCTCCATGGTAGTCA
G-Q-R	*MiGPPS*	Reverse	ACCGTGGGACGAAACCTCTTTCC
F-Q-F	*MiFPPS*	Forward	TGGGAAAGCAGATCCAGCCTGTGT
F-Q-R	*MiFPPS*	Reverse	TGCACTTTCATACTCTGCAAACGCA
GG-Q-F	*MiGGPPS*[a]	Forward	GACTGCTGGCAAAGATTTGGTGGCT
GG-Q-R	*MiGGPPS*[a]	Reverse	GGCGGCTTTCTCCTGATCAAAACCA
E-Q-F	*MiEO*	Forward	AGGTGCTGTAACACCTCCAGGCT
E-Q-R	*MiEO*	Reverse	CCTGGCTGAAAGGAAATGGCCCC
EF-F	*Elongation factor 1α*	Forward	AATACGACTCACTATAGGGCAAGCAG
EF-R	*Elongation factor 1α*	Reverse	ATACGACTCACTATAGGGCTCCTTCTC

[a] Later characterized as *GPPS*

2.13. qRT-PCR

Quantitative PCR was performed with Brilliant SYBR Green QPCR Master Mix (Stratagene, USA) with elongation factor 1α (EF1α) as a normalizing gene. Primers used for amplifying a fragment of *MiGPPS*, *MiFPPS*, *MiGGPPS* (*MiGPPS2*), *MiEO* and *MiEF1α* were as shown in Table 2.4. For all of the five genes, at least three amplicons were sequenced to confirm the primer specificity. Transcript abundance was quantified with a Mx3000P Real Time PCR Thermocycler (Stratagene) using a program with 45 cycles of 95 °C for 30 s, 63 °C for 30 s and 72 °C for 30 s, followed by a melting curve analysis of transcripts. The relative transcript abundance of the raw stage (0 DAH) of mango was considered 1 and the fold difference for the rest of the tissues was

calculated. Each measurement was repeated with four independent biological replicates, each of which was represented by at least two technical replicates. Ripening stages were compared to each other for the relative transcript abundance of each of the genes between the ripening stages and localities by ANOVA with the aid of Fisher's LSD at $p \leq 0.05$ using StatView software, version 5.0 (SAS Institute Inc., USA).

Results


GCACATCAGGGGCACAT
TCACTTGGACTTCACTT
GGGATCTTTAAGGGATC
CGGACATACATCGGACA
GCACATCAGGGGCACAT
TCACTTGGACTTCACTT
GGGATCTTTAAGGGATC
CGGACATACATCGGACA

3.1 Geographic variation in the Alphonso mango flavor volatiles

Three locations, namely, Dapoli, Deogad and Vengurle spanning the Konkan region of Alphonso mango cultivation as detailed in the Materials and methods section were covered for the volatile analysis. At each location, four trees and four stages of ripening were selected for this analysis to get statistically valid data. The details of the results obtained are given below.

3.1.1 Detection of volatiles

Thirty volatile compounds were detected, out of which twenty were quantitated in the fruits of four ripening stages from the three different localities (Fig. 3.1) (Table 3.1). Compounds were classified into four classes, viz. monoterpenes (13 compounds), sesquiterpenes (5 compounds), lactones (8 compounds), furanones (2 compounds) and others (2 compounds). Monoterpenes were found in the highest concentration (2.55 to 374 μg g^{-1}) in all the 48 tissues analyzed. (*Z*)–Ocimene, one of the monoterpenes, showed dominance throughout the range of the tissues studied (Table 3.1). Ten compounds, *viz.*, 2-hexanol, camphene, β-pinene, α-methylbutyrolactone, γ-valerolactone, β-terpinene, 4-carene, ocimene epoxide, β-cyclocitral and β-ionone which were detected in trace levels only in some tissues were not quantified. Furaneol, one of the most important flavor compounds in mango identified in our previous studies (Pandit et al., 2009a; Pandit et al., 2009b) could not be quantified because of its inconsistent detection.

3.1.2. Ripening related changes

All the 12 mono- and sesquiterpenes were detected in all the four ripening stages. For all the three localities, the highest concentration of total monoterpenes was found in the ripe fruits (Fig. 3.2). When the trend of appearance of each of the monoterpenes through the ripening stages was compared statistically, for Dapoli, all the monoterpenes except p-cymene showed the highest quantity in the ripe fruit. In case of Deogad fruits,

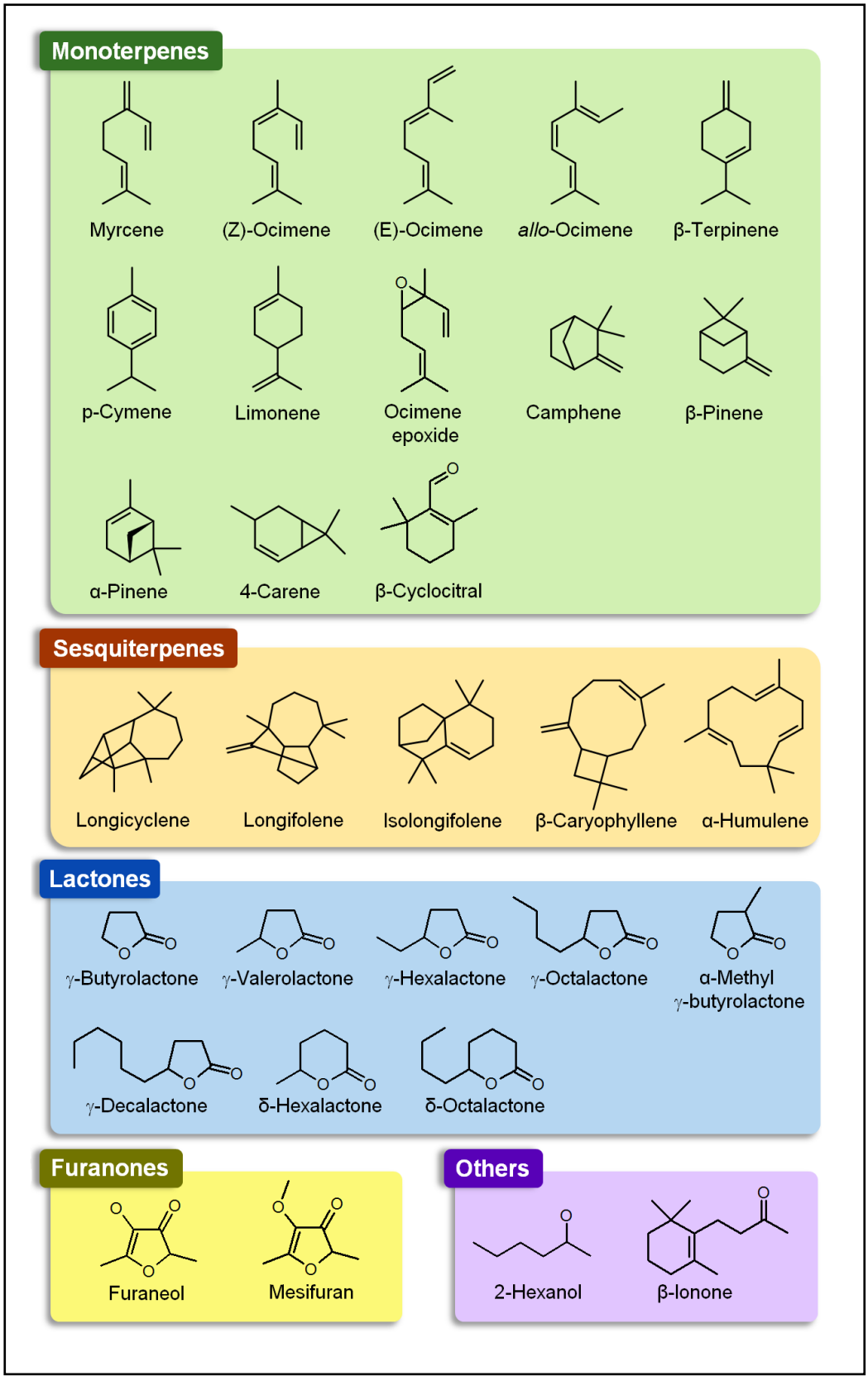


Figure 3.1

Classification and chemical structures of the volatile compounds detected in Alphonso mango

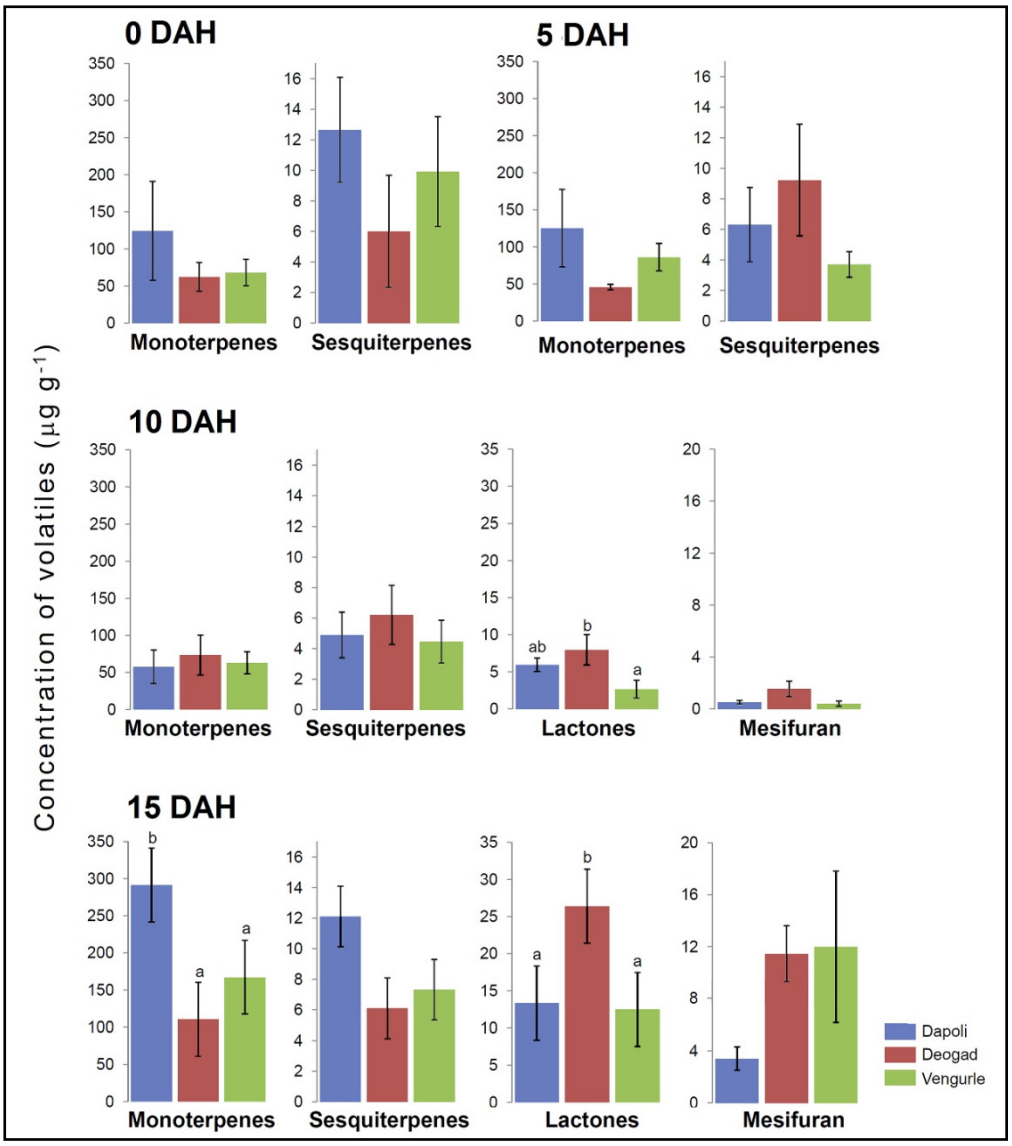


Figure 3.2

Concentration (µg g^{-1} tissue) of various volatile classes in the ripening fruits of Alphonso from three different localities, Dapoli, Deogad and Vengurle in the Konkan region, Maharashtra, India. Lactones and furanones were not detected in 0 DAH and 5 DAH fruits. The columns having different alphabets on the top indicate significantly different ($p \leq 0.05$) values.

although the highest quantity of all except p-cymene was detected in the ripe fruit, none of the compounds differed statistically between the ripening stages for their quantity. For Vengurle, all the monoterpenes, except p-cymene and

Table 3.1

Volatile composition (μg g^{-1} tissue) of ripening fruits (DAH: days after harvest) of Alphonso mango from three cultivation localities, Dapoli, Deogad and Vengurle in Maharashtra, India. Values shown are average of four plants sampled from each locality and those followed by ± symbol indicate standard error of measurements. Compounds have been ordered according to their retention indices.

Compounds	0 DAH			5 DAH			10 DAH			15 DAH		
	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle
α-Pinene[a]	0.14±0.05	0.09±0.03	0.06±0.01	0.15±0.05	0.07±0.01	0.09±0.02	0.05±0.03	0.11±0.03	0.06±0	0.3±0.08	0.14±0.02	0.23±0.02
γ-Butyrolactone	0	0	0	0	0	0	0.52±0.09	0.72±0.21	0.34±0.15	1.52±0.39	5.5±1.69	2.07±0.66
Myrcene[a]	0.84±0.42	0.43±0.13	0.51±0.13	0.89±0.37	0.36±0.03	0.54±0.08	0.4±0.15	0.49±0.17	0.45±0.12	2.18±0.32	0.71±0.12	1.17±0.28
p-Cymene[a]	0.13±0.05	0.04±0.03	0.15±0.08	0	0.04±0.04	0.04±0.02	0.04±0.02	0.06±0.06	0.02±0.01	0.07±0.04	0.02±0.02	0.03±0.03
Limonene[a]	0.19±0.04	0.13±0.03	0.18±0.04	0.13±0.06	0.14±0.05	0.22±0.03	0.11±0.02	0.15±0.01	0.11±0.02	0.3±0.03	0.18±0.02	0.21±0.02
(*Z*)-Ocimene	116±63.2	58.1±18.3	62.9±17.2	116±48.9	42.1±3.76	81.1±18.5	53.5±21.7	68.6±25.9	58.4±14.4	273±39.5	104±26.4	157±40.8
(*E*)-Ocimene	5.1±2.69	2.58±0.8	2.88±0.8	5.59±2.43	2.05±0.2	2.93±0.4	2.41±0.96	2.84±0.96	2.65±0.67	11.5±2.77	4.12±0.85	6.74±1.63
Mesifuran[a]	0	0	0	0	0	0	0.51±0.14	1.54±0.59	0.38±0.23	3.38±0.93	11.4±2.17	12.0±5.83
γ -Hexalactone[a]	0	0	0	0	0	0	1.33±0.16	1.81±0.43	0.52±0.23	2.64±0.43	4.57±0.53	2.29±0.42
δ-Hexalactone[a]	0	0	0	0	0	0	0.88±0.23	1.22±0.46	0.44±0.23	1.92±0.53	7.48±1.78	2.58±0.71
Allo-ocimene	1.45±0.69	0.74±0.23	0.76±0.22	1.79±0.71	0.66±0.1	0.72±0.15	0.73±0.3	0.99±0.29	0.88±0.21	3.17±0.36	1.23±0.25	1.72±0.57

Compounds	0 DAH			5 DAH			10 DAH			15 DAH		
	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle	Dapoli	Deogad	Vengurle
γ -Octalactone[a]	0	0	0	0	0	0	2.08±0.37	2.54±0.7	0.95±0.51	5.34±1.22	5.74±0.52	3.9±0.92
δ -Octalactone	0	0	0	0	0	0	1.04±0.13	1.58±0.36	0.42±0.13	1.7±0.28	2.86±0.16	1.39±0.29
Longicyclene	1.83±0.3	0.73±0.63	1.79±0.97	0.28±0.13	1.62±1.06	0.7±0.35	0.45±0.12	1.14±0.69	0.48±0.2	1.18±0.47	0.62±0.36	0.84±0.55
Isolongifolene[a]	0.65±0.12	0.25±0.1	1.14±0.9	0.23±0.05	0.6±0.15	0.36±0.14	0.47±0.09	1.06±0.66	0.54±0.03	1.38±0.68	0.6±0.26	0.77±0.42
Longifolene[a]	6.36±2.53	2.28±2.06	5.44±2.03	1.2±1	4.64±2.74	1.26±0.68	1.68±0.72	1.9±1.07	2.1±1.26	3.03±0.79	1.59±0.76	1.97±1.35
β-Caryophyllene[a]	2.43±1.14	1.75±0.67	0.99±0.26	3±1.21	1.5±0.18	0.87±0.22	1.5±0.53	1.38±0.46	0.9±0.15	4.15±0.66	2.11±0.51	2.48±0.69
Humulene[a]	1.36±0.59	0.98±0.38	0.55±0.16	1.58±0.61	0.87±0.1	0.51±0.11	0.77±0.29	0.72±0.25	0.43±0.06	2.37±0.4	1.17±0.32	1.25±0.36
γ -Decalactone[a]	0	0	0	0	0	0	0.08±0.04	0.07±0.01	0.07±0.01	0.2±0.01	0.23±0.06	0.22±0.07
Unidentified	0	0	0	0	0	0	0.59±0.2	0.7±0.28	0.85±0.18	0.36±0.11	2.85±1.08	0.6±0.23

[a] These compounds were identified by comparing their mass spectrum and Kovat's index with those of authentic external standards; whereas, rest of the compounds were identified by comparing their mass spectrum and Kovat's index with those reported in the literature

limonene showed significantly higher quantity in the ripe fruit as compared to the other stages (Table 3.1).

Out of the five sesquiterpenes detected, β-caryophyllene and humulene showed statistically higher values in the ripe fruit than any other stage for all the three localities. The pattern of appearance of the other three sesquiterpenes, longicyclene, isolongifolene and longifolene was quite inconsistent; each of them showed dominance at different stages through the localities, however, the difference between the stages was significant only for Dapoli (Table 3.1).

Lactones and mesifuran were detected as ripening related compounds and their trend of appearance through the ripening stages was similar for all the three localities. They were completely absent in raw and 5 DAH tissues and the highest quantity of total and each of these ripening related compounds was observed in the ripe fruit (Table 3.1). In addition to lactones and furanones, five unidentified ripening-related compounds were detected; out of which one was quantified (Table 3.1, Fig. 3.3a) whereas four others were not quantified because of their low amounts (Fig. 3.3b-e).

3.1.3. Cultivation-locality wise variation

Qualitatively the volatile composition of the fruits from all the three sampling localities selected for the present study was similar; there was only quantitative difference in the appearances of the volatiles.

3.1.3.1. *15 DAH*

Quantity of total monoterpenes in the ripe fruits from Dapoli (201-374 μg g^{-1}) was significantly higher than that of Deogad (57-187 μg g^{-1}) and Vengurle (73-282 μg g^{-1}). Similar trend was observed when the compounds were considered individually. Level of all the seven monoterpenes was significantly higher for Dapoli fruits as compared to Deogad (Fig. 3.2). Dapoli fruits also had higher content of all the monoterpenes when compared with Vengurle, and the difference was significant for myrcene, limonene, (*Z*)-ocimene and *allo*-

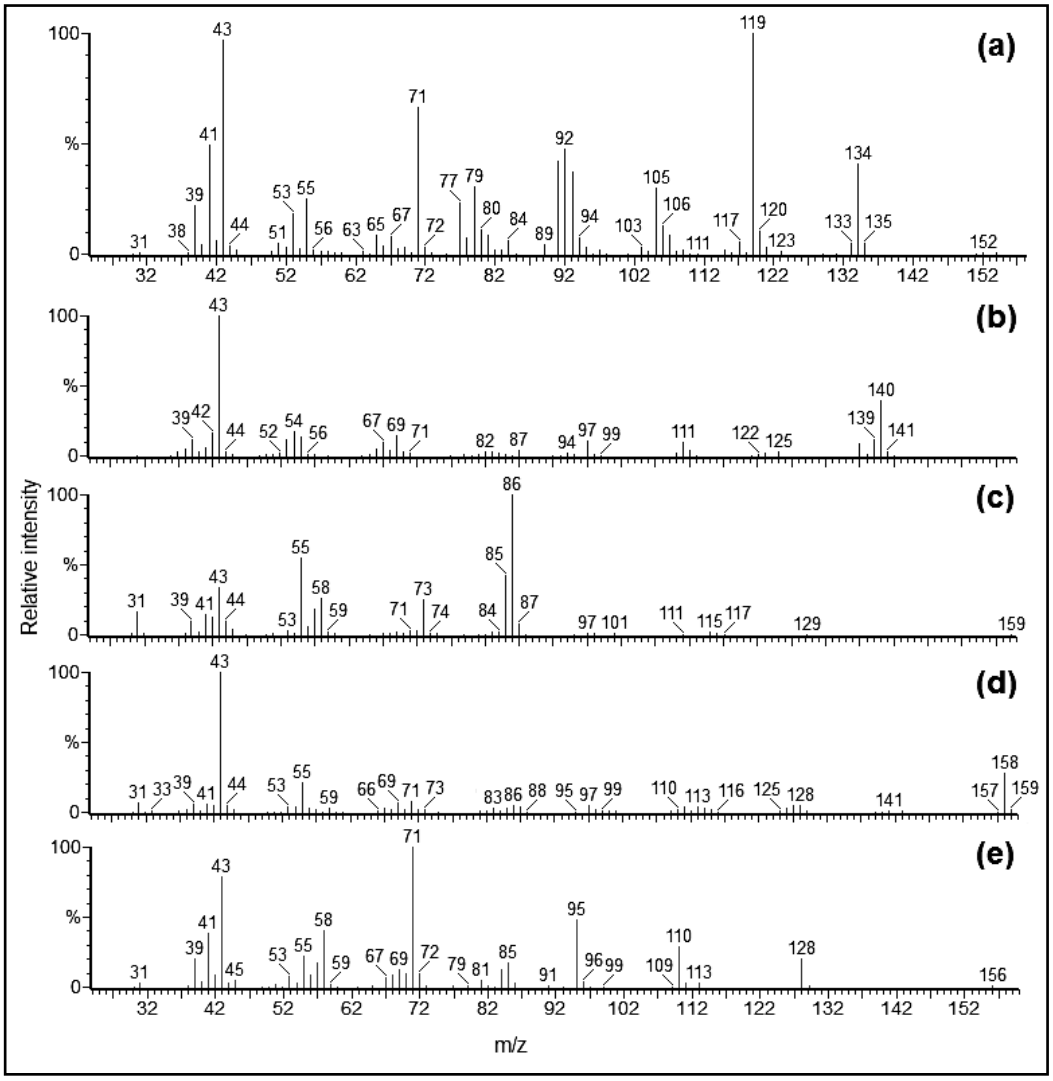


Figure 3.3
Mass spectra of an unidentified but quantified (a) and unidentified and unquantified (b-e) ripening-related compounds in Alphonso mango.

ocimene (Table 3.1). Vengurle fruits had higher level of all the monoterpenes as compared to Deogad.

