Chapter 6

Metabolite Profiling of Tuber in Sweet Potato

6.1 Introduction

With the mounting intimidation of increase in global population, critical need for augmentation of productivity in crop plants arises. This might be achieved by the infusion of novel genetic variability and improvement of the nutritional and industrial efficacy of the crop species. Efficient exploitation of genetic diversity remains a crucial factor in designing crops with improved agronomic traits and malleability to challenging environmental conditions. It has become possible to introduce and overexpress alien genes into plants and to design crop varieties with better agronomic attributes and adaptability to challenging environmental conditions. Metabolomics offers unique opportunities to accomplish the purpose of improving the current position of biological information(s) associated to the metabolites and more generally functional genomics. It is critical to achieve unbiased metabolic analyses and ultimately define the biochemical functions of primary and secondary metabolites [407, 408]. The term 'metabolome' implies the observable chemical profile or fingerprint of the metabolites in cell, tissues or organism [409]. In metabolite profiling, it is favorable to use a broad spectrum of chemical analyses, which are rapid, reproducible, and stable in time while necessitating only a very basic and simple sample processing. However, owing to the enormous diversity of primary and secondary plant metabolites, a comprehensive analysis of the metabolome is challenging. Consequently, exploring the metabolome necessitates the prioritization of distinct metabolite subsets on the basis of their physicochemical characteristics or abundance. Nonetheless, metabolite profiling strategies are instigated by a massive amount of individual targeted metabolite assays of high specificity and precision. Metabolite fingerprinting aims at acquiring global metabolite blueprints by NMR or MS-based applications which merely enable for suboptimal recovery of individual metabolites [293]. The magnitude of the metabolome varies significantly, depending on the organism studied. The plant kingdom has an estimated 200,000 primary and secondary metabolites. Metabolites represent diverse set of arrangements at atomic level than that of the proteome and transcriptome and this provides wide variations in chemical (molecular weight, polarity, solubility) and physical (volatility) characteristics. At genome level, pleiotropic effects may cause apparently distinct biochemical pathways to be affected (e.g., by genetic alterations). Therefore, analysis of all metabolites in a single analysis would be preferred [407]. GC-MS is an integrated system where volatile and thermally stable compounds are initially separated by gas chromatography (GC) and then eluting compounds are identified

conventionally by electron-impact mass spectrometers. In metabolomics, GC-MS has been described as one of the most efficient techniques. These approaches have been used to discriminate between two related genotypes as well as the genetically modified plants [293, 410].

Sweet potatoes are rich in antioxidants, such as phytophenolics and carotenoids, which also provide distinct flesh colors. The phytophenols and carotenoids often vary across the genotypes and are thought to be associated with genetic factors, which play a vital role in the formation of secondary metabolites [7]. Metabolites are the consequence of the interaction of the system's genome with its milieu, and are not merely the end product of gene expression but also form part of the regulatory system in an integrated manner [411]. So as to elucidate the factors pertaining to the varietal differences, and transgene mediated changes in the level of metabolites, a comprehensive analysis of phytochemicals as well as the metabolome profiling of wild type and transgenic tubers was performed. In addition, to nullify the environmental effect(s), wild types and transgenic lines were grown in precisely the same conditions. This study deals with the comparative analysis of the levels and types of secondary metabolites in wild types and the transformants.

6.2 Materials and Methods

6.2.1 Plant growth and maintenance, and tissue harvesting

The transgenic and wild type plants were grown in parallel in the greenhouse in identical conditions as described in **Section 4.2.1**. Mature tubers were harvested and pooled from eight different plants to normalize the growth and developmental effects, if any.

6.2.2 Determination of phytophenols and carotenoids

To determine the phytophenols, the extracts were prepared from lyophilized tuber samples. Total phenolic content (TPC) was determined by the method based on oxidation-reduction reaction by Folin-Ciocalteu reagent using gallic acid as standard.

Total flavonoids content (TFC) was determined by colorimetric method as described earlier [412]. The alcoholic tuber extract was diluted to a final volume of 5 ml with Milli-Q water and 0.3 ml 5% $NaNO_2$ was added. The mixture was then incubated at room temperature for 5 min. Subsequently, 0.3 ml 10% $AlCl_3$ was added followed by the addition of 2 ml 1 N

NaOH after 6 min. The solution was mixed thoroughly and the absorbance was measured at 510 nm against Milli-Q water as blank. Quercetin was used as standard and flavonoid contents were measured as quercetin equivalent.

The total anthocyanin content (TAC) was determined according to **Mancinelli et al. (1988)** with few modifications [413]. Approximately 0.5 g of tuber sample was extracted with 20 ml of acidified methanol (1% v/v HCl) for 24 h in darkness at 4°C with occasional shaking. Samples were then centrifuged at 5,000 x g for 15 min at 4°C. The supernatant was recovered and diluted to an appropriate concentration. The absorbance was read at 530 and 657 nm and total anthocyanins was estimated using the formula, A_{530} - $0.25A_{657}$ to recompense the absorption of chlorophyll degradation products at A_{530}.

Total carotenoid contents (TCC) were determined by the modified method of **Koala et al. (2013)** [414]. A known weight of dry powder was extracted with acetone-hexane (50:50, v/v) for TCC assay. Extracts were stirred vigorously, filtered and stored at 4°C in dark until further use. Absorbance of suitably diluted extracts was measured at 455 nm with β-carotene as standard.

6.2.3 qRT- PCR analysis of flavonoid pathway genes

The expression of key flavonoid pathway genes, chalcone synthase (*CHS*), chalcone flavanone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and UDP-glucose flavonoid 3-O-glucosyl transferase (*UFGT*) was analyzed by qRT-PCR. Total RNA was isolated by TriPure Isolation Reagent (Roche Diagnostics) following manufacturer's recommendation. cDNA were prepared using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). qRT-PCR analysis was performed using gene-specific primers: *CHSF* 5'-TGGGCCTGGGCTTACAATC-3' and *CHSR* 5'-CTTTGGGCCGGGCTTAA-3' (*CHS*); *CHIF* 5'-GCGGAGGAGTTGACGGATT-3' and *CHIR* 5'-TTCTCAAAGGGACCCGTAACG-3' (*CHI*); *F3HF* 5'-TATTCAAGGTGGCCGGACAA-3' and *F3HR* 5'-CAGCAGTTTGCATGCCAAGT-3' (*F3H*); *DFRF* 5'-TTATCGGCTCCTGGTTGGT-3' and *DFRR* 5'-TGTCCGCTTTCGGTAGTTC-3' (*DFR*); *ANSF* 5'-GCGTCCCGAACTCCATCAT-3' and *ANSR* 5'-CTTGCCGTTGCTGAGGATCT-3' (*ANS*); *UFGTF* 5'-GCCGCCACTCCAAACG-3' and *UFGTR* 5'-CATTCCTGGGATTACTTTCAGCTT-3' (*UFGT*). The expression data were normalized

against the expression levels of actin as an internal control using actin specific primers *Actin*F 5'-CTCCCCTAATGAGTGTGATGTGAT-3' and *Actin*R 5'-GAGCCCCATGAGAACATTACCA-3'. The primers were designed using the Primer Express Software v3.0.1. The qRT-PCR was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) using SYBR green dye. The analyses were done with two biological and three technical replicates. Mean of Ct values for target and endogenous control was considered for calculating the relative quantitation (RQ) value using comparative Ct ($2^{-\Delta\Delta Ct}$) method.

6.2.4 Metabolite profiling of tuber

Metabolite profiling of wild type (cv. OFSP-6) and two independent transgenic tubers (one each from COE and TOE lines) were performed by the method described in **Section 5.2.5**.