Similar to monoterpenes, total and individual sesquiterpenes were present in decreasing quantities from Dapoli (7.4-14 μg g^{-1}) to Vengurle (2.8-15 μg g^{-1}) to Deogad (1.5-9.1 μg g^{-1}). Nevertheless, the difference was insignificant with respect to total sesquiterpenes content (Fig. 3.2). Similarly, each of the sesquiterpenes, when considered independently, was present in decreasing amount from Dapoli to Vengurle to Deogad and the higher amounts of β-

caryophyllene and humulene in Dapoli than Deogad were statistically significant (Table 3.1).

Level of total lactones was significantly higher in Deogad (19-26 μg g^{-1}) as compared to Dapoli (8-19 μg g^{-1}) and Vengurle (7-19 μg g^{-1}) (Fig. 3.2). When considered individually, all the lactones occurred in higher quantity in Deogad fruits as compared to Dapoli and Vengurle and the difference was significant for four (γ-butyrolactone, γ-hexalactone, δ-hexalactone and δ-octalactone) of the six lactones (Table 3.1). The quantity of total and individual lactones was statistically similar for Dapoli and Vengurle (Fig. 3.2). The ratio of γ-lactones/δ-lactones was significantly higher for the fruits from Dapoli than those from Deogad; whereas, for Vengurle, this ratio was higher than that for Deogad and lower than that of Dapoli (Fig. 3.4). Mesifuran was the only furanone detected in the present study; its quantity was higher in Deogad and Vengurle as compared to Dapoli (Fig. 3.2).

When the quantity of each of the above-mentioned four chemical classes was compared to each other on a radar plot (Fig. 3.5), Deogad fruits

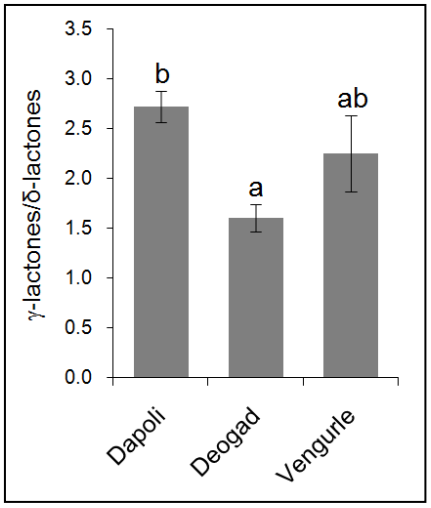


Figure 3.4

Ratio of quantities of γ-lactones/δ-lactones in the ripe fruits (15 DAH) of Alphonso mango. The columns having different alphabets on the top indicate significantly different ($p \leq 0.05$) values.

were clearly seen as lactone and mesifuran dominant as compared to Dapoli fruits which could be seen as mono- and sesquiterpene dominant. Vengurle region was similar to Deogad in terms of mesifuran content and to Dapoli in terms of lactone content. On the scale of terpenes, Vengurle fruits showed average quantities.

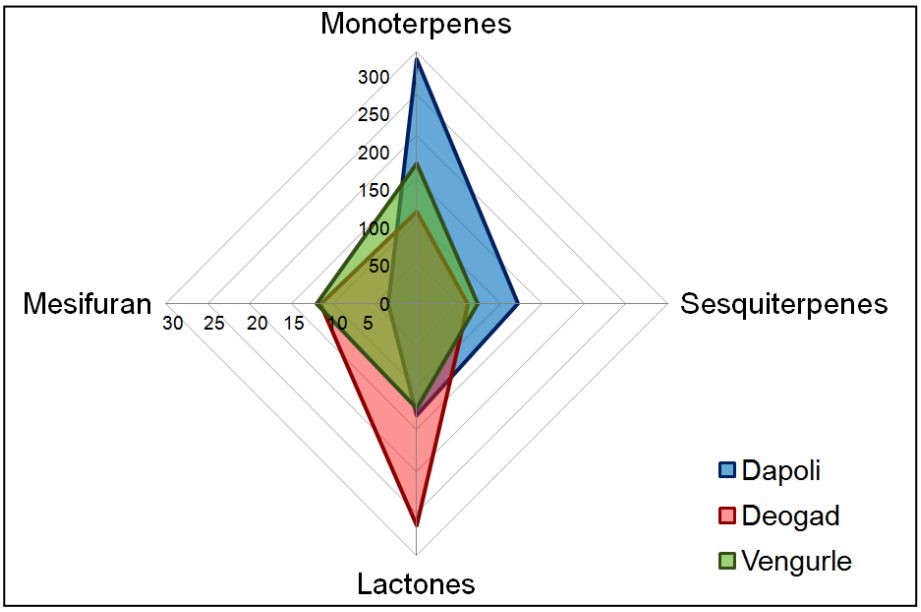


Figure 3.5
Radar plot of monoterpene, sesquiterpene, lactone and mesifuran content of ripe Alphonso fruits from three localities, Dapoli, Deogad and Vengurle in India. Values across the axis represent volatile concentration (µg g^{-1} tissue). Axis scale for sesquiterpenes and lactones is the same as that of mesifuran

3.1.3.2. *10 DAH*

When 10 DAH stage of each of the three localities was compared with each other, Deogad fruits had slightly higher level of the mono- and sesquiterpenes (32-153 and 2.8-11 µg g^{-1}) as compared to Dapoli (2.6-105 and 1.4-8.8 µg g^{-1}) and Vengurle (29-101 and 2.2-8.5 µg g^{-1}), but the difference was insignificant (Fig. 3.2). Also, all the individual monoterpenes and two sesquiterpenes, longicyclene and isolongifolene were present in the highest quantity in Deogad fruits (Table 3.1). Significantly higher amount of total lactones was detected in Deogad (3.3-13 µg g^{-1}) in comparison with Vengurle (0.3-5.5 µg g^{-1})

but not in comparison with Dapoli (3.3-7.7 μg g^{-1}) (Fig. 3.2). When considered individually, γ-hexalactone and δ-octalactone were detected in higher level in Deogad compared to the other two localities (Table 3.1). Deogad fruits contained higher amount of mesifuran than that of Dapoli and Vengurle.

3.1.3.3. *5 DAH*

Total monoterpene content of Dapoli fruits (22-220 μg g^{-1}) was higher than that of Deogad (34-51 μg g^{-1}) and Vengurle (41-133 μg g^{-1}) and sesquiterpene content of Deogad fruits (2.8-19 μg g^{-1}) was slightly higher than that of Dapoli (1.4-11.8 μg g^{-1}) and Vengurle (2.1-5.8 μg g^{-1}), but the difference was insignificant (Fig. 3.2). When considered individually, five out of seven monoterpenes along with the two sesquiterpenes were higher in Dapoli and the rest three sesquiterpenes were higher in Deogad; in this case also the difference was insignificant (Table 3.1).

3.1.3.4. *0 DAH*

Six of the seven monoterpenes and four of the five sesquiterpenes along with total mono- and sesquiterpenes were higher in Dapoli (38.2-320 and 5.5-21 μg g^{-1}) as compared to Deogad (4.4-90 and 0.6-17 μg g^{-1}) and Vengurle (47-117 and 1.3-18 μg g^{-1}); however, the difference was insignificant (Table 3.1, Fig. 3.2).

3.1.4. Principal component analysis

To analyze the complex data of quantitative distribution of volatiles across various geographic locations through various ripening stages, PCA was carried out by treating the data in various combinations.

The first dataset for PCA had quantity of four classes of volatiles, viz., monoterpenes, sesquiterpenes, lactones and furanones in the ripe fruit of all the plants from each of the three localities. Four principle components could extract all the 100% variation in the data. The first and the second principal components together accounted for 71% of the total observed variance (Fig.

3.6). PC1 had high positive loadings for mono- and sesquiterpenes and negative loading for lactones and a furanone. On the other hand, these ripening specific compounds contributed high positive loading to PC2 (Fig. 3.6, inset). Mono- and sesquiterpenes also depicted positive loading for PC2, but the magnitude was lower than that of lactones and furanones. In the score plot of the principle components, all the three localities, each represented by

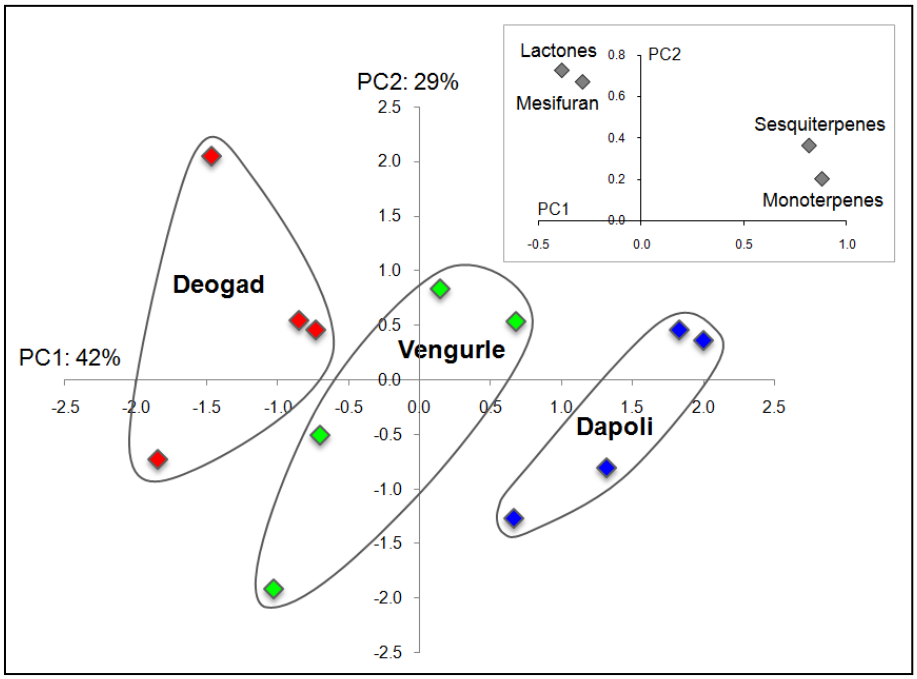


Figure 3.6
Score plot and loading plot (inset) of Principal Component Analysis of monoterpene, sesquiterpene, lactone and mesifuran content of the ripe (15DAH) fruits of Alphonso from three localities, Dapoli, Deogad and Vengurle in Konkan, India. Points in each group of localities represent each of the four plants sampled from the respective locality.

four plants could be clearly separated from each other, with no overlapping regions (Fig. 3.6). This separation was mainly across PC1, with Deogad and Dapoli localities having negative and positive scores, respectively and Vengurle group located at the centre of the plot. When score plot and loading plot were compared to each other, presence of Deogad plants across the second and the third quadrant could be easily correlated to the negative loading for lactones and mesifuran across PC1. Similarly, high positive score

of Dapoli plants across PC1 can be attributed to the high positive loadings for mono- and sesquiterpenes.

Principle components were also extracted from the data having quantities of the twenty volatiles in four ripening stages from all the three localities. In this case also, four principal components could extract 100% variation in the data; whereas the first two components explained 88% of the observed variation. In the score plot of the first two principal components, ripe fruits from all the three localities were similar in terms of high positive score for PC1. Across PC2, the localities showed clear differentiation with high positive score for Dapoli, high negative score for Deogad and a low negative score for Vengurle (Fig. 3.7a). 10 DAH stages of all the three localities were located in the third quadrant; whereas, 0 DAH and 5 DAH stages were mixed with each other and were placed in the second and third quadrant, with no clear differentiation between the localities.

The pattern of qualitative and quantitative appearance of each of the twenty volatile compounds through the ripening stages across the geographic locations is also depicted by the loading plot (Fig. 3.7b). Three groups could be clearly segregated on the plot and the one with high positive loading across PC1 and high negative loading for PC2, was the most clear group formed by the ripening related compounds, lactones and furanones. Close proximation of the compounds in this group indicated high correlation between their quantities across various samples. The second group lying in the first quadrant of the plot was formed by six monoterpenes and two sesquiterpenes (β-caryophyllene and humulene); whereas, the third cluster was constituted by three sesquiterpenes (longicyclene, isolongifolene and longifolene) with p-cymene placed near this group.

3.1.5. Variation in terms of flavor

The minimum concentration at which any volatile compound can be detected is called odor detection threshold and the relative aroma contribution of each compound or odor units are calculated by dividing concentration of the compound by its odor detection threshold (Goldstein, 2009). In the present

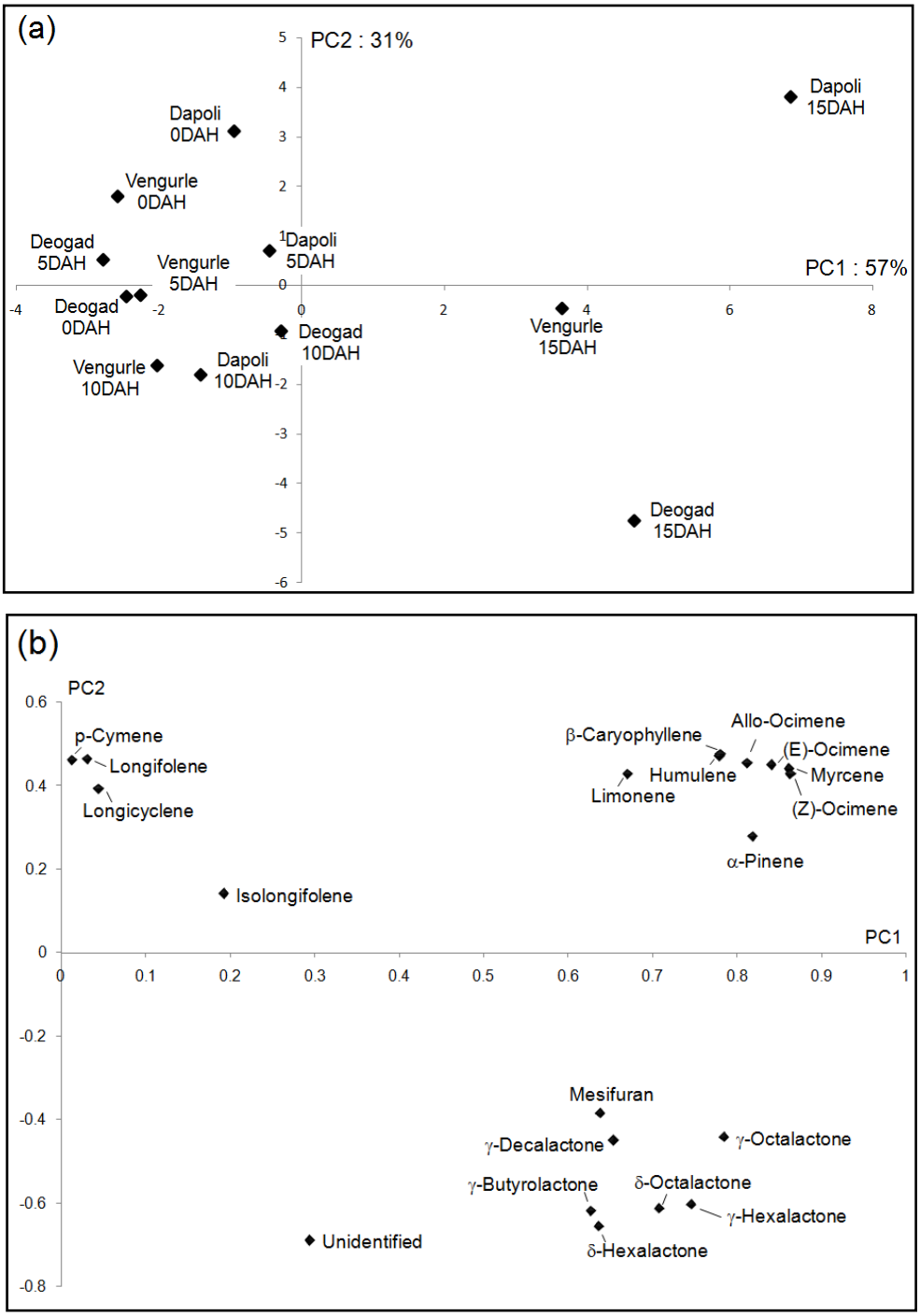


Figure 3.7

Score plot (a) and loading plot (b) of Principal Component Analysis for quantity of each of the 20 volatiles detected in the four ripening stages of the Alphonso fruits from three localities, Dapoli, Deogad and Vengurle in India (DAH: days after harvest). Average compound quantities of four plants were considered for each stage from each locality.

data, out of twenty volatiles quantified in the ripe fruit (15 DAH), odor units were calculated for twelve compounds for which odor detection threshold values were available in the literature (Table 3.2). Three compounds having the lowest odor detection threshold were mesifuran, α-pinene and γ-octalactone. When the odor detection thresholds of the compounds were correlated with their concentration in the Alphonso mesocarp, for all the three localities, the highest odor units were contributed by mesifuran followed by (*Z*)-ocimene and γ-octalactone. Since (*Z*)-ocimene has different odor character than mesifuran and γ-octalactone, ratios of its odor units were calculated with

Table 3.2

Odor units of some compounds in the ripe (15 DAH) fruits of Alphonso from three cultivation localities, Deogad, Dapoli and Vengurle, India. Values indicated are average of four plants for each locality.

Compound	ODT (ppb)	Dapoli	Deogad	Vengurle
α-Pinene	6	50	24	38
Myrcene	14	155	51	83
p-Cymene	150	0.5	0.1	0.2
Limonene	10	30	18	21
(*Z*)-Ocimene	55	4979	1889	2851
Mesifuran	0.03	112696	381455	399773
γ-Hexalactone	1600	1.7	2.9	1.4
γ-Octalactone	7	762	820	558
δ-Octalactone	400	4.3	7.1	3.5
β-Caryophyllene	64	65	33	39
Humulene	120	20	10	10
γ-Decalactone	11	18	20	20

these compounds. Deogad fruits had high ratio of mesifuran/ (*Z*)-ocimene and γ-octalactone/ (*Z*)-ocimene as compared to Dapoli; whereas, for Vengurle, these ratios were in between those for Deogad and Vengurle (Fig. 3.8).

In order to get insights into the biosynthesis of flavor volatiles and regulation of their variation through the ripening stages of Alphonso mango from different cultivation locations as detailed above, molecular studies were undertaken in the perspective of isolation and characterization of a few key genes involved in the biosynthesis of terpenes and furanones which are the two important components of Alphonso mango flavor.

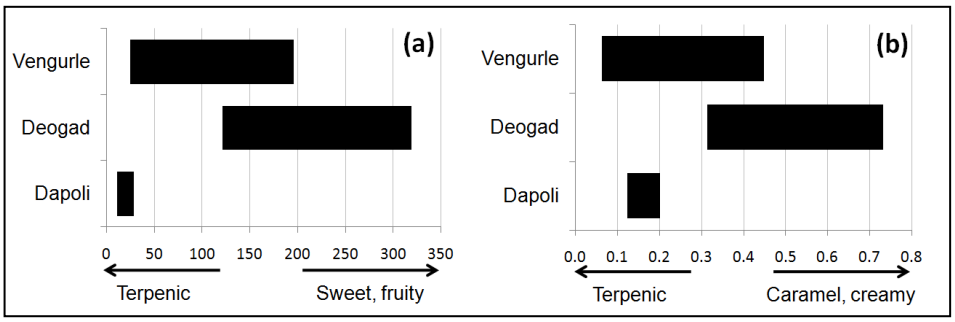


Figure 3.8

Odor unit ratios of mesifuran/(*Z*)-ocimene (a) and γ-octalactone/(*Z*)-ocimene (b) in the ripe Alphonso fruits from three localities, Dapoli, Deogad and Vengurle in India.

3.2. Molecular cloning and characterization of two geranyl pyrophosphate synthases from Alphonso mango

3.2.1. Isolation of *MiGPPS* (*MiGPPS1*) and *MiGGPPS* (*MiGPPS2*)

Based on the conserved regions of GPPS and GGPPS reported from the other plants, primers were designed and used to amplify an interdomain fragment of these genes from Alphonso mango. The fragments obtained showed high similarity to the respective genes reported from the other plants. Based on the nucleotide sequence of these fragments, gene specific primers were designed so as to have an overlapping region of 327 and 183 bp between 5' and 3' RACE fragments for GPPS and GGPPS, respectively. The full-length open

reading frames were finally obtained using terminal primers based on the sequences of the RACE fragments. The fragments obtained at the end of all of the above steps encoded an amino acid sequence without a stop codon. For GPPS, sequencing of at least 20 recombinant plasmids after RACE yielded variable sequences which differed from each other in terms of nucleotide substitutions. Utilizing these variations, variable primers were designed and were used to obtain 13 variable open reading frames of GPPS which shared about 93 to 99% sequence identity with each other at the amino acid level (Table 3.3). Out of these sequences, only one was used for the further analysis.

The 1266 bp long complete open reading frame (ORF) of *MiGPPS* (*MiGPPS1*), flanked by 112 bp untranslated region (UTR) at the 5' end and 242 bp UTR at the 3' end, encoded a protein of 421 amino acids with the predicted molecular weight of 46.2 kD and the isoelectric pH of 6.19. Similarly, the 987 bp long ORF of *MiGGPPS* (*MiGPPS2*), flanked by 343 bp UTR at the 5' end and 144 bp UTR at the 3' end encoded a protein of 328 amino acids with the predicted molecular weight of 35.6 kD and isoelectric point of 5.5. The *in silico* translated *MiGPPS* (*MiGPPS1*) showed the highest sequence similarity with the GPPS from *Quercus robur* (80% identity), *Arabidopsis* (71% identity) and *Picea abies* (66% identity) (Fig. 3.9). The putative protein sequence of MiGGPPS (MiGPPS2) on the other hand showed the highest sequence similarity to the GGPPS from *Corylus avellana* (84% identity) and *Lupinus albus* (79% identity), the large subunit (LSU) of the heterodimeric G(G)PPS from *Humulus lupulus* (81% identity) and the LSU of heterodimeric GPPS from snapdragon (78% identity).