6.2.5 Statistical analysis

Statistical significance of the data was analyzed by the unpaired student's *t*-test method using Graphpad prism 5 software. $P < 0.05$ was considered to be statistically significant, and the results are expressed as mean ± SE.

6.3 Results

6.3.1 Determination of phytophenols and carotenoids

Sweet potatoes are rich in antioxidants, such as phytophenolics and carotenoids, which also provide distinct flesh colors. The Hunter 'L' 'a' 'b' color evaluation (as described in Chapter 5, Section **5.3.2)** instigated to check the level of phytophenols and carotenoids. To examine the disparity at the level of metabolites, a comprehensive biochemical analyses was carried out. Detailed analyses revealed contrasting trends at the level of phytophenols and carotenoids. While, TPC in WFSP-WT was 1.58 mg GAE/g, it was 1.49 mg GAE/g in OFSP-6. Intriguingly, TFC was 0.705 mg QE/g in OFSP-6 as against 0.617 mg QE/g in WFSP-WT, which was significantly higher ($p < 0.05$). Furthermore, the TPC as well as TFC contents were considerably higher in transgenic lines. The TPC content of different transgenic line was in the range of 1.59-1.88 mg GAE/g and the TFC content was found to be in the range of 1.04-2.25 mg

QE/g. The fold increase of TPC and TFC was upto 1.25- to 3.19-fold, respectively in transgenic lines (**Fig. 6.1 A and B**).

Anthocyanin is among the important groups of phenolic compounds that contribute to the characteristic color. As these constitute a subcategory of flavonoids, the TAC of both the wild type cultivars was anticipated to follow the same trend of TFC. The TAC of OFSP-6 was significantly higher (0.016 mg/g) than that of WFSP-WT (0.012 mg/g). The TAC content of transgenic lines was in the range of 0.02-0.09 mg/g which was significantly higher than that of wild type counterpart cv. OFSP-6 (**Fig. 6.1 C)**. A higher TCC was expected in OFSP-6 as intensity of orange color is attributed to the carotenoid content of an individual cultivar(s) [415]. As expected, TCC of OFSP-6 was found to be 0.047 mg BCE/g which was 8-fold higher than that of WFSP-WT (0.006 mg BCE/g). However, TCC content in most of the transgenic lines was not significantly different ($p > 0.05$) except in case of high transgene expressing lines (**Fig. 6.1 D**).

6.3.2 Differential expression of flavonoid pathway genes

As flavonoids are ubiquitous in nature and major contributors of color and flavor, we investigated the relative transcript accumulation of the key flavonoid pathway genes viz., *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT* to elucidate the underlying molecular mechanism and interaction of gene products responsible for varietal differences (**Fig. 6.2 A**). Transcript analyses revealed a low transcript abundance of the early pathway genes viz., *CHS* and *CHI* (**Fig. 6.2 B and C**) and the late pathway genes viz., *DFR, ANS* and *UFGT* in WFSP-WT when compared with OFSP-6, whereas the transcript abundance was invariably higher in transgenic lines than that of OFSP-6 (**Fig. 6.2 E-G**). Interestingly, though the *F3H* transcript was slightly induced in WFSP-WT, the TFC was found to be low. However, both the COE and TOE lines followed the same trend (**Fig. 6.2 D**). This discrepancy might be due to the post-transcriptional and/or post-translational modification(s) of the candidate genes.

6.3.3 Metabolite profiling of tuber

The biosynthesis of secondary metabolites in plants represents an intricate cellular network involving the transcription, translation and post-translational modification of several gene products. Global analysis of plant metabolomes is a difficult task owing to the huge number

and diversity of primary and secondary metabolites present in plant tissues. Nonetheless, it is imperative to explore the metabolome of a crop species since most of these metabolites are responsible for the organoleptic and qualitative properties of foods originating from such crops [416]. In addition, subtle changes in the genome and proteome may be reflected at the metabolome level. The extreme quantitative and qualitative variations in metabolites have made plants the ideal models for dissecting the biosynthetic pathways and regulation of metabolites. Furthermore, metabolome of a crop species may be affected by natural or developmental variations [417]. Therefore, to negate the effect of such variations, the tuber metabolome was developed from the wild type as well as from the transgenic lines grown in parallel, and the mature tubers were harvested precisely at the same developmental stage. GC-MS approach was used to develop a robust sweet potato tuber metabolome and to precisely detect transgene mediated metabolic shift(s) in transgenic plants.

6.3.3.1 Functional classification and significance

All the metabolites were searched on Chemspider (http://www.chemspider.com/) and PubChem (http://pubchem.ncbi.nlm.nih.gov/) for their molecular structure, putative function and distinct metabolic pathways, and were classified into five groups. The largest class in wild type as well as in the transgenic events was organic acid followed by other classes, sugar, amino acids and sugar alcohol (**Fig. 6.3 A-C**). The largest class "Organic acid" comprised 45% in wild type, 44% in COE line and 39% in TOE line. This class includes few of the well known metabolites putatively known for their medicinal as well as economic values further strengthening the medicinal and economic properties of sweet potato. Ricinoleic acid co-eluted at the same retention time in wild type (18.221) and transgenic lines (18.223). Ricinoleic acid is the major constituent of castor oil, accounting for about 90%. It is well known for its laxative properties having a pro-or anti-inflammatory action following acute or repetitive local application, respectively [418]. Vegetable oil-based organogels are also produced using ricinoleic acid, which had gained considerable attention recently. The comprehensive exploitation of organogels include environmental cleanup, protected transport of flammable liquids, aerogel formation, ingredients in lubricants and coatings, and applications in pharmaceutical and food technologies. In food industry, efforts have been made pertaining to the principles of organogelation to structure oils without the need for high levels of saturated or trans fatty acids, which have