3.2.2. Phylogenetic analysis

To understand the evolutionary relationships of MiGPPS (MiGPPS1) and MiGGPPS (MiGPPS2) with the short chain prenyltransferases from the other plants, phylogenetic analysis was carried out using the deduced amino acid sequences of MiGPPS (MiGPPS1), MiGGPPS (MiGPPS2) and a few

Table 3.3

Variable amino acids in the *in silico* translated open reading frames of *GPPS* from mango

Variant	Amino acids																																										
	103	104	105	106	119	127	148	197	222	227	251	252	255	258	260	265	285	286	290	295	327	329	332	340	350	351	358	360	362	366	371	381	392	393	396	398	399	406
G1	V	T	E	V	I	R	D	F	L	V	E	Q	S	H	M	C	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G2	V	T	E	V	M	R	D	F	L	V	E	E	S	H	M	Y	S	T	K	Y	I	L	I	V	V	D	L	I	I	Y	G	T	D	T	E	S	D	A
G3	V	T	E	V	M	R	E	L	L	V	D	Q	S	Y	M	Y	S	A	M	F	I	H	V	M	I	D	S	V	V	Y	R	T	D	A	K	D	N	S
G4	V	T	E	V	M	R	E	F	L	V	D	Q	S	Y	M	Y	S	A	M	F	I	H	V	M	I	N	S	V	V	Y	R	M	D	A	E	H	N	S
G5	–	–	–	–	M	R	D	F	L	V	E	E	S	H	I	Y	S	T	K	Y	I	L	I	M	V	D	L	I	I	Y	G	T	D	T	E	S	D	A
G6	–	–	–	–	I	H	D	F	L	V	E	Q	S	H	M	Y	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G7	–	–	–	–	I	R	D	F	L	A	E	Q	S	H	M	Y	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	G	T	E	S	D	A
G8	–	–	–	–	I	R	D	F	S	V	E	Q	S	H	M	Y	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G9	–	–	–	–	I	R	D	F	L	V	E	Q	S	H	M	Y	P	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G10	–	–	–	–	I	R	D	F	L	V	E	Q	S	H	M	Y	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G11	–	–	–	–	M	R	D	F	L	V	E	E	S	H	M	Y	S	T	K	Y	I	L	I	M	V	D	L	I	I	H	G	T	D	T	E	S	D	A
G12	–	–	–	–	I	R	D	F	L	V	E	Q	G	H	M	Y	S	T	K	Y	V	L	V	M	V	D	S	I	I	Y	R	T	D	T	E	S	D	A
G13	–	–	–	–	M	R	E	L	L	V	D	Q	S	Y	M	Y	S	A	M	F	I	H	V	M	I	N	S	V	V	Y	R	M	D	A	E	H	N	S

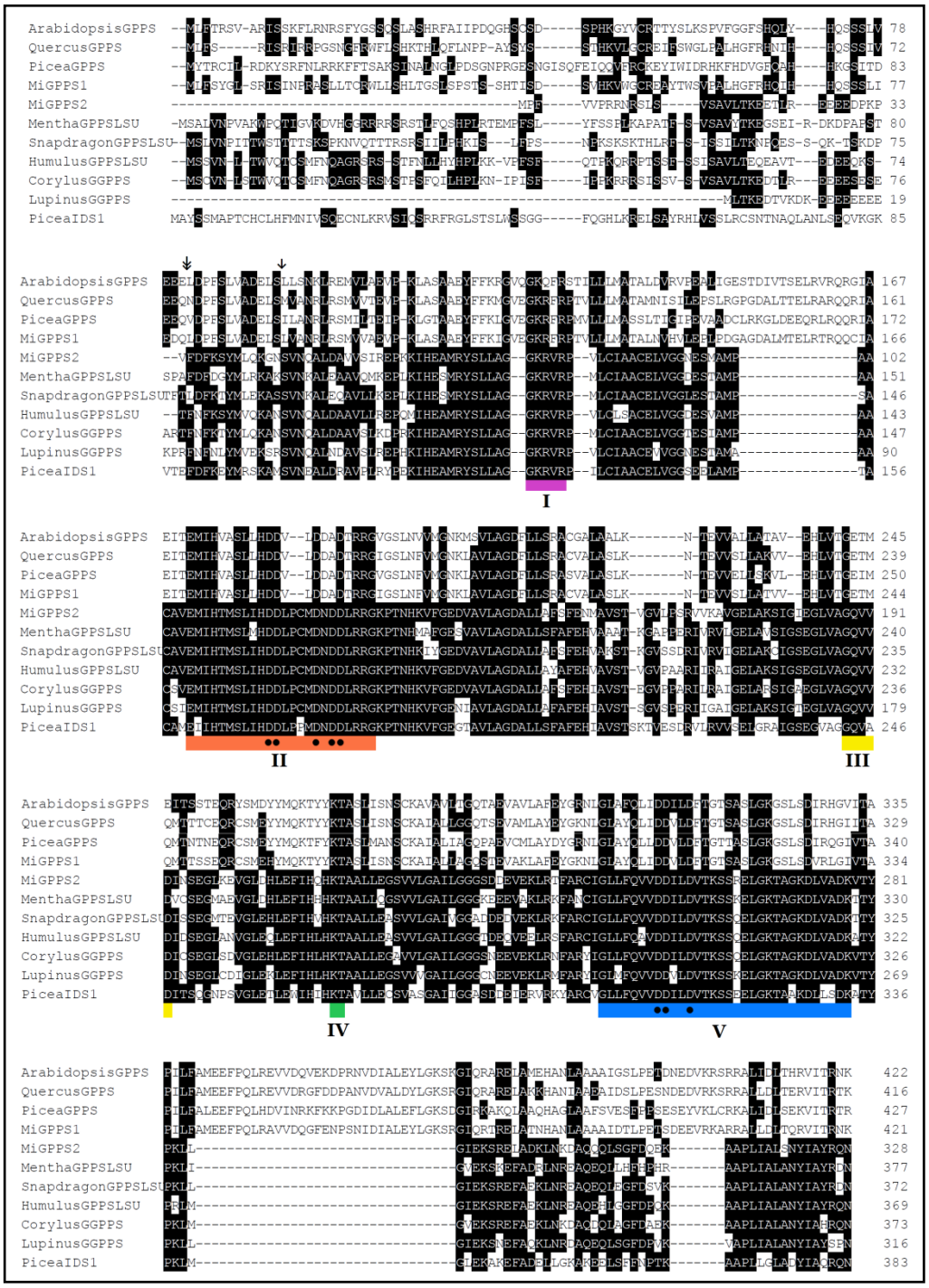


Figure 3.9

Alignment of *in silico* translated sequences of *MiGPPS1* and *MiGPPS2* with the closest characterized orthologs. Five regions which are conserved among the prenyltransferases (I-V) are indicated by purple, coral, yellow, green and blue color, respectively. Aspartate residues of FARM and SARM of region II and V, respectively, are indicated by black filled circles. The truncation site for MiGPPS1 is indicated by an arrow; whereas that of MiGPPS2 is shown by the arrow with two heads.

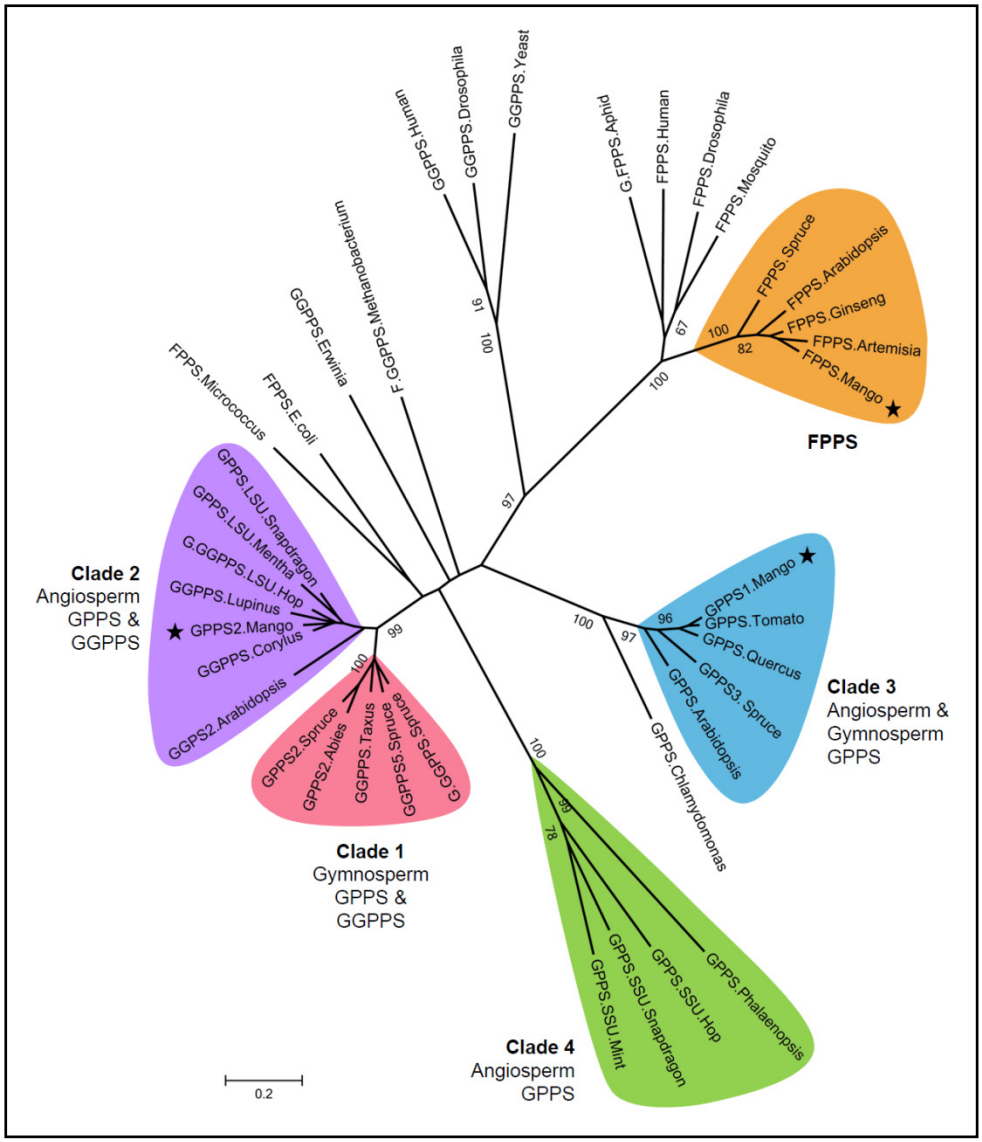


Figure 3.10

Neighbour-joining tree between a few functionally characterized isopentenyl diphosphate synthases from the other organisms and from mango (indicated by star symbol). The values at the branches indicate the bootstrap values obtained with 1000 replicates.

NCBI accession numbers of the sequences used were: GPPS: Arabidopsis, CAC16849; *Abies grandis*, AAN01134; *Quercus robur*, CAC20852; Spruce (GPPS2), ACA21458; Spruce (GPPS3), ACA21459; Tomato, ABB88703; *Phalaenopsis bellina*, ABV71395; Mint (LSU), AAF08793; Snapdragon (LSU), AAS82860; Hop (LSU), ACQ90682; Mint (SSU), AF182827; Snapdragon (SSU), AAS82859; Hop (SSU), ACQ90681; Aphid (G/FPPS), AAY33491; *Chlamydomonas reinhardtii*, XP_001691069; FPPS: Arabidopsis, CAB80990; Spruce, ACA21460; *Panax ginseng*, AAY87903; *Artemisia spiciformis*, AAP74719; Drosophila, NP_477380; Mosquito, XP_308653; Human, NP_001995; *Escherichia coli*, NP_414955; *Micrococcus luteus*, AA25265; *Methanothermobacter marburgensis* (F/GGPPS), YP_003849447; GGPPS: Arabidopsis, NP_179960; *Corylus avellana*, ABW06960; *Taxus canadensis*, AAD16018; *Lupinus albus*, AAA86688; Spruce (GGPPS5), ACA21461; Spruce (G/GGPPS), ACZ57571; Drosophila, AAC05273; Human, NP_004828; *Erwinia uredovora*, P21684; Yeast, NP_015256.

functionally characterized isopentenyl diphosphate synthases from the other organisms (Fig. 3.10). The analysis indicated that plant GPPS are closer to GGPPS than to FPPS which formed a clearly distinct cluster. GPPSs were scattered in four different clades and were accompanied by GGPPS in clade 1 (formed by gymnosperm GPPS and GGPPS) and clade 2 (formed by angiosperm GGPPS, GPPS-LSU and MiGGPPS (MiGPPS2)). Clade 3 contained the angiosperm and gymnosperm GPPS including MiGPPS (MiGPPS1). Clade 4, on the other hand had the small subunit of GPPS which along with the LSU of clade 2 forms a functional heterodimeric GPPS in angiosperms.

3.2.3. Recombinant expression and *in vitro* enzymatic assays

The proteins of GPPS and GGPPS have a plastid targeting sequence at their N-terminal, since biosynthesis of mono- and diterpenes is thought to be localized in the plastids. But analysis of the *in silico* translated amino acid sequences of *MiGPPS* (*MiGPPS1*) and *MiGGPPS* (*MiGPPS2*) by the TargetP program (http://www.cbs.dtu.dk/services/TargetP) did not give clear indication of the presence of such transit peptide. Still, for both *MiGPPS* (*MiGPPS1*) and *MiGGPPS* (*MiGPPS2*), the truncation sites were decided based on their alignment with the sequences reported earlier (Fig. 3.9). The complete ORFs as well as the ORFs truncated at the 5' end were cloned in the pEXP5 CT TOPO vector for expressing them as $(his)_6$-tagged recombinant proteins. To know whether the protein would be soluble upon recombinant expression in *E. coli*, sequence based prediction was carried out on the ProSO server (Smialowski et al., 2007). In agreement with the prediction that none of these proteins would be soluble in *E. coli*, no much protein was obtained in the soluble fraction for MiGPPS (MiGPPS1) and MiGGPPS (MiGPPS2) during the initial experiments. Out of the several modifications made in the experiments for obtaining soluble protein (such as inducing at lower OD_{600}, using lower concentration of IPTG, giving heat shock before induction, modifying content of the growth media), inclusion of 1 mM sorbitol and 2.5

mM betaine in the LB media (Blackwell and Horgan, 1991) yielded active protein (see below) in the soluble fraction (Fig. 3.11).

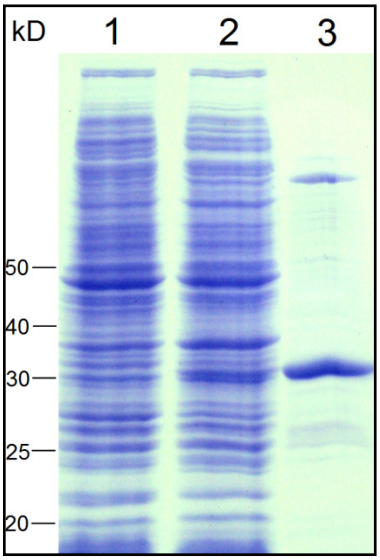


Figure 3.11

SDS-PAGE analysis of the crude lysate of the cells carrying empty vector (lane 1), crude lysate of the cells carrying *MiGPPS2* construct (lane 2) and the purified recombinant MiGPPS2 protein.

To get functional insights into the recombinant proteins, *in vitro* assays were carried out by incubating the recombinant proteins with IPP and DMAPP substrates and the products were analyzed by LC-MS/MS. None of the isopentenyl diphosphate products were detected in the assays with the soluble fraction of the full-length and the truncated versions of MiGPPS (MiGPPS1) and MiGGPPS (MiGPPS2) obtained with the LB media. For MiGPPS (MiGPPS1), when sorbitol and betaine were included in the LB media, the soluble fraction of the truncated version showed the formation of GPP along with about 7-12% (of the total) FPP at the optimum conditions (Fig. 3.12). The same medium was used for truncated version of MiGGPPS (MiGPPS2) and the purified protein, surprisingly, did not show any GGPP forming activity, rather GPP was detected as a main product with about 8-16% (of the total) FPP at the optimum conditions. None of the isopentenyl diphosphate products were detected with the full-length versions of MiGPPS

(MiGPPS1) and MiGGPPS (MiGPPS2) and with the protein expressed from an empty vector, confirming the *in vitro* activities of the recombinant proteins.

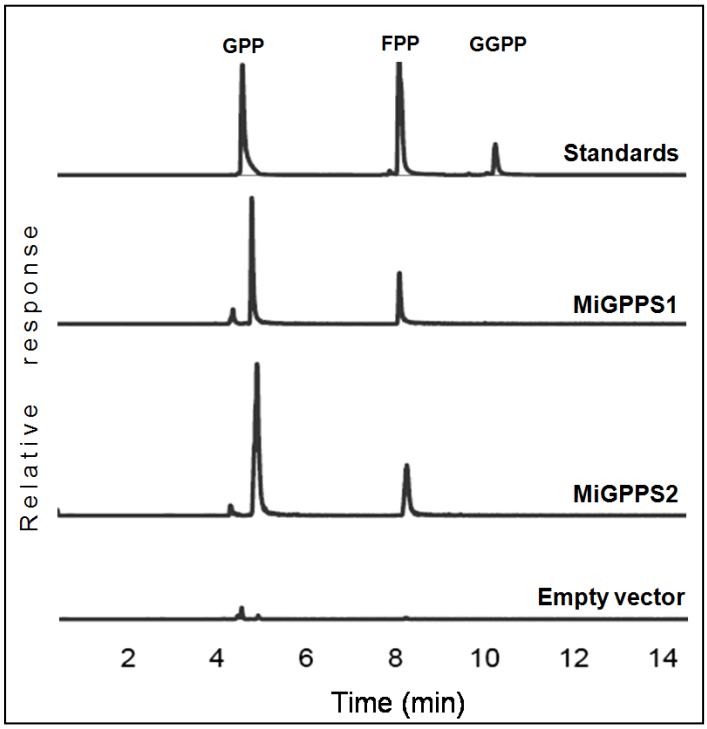


Figure 3.12
LC-MS/MS chromatogram of the standards of GPP, FPP and GGPP and of the *in vitro* assays with the protein expressed from MiGPPS1, MiGPPS2 and the empty vector.

3.2.4. Complementation assay for MiGGPPS (MiGPPS2)

MiGGPPS (MiGPPS2) showed the closest sequence similarity with the GGPPS reported from the other plants but no GGPP was detected in the *in vitro* assays. To confirm the absence of GGPP synthase activity of the MiGGPPS (MiGPPS2), complementation assay was carried out by co-transforming *MiGGPPS* (*MiGPPS2*) with pACCARΔcrtE. *PaIDS5* from *Picea abies,* which has been shown to be a functional GGPPS, was used as a positive control. The colonies obtained with the co-transformation of *PaIDS5* with pACCARΔcrtE showed the formation of yellow pigments; whereas, no yellow colored colonies were seen with *MiGGPPS* (*MiGPPS2*) -

pACCARΔcrtE cotransformation (Fig. 3.13). This confirmed that MiGGPPS (MiGPPS2) does not exhibit GGPP forming activity *in vivo* also. Henceforth, MiGPPS and MiGGPPS were named as MiGPPS1 and MiGPPS2, respectively.

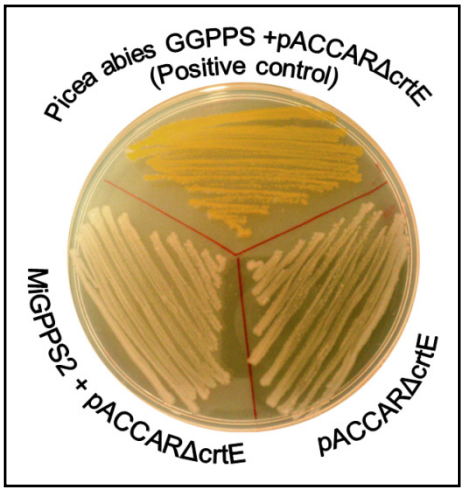


Figure 3.13
Complementation assay for the confirmation of absence of GGPP synthase activity of MiGPPS2. Functionally characterized GGPPS from *Picea abies* was used as a positive control.

3.2.5. Biochemical characterization

To know the optimum biochemical requirements of MiGPPS1 and MiGPPS2, *in vitro* assays were carried out by measuring the activity at varying temperature, $MgCl_2$ concentration and pH. Both the enzymes required the temperature of 40 °C for the optimum activity and more than 60% of the optimum activity was retained at 30 and 35 °C (Fig. 3.14). The activity sharply reduced above the temperature of 40 °C. The $MgCl_2$ concentration of 6 mM was required for both of the enzymes for exhibiting the maximum activity. MiGPPS1 showed more than 80% of the optimum activity at the $MgCl_2$ concentration of 3-15 mM; similar extent of activity was exhibited by MiGPPS2 between 6 and 15 mM $MgCl_2$. None of these enzymes showed any activity in the absence of Mg^{2+} or in the presence of other divalent metal ions

such as Mn^{2+}, Zn^{2+} or Ca^{2+}. Both MiGPPS1 and MiGPPS2 were optimally active between pH 7 and 8.

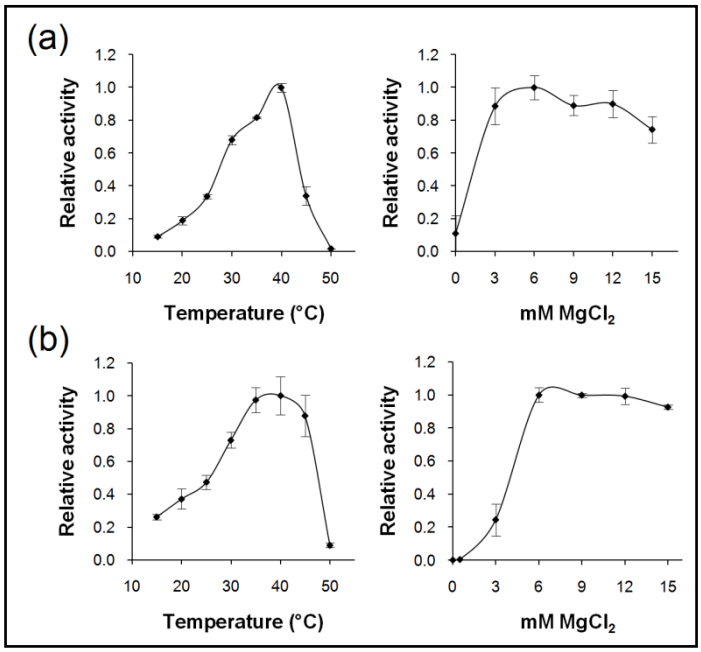


Figure 3.14

Optimum temperature and $MgCl_2$ concentration requirement of recombinant MiGPPS1 (a) and MiGPPS2 (b). For each enzyme and each parameter, peak area of GPP in the assay showing maximum activity was considered 1 and the relative values were calculated for the other assays.