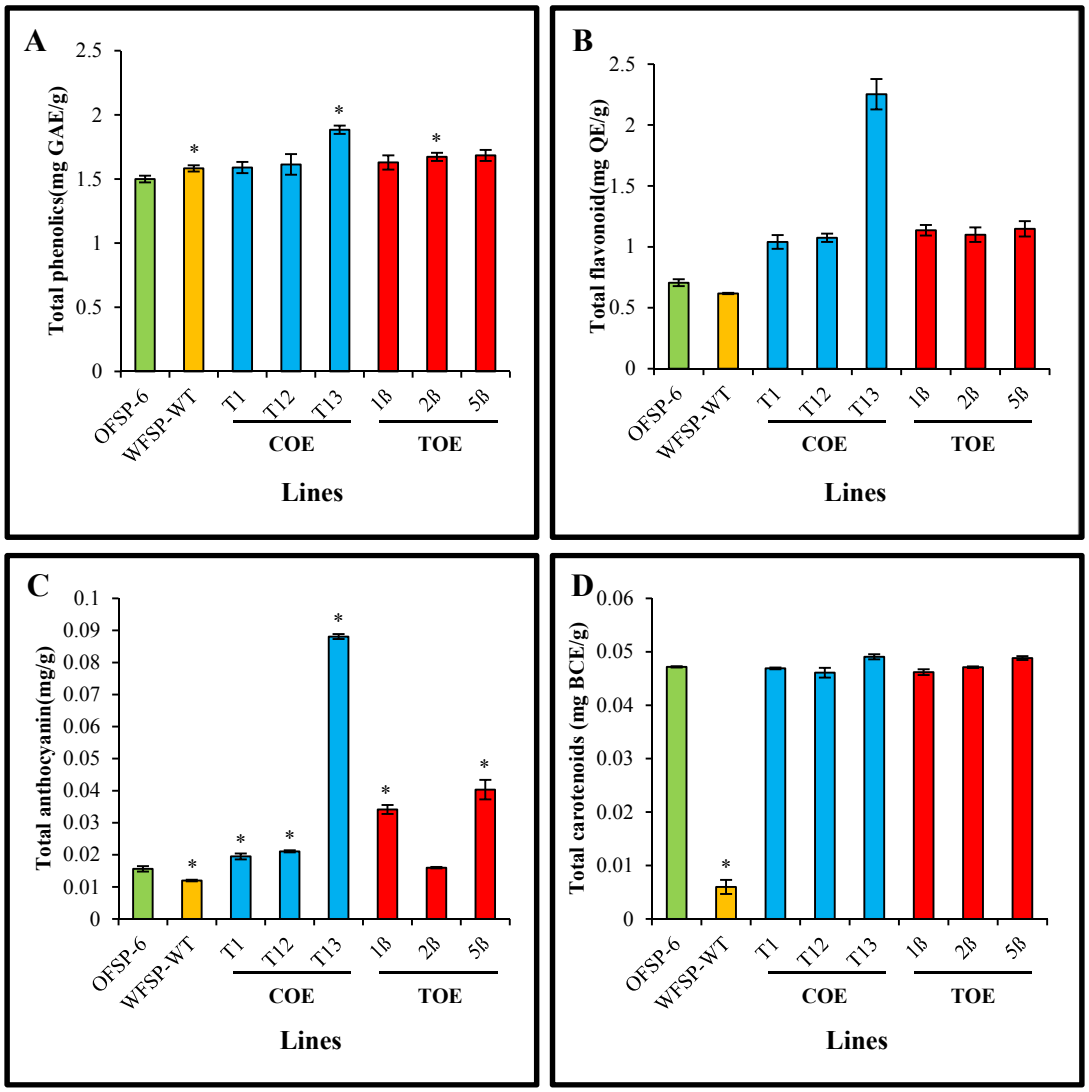

Fig. 6.1. Determination of phytophenols and carotenoids. A comparative analysis of total phenolic content (**A**), flavonoids (**B**), anthocyanin (**C**) and carotenoids (**D**) was carried out on dry basis in wild types (cv. OFSP-6 & WFSP-WT) and transgenic (COE & TOE) lines. Values are presented as means ± S.E. (n=3) of a composite sample of four to six tubers. GAE: gallic acid equivalent; QE: quercetin equivalent; BCE: of β-carotene equivalents; * indicates the level of significance at $p < 0.05$.

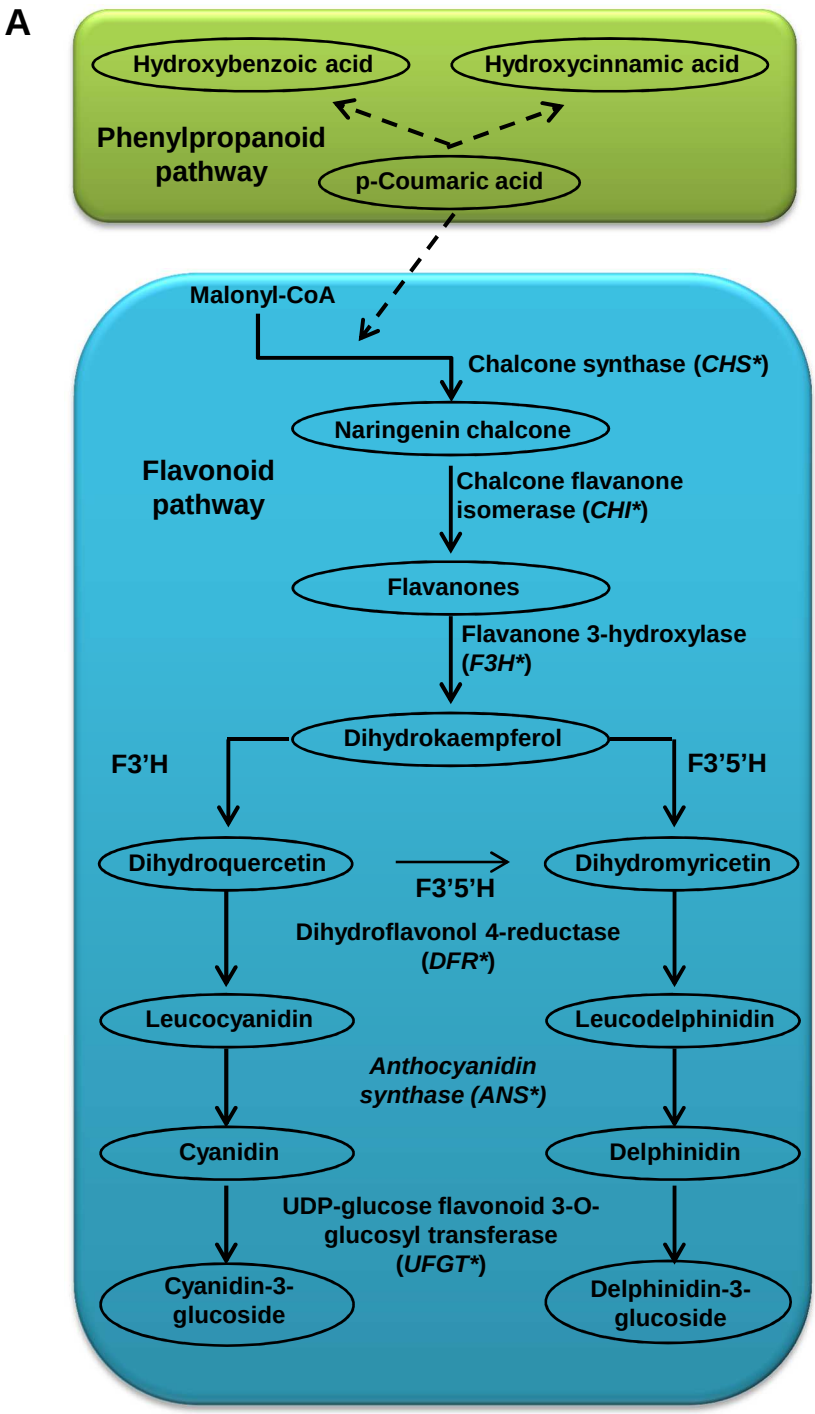

Fig. 6.2. Transcript abundance of flavonoid pathway genes. The candidate genes selected for expression analysis are shown with asterisk (*) (**A**).