3.2.6. Homology modeling of MiGPPS2

In order to get insights into the structural features of MiGPPS2 which are responsible for the observed GPP synthase activity of this GGPPS-like protein, homology based model of MiGPPS2 was generated. LSU of mint GPPS which shows 78% sequence identity with MiGPPS2 was used as a template. Assessment of the structure by Ramachandran plot showed the presence of 98.3% residues in the favoured region and 1.7% residues in the allowed region (Fig. 3.15). The structure was further evaluated by Protein Structure Analysis tool yielding the Z-score of -8.18 (Fig. 3.16) and the negative energy values for all the residues in the energy plot (Fig. 3.17). The

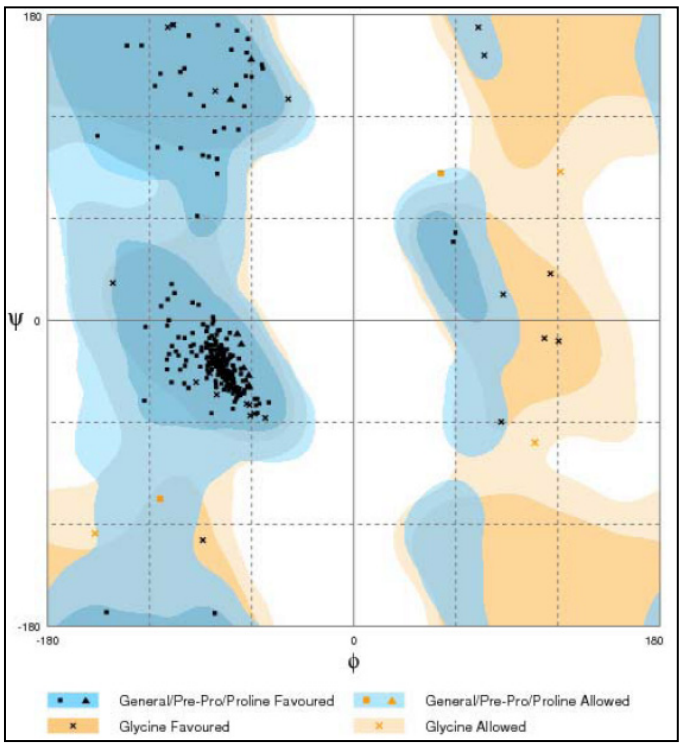


Figure 3.15

Ramachandran plot assessment of the homology model generated for MiGPPS2

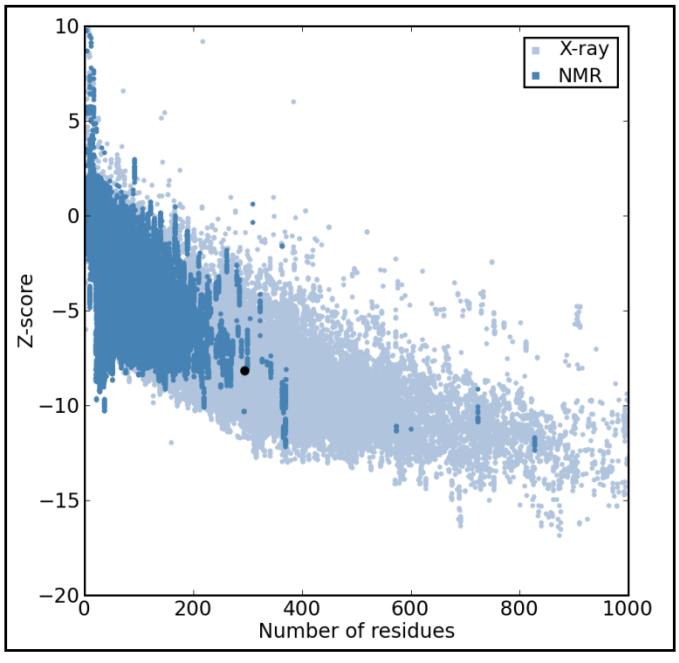


Figure 3.16

Z-score plot of the homology model generated for MiGPPS2 indicated by the black filled circle

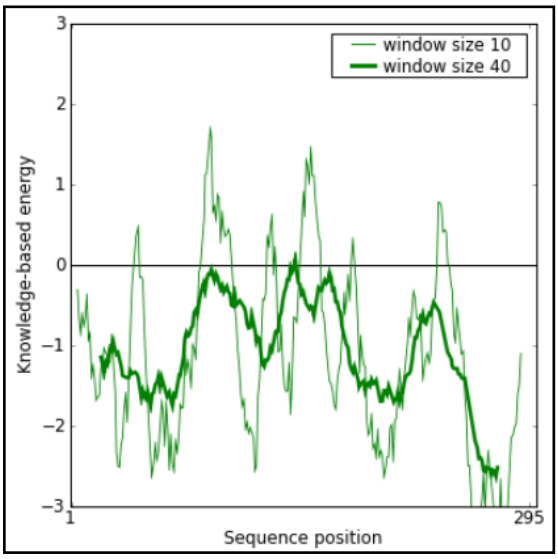


Figure 3.17

ProSA energy profile of the homology model generated for MiGPPS2

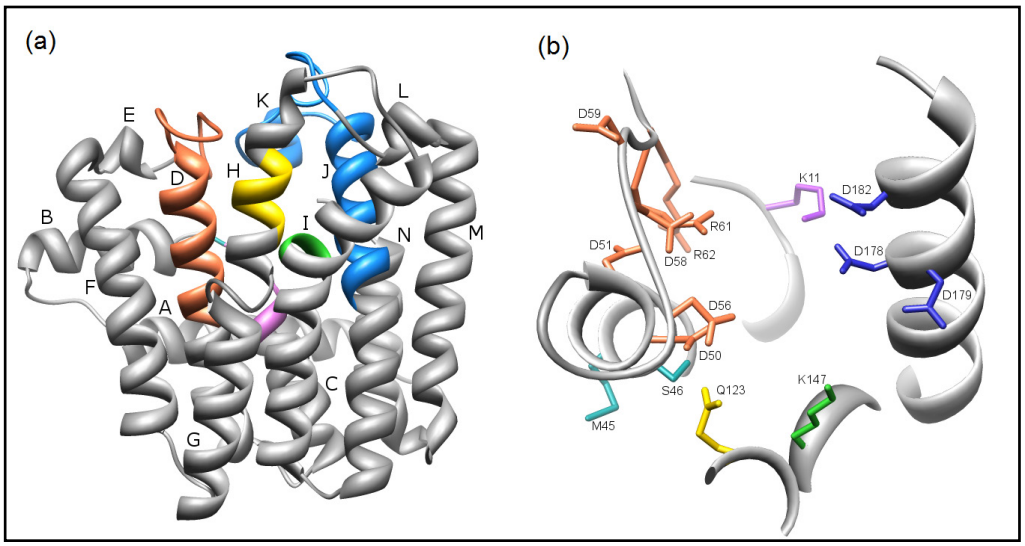


Figure 3.18

Homology model of MiGPPS2 generated using mint GPPS (PDB ID: 3KRF) as a template. (a) Overall structure of MiGPPS2 showing the 14 helices (A-N) and the five conserved regions (I-V) in the colors same as those used in Figure 3.9. (b) Top-view of the model showing central reaction cavity and part of the structure harbouring the residues involved in the catalysis. Side chains of only some of the important residues in the conserved domains are indicated in the colors same as those used in Figure 3.9. Side chains of the residues (M45 and S46) at the position corresponding to the CLD region of FPPS and GGPPS are shown in dark cyan color.

root mean square deviation (RMSD) between the modeled structure of MiGPPS2 and the template (LSU of mint GPPS) was 0.55 Å. Overall, all of these assessments indicated good quality of the model generated for MiGPPS2. The structure of MiGPPS2 composed of 14 helices (A to N) that surrounded the central reaction cavity (Fig. 3.18). Last two residues of helix D and a loop between helix D and E had the first aspartate rich motif (FARM); whereas, helix J had the second aspartate rich motif SARM. Side chains of all the aspartate residues of FARM and SARM, which were present facing each other in the central reaction cavity, and those of the two arginine residues proceeding FARM, pointed towards the central activity cavity suggesting their role in the catalytic activity.

3.2.7. Expression profiling

In order to understand actual contribution of *MiGPPS1* and *MiGPPS2* to the Alphonso flavor, transcripts of these genes were profiled through the ripening stages of Alphonso mango from three cultivation localities that are known to exhibit geographic variation in the flavor of Alphonso fruits.

3.2.7.1. *MiGPPS1*

For all of the three localities, Dapoli, Deogad and Vengurle, the fruits of 10 days after harvest (10 DAH) showed the highest expression (Fig. 3.19). The transcript level was about 3.4 (Vengurle) to 17 (Deogad) fold higher in 10 DAH stage than the raw (0 DAH) stage. Although the expression was higher in the ripe (15 DAH) stage as compared to the raw stages, there was a slight reduction in the expression during the transition from 10 DAH to 15 DAH stage. No uniform difference between the localities through the ripening stages could be seen for the expression level of *MiGPPS1*, but the overall pattern was similar for all the three localities. Deogad fruits had the higher expression level in 5 DAH and 10 DAH stage and the lower expression level in the 0 DAH and 15 DAH stage as compared to Dapoli and Vengurle; however, this difference was only 1.2 to 3 folds. Expression of *MiGPPS1* was also assessed in the raw and ripe exocarp of the fruits from Deogad; ripe skin

had about 5 fold higher transcripts as compared to the raw skin. The transcript level was 3.9 and 4.4 folds higher in the exocarp as compared to the mesocarp for the raw and ripe stages, respectively.

3.2.7.2. *MiGPPS2*

For all of the three localities, Dapoli, Deogad and Vengurle, the fruits of 10 days after harvest (10 DAH) showed the highest expression (Fig. 3.19). This transcript level was about 7.2 (Vengurle) to 18 (Deogad) fold higher in 10 DAH stage than the raw (0 DAH) stage. There was a 4.5 (Deogad) to 9.5 (Vengurle) fold reduction in the expression during the transition from 10 DAH to 15 DAH stage. When localities were compared to each other, Deogad fruits had higher expression level in 5 DAH and 15 DAH stage and lower expression levels in the 0 DAH and 10 DAH stage as compared to Dapoli and Vengurle. Nevertheless, the overall pattern of *MiGPPS2* transcripts was similar for all the three localities.

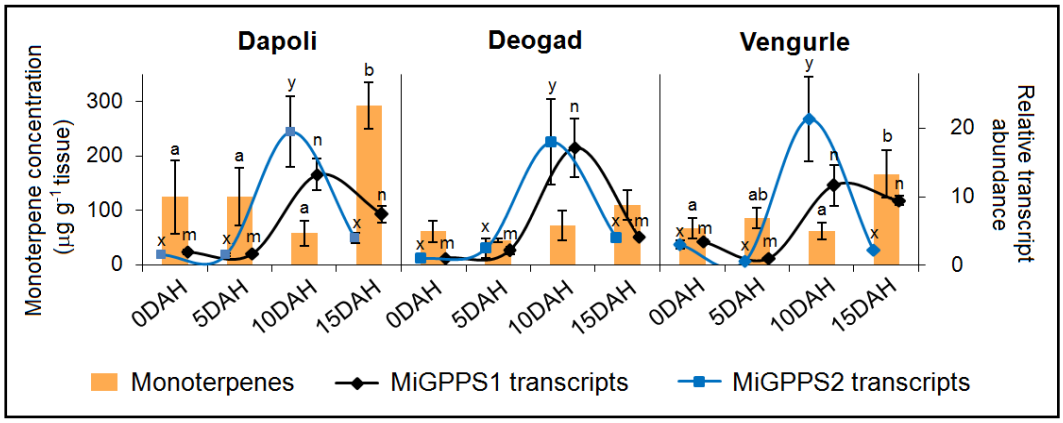


Figure 3.19

Relative abundance of *MiGPPS1* and *MiGPPS2* transcripts in the ripening stages of Alphonso fruits from three cultivation localities, Dapoli, Deogad and Vengurle in India (DAH: days after harvest).

Values represented are average of four independent biological replicates each of which was represented by at least two technical replicates. Alphabets indicate the significance of ANOVA ($p \leq 0.01$) for the comparison between the ripening stages for the levels of monoterpenes (a, b, etc.) and the relative transcript abundance of *MiGPPS1* (m, n, etc.) and *MiGPPS2* (x, y, etc.); the values having different letter are significantly different from each other. Alphabets are indicated only at the stages where the difference between the localities is significant. Values for each of the three systems (monoterpene level and transcript abundance of *MiGPPS1* and *MiGPPS2*) are staggered at each ripening stage for clear representation

3.3. Isolation and functional characterization of the farnesyl pyrophosphate synthase from Alphonso mango

3.3.1. Isolation of cDNA encoding farnesyl pyrophosphate synthase from mango

By homology based PCR-approach, the cDNA showing high sequence similarity to the plant farnesyl pyrophosphate synthase was isolated from the ripe fruit of Alphonso Mango. Terminal regions of the cDNAs were obtained by rapid amplification of cDNA ends (RACE) which was followed by isolation of the full-length cDNA having the length of 1380 bp, which was named as *MiFPPS* (*Mangifera indica FPPS*). The first in-frame ATG at the 5' end was considered as a start codon. The complete open reading frame of 1029 bp was flanked by 40 untranslated nucleotides at 5' end and 259 nucleotides including the AATAAAA motif between stop codon and the polyadenylation sequences at the 3' end. The reading frame encoded a protein of 343 amino acids with the calculated molecular weight of 39.5 kD and a isoelectric point of 5.35. The *in silico* translated amino acid sequence showed the highest homology with the FPPS from *Panax ginseng* (86% identity) (Kim et al., 2010), *Centella asiatica* (85% identity) (Kim et al., 2005) and *Hevea brasiliensis* (84% identity) (Adiwilaga and Kush, 1996). Alignment of the *in silico* translated MiFPPS with the FPPS sequences from other plants revealed the presence of five regions which are conserved among prenyltransferases (Chen et al., 1994). Out of these, regions II and V contained FARM (first aspartate rich motif, between 141 and 145 amino acids) and SARM (second aspartate rich motif, between 280 and 284 amino acids), respectively which are essential for substrate binding and catalysis (Fig. 3.20).

To know the genomic organization of *MiFPPS*, PCR was carried out on the genomic DNA of Alphonso using the terminal primers. The resulting fragment of about 3.8 kb was cloned and its sequence was determined by primer walking. The genomic sequence of *MiFPPS* showed the presence of 11 introns having the total size of 2717 bp (Fig. 3.21). The shortest and the longest introns had the sizes of 87 bp and 897 bp, respectively. Almost all the

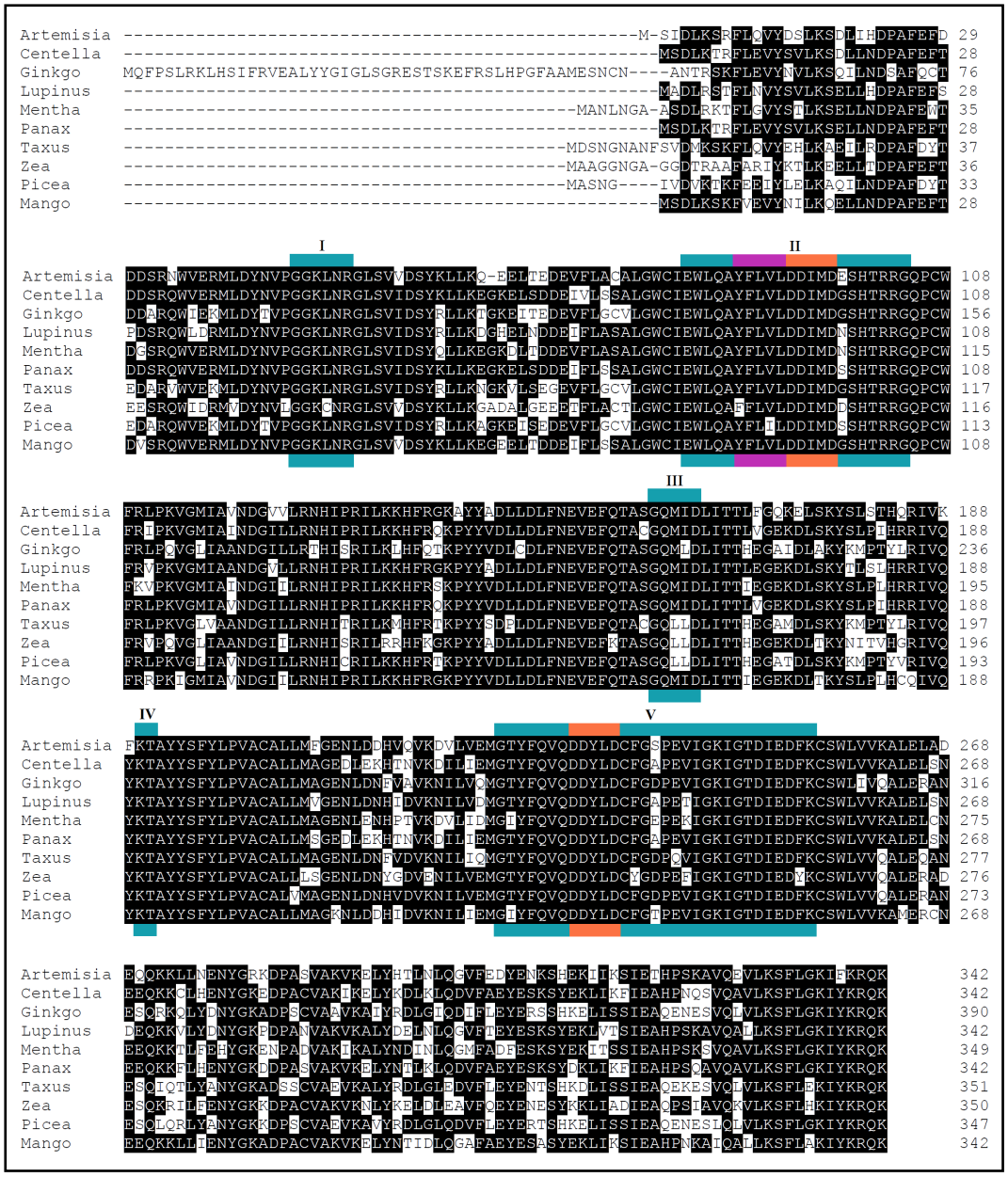


Figure 3.20

Alignment of the *in silico* translated sequence of *MiFPPS* with the closest characterized sequences from the other plants. Five regions which are conserved among the isoprenyl diphosphate synthases (I-V) are indicated in the dark cyan color; FARM and SARM in the region II and V are indicated in coral color and the chain length determining stretch in the region II is indicated in the purple color. NCBI accession numbers of the sequences were: AAP74719 (*Artemisia tridentate*), AAV58896 (*Centella asiatica*), AAR27053 (*Ginkgo biloba*), AAA86687 (*Lupinus albus*), AAK63847 (*Mentha x piperita*), AAY87903 (*Panax ginseng*), AAS19931 (*Taxus x media*), NP_001105039 (*Zea mays*) and ACA21460 (*Picea abies*).

introns followed the "GT-AG" rule (Breathnach and Chambon, 1981); in that, the introns began with GT at the 5' ends and ended with AG at the 3' end.

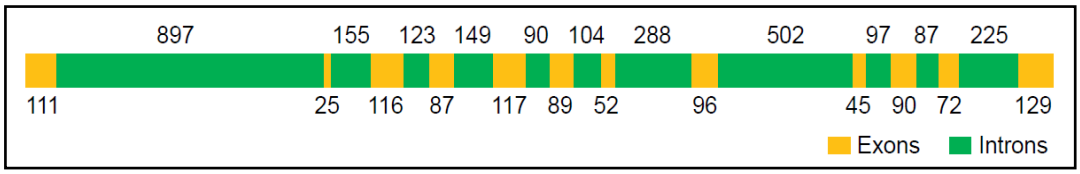


Figure 3.21

Genomic organization of *MiFPPS*. Number on the top and the bottom indicate sizes (bp) of the introns and the exons, respectively.

3.3.2. Recombinant expression and enzymatic assays of MiFPPS

Full-length open reading frame of *MiFPPS* with the start codon and without the stop codon was cloned in the pEXP5 CT TOPO vector. Before actual expression, the deduced amino acid sequence of MiFPPS was analyzed on the PROSO server to predict the solubility of the recombinantly expressed protein in *E. coli* (Smialowski et al., 2007). The prediction that MiFPPS would be soluble upon heterologous expression with the probability of 0.507 appeared correct and good amount of reco mbinant protein was obtained in the soluble fraction upon expression (Fig. 3.22). Purification of the crude soluble fraction

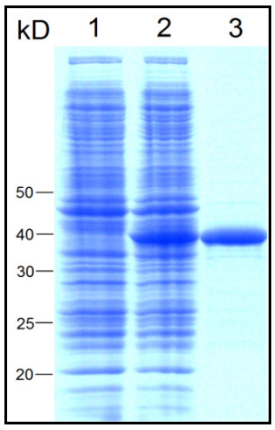


Figure 3.22

SDS-PAGE analysis of the crude lysate of the cells carrying empty vector (lane 1), crude lysate of the cells carrying *MiFPPS* construct (lane 2) and the purified recombinant MiFPPS protein. Sizes of the proteins in the molecular marker are indicated on the left.

through Ni-NTA agarose matrix yielded MiFPPS protein of the expected size. Enzymatic assays were carried out using the purified recombinant protein and IPP and DMAPP substrates along with Mg^{2+} as a divalent metal ion cofactor and the products were analyzed by LC-MS/MS. MiFPPS clearly produced *E,E*-FPP as its main product along with about 1-3% of GPP. The very minute amounts of GPP and FPP detected in the assays with the protein expressed from an empty vector can be attributed to the activity of the enzymes from *E. coli* (Fig. 3.23). Neither FPP nor GPP were detected in the enzyme assays with the boiled protein, without protein and without substrates, confirming the FPP synthase activity of MiFPPS.