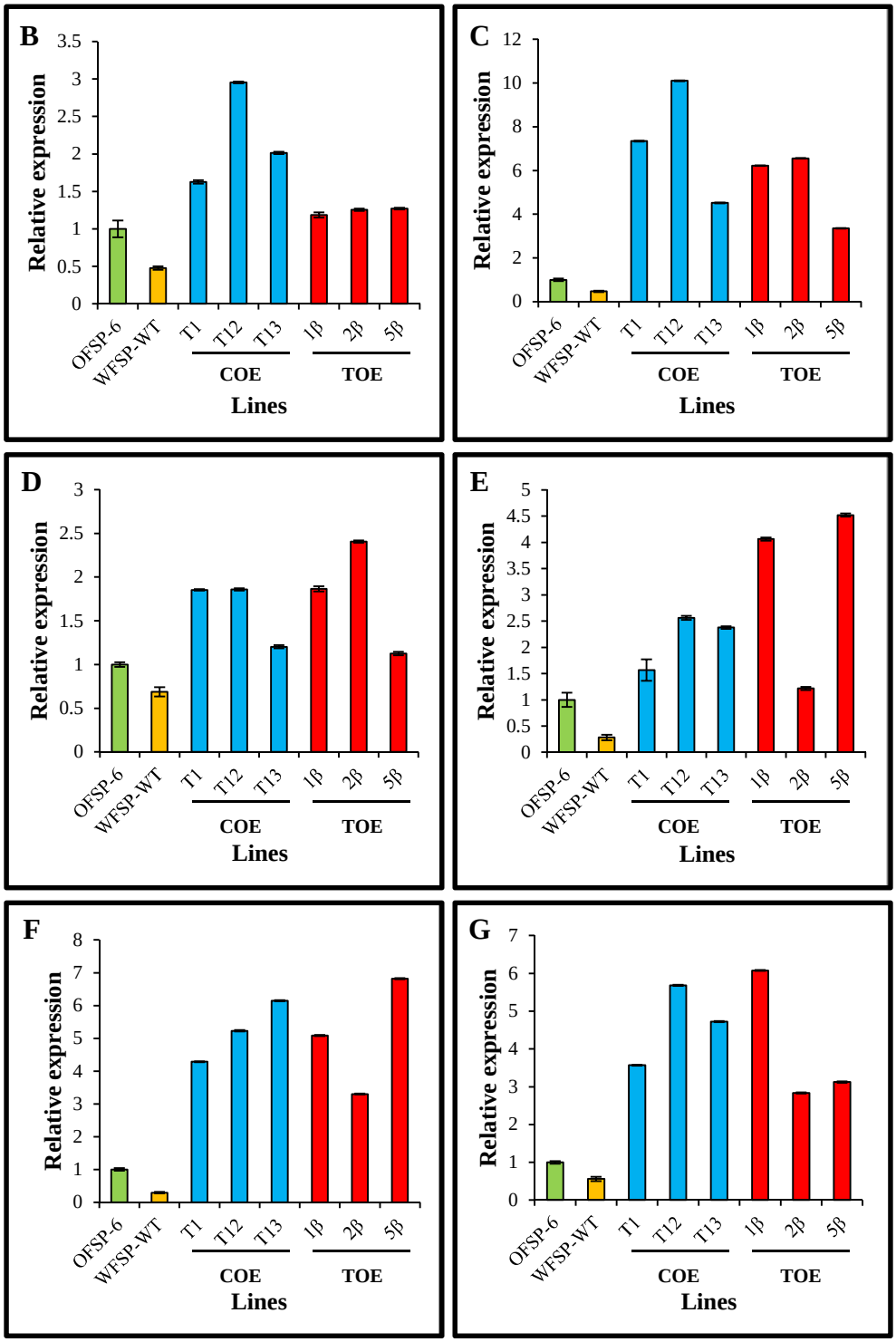

Fig. 6.2. Transcript abundance of flavonoid pathway genes. The relative abundance of chalcone synthase (*CHS*) (**B**), chalcone flavanone isomerase (*CHI*) (**C**), flavanone 3-hydroxylase (*F3H*) (**D**), dihydroflavonol 4-reductase (*DFR*) (**E**), anthocyanidin synthase (*ANS*) (**F**), and UDP-glucose flavonoid 3-O-glucosyl transferase (*UFGT*) (**G**) was determined in wild types and two independent transgenic lines by qRT-PCR. The mean values of three replicates were normalized using actin as internal control. Values represent mean ± SE.

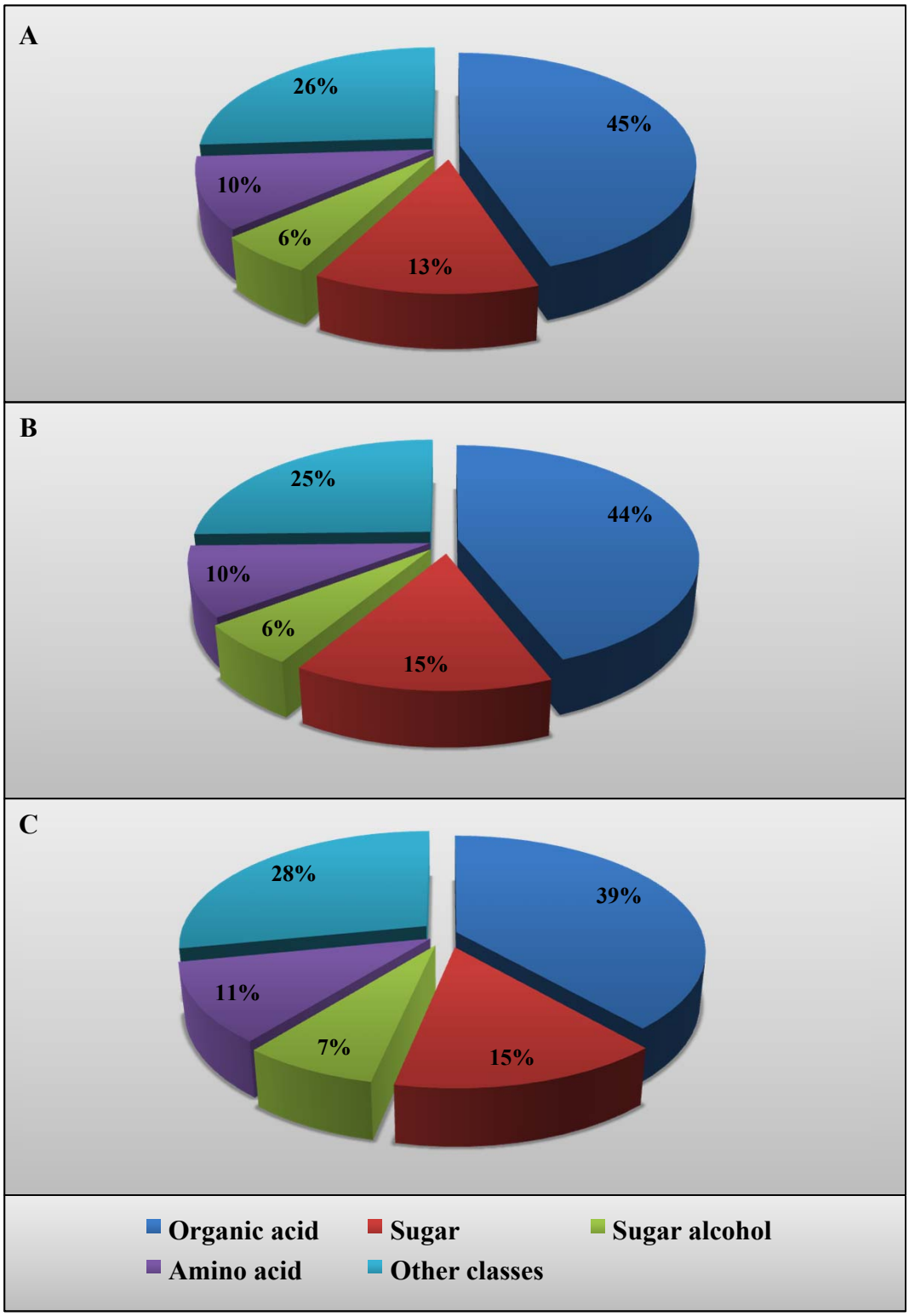

Fig. 6.3. Functional classification of metabolites. All the metabolites identified by GC-MS analysis was classified into five groups as represented in the pie chart in wild type (cv. OFSP-6) (**A**), COE (**B**) and TOE (**C**) lines.

predictable implications in cardiovascular diseases. There is active research as well in using organogels to minimize oil migration in composite food products and for enhanced stability and delivery of lipophilic bioactive molecules [419]. Glucaric acid was another important metabolite observed in wild type (RT- 15.27) as well as in the transgenic lines (RT-15.271). Glucaric acid is an organic acid with potential anticarcinogenic properties and can eliminate carcinogens, steroids and toxins in the liver [420]. Moreover, ferulic acid (RT- 17.78) and azelaic acid (12.575) were found to be prominent among other organic acids across the wild type and transgenic lines (**Table 6.1-6.3**). Ferulic acid is a phenolic acid, an effective ubiquitous plant antioxidant that is the metabolic byproduct of phenylalanine and tyrosine. It occurs mostly in seeds and leaves either in its free form or covalently bound to lignin and other biopolymers. It can readily form a resonance stabilized phenoxy radical which accounts for its potent antioxidant potential owing to its phenolic nucleus and an extended side chain conjugation. UV absorption by ferulic acid catalyzes stable phenoxy radical formation and thus potentiates its capability to scavenge the free radical chain reactions. Addition of ferulic acid considerably increased and improved the stability of the vitamins C and E, and doubled the photoprotection to solar simulated irradiation of skin [421, 422]. Additionally, azelaic acid is saturated dicarboxylic acid which is implicated in defense responses following infection. It acts as a signal that induces the accumulation of salicylic acid, a vital element of a plant's defense response. It acts against acne bacteria that infect skin pores and thus used in treatment of mild to moderate acne as well, both comedonal acne and inflammatory acne. It also decreases the production of keratin, which is a natural substance that promotes the growth of acne bacteria [423]. Other important members of this group were docosanoic acid, ribonic acids, malic acid, xylonic acid etc. Additionally, a diverse group of aliphatic and aromatic acids, and flavonoids had also been characterized. From the aliphatic acids, it contained a significant concentration of palmitelaidic, oleic, tetracosanoic, hexacosanoic acids and 2,3,4,5-tetrahydroxypentanoic acid-1,4-lactone. A high concentration of the aromatic acids, ferulic acid and olean-12-en-28-oic acid was also observed. Olean-12-en-28-oic acid, a pentacyclic triterpenoid and some analogous triterpenoids are known to induce apoptosis possibly via activating caspase-3 as a prime apoptosis protease in the tumor cells and having potential anti-tumoural activities [424].

Second largest class of metabolites was the “Other classes” which covers 26%, 25% and 28% of total metabolites in wild type (cv. OFSP-6), COE and TOE lines, respectively. This class

includes various intermediate products of different metabolic pathways and their byproducts such as butanal, dodecanol, isododecyl alcohol, galactose oximes, uridin, stigmasterol, tocopherol etc. Few of the metabolites and/or their isoforms eluted at different retention time in wild type as well as in the transgenic events (**Fig. 6.3 A-C**; **Table 6.1-6.3**). This class includes different phytosterols (*e.g.* ergostane, stigmasterol, campesterol, stigmast-5-en-3-ol etc.), terpenes and their derivative (*e.g.* betulin, solanesol, amyrin etc.), phenols (tocopherol), nucleoside (uridin) and alcohol derivatives. Phytosterols encompass plant sterols and stanols, are steroid compounds similar to cholesterol which vary only in carbon side chains and/or presence or absence of a double bond. Even though phytosterols and cholesterol have analogous chemical structures, they differ strikingly in their synthesis, intestinal absorption, and metabolic fate. Phytosterols restrain intestinal cholesterol absorption, thereby lowering total and low-density lipoprotein (LDL) cholesterol levels [425]. Additionally, evidence for valuable properties of plant sterols on disorders such as cutaneous xanthomatosis, colon cancer and prostate hyperplasia has also been established and these compounds are known to inhibit lung, stomach, ovarian and breast cancers [426]. Moreover, anti-diabetic effect of stigmast-5-en-3-ol, a plant phytosterol found commonly in many plants has been established [427]. It has also been indicated by several studies that stigmasterol may be helpful for the prevention of certain cancers, including ovarian, prostate, breast, and colon cancers [428]. Interestingly, a small number of terpenes and their derivative were also found to be present in both the transgenic events and in the wild type metabolome. Presence of betulin and solanesol reconfirmed and substantiated the findings and possible use of sweet potato or its purified compounds as curatives. Nonetheless, very little is known about the mechanism of action of betulin hitherto, but it has also been reported to prevent and to some extent reverse liver fibrosis *in vivo* [429]. Conversely, solanesol was also found in high concentration which is a vital bioactive component and has been used as an antiulcer or for the mitigation of hypertension. In addition, solanesol is an indispensable therapeutic intermediate in the industrial synthesis of coenzyme Q10, which is an exceptional remedy in cardiovascular disease, cancer, and atherosclerosis [430]. In addition, amyrins, a pentacyclic triterpene putatively known for its anti-inflammatory effects, was also found to be a common metabolite across the wild type and transgenic lines. Amyrin has been known as well for the amelioration of L-arginine-induced acute pancreatitis [431].

Table 6.1: List of metabolites in wild type (cv. OFSP-6)

Organic acid	
NAME	RT
Butanedioic acid, bis(trimethylsilyl) ester	6.565
Propanoic acid, 2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	6.886
2-Butenedioic acid (E)-, bis(trimethylsilyl) ester	7.031
Nonanoic acid, trimethylsilyl ester	7.183
SILANOL, TRIMETHYL-, PHOSPHATE	8.808
Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester	9.058
Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	9.515
Undecanoic acid, trimethylsilyl ester	9.748
Propanoic acid, 3-[bis(trimethylsilyl)amino]-2-methyl-, trimethylsilyl ester	9.915
L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester	10.038
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone, tris(trimethylsilyl)-	10.776
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone	10.921
Gluconic acid, 2-methoxime, tetra(trimethylsilyl)-, trimethylsilyl ester	11.128
ARABINONIC ACID, 2,3,5-TRIS-O-(TRIMETHYLSILYL)-, .GAMMA.-LACTONE	11.541
Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)	12.08
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.282
Undecanoic acid, tert-butyldimethylsilyl ester	12.326
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.428
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.522
Azelaic acid, bis(trimethylsilyl) ester	12.575
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	12.858
[1,1'-BIPHENYL]-4-CARBOXYLIC ACID, TRIMETHYLSILYL ESTER	12.953
2-Thiobarbituric acid, S-trimethylsilyl-, bis(trimethylsilyl) ether	13.424
Gulonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	14.232
cis-9-Hexadecenoic acid, trimethylsilyl ester	14.818
1,2,3-PROPANETRICARBOXYLIC ACID, 2-[(TRIMETHYLSILYL)OXY]-	14.858
Hexadecanoic acid, trimethylsilyl ester	15.005
GLUCARIC ACID, 2,3,4,5-TETRAKIS-O-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	15.27
Tetradecanoic acid, dimethyl(isopropyl)silyl ester	15.314
Heptadecanoic acid, trimethylsilyl ester	15.573
TRIMETHYL({2,3,4,5,6-PENTAKIS[(TRIMETHYLSILYL)OXY]CYCLOHEXYL}OXY)SILANE	15.826
OELSAEURE, TRIMETHYLSILYLESTER	16.566
OLEIC ACID, TRIMETHYLSILYL ESTER	16.626
Octadecanoic acid, trimethylsilyl ester	16.783