3.3.3. Biochemical characterization of MiFPPS

To study the optimum biochemical requirements of the recombinant enzyme, assays were carried out at varying temperature, pH and $MgCl_2$ concentration. Optimum temperature for the activity of recombinant enzyme was found to

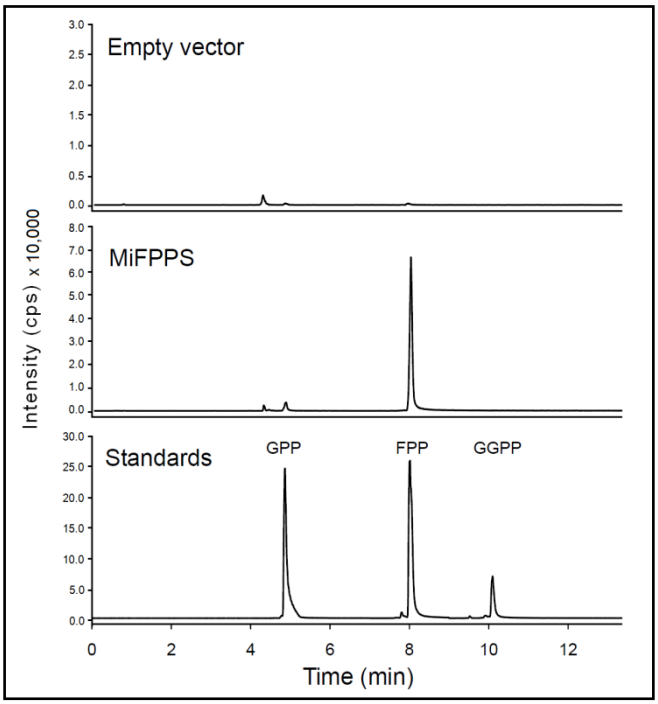


Figure 3.23
LC-MS/MS chromatogram of the *in vitro* assay with the protein expressed from the empty vector, of the *in vitro* assay with the purified MiFPPS and of the standards of GPP, FPP and GGPP

be 25 °C and more than 75% of the optimum activity was retained at 15 °C and 20 °C; however, the activity sharply reduced beyond 25 °C (Fig. 3.24). When the activity of MiFPPS was assayed at varying pH, maximum FPP production was observed at pH 7.5 with retention of more than 80% activity between pH 7 and 9; however, only about 10% of the optimum activity was detected below pH 7. The enzyme also required Mg^{2+} as a divalent metal ion cofactor for its activity. The optimum $MgCl_2$ concentration was found to be 0.3 mM and the enzyme was fairly active with more than 75% of the maximum activity till 1 mM $MgCl_2$ after which the activity reduced slowly. Strikingly, MiFPPS specifically required Mg^{2+} for catalysis and was found to be inactive with other divalent metal ions such as Mn^{2+}, Zn^{2+} and Ca^{2+}. To test whether the enzyme shows any specificity towards allylic substrates, GPP was added as an allylic substrate instead of DMAPP. MiFPPS showed the geranyl transferase activity by recognizing GPP as an allylic substrate to produce FPP.

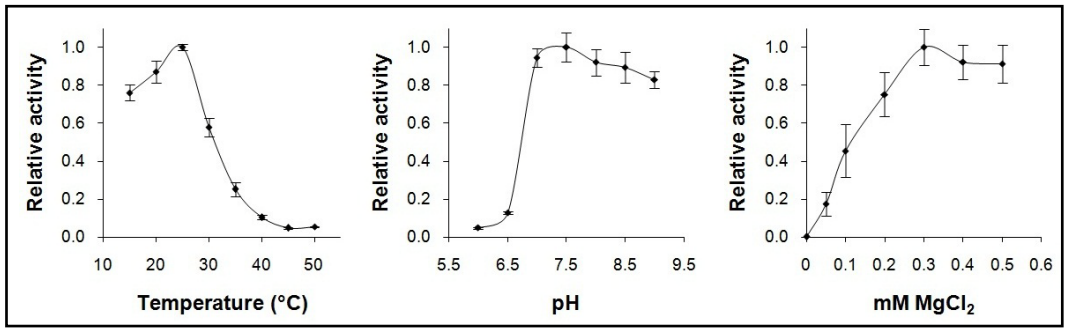


Figure 3.24

Optimum temperature, pH and $MgCl_2$ concentration requirement of recombinant MiFPPS. For each parameter, peak area of FPP in the assay showing maximum activity was considered 1 and the relative values were calculated for the other assays.

3.3.4. Structural features of MiFPPS

To get the structural insights into MiFPPS enzyme, we performed homology modeling with the avian FPPS as a template (1UBV) (Tarshis et al., 1994) which showed 50% sequence identity with MiFPPS. Quality of the generated model was assessed by Ramachandran plot which showed the presence of 98% residues in the allowed region (Fig. 3.25). Further evaluation of the

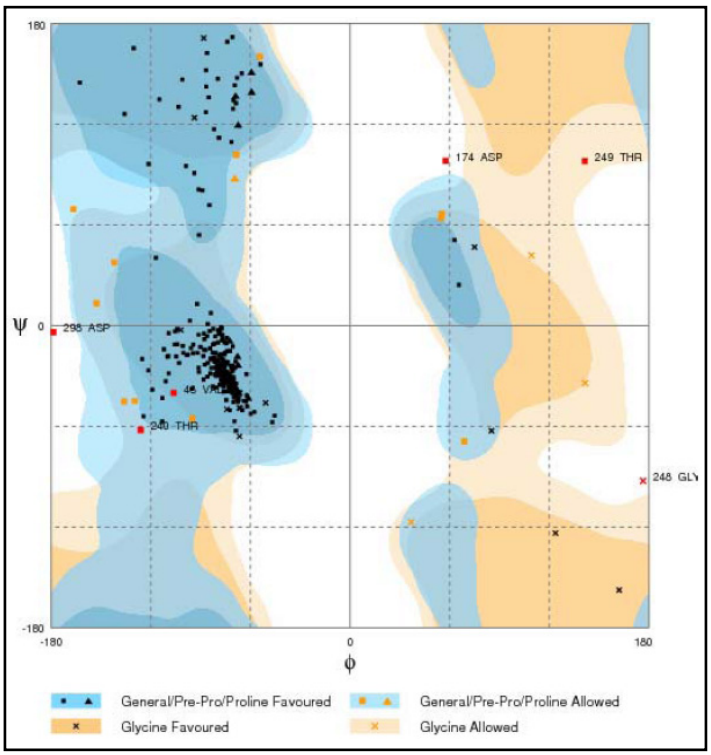


Figure 3.25

Ramachandran plot assessment of the homology model generated for MiFPPS

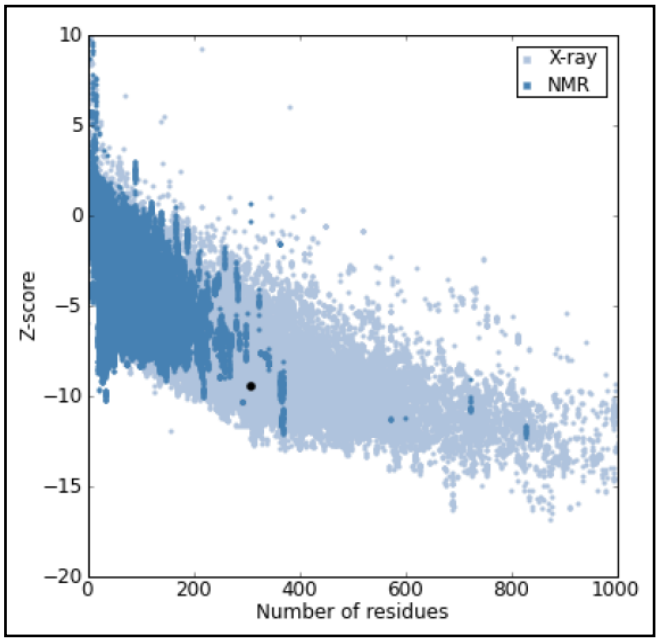


Figure 3.26

Z-score plot of the homology model generated for MiFPPS indicated by the black filled circle.

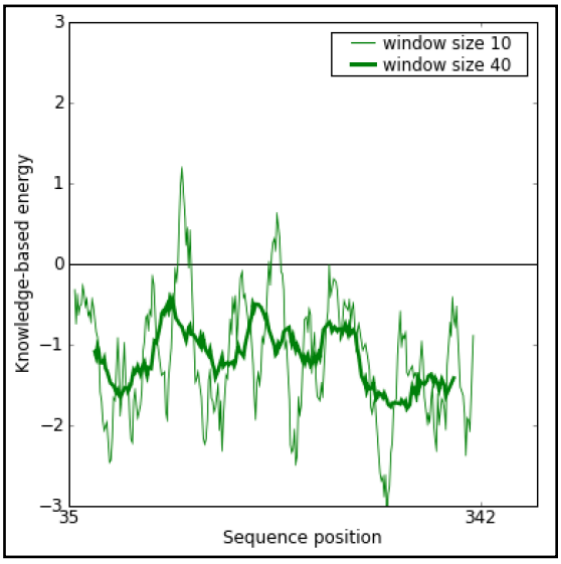


Figure 3.27
ProSA energy profile of the homology model generated for MiFPPS

structure by ProSA-web yielded a Z-score of -9.46 (Fig. 3.26) and the negative energy values for all the residues in the energy plot (Fig. 3.27). RMSD for superimposition of the modeled structure of MiFPPS with the template (avian FPPS) was 0.61 Å. Overall, all of these assessments point towards good quality of the model generated for MiFPPS. The avian FPPS has 10 helices (A-J); the structure of MiFPPS at the region corresponding to helix A of avian FPPS could not be determined because of low sequence identity of this region. In the modeled structure of MiFPPS, other nine helices (B-J) surrounded the central reaction cavity (Fig. 3.28a). All the five regions conserved in isoprenyl diphosphate synthases lined the substrate binding cavity. FARM and SARM were present on the opposite walls of the cavity on the D and H helix, respectively. Carboxylate side chains of all the six aspartate residues in FARM and SARM were extended into the substrate binding cleft. Side-chain of the phenylalanine residue present before FARM and known to be involved in the chain-length determination of the product also protruded into the activity cavity (Fig. 3.28b).

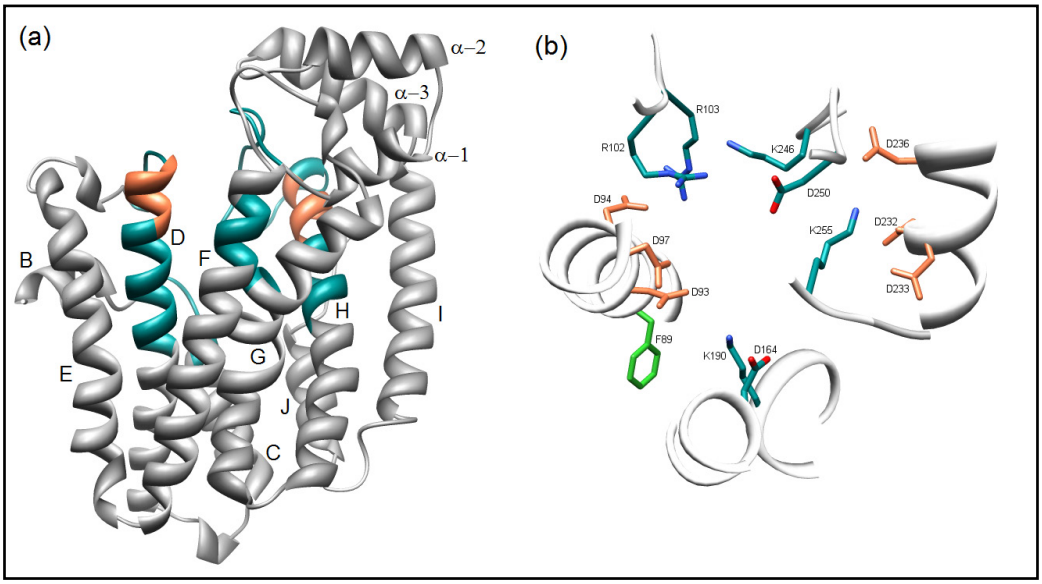


Figure 3.28

Homology model of MiFPPS generated using Avian FPPS (1FPS) as a template. (a) Overall structure of MiFPPS showing the five conserved regions (I-V) in the dark cyan color and FARM and SARM in the region II and V in the coral color. The helical regions have been labelled by alphabets A to J; α-1, α-2 and α-3 are the short helices between helices H and I. (b) Top-view of the model showing central reaction cavity and part of the structure harbouring the residues involved in the catalysis. Side chains of the aspartate residues in the FARM and SARM are indicated in the dark cyan color, those of the other important residues in the conserved regions are shown in the coral color and that of phenylalanine in the chain-length determining region is indicated in the green color.

3.3.5. Expression profiling of *MiFPPS* through ripening stages

In order to get insights into involvement of *MiFPPS* in the regulation of sesquiterpene biosynthesis, transcripts of *MiFPPS* were profiled through the four ripening stages of Alphonso mango from three cultivation localities in India that exhibit geographic variation in terpene content (Fig. 3.29). When the ripening stages were compared to each other, for all the three localities the level of expression of *MiFPPS* was statistically similar for the raw (0 days after harvest) and 5 DAH fruits. About 6.6 (Vengurle) to 16 (Dapoli) fold increase in the expression was observed at the 10 DAH stage as compared to the raw

fruits (Fig. 3.29). Although the transcripts were present in the higher amounts in the ripe (15 DAH) stage than the raw stage, the level was reduced by about half in comparison with 10 DAH. When the localities were compared to each other for the expression level of *MiFPPS*, Deogad fruits had the significantly higher level of the transcripts in the ripe fruits, Dapoli and Deogad in the 10 DAH fruits; whereas Deogad and Vengurle in the raw fruits as compared to the other localities. Still, the overall pattern of *MiFPPS* transcripts was similar for all the three localities.

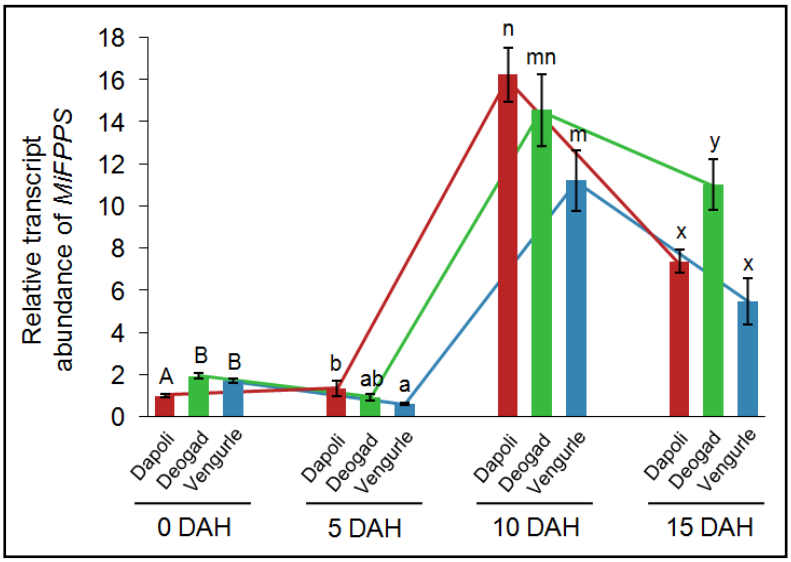


Figure 3.29

Abundance of MiFPPS transcripts relative to EF1α in the ripening stages of Alphonso fruits from three cultivation localities, Dapoli, Deogad and Vengurle, in India (DAH: days after harvest). Values represented are average of four independent biological replicates each of which was represented by at least two technical replicates. Alphabets over the columns indicate the significance of ANOVA ($p \leq 0.05$) for the comparison between localities for each ripening stages; the values having different letter are significantly different from each other. The ANOVA based comparison is independent for each ripening stage and therefore, is represented by different series of alphabets (0 DAH: A, B, C; 5 DAH: a, b, c; 10 DAH: m, n, o and 15 DAH: x, y, z).

3.4. Molecular characterization of an oxidoreductase catalyzing furaneol biosynthesis in 'Alphonso' mango

3.4.1. Isolation of *MiEO*

In order to isolate the genes involved in the biosynthesis of furaneol (enone oxidoreductase), degenerate primers were designed based on the alignment of the sequences of the putative quinone oxidoreductase from *Fragaria x ananassa* (AY048861), *Vigna radiata* (U20808) and *Helianthus annuus* (AF384244) and were used for amplification over the cDNA prepared from the ripe fruits of Alphonso mango. Sequences of the fragments obtained showed high similarity to those of quinone oxidoreductase from the other plants. For obtaining the full-length open reading frame of this gene, gene specific primers were designed and were used for rapid amplification of cDNA ends (RACE). The 5' and 3' RACE fragments thus obtained again showed high similarity to the respective terminal regions of the mRNA sequences of the orthologous quinone oxidoreductases. Based on these alignments, terminal primers were designed and the full-length cDNA of mango enone oxidoreductase (*MiEO-Mangifera indica* enone oxidoreductase) was obtained by amplification over the cDNA prepared from the ripe fruits.

The complete open reading frame (ORF) of *MiEO* was 1143 bp long and was flanked by a 40 bp UTR at the 5' end and 115 bp UTR at the 3' ends. The ORF encoded a protein having 381 amino acids, the calculated molecular weight of 40.6 kD and the pI of 8.61. The similarity of the *in silico* translated amino acid sequence of MiEO was 79% with the chloroplastic alkenal/one oxidoreductase (AOR) from *Cucumis sativus* (CsAOR) (Yamauchi et al., 2011), 73% with the enone oxidoreductase (EO) from *Solanum lycopersicon* (SlEO) (Klein et al., 2007), 72% with the EO from *Fragaria x ananassa* (FaEO) (Raab et al., 2006) and 71% with the AOR from *Arabidopsis thaliana* (AtAOR) (Yamauchi et al., 2011). MiEO also showed high sequence similarity to many uncharacterized sequences reported on the NCBI database (Table 3.3). The putative amino acid sequence of MiEO showed the presence of the conserved GxGxxG domain which is involved in binding with NADP (Edwards et al.,

Table 3.3

Uncharacterized sequences from the NCBI database showing high identity with *MiEO*

Accession number	Plant	Putative annotation	Sequence identity with *MiEO*
XP_002525379	*Ricinus communis*	Alcohol dehydrogenase	94 %
ABK96279	*Populus trichocarpa* x *Populus deltoides*	Unknown	90%
XP_002323668	*Populus trichocarpa*	Unknown	90%
ADN33837	*Cucumis melo*	Alcohol dehydrogenase	89%

1996) (Fig. 3.30). Structural features of the putative protein of MiEO were studied by Hierarchical Neural Network analysis (Combet et al., 2000; Guermeur et al., 1999). This analysis reaveled that MiEO was composed of 36.25% alpha helix, 44.66% random coil and 19.09% extended strand structures (Fig. 3.31).

3.4.2. Recombinant expression and *in vitro* enzymatic assays

Similar to CsAOR, AtAOR and SlEO, N-terminal region of *in silico* translated MiEO was characterized by the presence of putative chloroplast targeting peptide as revealed by analysis of the sequence by ChloroP program (Table 3.4), suggesting that the MiEO protein might be localized in the chloroplast, as was shown for CsAOR (Yamauchi et al., 2011). To get functional insights into MiEO, the complete open reading frame without the 5'-nucleotide stretch corresponding to the putative chloroplast-targeting peptide (Fig. 3.30) was cloned in pCRT7/NT-TOPO vector and the ORF was expressed in *E. coli* as $(his)_6$-tagged protein. The enzymatic activity of the purified recombinant MiEO protein was assessed using fructose-1,6-diphosphate as a substrate and NADH as a reducing agent and the product formation was examined by gas chromatography-mass spectrometry. As has been observed previously (Raab et al., 2006), in the initial experiments, furaneol was detected in the assays with the protein expressed from the plasmid having the reverse-oriented insert

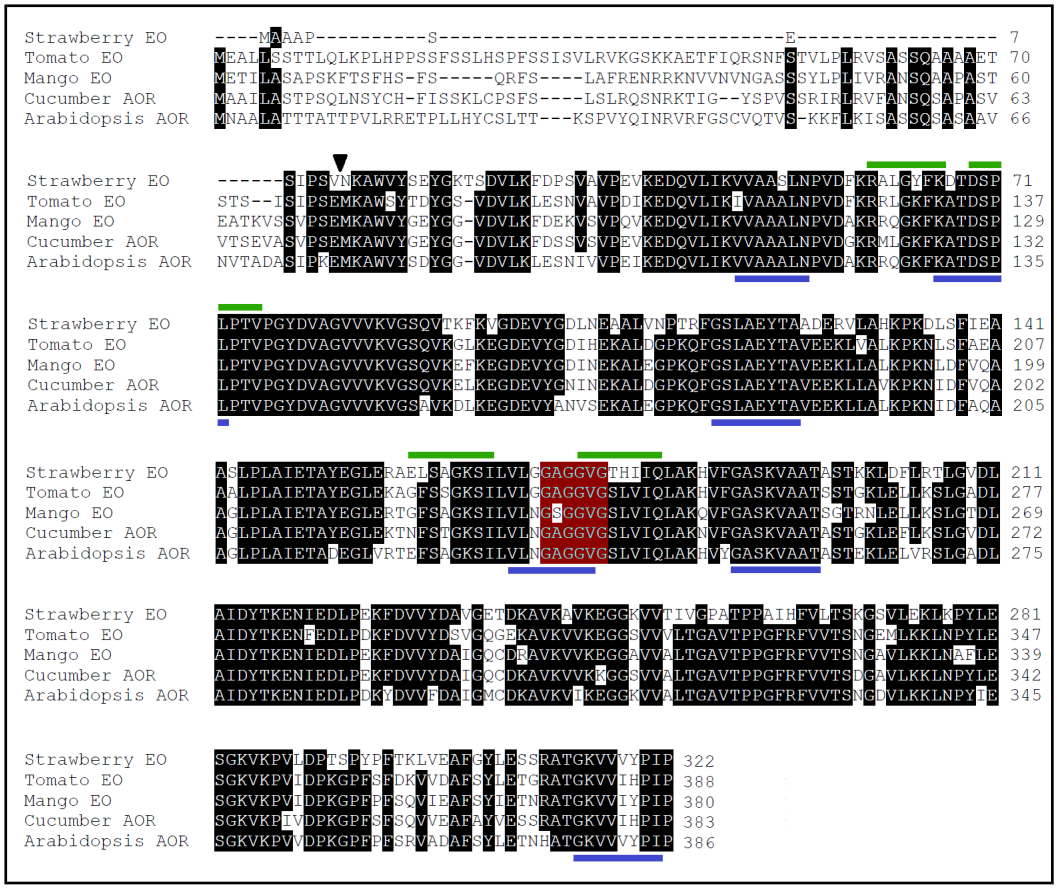


Figure 3.30

Alignment of the *in silico* translated sequence of *MiEO* with the closest characterized homologs from the other plants. Regions of the alignment corresponding to the nucleotide sequence used for designing degenerate primers is marked by the blue lines below the alignment, and that used for designing gene specific primers for RACE is indicated by the green lines above the alignment. The conserved NAD(P)H-binding domain is highlighted in the maroon color. Arrow head indicates the truncation site for removing the putative chloroplast targeting sequence.