9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	16.948
Hexadecanoic acid, tert-butyldimethylsilyl ester	17.111
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	17.19
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	17.322
cis-15-Tetracosenoic acid, trimethylsilyl ester	17.533
Nonadecanoic acid, trimethylsilyl ester	17.619
Ferulic acid, trimethylsiloxy, trimethylsilyl ester	17.78
Heptadecanoic acid, tert-butyldimethylsilyl ester	17.955
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	18.221
cis-13-Eicosenoic acid, trimethylsilyl ester	18.3
Eicosanoic acid, trimethylsilyl ester	18.444
17-Octadecynoic acid, tert-butyldimethylsilyl ester	18.582
trans-9-Octadecenoic acid, tert-butyldimethylsilyl ester	18.672
Octadecanoic acid, tert-butyldimethylsilyl ester	18.827
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	19.049
Heneicosanoic acid, trimethylsilyl ester	19.39
9,12-Octadecadienoic acid, tert-butyldimethylsilyl ester, (Z,Z)-	19.485
Nonadecanoic acid, tert-butyldimethylsilyl ester	19.871
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	20.034
Docosanoic acid, trimethylsilyl ester	20.544
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	20.725
10-Undecenoic acid, tert-butyldimethylsilyl ester	20.797
Tetracosanoic acid, trimethylsilyl ester	21.969
Tetracosanoic acid, trimethylsilyl ester	23.77
Docosanoic acid, tert-butyldimethylsilyl ester	24.795
Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	25.284
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	26.11
Tricosanoic acid, tert-butyldimethylsilyl ester	26.592
Hexacosanoic acid, trimethylsilyl ester	27.217
Tetracosanoic acid, tert-butyldimethylsilyl ester	27.727
Hexacosanoic acid, trimethylsilyl ester	28.166
Pentacosanoic acid, tert-butyldimethylsilyl ester	28.577
THREONIC ACID, 2,3-BIS-O-(TRIMETHYLSILYL)-, .GAMMA.-LACTONE, D-	28.7
10,12-Tricosadiynoic acid, trimethylsilyl ester	29.1
Olean-12-en-28-oic acid, 3-(acetyloxy)-, methyl ester, (3.beta.)	32.996
Other classes	
2,4(1H,3H)-Pyrimidinedione, dihydro-1,3-bis(trimethylsilyl)-	9.19
BUTANAL, 2,3,4-TRIS[(TRIMETHYLSILYL)OXY]-	9.366
1,2-Epoxy-3,4-dihydroxycyclohexano[a]pyrene	11.36

1-Dodecanol, 3,7,11-trimethyl	11.751
Isododecyl alcohol, trimethysilyl derivative	11.768
({4,5-BIS[(TRIMETHYLSILYL)OXY]TETRAHYDRO-3-FURANYL}OXY)(TRIMETHYL)SILANE	12.783
GALACTOSE OXIME 6TMS	13.836
(1E,3Z)-1-PHENYL-3-(TRIMETHYLSILYLOXY)-1,3-PENTADIENE	14.114
alpha.-D-Glucopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate	15.767
TRIMETHYL({2,3,4,5,6-PENTAKIS[(TRIMETHYLSILYL)OXY]CYCLOHEXYL}OXY)SILANE	15.826
2,4'-Bis(trimethylsilyloxy)diphenylmethane	16.111
Silane, dimethylbis[(trimethylsilyl)methyl]	16.2
1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl	17.917
1,5,9-Decatriene, 2,3,5,8-tetramethyl-	17.925
Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	18.804
Tocopherol-.beta.-tms-derivative	26.883
Cholesta-2,4-diene	27.242
STIGMAST-5-EN-3-OL, (3.BETA.)	27.546
Stigmast-5-en-3-ol, oleate	27.893
9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)	28.065
alpha.-Tocopherol (vitamin E), trimethysilyl derivative	28.313
SILANE, [[(3.BETA.)-CHOLEST-5-EN-3-YL]OXY]TRIMETHYL-	28.4
4.alpha.,5-Cyclo-A-homo-5.alpha.-cholestan-6-one	28.55
Silane, trimethyl[(3,7,11-trimethyl-2,6,10-dodecatrienyl)oxy]-	28.876
SOLANESOL	28.881
Campesterol tms	29.218
TETRAHYDRODAMMARADIENOL	29.231
Stigmasterol trimethylsilyl ether	29.438
XYLOPYRANOSIDE, METHYL 2,3,4-TRIS-O-(TRIMETHYLSILYL)-, .ALPHA.-D-	29.667
STIGMAST-5-EN-3-OL, (3.BETA.)-	29.75
Betulin	29.767
beta.-Sitosterol trimethylsilyl ether	29.88
Silane, (1,1-dimethylethyl)dimethyl(octacosyloxy)	30.048
1,2,2,3,4,4,5,5,6,6-Decamethylhexasilinane-1,3-diol	30.266
Ergostane-5,25-diol, 3,6,12-tris[(trimethylsilyl)oxy]-, 25-acetate, (3.beta.,5.alpha.,6.beta.,12.beta.)-	30.404
TMS ETHER OF 2-MONOOLEGLYCEROL	31.03
Silane, (9,19-cyclo-9.beta.-lanost-24-en-3.beta.-yloxy)trimethyl-	31.61
14,17-Nor-3,21-dioxo-.beta.-amyrin, 17,18-didehydro-3-dehydroxy-	31.725
METHYL 3-TRIMETHYLSILYLOXYTETRADECANOATE	32.05

SOLANESOL	32.361
SUGAR	
NAME	RT
d-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	11.454
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.211
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.576
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.683
d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)	13.767
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	19.7
D-Turanose, heptakis(trimethylsilyl)-	21.002
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	21.362
D-Turanose, heptakis(trimethylsilyl)-	22.16
D-Turanose, heptakis(trimethylsilyl)-	22.351
D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)-	22.988
D-Turanose, heptakis(trimethylsilyl)-	23.306
D-Turanose, heptakis(trimethylsilyl)-	23.511
Melibiose, octakis(trimethylsilyl)	27.352
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	27.627
GLUCOPYRANOSIDE-6,6-D2, METHYL-TETRAKIS-O-(TRIMETHYLSILYL)-	28.726
Hexopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)	30.401
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.688
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.791
beta.-D-Glucopyranose, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, 1-(trimethylsilyl)-1H-indole-3-acetate	31.895
AMINO ACID	
Glycine, N,N-bis(trimethylsilyl)-, trimethylsilyl ester	6.471
L-Leucine-2TMS	6.63
Serine tritms	7.305
N,O,O-Tris(trimethylsilyl)-L-threonine	7.688
l-Aspartic acid, bis(trimethylsilyl) ester	8.142
D,L-Alanine, N-(tert-butyldimethylsilyl)-N-methyl-, tert-butyldimethylsilyl ester	8.347
Valylvaline, N,N'-dimethyl-n-propoxycarbonyl-, butyl ester	8.351
L-ASPARTIC ACID, N-(TRIMETHYLSILYL)-	9.457
Phenylalanine-2TMS	9.81
L-Asparagine, N2-trimethylsilyl-, trimethylsilyl ester	10.417

D,L-Alanine, N-(tert-butyldimethylsilyl)-N-methyl-, tert-butyldimethylsilyl ester	10.561
Glutamine, tris(trimethylsilyl)-	10.651
L-Asparagine, N,N2-bis(trimethylsilyl)-, trimethylsilyl ester	11.293
L-Lysine-4TMS	11.88
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	12.858
L-Methionine-2TMS	22.611
SUGAR ALCOHOL	
NAME	RT
Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)	11.643
Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	11.776
l-(-)-Arabitol, pentakis(trimethylsilyl) ether	11.976
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)	12.154
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)	14.018
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)	14.13
Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	15.67
Myo-Inositol, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, bis(trimethylsilyl) phosphate	18.578
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	20.265