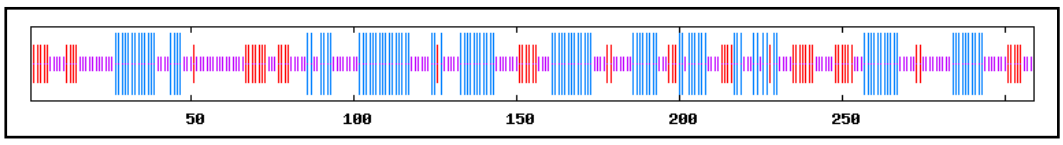


Figure 3.31

Hierarchical Neural Network analysis of the *in silico* translated sequence of *MiEO*. Alpha helices, random coils and extended strands are indicated in blue, violet and red color, respectively.

Table 3.4

Analysis of the *in silico* translated sequence of *MiEO* for the presence of putative chloroplast targeting peptide by ChloroP program (cTP: chloroplast targeting peptide)

Score	cTP	CS-score	cTP-length
0.525	Yes	4.931	58

indicating the activity of the *E. coli* proteins. However, increasing the stringency of the wash solution to 40 mM imidazole during the purification of protein by affinity chromatography using Ni-NTA agarose spin columns resulted in diminishing of this oxidoreductase activity originating from *E. coli*. The MiEO protein purified and assayed in this way clearly showed the presence of furaneol in the GC-MS analysis (Fig. 3.32). Although fructose-

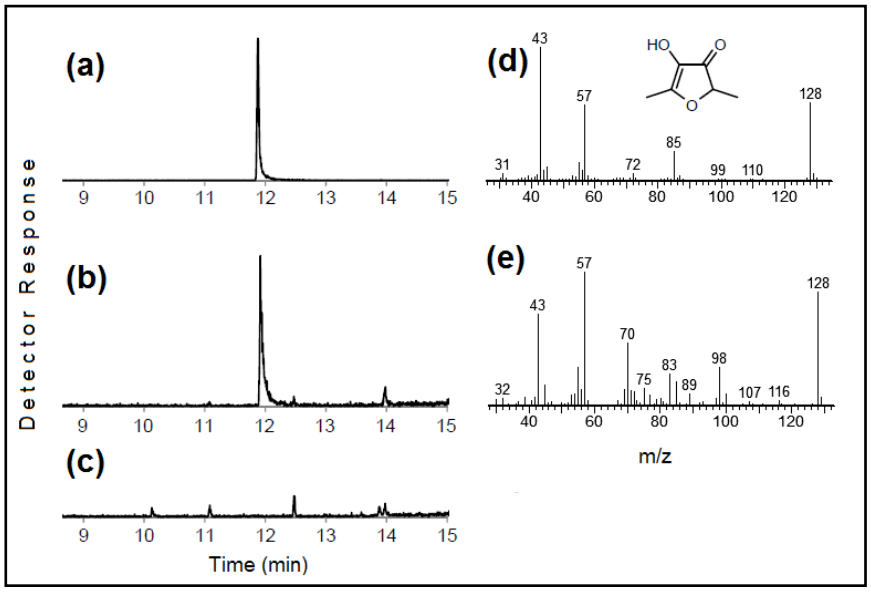


Figure 3.32

GC-MS chromatograms of the ion of *m/z* 128 for the authentic furaneol (a), for the assay having the protein expressed from *MiEO* (b) and for the assay having the protein expressed from the plasmid carrying reverse-oriented insert (c). In a separate analysis, total ions were monitored; the spectra represented are of the authentic furaneol (d) and of the furaneol detected in the assay with MiEO (e).

1,6-diphosphate is not a direct natural precursor of furaneol, the enzyme from strawberry, FaEO was shown to be able to covert fructose-1,6-diphosphate to furaneol via an intermediate, HMMF (Raab et al., 2006). Detection of furaneol in the assays carrying purified MiEO along with fructose-1,6-diphosphate as substrate and the absence of furaneol in the assays carrying boiled protein thus confirmed the furaneol forming activity of MiEO.

3.4.3. Expression profiling of *MiEO*

To get insights into actual role of *MiEO* in the profile of furanones observed through the ripening Alphonso fruits, transcripts of *MiEO* were profiled through the ripening stages of Alphonso. The highest expression of *MiEO* was detected at the 10 DAH (days after harvest) stage of the ripening fruits (Fig. 3.33). There was reduction in the expression of *MiEO* during the transition from 10 DAH to 15 DAH (ripe) fruits. Although furaneol and mesifuran are completely absent in the raw fruits (0 DAH), the expression level was only

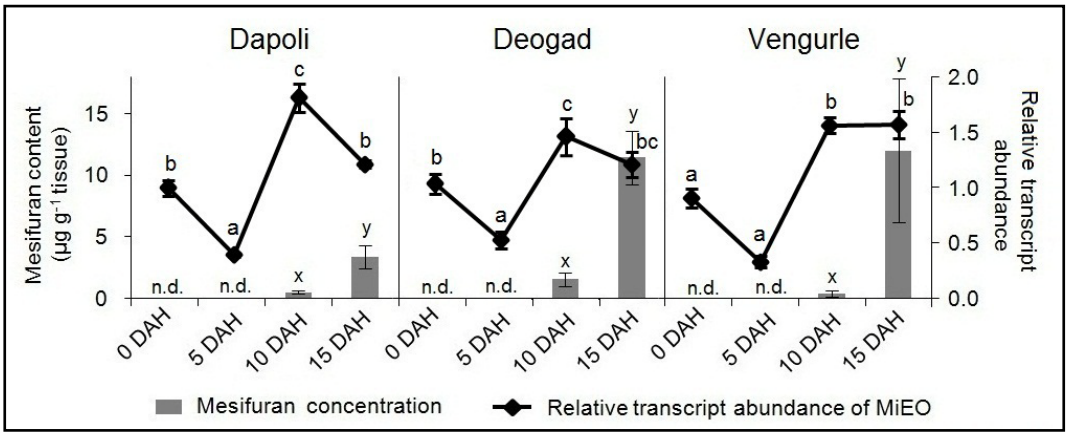


Figure 3.33

Mesifuran content and relative abundance of *MiEO* transcripts in the ripening fruits of Alphonso mango from the three cultivation localities, Dapoli, Deogad and Vengurle in India (DAH: days after harvest). The relative transcript abundance of *MiEO* for the raw stage (0 DAH) from Dapoli was considered 1 and the fold difference for the rest of the tissues was calculated. Each measurement was repeated with four independent biological replicates, each of which was represented by at least two technical replicates. Alphabets indicate the significance of ANOVA ($p \leq 0.01$) for the comparison between the ripening stages for the levels of mesifuran (x, y, etc.) and the relative transcript abundance of *MiEO* (a, b, etc.); the values having different letter are significantly different from each other.

about 1.5 folds lower than the ripe fruits (Fig. 3.33). There was about two fold reduction in the expression during the transition of fruits from 0 to 5 DAH stage. As detailed in the section 3.1, Alphonso mango shows geographic variation in the furanone content. Out of the three localities which were studied for the content of volatiles, Dapoli was characterized by the lowest amount of mesifuran in the ripe fruits; whereas, for 10 DAH stage, the highest amount of mesifuran was detected in the fruits from Deogad (Fig. 3.33). To know if there is any contribution of *MiEO* to such geographic variation, expression of *MiEO* was also analysed in the ripening fruits of mangoes from these cultivation locations. Although there were some differences between the localities for the level of *MiEO* transcripts, the pattern was uniform but could not be correlated with the varied mesifuran content through the localities.

Discussion

4.1 Geographic variation influences flavor volatile chemistry of Alphonso mango

Flavor is one of the most important characters of fruits and vegetables that decide acceptability and preference of these important food items by humans. For plants, flavor forms one of the most important phenotypes of the fruits that plays a vital functional role by attracting seed disseminators and repelling pathogens thereby increasing shelf-life of the fruits (Lanciotti et al., 2004). Qualitative and quantitative nature of the flavor depends on various parameters including intrinsic factors such as genotype and physiological state of the plant as well as extrinsic factors such as abiotic and biotic conditions (Dudareva et al., 2006; Pandit et al., 2009a; Vallat et al., 2005). Alphonso mangoes which are highly rich in flavor volatiles and which are thought to vary in their flavor with cultivation locality form one of the best systems to study regulation of flavor chemistry.

4.1.1. Nature of the geographic variation in volatiles

To have the crop of consistent quality, Alphonso mango trees are propagated by asexual propagation method, grafting; hence are genetically identical. To analyze the locality based variation in the flavor volatiles of Alphonso mango, which is thus likely to be the effect of only extrinsic conditions such as variable geo-climatic factors, four plants representing four biological replicates were analyzed from each locality representing the Konkan region. The gas chromatographic analysis of the mesocarp tissue revealed that fruits from all the three cultivation localities are rich in monoterpenes and ripe fruits are characterized by the unique presence of lactones and furanones in them. These results are consistent with the previous study from our laboratory (Pandit et al., 2009a; Pandit et al., 2009b) and the other reports which also have shown (*Z*)-ocimene as the dominant compound in the volatile blend of Alphonso (Idstein and Schreier, 1985). Concentration of mono- and sesquiterpenes detected (2.6 to 374 μg g^{-1} and 0.6 to 20.8 μg g^{-1}, respectively)

was quite low as compared to the previous report (Pandit et al., 2009b) in which whole fruit (except endocarp) was analyzed for the volatile composition as compared to the only mesocarp in the present study. This suggested that the Alphonso exocarp might contain much higher concentration of terpenes than mesocarp which has been confirmed in studies carried out in our laboratory. For all the three localities, higher amounts of total and individual monoterpenes were detected in the ripe fruits as compared to the other stages. This also is consistent with the earlier report on the fruits from Deogad region (Pandit et al., 2009b). Furaneol which has been reported earlier as an important flavor constituent (Pandit et al., 2009a; Pandit et al., 2009b) could not be quantified because of its inconsistent detection which can be attributed to its instability at high operating temperatures during GC (Siegmund et al., 2010). Because of this fact, furaneol is more commonly analysed by the techniques such as HPLC (Perez et al., 1996).

Principle component analysis (PCA) is a powerful tool to reduce dimensionality in the multivariate data. Through PCA, the multidimensional data is converted into the data which can be represented by minimum number of variables, still retaining variability in the original data (Ringner, 2008). For the present data also, PCA turned out to be an efficient method of extracting variability in the volatiles through ripening stages of Alphonso from multiple localities. One of the principle components (PCs) of the PCA of quantities of compounds in the ripening stages from three localities represented transition of the fruits towards the ripe stage with synthesis of the new compounds (lactones and furanones) and increase in quantities of the already-present volatiles (terpenes). The other PC underlined clear discrimination between localities for the volatiles in the ripe fruits. On the other hand, both of the above PCs supported absence of grouping of localities for the unripe stages. In the loading plot of the same PCA, terpene volatiles formed two distinct clusters. This points towards the possibility of presence of at least two types of terpene synthases in Alphonso fruits. Each of them might be regulated in a specific manner giving rise to uniquely separate profile of their products through the ripening stages for different localities. Furthermore, similar profile

of all the monoterpenes through the set of tissues studied as suggested by clustering of all the monoterpenes (except p-cymeme) in a single group, further indicated the possibility of regulation of their biogenesis at a common upstream step such as geranyl pyrophosphate synthase which provides direct precursors for monoterpenes.

4.1.2. Geographic variation in terms of flavor

Comparative quantitative analysis of volatile compounds within a single tissue and between different tissue samples can be very informative in the perspective of plant biochemistry. For example, it can shed light on the most active pathways among array of biosynthetic routes of volatiles or can give a hint towards the pathways triggered during physiological processes such as ripening (Pech et al., 2008). Nevertheless, when volatiles are studied from tissues such as edible fruit, the analysis cannot be considered functional without dissecting relative importance of these compounds in the human's viewpoint in terms of the flavor. Because of differential interactions of various volatile compounds with their receptors in human olfactory system, these chemicals are sensed in different magnitudes, as well as each of them has its own characteristic response.

Among the present set of compounds, mesifuran, (*Z*)-ocimene and γ-octalactone had the highest odor units and thus they were the major contributors to the Alphonso flavor. Out of these, mesifuran has coumarin-like fruity odor, (*Z*)-ocimene has citrus-like terpenic odor, whereas γ-octalactone has coconut-like creamy odor. Thus, having the high ratio of mesifuran/ (*Z*)-ocimene and γ-octalactone/ (*Z*)-ocimene, Deogad fruits can be described as relatively "pleasant" and "fruity" in terms of the flavor as compared to Dapoli fruits which can be said to be more "terpenic" because of the low ratios of above mentioned compounds (Fig. 3.8). Furthermore, the two types of lactones detected in the present study, γ-lactones and δ-lactones, are thought to interact with each other to affect nature of the flavor (Hashimoto et al., 2008) and ratio of γ- and δ-lactones is thought to play an

important role in establishing the flavor quality (Heath, 1981). The lower ratio of γ-lactones/δ-lactones observed in the fruits from Deogad and the higher ratio in the fruits from Dapoli can also, thus contribute to the varied flavor of Alphonso observed among these locations.

4.1.3. Source of the geographic variation

Plants are one of the most dynamic entities among the living organisms that adapt themselves according to the surrounding biotic as well as abiotic environment. Such an ability of plants to show phenotypic changes in different environments, especially under differential abiotic conditions, is termed as phenotypic plasticity (Majetic et al., 2009). Till now, existence of geographic variation or phenotypic plasticity in terms of volatiles has been shown mainly in the floral scents (Dotterl et al., 2005; Knudsen, 2002; Svensson et al., 2005) and in the essential oils in the vegetative tissue (Adams, 2001; Neffati et al., 2009). However, in most of these cases, plants belonged to the natural populations and hence they can show some genetic variation, which along with the varied environmental conditions could account for the observed variation in the volatile composition (Majetic et al., 2009). Since Alphonso plants are genetically identical because of their propagation by grafting, the difference observed in the volatiles at different cultivation localities is likely to be regulated by the external environmental factors. Furthermore, cultivation practices and the post-harvest treatment to the Alphonso fruits throughout the broad cultivation region, Konkan, are the same. Therefore, the influence of the external factors is probably exerted during fruit development and in terms of flavor variation; this effect is reflected 15 days after the mature fruit has been detached from the plant.

Fruit ripening is one of the most important processes in the life cycle of the trees. During this phase, fruits get their functional dimension by becoming attractive to seed dispersing agents, which is achieved by increase in sweetness and nutritive value and synthesis of coloring and flavoring compounds. In the case of Alphonso mango, changes in the flavor volatiles along fruit ripening were very clear. Furanones and lactones were completely absent in the raw

fruits and they were synthesized in increasing amount during fruit ripening till 15 DAH stage. Although ripening was also characterized by the increase in the concentration of terpenes, these compounds were already present in the raw fruits as well as in the vegetative tissue such as leaves as shown previously (Pandit et al., 2009b). In the light of these observations, there appears to be a different molecular mechanism for the regulation of biosynthesis of terpenes and lactones, which can also account for the differential behavior of these two classes of volatiles among different geographic locations.

In climacteric fruits, most of the ripening related changes are induced by ethylene. Indeed, in some of the mango cultivars, ethylene production was also observed prior to attaining harvest maturity (Cua and Lizada, 1990), pointing towards developmental control of ripening. Both developmental and climacteric ethylene production are regulated at the rate-limiting step in its biosynthetic pathway catalyzed by 1-aminocyclopropane-1-carboxylate synthase (ACC synthase), which has thus shown to be the controller of ripening-related changes (Argueso et al., 2007). In addition, in tomato (Vrebalov et al., 2002) and banana (Elitzur et al., 2010; Liu et al., 2009) it was shown that MADS-box transcription factors play a vital role in developmental control of the ripening-related changes. This involvement of MADS transcription factors in the ripening is thought to be through their regulatory action on endogenous ethylene production (Elitzur et al., 2010) which further induces expression of flavor and other ripening-related genes (Klee, 2004).

In case of Alphonso mangoes, no variation in terms of volatile composition was observed among the localities at non-ripe stages and the clear difference was seen at the ripe stage. It is possible that the geographic difference might also exist at the non-ripe stages of the fruits in terms of the levels of precursors of the volatiles and during the process of ripening this difference is transformed into the variation observed in the volatiles. Although, it is difficult to pinpoint the exact environmental factors that are responsible for such phenotypic plasticity, there are certain known conditions that vary between sampling localities selected for the present study. For

example, plantation region at Deogad is hilly and the soil is lateritic and rocky and thus has much lower moisture retention capacity as compared to Dapoli and Vengurle (Burondkar et al., 2000; and personal observation). It was also shown that at Deogad, heat units required for complete maturation of the fruits are gained much rapidly as compared to Vengurle (Burondkar et al., 2000). It is possible that such differential environmental clues at various geographic locations might be picked up by the upstream regulatory switches such as MADS-box transcription factors during fruit development. The final important components of this dogmatic cascade, which might be acted upon by these regulatory elements to create the flavor variation, are obviously the genes actually involved in the biosynthesis of flavorants. To understand the roles and the regulation of such genes, a few key biosynthetic genes of terpenes and furanones, which are two of the most important flavor compounds in this fruit were isolated and characterized from Alphonso mango.

4.2. Two diverse geranyl diphosphate synthases are involved in GPP formation in 'Alphonso' mango

Monoterpenes form the important class of volatile compounds produced by plants. These chemicals are best represented in the blend of floral volatiles and essential oils of plants. Monoterpenes also play an important role as flavor compounds of many fruits giving a characteristic identity to that fruit. Many studies, as detailed in the section 1.7, have till now been carried out to understand the technical composition of mango flavor indicating the dominance of monoterpenes in mango fruits. On the other hand, there is no information available on the biosynthesis of monoterpenes in mango. Except an earlier report from our laboratory (Pandit et al., 2010), neither the enzymes involved in the monoterpene biogenesis have been studied nor are the putative sequences of any flavor genes reported. In order to understand biogenesis and regulation of monoterpenes in mango fruits, we isolated and characterized the genes encoding two geranyl pyrophosphate synthases from Alphonso mango.

4.2.1. *MiGGPPS* (*MiGPPS2*) encodes a functional GPP synthase

The biosynthesis of terpenes is characterized by the promiscuity of the enzymes involved in the pathway (Christianson, 2007). Many of the prenyl transferases reported till now are shown to produce multiple isopentenyl diphosphate products. For example, one isopentenyl diphosphate synthase from *Picea abies* was shown to produce both, GPP and GGPP (Schmidt et al., 2010). Similar bifunctional activity was also shown to be associated with the heterodimeric G(G)PPS from *Humulus lupulus* (Wang and Dixon, 2009). Although GGPPS is not generally responsible for the production of volatile terpenes, the possibility of its contribution to the biosynthesis of mono- and/or sesquiterpenes could not be ruled out in the light of above-mentioned studies. Thus, in addition to *GPPS*, the sequence similar to *GGPPS* was also cloned from the Alphonso mangoes and the encoded enzyme was assessed for its biosynthetic properties. Surprisingly, in the *in vitro* assays of this putative GGPPS, no GGPP was detected, rather GPP was found to be a major product in addition to small amount of FPP. Since such a production of GPP and FPP instead of GGPP could be the consequence of an *in vitro* reaction, it was necessary to confirm the observed activity by an *in vivo* assay. The absence of GGPP yielding activity with the putative GGPPS was verified by the "*in bacterio*" complementation assay. The plasmid pACCARΔcrtE has been constructed by cloning the *Erwinia uredovora* genes of caratogenesis (*crt*) cluster except *GGPPS* (Sandmann et al., 1993). When pACCATΔcrtE is complemented by the functional GGPPS, yellow colored zeaxanthin diglucoside pigment is formed. The putative GGPPS coding sequence from mango analyzed in this manner did not produce yellow-colored colonies confirming the absence of GGPP synthase activity with the putative GGPPS from mango. This finding along with the GPP synthase activity detected in *in vitro* assays resulted in nomenclature of the putative *GGPPS* as *MiGPPS2*.

4.2.2. Biochemical properties of MiGPPS1 and MiGPPS2

In contrast to the low sequence identity between MiGPPS1 and MiGPPS2, biochemical properties of these two enzymes were much similar. The relatively higher optimum temperature (40 °C) of MiGPPS1 and MiGPPS2

can be attributed to the higher temperature observed in the ripening fruits of Alphonso mangoes (Kumar et al., 1990). During the enzymatic reaction of prenyltransferases, binding of divalent metal ions such as Mg^{2+} to the allylic substrate is necessary for the dissociation of pyrophosphate moiety of the allylic molecule. Mg^{2+} is also required for the binding of the substrate molecules to the enzyme (King and Rilling, 1977). These facts could clearly explain the absence of the activity of MiGPPS1 and MiGPPS2 in the absence of $MgCl_2$. Many of the GPPSs reported till now were at least partially active with Mn^{2+} as a cofactor instead of Mg^{2+} (Croteau and Purkett, 1989; Tholl et al., 2001) ; whereas, some of the GPPSs preferred Mn^{2+} over Mg^{2+} (Clastre et al., 1993; Suga and Endo, 1991). On the contrary, none of the MiGPPS1 and the MiGPPS2 showed any activity when Mn^{2+} was used as a divalent metal ion. Since mango fruits contain more than 600 fold higher concentration of magnesium as compared to manganese (Malik et al., 2004), the lack of activity with Mn^{2+} can be explained as an adaptation of MiGPPSs to the higher concentration of Mg^{2+} in the fruits. The activity profile with the varying Mg^{2+}concentration, the optimum Mg^{2+} concentration of 6 mM and the pH optima between 7 and 8 of MiGPPS1 and MiGPPS2 was quite similar to the GPPS reported from *Abies grandis* (Tholl et al., 2001).

As detailed in the Results section 3.1, the highest concentration of monoterpenes was detected in the ripe fruits (15 DAH) for all the three localities, Dapoli, Deogad and Vengurle. Among these three localities, Dapoli had the highest concetration of monoterpenes and Deogad had the lowest concentration at 15 DAH stage. The expression pattern for all the three localities through the ripening stages was quite similar to each other with the expression optima at 10 DAH for both *MiGPPS1* and the *MiGPPS2*. This suggests the key ripening related role of these two genes in Alphonso mango fruits. The highest expression at 10 DAH stage can be hypothesized as a preparative expression for the 15 DAH stage containing the highest amount of monoterpenes. The absence of clear difference between the localities for the transcript abundance of *MiGPPS1* and *MiGPPS2* could be surmised to be

because of regulation of monoterpene biosynthesis at the other check-points such as monoterpene synthase or involvement of these genes in the additional functions.

4.2.3. Functions of *MiGPPS1* and the orthologous genes

Among the four G/GGPPS clades, the enzymes of clade 1, 2 and 4 were shown to be involved in the biosynthesis of respective isopentenyl diphosphates and/or terpenes. Expression of *GPPS-SSU* of clade 4 was highly correlated with the volatiles and the expression of monoterpene synthase in hop and in the other plants (Wang and Dixon, 2009). The enzymes belonging to clade 1 were also shown to be involved in the terpene production induced upon methyl jasmonate treatment (Hefner et al., 1998; Schmidt and Gershenzon, 2007, 2008; Schmidt et al., 2010). Similarly, methyl jasmonate induced taxol biosynthesis in *Corylus avellana* and the spatial variation in the floral volatiles of hop was paralleled by the similar pattern of expression of *GGPPS* and *GPPS-LSU*, respectively, which group together in clade 2 (Wang and Dixon, 2009; Wang et al., 2010). Although the enzymes belonging to clade 3 have also been shown to have GPP synthase activity *in vitro*, no correlation between *in planta* expression of these genes and the phenotype has been found. For example, although there was an increase in the monoterpene-rich oleoresins upon methyl jasmonate treatment in spruce, the expression level of *GPPS3* belonging to clade 3 was unaltered (Schmidt and Gershenzon, 2007). Similarly, silencing of *GPPS* in tomato resulted in dwarfed plants, which was further shown to be because of reduced gibberellin content (van Schie et al., 2007). These studies point towards involvement of members of clade 3 in the functions in addition to/other than monoterpene biosynthesis. Thus, in addition to the explanation as a preparative expression for synthesizing the highest amount of monoterpenes at the ripe stage, the maximum expression of *MiGPPS1* observed at the 10 DAH stage can be attributed to the hypothetical involvement of *MiGPPS1* in the additional functions as detailed above.

4.2.4. MiGPPS2 and its product chain-length determination

The amino acid sequence of GPPS and GGPPS show higher homology to each other than to FPPS, although there is a difference of C10 between the products formed by GPPS and GGPPS (Hsiao et al., 2008). This fact is also evident from the phylogenetic tree constructed in the present work where FPPS formed a distinct cluster; whereas, GPPS and GGPPS were clustered close to each other. MiGPPS2 grouped with the angiosperm GGPPS and GPPS-LSU forming clade 2. The large subunit of GPPS is inactive by itself and it forms an active enzyme only with another small subunit (Nagegowda, 2010). Thus, MiGPPS2 is the only member of clade 2 which by itself formed an active GPPS presumably as a homodimer, since single recombinant polypeptide could show the activity in our studies.

In contrast to GGPPS and FPPS, which have been well understood for the chain-length determining mechanism, not much is known about the means by which GPPS restrict its products length to C10. In case of GGPPS and FPPS, it has been shown that the two amino acids at the -5 and -4 position before FARM form the chain-length determining region. If the residues in this region are bulky and aromatic like phenylalanine and tyrosine, their side-chains protrude into the reaction cavity which blocks the further chain elongation leading to formation of shorter chain products such as FPP. In case of almost all GPPS reported till now, the corresponding amino acids were much smaller such as alanine/methionine and serine. The chain-length determination mechanism of GPPS thus appears to be different than that of FPPS and GGPPS. In case of the heterodimeric GPPS the unawareness about the product chain-length determining mechanism is further aggravated by the fact that the catalytic LSU shares very high sequence homology with the GGPPS. More importantly, the residues in the chain-length determining (CLD) region of GGPPS are also conserved among the GPPS-LSU and still there is a difference of C10 between the products of these two enzymes (Burke et al., 1999). Such high sequence similarity of GPPS-LSU with GGPPS is compensated by the interactions between LSU and SSU, which results in the

remodeling of the reaction cavity and restriction of the product length to GPP (Burke and Croteau, 2002; Chang et al., 2010; Orlova et al., 2009). MiGPPS2 is also highly similar to GGPPS and GPPS-LSU, nonetheless, it does not produce GGPP but synthesizes GPP and small amounts of FPP in the absence of any regulatory SSU. In MiGPPS2, the residues in the region corresponding to the CLD stretch were smaller (methionine and serine) and hence, in the modeled structure of MiGPPS2, none of these residues appear to have their side chains protruding into the activity cavity (Fig. 3.18). This underlines the possibility of regulation of the product size of MiGPPS2 by some other residues. The mutagenesis studies on *FPPS* and *GGPPS* have indicated that alterations of amino acids other than those of the active site and the CLD region can also result in the different products (Hemmi et al., 2003; Hirooka et al., 2000; Kawasaki et al., 2003; Lee et al., 2004). In addition, it was shown that out of merely eight relevant amino acid differences between the GPPS and GGPPS reported from grand fir, only one lie in the conventional CLD region (Burke et al., 2004). Conversion of each of this residue in GPPS, individually to the corresponding residue in GGPPS does not affect the product distribution indicating that more than one amino acid is involved in the chain-length determination. Although no obvious amino acid differences in the alignment between and in the structure of MiGPPS2, mint GPPS-LSU (Chang et al., 2010) and *Sinapis* GGPPS (Kloer et al., 2006) could be spotted, in the light of above-mentioned studies, an unexpected activity of the MiGPPS2 as a GPP synthase instead of GGPP synthase might be due to the cumulative effect of several minor amino acid differences distant from the active site and the conventional CLD region.

4.3. Farnesyl pyrophosphate synthase might be involved in the development of flavor and other metabolites in Alphonso mango

Sesquiterpenes formed another subclass of the terpenic compounds which dominated the flavor of Alphonso mango. One of the most important branch-point steps in the biosynthesis of these compounds is catalyzed by farnesyl pyrophosphate synthase (FPPS), which is thus shown to be a rate limiting step

in the terpenoid biosynthetic pathway (Grunler et al., 1994). In order to understand contribution of FPPS to the mango flavor, the gene encoding this enzyme was isolated and characterized from the fruits of Alphonso mango.

Using the degenerate primers based on the conserved regions of the farnesyl pyrophosphate synthases reported from the other plants turned out to be an effective strategy for isolating *FPP*S from non-model plant such as mango, from which no previous sequence information was available. When ends of the cDNAs were amplified by RACE, the obtained fragments showed sequence similarity to the 5' and 3' ends of the full-length *FPPS* genes reported from the other plants. Also, no intervening stop codons were detected upon *in silico* translation of the DNA sequence constructed from these fragments. These observations suggested that the obtained overlapping 5' and 3' fragments represent the complete open reading frame of *MiFPPS*. Genomic organization of plant *FPPS* has till now been reported only in Arabidopsis (Cunillera et al., 1996).The gene structure of *MiFPPS*, showing the presence of 11 introns and 12 exons is consistent with that of Arabidopsis *FPPS*.

4.3.1. Biochemical properties of MiFPPS

In consistence with the sequence-based characterization of the isolated cDNA as a FPPS, recombinantly expressed MiFPPS produced FPP as its major product in an *in vitro* assay using IPP with DMAPP as an allylic substrate. It is well-known that during the biogenesis of isopentenyl pyrophosphates higher than GPP, additions of two or more units of IPP to DMAPP occur sequentially. DMAPP condenses with the first molecule of IPP to produce GPP which remains enzyme bound (Elitzur et al., 2010) and is used as an allylic substrate for the subsequential addition of another molecule of IPP. Thus, in addition to the explanation as the activity of *E. coli* enzymes, detection of the trace amounts of GPP (1-3% of the total) during the assay with MiFPPS can be hypothesized as a consequence of an *in vitro* reaction. Such by-production of GPP by FPPS is also consistent with the earlier reports (Delourme et al., 1994; Hemmerlin et al., 2003; Ogura et al., 1985; Pan et al., 1996; Schmidt and Gershenzon, 2007; Xiang et al., 2010).

During the enzymatic reaction of prenyltransferases, binding of divalent metal ions such as Mg^{2+} to the allylic substrate is necessary for the dissociation of pyrophosphate moiety of the allylic molecule. Mg^{2+} is also required for the binding of the substrate molecules to the enzyme (King and Rilling, 1977). These facts clearly explained the absence of the activity of MiFPPS in the absence of $MgCl_2$. Few of the FPPSs reported till now were at least partially active with Mn^{2+} as a cofactor instead of Mg^{2+} (Hemmerlin et al., 2003; Ogura et al., 1985). Strikingly, MiFPPS did not show any activity when Mn^{2+} was used as a divalent metal ion. Since mango fruits contain more than 600 fold higher concentration of magnesium as compared to manganese (Malik et al., 2004), the lack of activity with Mn^{2+} can be explained as an adaptation of MiFPPS to the higher concentration of Mg^{2+} in the fruits. The absence of the activity with the other two divalent cations, Zn^{2+} and Ca^{2+} is consistent with most of the isopentenyl diphosphate synthases reported till now. The optimum pH of 7.5 observed for MiFPPS is also similar to the near-neutral optimal pH observed for the FPPS from pumpkin (Ogura et al., 1985), *Ricinus communis* (Green and West, 1974) and cotton (Widmaier et al., 1980). At the unripe stage, pH of the Alphonso fruit pulp is highly acidic and there is sharp increase in the fruit pH during ripening (Yashoda et al., 2006). The increased activity of MiFPPS during fruit ripening can thus result from both the increase in the expression level of *MiFPPS* as well as shifting of the physiological pH towards the optimum range of MiFPPS.

4.3.2. Structural feature of MiFPPS

For carrying out similar biochemical function, certain amino acids of a particular enzyme are highly conserved among different plants. Isopentenyl diphosphate synthases which carry out additions of IPP units to the allylic substrate also show the presence of five conserved regions (I-V). MiFPPS contained all of these regions and region II and V had first and second aspartate-rich motifs (FARM and SARM), which are highly essential for the catalytic activity of prenyltransferases. Aspartate residues in these regions are involved in binding with the pyrophosphate moiety of substrate through Mg^{2+}

bridges (King and Rilling, 1977). It was also shown that replacement of aspartate residues in these regions with the other amino acids results in drastic reduction in the enzymatic efficiency (Joly and Edwards, 1993; Marrero et al., 1992; Song and Poulter, 1994). In the modeled structure of MiFPPS, intervention of the carboxylic side chains of aspartate residues into the substrate binding cleft supports their role in binding pyrophosphate moieties of the substrate. A stretch of about seven amino acids before FARM forms chain-length determining (CLD) region of the prenyltransferases. Nature of amino acids at the fourth and the fifth position before FARM in this region decides chain-length of the products. If these amino acids are aromatic, their bulky side chains protrude into the activity cavity preventing further chain elongation of the prenyl pyrophosphate product and such enzymes usually synthesize shorter products such as GPP or FPP (Wang and Ohnuma, 1999). On the other hand, in the longer isoprenyl diphosphate synthases such as GGPPS, these amino acids are usually smaller (Ohnuma et al., 1996). In case of MiFPPS, these chain-length determining residues were tyrosine and phenylalanine and in the modeled structure, the side-chain of phenylalanine was observed to protrude in the central cavity (Fig. 3.28) supporting the functional characterization of MiFPPS as a farnesyl pyrophosphate synthase. Further, FPPSs are classified into two types based on their chain-length determining mechanisms. In Type I FPPS, two aromatic amino acids in the CLD region are solely responsible for determining the product length and FARM is constituted by DDX_2D; whereas, in Type II FPPS, only the 4^{th} amino acid upstream of FARM is aromatic and FARM is formed by DDX_4D (Ohnuma et al., 1997). Based on these reports and the sequence analysis, MiFPPS could be classified as Type I FPPS.

4.3.3. Probable functions of MiFPPS

Transcripts profiling of *MiFPPS*, carried out to know its expression behavior through the ripening process and along the cultivation localities, revealed that the profile was consistent through the ripening process for all the three localities. Such consistency suggested a key ripening related role of MiFPPS.

Its highest expression was observed at 10 DAH stage. This is an important stage in the Alphonso ripening that displays turning (yellowish) color and start of the fruit softening. In tomato, it was shown that during ripening, especially, during the turning stage (between mature green and red ripe stage) there was a substantial accumulation of the sterols (Chow and Jen, 1978; Whitaker, 1988), which has been hypothesized to play a role in the structural changes in the cell membrane associated with the fruit softening. Similar ripening-related accumulation of sterols has also been reported in apple (Galliard, 1968). Although no reports are available for mango, being a climacteric fruit such accumulation of sterol during mango ripening is very likely and can support the rise in *MiFPPS* transcripts at the 10 DAH stage. Secondly, in tobacco the overexpression of yeast *FPPS* resulted in the increased accumulation of not only sterols but also of carotenoids (Daudonnet et al., 1997). Considering that the carotenoids are the major components of ripe mango color, role of MiFPPS in this aspect demands investigation. In the earlier studies from our laboratory, we have reported that cultivar Alphonso belongs to the group of monoterpene dominant mangoes (Pandit et al., 2009a; Pandit et al., 2009b). Sesquiterpenes are the secondary volatile flavorants in Alphonso and they do not show a clear abundance pattern over the process of ripening. This suggests that the role of MiFPPS might be more prominent in the biosynthesis pathways of compounds such as sterols than in that of volatile sesquiterpenes.

In order for an enzyme to be able to function in the temporally and spatially diverse cellular environment, plants contain multiple isoforms of single enzyme. This is also true with respect to FPPS; many plants have been reported to contain different types of FPP synthases that vary in their expression level and function. Two *FPPS* genes are reported from *Arabidopsis* (Cunillera et al., 1996); one was shown to be constitutively expressed in all the tissues throughout the development; whereas, the other was expressed only in the particular organs at a specific stage and lack of its expression was shown to be correlated with the reduction in sitosterol content (Closa et al., 2010). Out of the three genes of *FPPS* found in *Artemisia tridentata*, one was shown to

produce different products than the other two which in turn were shown to be different from each other in terms of the biochemical requirements and the expression level (Hemmerlin et al., 2003). In additions, plants such as *Lupinus albus* (Attucci et al., 1995), *Parthenium argentatum* (Pan et al., 1996), rice (Sanmiya et al., 1997), *Panax ginseng* (Kim et al., 2010) and tomato (Gaffe et al., 2000) are also known to have at least two copies of *FPPS*.

In the light of these facts, there is a need of exploration of mango transcriptome and genome for the possible presence of the other isoforms of FPPS and of the mango metabolome for the additional phenotype (for example, sterols) of MiFPPS. Studies on the genes of other check-points of the sesquiterpene biosynthetic pathway such as sesquiterpene synthase, and the correlation studies between various genes of the pathway and their products in the context of metabolic flux can give very useful insights into the biogenesis and regulation of this largest sub-group of terpenes.

4.4. Multifunctional nature of an oxidoreductase catalyzing furaneol biosynthesis in 'Alphonso' mango

Mono- and sesquiterpenes are the quantitatively dominant compounds of the Alphonso mango flavor. Nonetheless, these chemicals are present throughout the developing and ripening stages of the fruits and even in the flowers and the leaves (Pandit et al., 2009b). On the other hand, in terms of the flavor changeover, ripening of Alphonso mango fruits is characterized by *de novo* synthesis of some compounds, out of which furanones form an important component. These compounds are not detected in any other part of the mango plant and in the fruits also, they are highly restricted to the ripe stage. In addition to having the sweet and pleasant odor, furaneol and mesifuran, two furanones detected in the Alphonso fruits, are characterized by their low odor detection threshold; suggesting that their odor can be detected at a very low concentration (Pino and Mesa, 2006). This makes the contribution of furanones to the Alphonso mango flavor, more than 20 folds greater than any other volatile compound, in terms of the odor units. To get the insights into

the biosynthesis and regulation of furanones in Alphonso mango, complete open reading frame of the gene encoding enone oxidoreductase which catalyzes the formation of furaneol was isolated and characterized from the ripe fruits of Alphonso mango.

4.4.1. Furaneol biosynthesis in Alphonso mango

Mango is only the third plant, after strawberry and tomato, which has been studied for the biosynthesis of furaneol; specifically, for the gene of enone oxidoreductase which encodes the final enzyme of furaneol biosynthesis pathway. Since ripe Alphonso mango fruits contain high amounts of furanones, the most likely *in planta* function of MiEO, which produces furaneol in the *in vitro* assays, is the biosynthesis of furaneol. In the ripening fruits of Alphonso mango, the peak level of furanones is detected at the ripe stage (15 DAH) (Pandit et al., 2009b); whereas, the highest expression of *MiEO* was seen at 10 DAH stage. This can be surmised as a preparative transcript accumulation for the synthesis of highest amount of furanones at the 15 DAH stge. However, in strawberry, it was shown that the expression of similar gene, *FaEO*, was highly correlated with the furanone levels in the same ripening stage of the fruits (Raab et al., 2006). Several reasons for such discrepancies between strawberry and mango could be accounted. Most importantly, there is a difference in the ripening physiology of these two fruits, strawberry is non-climacteric; whereas, mango is a climacteric fruit and noteworthy differences in the expression of various genes are observed among these two types of fleshy fruits (Lee et al., 2010). Secondly, the level of furanones observed in mango is much lower than that in strawberry; also the precursor of furaneol, HMMF, is not detected in the mango fruits (Klein 2007). In addition to this, in contrast to the geographic differencce observed in the furanone content (section 3.1.3), no clear discrepancy was observed in the expression level of *MiEO* in the fruits sampled from different cultivation locations. All these observations point towards involvement of MiEO in the functions in addition to biosynthesis of furaneol.

4.4.2. Other possible functions of MiEO

In addition to strawberry EO (FaEO), MiEO shows high sequence identity with some enzymes from the other plants. One such enzyme, CsAOR from *Cucumis sativus*, which shows 79% sequence identity with MiEO, catalyses the reduction of α, β-unsaturated alkenals/alkenones in the *in vitro* reactions (Yamauchi et al., 2011). Similar scavenging oxidoreductase activity was also shown to be associated with the enzyme from Arabidopsis (AtAOR). The unsaturated aldehydic/ketonic substrates of AORs, generated by lipid peroxidation, are highly reactive chemicals that can damage cellular activities by reacting with various biomolecules. Enzymes such as CsAOR and AtAOR are thus important for maintaining cellular processes by converting these harmful carbonyls into the saturated compounds (Yamauchi et al., 2011).

Analysis of the putative amino acid sequence of MiEO strongly suggests that MiEO might be localized in chloroplasts. This prediction can also be supported by the fact that chloroplasts are rich in fructose-1,6-diphosphate, the starting substrate for furaneol biosynthesis, accumulated by various pathways (Bouvier and Camara, 2007). Chloroplasts are also the centre of production of highly reactive chemicals because of high metabolic activity of this organelle, which can mainly be attributed to the process of photosynthesis. The increased rate of chemical reactions in chloroplasts of the fruits also results from the physiological transition of chloroplasts into chromoplasts, which is the most remarkable feature of fruit ripening. This conversion is characterized by various cellular changes such as dismantling of the thylakoid membrane system which is brought about by degradation of its membrane lipids and of chlorophylls, biosynthesis of carotenoides, reduction in the amount of the proteins involved in photosynthesis, etc. (Bouvier and Camara, 2007). Some of these metabolic processes, especially the degradation of membrane lipids, are known to yield highly reactive compounds such as unsaturated carbonyls and reactive oxygen species (Yamauchi et al., 2008). As a specific example, malondialdehyde, which is produced in the chloroplasts of the plants (Yamauchi et al., 2008), is one of the most harmful unsaturated carbonyls that shows increased level in the Alphonso mango in

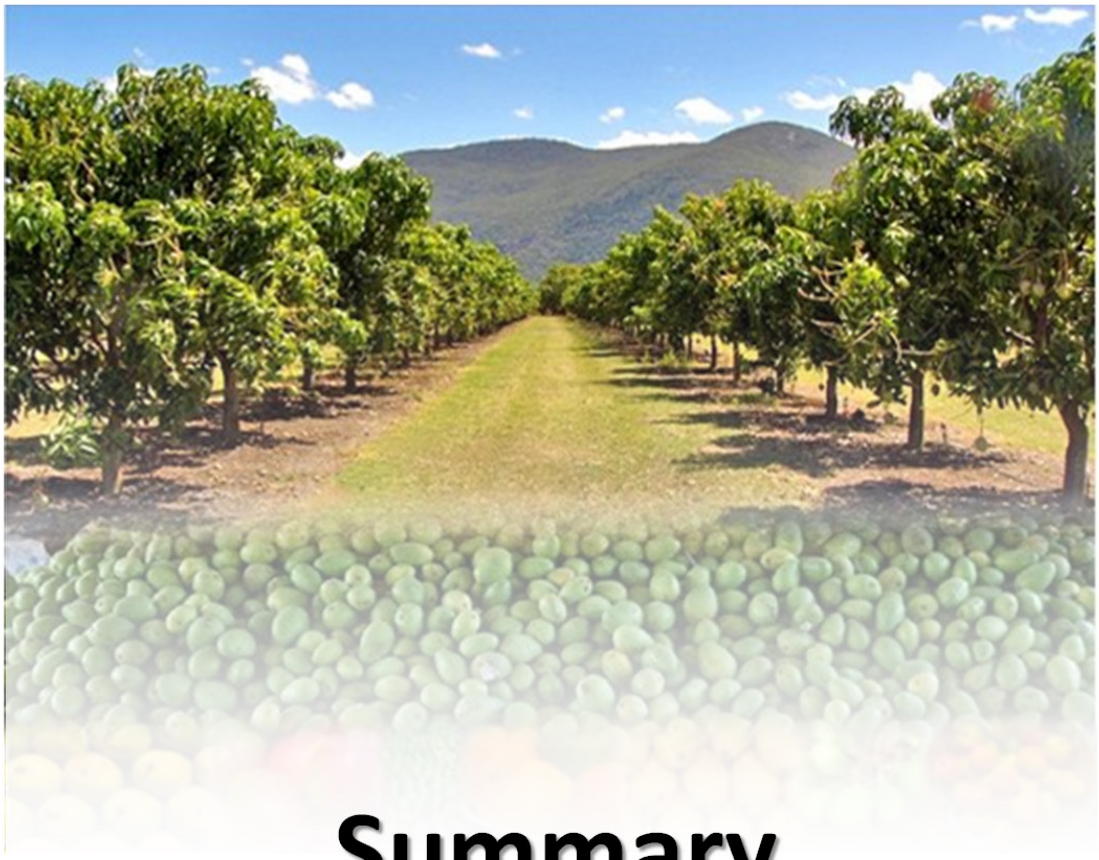
the physiological disease such as spongy (Nagamani et al., 2010). This makes the need of having the scavenging enzymes more in the chloroplasts/chromoplasts than any other organelle of the ripening fruits. Since the chloroplast-located enzymes from the other plants, CsAOR and AtAOR, which are highly similar to MiEO have been shown to be involved in scavenging of the reactive compounds, it is possible that MiEO also might be involved in such processes in addition to biosynthesis of furaneol. This hypothesis is also supported by the fact that HMMF, the precursor of furaneol, is not detected in the mango fruits (Klein et al., 2007). Although this could be because of the low level of HMMF in mango fruits, this observation along with the report of *MiEO*-like transcripts in the plants which have till now not been reported to contain furaneol (Table 3.3) and the absence of correlation between the transcript abundance of *MiEO* and the level of furanones in the mango fruits point towards the multifunctional nature of MiEO.