Table 6.2: List of metabolites in COE transgenic line

Organic acid	
NAME	RT
PROPANOIC ACID, 2,3-BIS[(TRIMETHYLSILYL)OXY]-, TRIMETHYLSILYL ESTER	6.89
2-Butenedioic acid (Z)-, bis(trimethylsilyl) ester	7.033
Nonanoic acid, trimethylsilyl ester	7.186
Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester	9.059
Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	9.515
Undecanoic acid, trimethylsilyl ester	9.749
Propanoic acid, 2-methyl-2,3-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	9.915
L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester	10.037
Pentonic acid, 3-deoxy-2,4,5-tris-O-(trimethylsilyl)-, trimethylsilyl ester	11.058
Gluconic acid, 2-methoxime, tetra(trimethylsilyl)-, trimethylsilyl ester	11.129
Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	11.417
D-Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone	11.533
Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone, D-	12.067
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.282
Undecanoic acid, tert-butyldimethylsilyl ester	12.326
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.425
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.523
Azelaic acid, bis(trimethylsilyl) ester	12.575
1,1'-BIPHENYL]-4-CARBOXYLIC ACID, TRIMETHYLSILYL ESTER	12.956
Tetradecanoic acid, trimethylsilyl ester	13.063
2-Thiobarbituric acid, S-trimethylsilyl-, bis(trimethylsilyl) ether	13.425
D-Glycero-L-manno-Heptonic acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)	14.122
Gulonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	14.235
L-ASPARTIC ACID, N-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	14.312
D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)	14.661
PALMITELAIDIC ACID 1TMS	14.817
1,2,3-PROPANETRICARBOXYLIC ACID, 2-[(TRIMETHYLSILYL)OXY]-, TRIS(TRIMETHYLSILYL) ESTER	14.858
Hexadecanoic acid, trimethylsilyl ester	15.005
GLUCARIC ACID, 2,3,4,5-TETRAKIS-O-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	15.271
Tetradecanoic acid, dimethyl(isopropyl)silyl ester	15.315
Gulonic acid, 2,3,5,6-tetrakis-O-(trimethylsilyl)-, lactone	15.368
Heptadecanoic acid, trimethylsilyl ester	15.572
Heptadecanoic acid, trimethylsilyl ester	15.904

OELSAEURE, TRIMETHYLSILYLESTER	16.565
OLEIC ACID, TRIMETHYLSILYL ESTER	16.625
Octadecanoic acid, trimethylsilyl ester	16.782
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	16.946
Hexadecanoic acid, tert-butyldimethylsilyl ester	17.107
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	17.324
Nonadecanoic acid, trimethylsilyl ester	17.619
Ferulic acid, trimethylsiloxy, trimethylsilyl ester	17.783
Isopimaric acid TMS	17.907
9-Octadecenoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester	17.945
Heptadecanoic acid, tert-butyldimethylsilyl ester	17.952
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	18.223
cis-13-Eicosenoic acid, trimethylsilyl ester	18.3
Eicosanoic acid, trimethylsilyl ester	18.443
17-Octadecynoic acid, tert-butyldimethylsilyl ester	18.582
trans-9-Octadecenoic acid, tert-butyldimethylsilyl ester	18.672
Octadecanoic acid, tert-butyldimethylsilyl ester	18.813
Heneicosanoic acid, trimethylsilyl ester	19.389
9,12-Octadecadienoic acid, tert-butyldimethylsilyl ester, (Z,Z)-	19.487
Nonadecanoic acid, tert-butyldimethylsilyl ester	19.873
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	20.033
Docosanoic acid, trimethylsilyl ester	20.543
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	20.727
Tetracosanoic acid, trimethylsilyl ester	21.968
Tetracosanoic acid, trimethylsilyl ester	23.764
Docosanoic acid, tert-butyldimethylsilyl ester	24.796
Tricosanoic acid, tert-butyldimethylsilyl ester	26.602
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	26.112
Hexacosanoic acid, trimethylsilyl ester	27.208
Tetracosanoic acid, tert-butyldimethylsilyl ester	27.73
Hexacosanoic acid, trimethylsilyl ester	28.165
THREONIC ACID, 2,3-BIS-O-(TRIMETHYLSILYL)-, .GAMMA.-LACTONE, D-	28.7
Olean-12-en-28-oic acid, 3-(acetyloxy)-, methyl ester, (3.beta.)-	32.992
Other classes	
1-Butanol, 2-methyl-, acetate	7
1,2-Ethanediol, monoacetate	7.008
SILANOL, TRIMETHYL-, PHOSPHATE	8.816
2,4(1H,3H)-Pyrimidinedione, dihydro-1,3-bis(trimethylsilyl)-	9.19

BUTANAL, 2,3,4-TRIS[(TRIMETHYLSILYL)OXY]-, (R*,R*)-	9.367
PENTITOL, 3-DESOXY-TETRAKIS-O-(TRIMETHYLSILYL)-	10.776
3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	10.919
1,2-Epoxy-3,4-dihydroxycyclohexano[a]pyrene	11.35
1-Dodecanol, 3,7,11-trimethyl	11.754
GALACTOSE OXIME 6TMS	13.836
TRIMETHYL({2,3,4,5,6-PENTAKIS[(TRIMETHYLSILYL)OXY]CYCLOHEXYL}OXY)SILANE	15.673
TRIMETHYL({2,3,4,5,6-PENTAKIS[(TRIMETHYLSILYL)OXY]CYCLOHEXYL}OXY)SILANE	15.826
2,4'-Bis(trimethylsilyloxy)diphenylmethane	15.827
2,4'-Bis(trimethylsilyloxy)diphenylmethane	16.113
1,5,9-DECATRIEN, 2,3,5,8-TETRAMETHYL	17.925
ANDROSTANE, SILANE DERIV.	19.033
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	20.267
Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	25.287
Tocopherol-.beta.-tms-derivative	26.884
STIGMAST-5-EN-3-OL, (3.BETA.)	27.546
Stigmast-5-en-3-ol, oleate	27.891
9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)	28.063
alpha.-Tocopherol (vitamin E), trimethysilyl derivative	28.311
SILANE, [[(3.BETA.)-CHOLEST-5-EN-3-YL]OXY]TRIMETHYL-	28.4
9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-	28.55
6,7-DIHYDROXYCOUMARIN-.BETA.-D-GLUCOPYRANOSIDE, PENTA-TMS	28.87
Campesterol tms	29.217
TETRAHYDRODAMMARADIENOL	29.228
Stigmasterol trimethylsilyl ether	29.436
XYLOPYRANOSIDE, METHYL 2,3,4-TRIS-O-(TRIMETHYLSILYL)-, .ALPHA.-D-	29.667
Betulin	29.769
beta.-Sitosterol trimethylsilyl ether	29.874
Silane, (1,1-dimethylethyl)dimethyl(octacosyloxy)	30.045
3.beta.,4.beta.-Bis(trimethylsiloxy)cholest-5-ene	30.405
TMS ETHER OF 2-MONOOLEGLYCEROL	31.028
14,17-Nor-3,21-dioxo-.beta.-amyrin, 17,18-didehydro-3-dehydroxy-	31.745
beta.-D-Glucopyranose, 2,3,4,6-tetrakis-O-(trimethylsilyl)-, 1-(trimethylsilyl)-1H-indole-3-acetate	31.756
SOLANESOL	32.356