Detailed enzymatic characterization of MiEO using various unsaturated substrates, transcript measurement of *MiEO* in the developing fruits and the vegetative tissue of mango as well as studies on *MiEO* by the approaches such as its expression in the model plant system can shed light on the exact contribution of this enzyme to the mango metabolome.

4.5. Concluion

With the objective of studying chemical basis for the variation observed in the flavor of Alphonso mango, ripening fruits of Alphonso mango from three cultivation localities, Dapoli, Deogad and Vengurle in Maharashtra, India, were analyzed for the volatile composition. The ripe fruits from Dapoli contained the highest amount of monoterpenes as compared to those from Deogad; whereas, the levels of lactones and furanones were higher in Deogad as compared to Dapoli. To study the biosynthesis of flavor volatiles and regulation of their variation through Alphonso ripening and the cultivation locations, the genes encoding key enzymes of the biosynthesis of terpenes (GPPS and FPPS) and furanones (enone oxidoreductase) were isolated, the

complete open reading frames were expressed in *E. coli* and the recombinant proteins were characterized enzymatically. The expression profiling of these gene in the ripening fruits of Alphonso mango depicted their ripening-related pattern of expression suggesting key ripening-related role of these genes in Alphonso mango.



Summary and Future Prospects

SUMMARY AND FUTURE DIRECTIONS

Alphonso is one of the highly popular mangoes in India that is thought to show cultivation-locality dependent variation in its flavor. To get chemical insights into such variation, ripening fruits of Alphonso were analyzed for the content of volatiles by gas chromatography. Three localities, Dapoli, Deogad and Vengurle belonging to north, central and south Konkan were selected for sampling the fruits. Ripe fruits from Deogad had lower content of mono- and sesquiterpenes and higher content of lactones and furanones as compared to the fruits from Dapoli; whereas fruits from Vengurle had average quantities of these chemicals in comparison with Deogad and Dapoli fruits. This variation was clearly reflected as separate clustering of the localities in the Principal Component Analysis. The localities were indistinguishable from each other in terms of raw fruit volatiles. This study exemplifies a case of phenotypic plasticity; since the plants chosen were clonally propagated, such geographic variation in the volatiles can be attributed to varied abiotic conditions in these three localities.

Biosynthesis of flavor volatiles in the Alphonso mangoes was studied with respect to homology-based isolation and characterization of the genes encoding two geranyl diphosphate synthase (*MiGPPS1* and *MiGPPS2*), a farnesyl pyrophosphate synthase (*MiFPPS*) and an enone oxidoreductase (*MiEO*) from the ripe fruits of Alphonso mango. GPPS and FPPS are the important branch-point enzymes involved in the biosynthesis of terpenes, which quantitatively dominate the mango flavor; whereas, enone oxidoreductase catalyzes the production of furaneol, an important ripening-related flavor compound which also acts as a precursor for another flavorant, mesifuran, in Alphonso mango. Complete open reading frames of these genes were isolated from the ripe fruit of Alphonso mango and heterologously expressed in *E. coli*. The purified recombinant proteins were assayed for the enzymatic activity. MiGPPS1, MiFPPS and MiEO, which showed high sequence similarity to the respective proteins from the other plants, produced the expected products, GPP, FPP and furaneol, respectively, in the *in vitro*

reactions. Although MiGPPS2 showed high sequence similarity with the plant GGPPS and the large subunit of heteromeric GPPS, it did not produce GGPP, but synthesized GPP without the need of any other subunit. The absence of GGPP synthase activity with MiGPPS2 was confirmed by the carotenogenic complementation assay.

The actual contribution of *MiGPPS1*, *MiGPPS2*, *MiFPPS* and *MiEO* to the Alphonso volatiles was assessed by measuring the relative transcript abundance of these genes in the ripening Alphonso fruits from Dapoli, Deogad and Vengurle by real-time quantitative PCR. All the four genes showed maximum expression at the 10 days after harvest stage at all the three locations. This can be hypothesized as a preparative expression for the next stage (15 DAH), which shows the highest concentration of terpenes and furanones. The pattern of expression of these genes across the three geographic locations, Dapoli, Deogad and Vengurle, which show variation in the volatiles, was quite similar. This suggests key ripening-related role of these genes in the Alphonso mango. In some of the plants these genes are also shown to be involved in the functions in addition to the biosynthesis of volatiles. For example, FPPS is well-known to be required for sterol biosynthesis; GPPS is involved in the gibberellin biosynthesis in tomato; whereas, the gene showing high similarity to the enone oxidoreductase is involved in the detoxification reactions in cucumber. In the light of these facts, in addition to the explanation as a preparative expression for the 15DAH stage, the highest expression of these genes at 10DAH stage can be hypothesized to be because of the involvement of these genes in additional functions in mango.

Future directions

This is the first study to address the chemical basis of cultivation locality dependent flavor variation observed in Alphonso mango. Also, the biosynthesis of volatiles in mango was studied for the first time. Apart from giving useful insights into the biology of the pleasant flavor of Alphonso mango, this study opens up new avenues of further research on mango flavor;

many questions are raised in addition to those being addressed. Some of the objectives with which future research on mango flavor can be continued are discussed below.

- Biosynthesis of lactones
    - This study highlights the abundance of numerous lactones in ripe fruits of Alphonso mango. No such diversity and high concentration of lactones is found in any other fruit. Also neither the genes, nor the enzymes involved in lactone biogenesis are known from any plant. In this scenario, Alphonso mango would be an ideal system to study biosynthesis of lactones.
- Effect of exogenous ethylene treatment on ripening of Alphonso mango
    - Analysis of volatile flavorants and the expression profiling of various flavor- and ripening related genes in ethylene-treated and control Alphonso fruits.
- Analysis of the glycosidically bound flavor compounds
    - Chemical analysis for the content of glycosides and biochemical studies on the enzymes and the genes of glycosyltransferases and glycosidases which are involved in the metabolism of glycosides of the volatiles, which represent an important part of flavor.
- Studies on the regulatory mechanism of the flavor biosynthesis
    - Analysis of the promoter regions of the genes studied in this thesis and the associated transcription factors with respect to their role in the regulation of flavor biogenesis during ripening and creation of flavor variation between different cultivation localities.
- Studies on the above-mentioned parameters in relation to the physiological disease such as spongy in Alphonso mango.

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APPENDIX 1

Nucleotide sequences of the *Mangifera indica* flavor genes

>JN035297 *Mangifera indica geranyl pyrophosphate synthase 1* (*MiGPPS1*), mRNA, complete cds

ATGTTATTTTCTTATGGCCTTTCTCGGATTTCAATAAATCCTAGAGCCTCCTTGTTGACTTGTCGT
TGGCTTCTCTCGCATCTGACTGGCTCCCTGAGCCCTTCCACTTCTTCACACACTATCAGTGACTCA
GTTCATAAGGTTTGGGGTTGCAGAGAAGCTTATACGTGGAGTGTTCCTGCCTTGCATGGTTTTAGA
CACCAAATTCATCACCAAAGCAGCTCCCTAATTGAGGATCAACTCGACCCATTTTCCCTTGTTGCT
GATGAACTATCACTTGTTGCTAACAGGCTGCGCTCCATGGTAGTCACTGAGGTACCCAAGCTTGCC
TCAGCAGCTGAGTATTTCTTCAAAATGGGAGTGGAGGGAAAGAGGTTTCGTCCCGCGGTTTTATTG
TTGATGGCAACAGCCTTGAATGTGCATGTACTTGAGCCACTTCCTGAAGGTGCAGGAGATGCTTTG
ATGACTGAGCTACGTACAAGACAACAATGTATAGCTGAGATTACTGAGATGATCCATGTAGCAAGC
CTTCTTCACGATGATGTCTTGGATGATGCAGATACAAGGCGTGGCATTGGTTCGTTAAATTTAGTA
ATGGGGAATAAGTTAGCTGTATTAGCGGGAGATTTTCTTCTATCTCGCGCTTGTGTTGCCCTTGCT
TCATTGAAAAACACAGAGGTTGTATCATTACTGGCAACAGTTGTAGAGCATCTTGTTACCGGTGAA
ACAATGCAAATGACTACTTCATCTGATCAACGGTGTAGCATGGAATATTATATGCAAAAAACATAC
TACAAGACTGCTTCATTGATATCAAATAGCTGCAAGGCAATTGCTCTTCTTGCTGGGCAATCAGCA
GAAGTTGCAATGTTGGCTTTTGAGTTTGGAAAAAATCTGGGACTGGCCTACCAATTAATAGATGAC
GTTCTTGATTTCACGGGCACATCAGCTTCACTTGGAAAGGGATCTTTATCGGACATACGGCATGGA
ATTGTAACGGCTCCTATACTGTTTGCAATGGAAGAATTCCCCCAGTTGCGTGCAGTTATTGATCAG
GGCTTTGAAAATCCTTCAAACGTCGATGTCGCTCTTGAATACCTTGGCAAGAGTCGGGGAATACAA
AGGACGAGAGAGCTAGCGACAAACCATGCCAACCTTGCTGCAGCTGCCATCGATGCTCTACCCAAA
ACTGACAATGAAGAAGTAAGAAAGTCAAGACGGGCACTTTTAGATCTAACTCAAAGAGTCATCACA
AGAAATAAATGA

>JN035298 *Mangifera indica geranyl pyrophosphate synthase 2* (*MiGPPS2*), mRNA, complete cds

ATGCCCTTTGTCGTGCCAAGACGAAACAGATCCCTGTCAGTTTCCGCTGTTCTCACCAAAGAAGAA
ACTCTCAGGGAAGAAGAGGAAGACCCAAAACCCGTGTTCGATTTCAAGTCTTATATGCTTCAGAAA
GGCAATTCTGTTAACCAGGCCCTTGACGCCGTCGTTTCAATCCGTGAACCCAAAAAAATTCACGAA
GCTATGAGGTATTCTCTTCTAGCGGGCGGCAAGCGTGTAAGACCGGTGCTCTGCATCGCTGCGTGT
GAACTTGTTGGTGGTAATGAGTCCATGGCGATGCCGGCCGCTTGTGCTGTTGAAATGATTCACACC
ATGTCCTTGATTCATGATGATCTACCTTGTATGGATAACGACGACCTTCGTCGGGGAAAACCGACA
AACCACAAAGTTTTCGGTGAAGACGTCGCCGTTTTAGCCGGTGATGCACTTCTTGCCTTTTCGTTT
GAAAACATGGCTGTTTCTACGGTTGGCGTTCTGCCTTCGAGGGTGGTCAAAGCAGTTGGAGAATTA
GCGAAATCGATTGGCATTGAGGGTCTTGTTGCCGGCCAAGTTGTGGATATAAACTCTGAAGGTTTA
AAAGAAGTGGGCTTAGATCATCTTGAATTTATTCATCAGCATAAGACAGCTGCATTACTGGAAGGA
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GCTCGGTGTATTGGGTTGTTGTTTCAGGTGGTTGATGATATTCTTGATGTGACAAAGTCATCTCGG
GAACTTGGAAAGACTGCTGGCAAAGATTTGGTGGCTGATAAAGTCACTTATCCTAAGTTGTTGGGG
ATTGAAAAATCAAGGGAATTAGCTGACAAGTTAAATAAAGATGCTCAACAACAATTGTCTGGTTTT
GATCAGGAGAAAGCCGCCCCTTTGATTGCTTTGTCTAATTATATTGCCTATAGGCAAAATTGA

>JN035296 *Mangifera indica farnesyl pyrophosphate synthase* (*MiFPPS*), mRNA, complete cds

ATGAGTGATTTGAAGTCCAAGTTCGTGGAGGTTTACAATATCTTAAAACAGGAGCTTCTCAATGAC
CCTGCCTTTGAATTTACTGACGTTTCTCGCCAATGGGTCGAACGTATGCTGGATTACAATGTTCCT
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CTAACAGATGATGAAATTTTTCTTTCATCTGCACTTGGCTGGTGTATCGAATGGCTTCAGGCTTAT
TTTCTTGTTCTTGATGATATCATGGATGGCTCACATACACGTCGTGGTCAACCTTGCTGGTTCAGA
CGTCCGAAGATTGGTATGATTGCCGTAAATGATGGCATAATACTTCGCAACCATATCCCAAGAATT
TTGAAGAAGCATTTTAGGGGAAAGCCTTATTATGTGGACTTGTTGGATTTATTTAATGAGGTCGAA
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CCGGTTGCTTGTGCTTTACTGATGGCAGGCAAAAATCTTGATGATCACATTGATGTCAAGAACATT
CTTATTGAAATGGGAATCTATTTTCAAGTACAGGATGATTATCTAGATTGTTTTGGCACTCCTGAA
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GTTGCAAAAGTAAAAGAGCTTTACAATACTATCGATCTTCAGGGTGCGTTTGCAGAGTATGAAAGT
GCAAGTTATGAAAGGTTAATCAAATCCATTGAAGCTCATCCCAATAAGGCCATTCAAGCTTTGTTG
AAGTCATTTTTAGCCAAGATATATAAGAGGCAGAAGTAG

>*Mangifera indica farnesyl pyrophosphate synthase* (*MiFPPS*), genomic DNA

ATGAGTGATTTGAAGTCCAAGTTCGTGGAGGTTTACAATATCTTAAAACAGGAGCTTCTCAATGAC
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TTGTTTAAGATAAACTTCAGCCTACTTTAATTCAATGGACATAGATTTGTGTTGTAAACAGTGGGC
TGAAAGCATTTGGTATTTTTCATAGAGTTGAAATCAATAGTATCGTGGCTGAAAGAAATGAGACGA
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GGGCTCCTAGTTGGAATTTTCAGGGGATATGAATTGATTTGAATTGGCTAATCTGTTAAAGATTAT
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TGCATCTGCTTGTCATGTTAATGTGATCAACATTTTACGGGGTATTCTTGTAGGGTGCGTTTGCAG
AGTATGAAAGTGCAAGTTATGAAAGGTTAATCAAATCCATTGAAGCTCATCCCAATAAGGCCATTC
AAGCTTTGTTGAAGTCATTTTTAGCCAAGATATATAAGAGGCAGAAGTAG

>*Mangifera indica enone oxidoreductase* (*MiEO*), mRNA, complete cds

ATGGAAACCATACTAGCATCAGCACCTTCTAAATTCACAAGCTTCCACTCATTTTCTCAGAGGTTC
TCCCTCGCCTTCCGGGAAAATAGAAGGAAAAACGTTGTCAACGTTAACGGAGCGTCATCGTCTTAC
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AGTGTACCCAGTGAAATGAAAGCGTGGGTGTATGGAGAATATGGAGGAGTCGATGTATTGAAATTT
GATGAGAAAGTTTCTGTGCCTCAAGTGAAGGAAGATCAGGTGTTGATCAAGGTTGTTGCTGCGGCT
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CAAGTATTTGGTGCTTCAAAAGTTGCCGCAACTTCAGGAACCAGAAACTTGGAACTTTTGAAGAGC
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GAGCTCCCAACGCGTT

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Academic Qualifications

Master of Science (M.Sc.)	Biochemistry, 2003, First Class, University of Pune, Pune, India
Bachelor of Science (B.Sc.)	Chemistry, 2001, First Class with Distinction, University of Pune, Pune, India

Other Qualifications

- Completed the distance learning "General Course on Intellectual Property" of the World Intellectual Property Organization (WIPO) from October 1 to November 15, 2010
- Test of English as a Foreign Language (TOEFL) 2005; Score (CBT): 260/300
- National Eligibility Test for Junior Research Fellowship (NET, JRF) 2005, conducted by Council of Scientific and Industrial Research and University Grant Commission (CSIR-UGC), Government of India.
- National Eligibility Test for Lecturership (NET, LS) 2004, conducted by Council of Scientific and Industrial Research and University Grant Commission (CSIR-UGC), Government of India.
- Graduate Aptitude Test for Engineering (GATE) 2003 in Life Sciences; Percentile Score: 82.7

Professional Work Experience

August 2003 to April 2006: Worked as a Project Assistant II on the project entitled "Biomolecular Prospecting of *Symplocos* and *Gaultheria* species" at National Chemical Laboratory, India. The project involved biodiversity analysis in the species of *Symplocos* and *Gaultheria*, which are traditional medicinal plants of India, using the molecular markers along with a part of Natural Product Chemistry which involved isolation of secondary metabolites from above plants and analysis of natural product diversity using techniques like HPTLC, GC-MS, etc.

Projects Undertaken

- As a part of M.Sc. Degree: Isolation and Purification of Invertase from *Achras sapota.* Duration: 4 months, part time, 2003; under the guidance of Dr. S. V. Amarapurkar, at the Department of Chemistry, University of Pune, Pune, India

- Summer Training Project on Polymerase Chain Reaction at the National Institute of Virology, Pune, India during May-June 2002.

Honors/Awards

- Short-Term Scholarship (May-November 2010) from German Academic Exchange (DAAD) for carrying out part of the Ph.D. work under the guidance of Prof. Jonathan Gershenzon at the Max Plank Institute of Chemical Ecology, Jena, Germany
- Junior Research Fellowship (2006-2011) from the Council of Scientific and Industrial Research, India for doing Ph.D. at the National Chemical Laboratory, India
- Best Poster Award for the poster "Volatiles of developing and ripening Alphonso mango" presented on the occasion of the National Science Day, 2009, at the National Chemical Laboratory, Pune.
- Ranked second in the Biology Course Work examination held in December 2007 as a part of Ph.D. studies at the National Chemical Laboratory, Pune.
- Kawathekar Trust Scholarship during two years of M.Sc.

Publications

- Pandit SS, **Kulkarni RS**, Giri AP, Köllner TG, Degenhardt J, Gershenzon J, Gupta VS, 2010, Expression profiling of various genes during the development and ripening of Alphonso mango, *Plant Physiology and Biochemistry* 48, 426-433.
- Pandit SS, **Kulkarni RS**, Chidley HG, Giri AP, Köllner TG, Degenhardt J, Gershenzon J, Gupta VS, 2009, Changes in volatile composition during fruit development and ripening of 'Alphonso' mango, *Journal of Science of Food and Agriculture*, 89, 2071-2081.
- Pandit SS, Chidley HG, **Kulkarni RS**, Pujari KH, Giri AP, Gupta VS, 2009, Cultivar relationships in mango based on fruit volatile profiles, *Food Chemistry*, 144, 363-372.
- Apte GS, Bahulikar RA, **Kulkarni RS**, Lagu MD, Kulkarni BG, Suresh HS, Rao PSN and Gupta VS, 2006, Genetic diversity analysis in *Gaultheria fragrantissima* Wall. (Ericaceae) from the two biodiversity hotspots in India using ISSR markers, *Current Science,* 91, 1634-1640.

Communicated

- **Kulkarni RS**, Chidley HG, Pujari KH, Giri AP and Gupta VS, Geographic variation in the flavor volatiles of Alphonso mango. Communicated to *Food Chemistry*, February 2011

Under preparation

- **Kulkarni RS**, Pandit SS, Chidley HG, Nagel R, Schmidt A, Gershenzon J, Giri AP and Gupta VS, Cloning and functional characterization of farnesyl pyrophosphate synthase from mango
- **Kulkarni RS**, Pandit SS, Chidley HG, Nagel R, Schmidt A, Gershenzon J, Giri AP and Gupta VS, Two diverse geranyl diphosphate synthases are involved in the monoterpene biosynthesis in mango
- **Kulkarni RS,** Schmidt A, Giri AP, Pujari KH, Gershenzon J and Gupta VS, Molecular characterization of an oxidoreductase catalyzing furaneol biosynthesis in 'Alphonso' mango

Conferences/workshops

- **Kulkarni RS**, Chidley HG, Pujari K, Schmidt A, Gershenzon J, Giri A and Gupta V. Biochemistry and Molecular Biology of Alphonso Mango Flavor. (Poster) World Congress on Biotechnology, 21-23 March 2011, Hyderabad, India.
- Attended International Workshop on Nocturnal Pollination: Patterns and Processes, 23-27 March 2009, Indian Institute of Science, Bangalore, India.
- Banu S, **Kulkarni RS**, Borse SG, Kulkarni BG, Lagu MD, Joshi SP and Gupta VS, Genetic and biomolecular prospecting of two medicinally potent species of *Symplocos* from the two biodiversity hotspots in India. (Poster) International conference on Plant Genomics and Biotechnology: Challenges and Opportunities, 26-28 October 2005, IGAU, Raipur, India.
- Apte GS, Lagu MD, **Kulkarni RS**, Banu S, Borse SG, Kulkarni BG, Joshi SP and Gupta VS "Genetic diversity in natural populations of *Gaultheria fragrantissima* in two biodiversity hotspots in India." Proc. of National Workshop on Biodiversity Resource Management and Sustainable Use, 11-15th October 2004. Madurai Kamaraj University, Madurai, India.

Personal Details

Date of birth:	06/06/1982
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Nationality:	Indian
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