SUGAR	
d-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	11.454
MANNOFURANOSIDE, METHYL 2,3,5,6-TETRAKIS-O-(TRIMETHYLSILYL)-, .ALPHA.-D-	12.758
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.214
2,3,4,6-Tetra-O-trimethylsilyl-1-[1,7-dicarba-closo-dodecaboran-(12)-1-yl]-D-glucopyranose	13.254
D-FRUCTOSE, O-METHYLOXIM, PENTAKIS-O-(TRIMETHYLSILYL)-	13.577
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.683
1,2,3,4,6-PENTAKIS-O-(TRIMETHYLSILYL)HEXOPYRANOSE	15.13
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	19.697
D-Turanose, heptakis(trimethylsilyl)	21.011
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	21.388
D-Turanose, heptakis(trimethylsilyl)	22.161
D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)	22.35
D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)-	22.987
D-Turanose, heptakis(trimethylsilyl)	23.307
D-Turanose, heptakis(trimethylsilyl)-	23.509
Melibiose, octakis(trimethylsilyl)	27.352
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)	27.624
GLUCOPYRANOSIDE-6,6-D2, METHYL-TETRAKIS-O-(TRIMETHYLSILYL)	28.721
Thymol-.beta.-d-glucopyranoside, tetrakis(O-trimethylsilyl)-	30.592
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.686
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)-	30.79
D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)-	31.882
AMINO ACID	
L-Leucine-2TMS	6.63
Serine tritms	7.305
N,O,O-Tris(trimethylsilyl)-L-threonine	7.687
l-Aspartic acid, bis(trimethylsilyl) ester	8.144
l-Norvaline, n-butoxycarbonyl-, butyl ester	8.348
Valylvaline, N,N'-dimethyl-n-propoxycarbonyl-, butyl ester	8.351
L-ASPARTIC ACID, N-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	9.457

Phenylalanine-2TMS	9.81
L-Asparagine, N2-trimethylsilyl-, trimethylsilyl ester	10.407
D,L-Alanine, N-(tert-butyldimethylsilyl)-N-methyl-, tert-butyldimethylsilyl ester	10.561
Glutamine, tris(trimethylsilyl)	10.65
L-Asparagine, N,N2-bis(trimethylsilyl)-, trimethylsilyl ester	11.293
L-Lysine-4TMS	11.88
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	12.86
L-Methionine-2TMS	22.611
SUGAR ALCOHOL	
Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)	11.643
Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	11.776
l-(-)-Arabitol, pentakis(trimethylsilyl) ether	11.976
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)	12.154
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)-	14.018
Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	15.671
D-Myo-Inositol, 1,2,4,5,6-pentakis-O-(trimethylsilyl)-, bis(trimethylsilyl) phosphate	18.576
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	30.404
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	31.031

Table 6.3: List of metabolites in TOE transgenic lines

Organic acid	
Name	**RT**
2-BUTENEDIOIC ACID (E)-, BIS(TRIMETHYLSILYL) ESTER	7.031
Nonanoic acid, trimethylsilyl ester	7.185
Malic acid, O-(trimethylsilyl)-, bis(trimethylsilyl)ester	9.057
Butanoic acid, 4-[bis(trimethylsilyl)amino]-, trimethylsilyl ester	9.515
Undecanoic acid, trimethylsilyl ester	9.748
PROPANOIC ACID, 2-METHYL-2,3-BIS[(TRIMETHYLSILYL)OXY]-, TRIMETHYLSILYL ESTER	9.914
L-Threonic acid, tris(trimethylsilyl) ether, trimethylsilyl ester	10.037
L-Asparagine, N,N2-bis(trimethylsilyl)-, trimethylsilyl ester	11.291
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.28
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.425
Ribonic acid, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, trimethylsilyl ester	12.521
Azelaic acid, bis(trimethylsilyl) ester	12.575
n-Tridecanoic acid, trimethylsilyl ester	12.867
1,1'-BIPHENYL]-4-CARBOXYLIC ACID, TRIMETHYLSILYL ESTER	12.95
Tetradecanoic acid, trimethylsilyl ester	13.065
2-Thiobarbituric acid, S-trimethylsilyl-, bis(trimethylsilyl) ether	13.424
D-Glycero-D-gulo-Heptonic acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)-, .gamma.-lactone	14.237
cis-9-Hexadecenoic acid, trimethylsilyl ester	14.818
Hexadecanoic acid, trimethylsilyl ester	15.005
GLUCARIC ACID, 2,3,4,5-TETRAKIS-O-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	15.271
Tetradecanoic acid, dimethyl(isopropyl)silyl ester	15.316
Arabino-hexaric acid, 3-deoxy-2,4,5-tris-O-(trimethylsilyl)-, bis(trimethylsilyl) ester	15.903
Heptadecanoic acid, trimethylsilyl ester	15.905
OELSAEURE, TRIMETHYLSILYLESTER	16.564
OLEIC ACID, TRIMETHYLSILYL ESTER	16.625
Octadecanoic acid, trimethylsilyl ester	16.782
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	16.945
Hexadecanoic acid, tert-butyldimethylsilyl ester	17.108
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	17.325
Nonadecanoic acid, trimethylsilyl ester	17.619
Ferulic acid, trimethylsiloxy, trimethylsilyl ester	17.783
Isopimaric acid TMS	17.908
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	18.223

cis-13-Eicosenoic acid, trimethylsilyl ester	18.3
Eicosanoic acid, trimethylsilyl ester	18.443
17-Octadecynoic acid, tert-butyldimethylsilyl ester	18.582
trans-9-Octadecenoic acid, tert-butyldimethylsilyl ester	18.672
Octadecanoic acid, tert-butyldimethylsilyl ester	18.825
Heneicosanoic acid, trimethylsilyl ester	19.389
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	19.697
Nonadecanoic acid, tert-butyldimethylsilyl ester	19.874
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	20.035
Docosanoic acid, trimethylsilyl ester	20.547
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	20.717
Tetracosanoic acid, trimethylsilyl ester	21.968
Tetracosanoic acid, trimethylsilyl ester	23.765
Ricinoleic acid, trimethylsiloxy, trimethylsilyl ester	26.111
Tricosanoic acid, tert-butyldimethylsilyl ester	26.602
Tetracosanoic acid, tert-butyldimethylsilyl ester	27.731
Hexacosanoic acid, trimethylsilyl ester	28.165
THREONIC ACID, 2,3-BIS-O-(TRIMETHYLSILYL)-, .GAMMA.-LACTONE, D-	28.7
Olean-12-en-28-oic acid, 3-(acetyloxy)-, methyl ester, (3.beta.)	32.99
Other classes	
1,2-Ethanediol, monoacetate	7.008
SILANOL, TRIMETHYL-, PHOSPHATE	8.808
2,4(1H,3H)-Pyrimidinedione, dihydro-1,3-bis(trimethylsilyl)-	9.19
BUTANAL, 2,3,4-TRIS[(TRIMETHYLSILYL)OXY]-, (R*,R*)-	9.366
3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	10.61
Amine, N,N,N-tris((trimethylsilyloxy)ethyl)	10.717
PENTITOL, 3-DESOXY-TETRAKIS-O-(TRIMETHYLSILYL)	10.777
1,2-Epoxy-3,4-dihydroxycyclohexano[a]pyrene	11.357
ERYTHRITOL-1-D1, TETRAKIS-O-(TRIMETHYLSILYL)	11.542
GALACTOSE OXIME 6TMS	13.841
Glucose oxime hexakis(trimethylsilyl)	14.019
Acetamide, N-[2-[5-methoxy-1-(trimethylsilyl)-1H-indol-3-yl]ethyl]-N-(trimethylsilyl)	14.109
TRIMETHYL({2,3,4,5,6-PENTAKIS[(TRIMETHYLSILYL)OXY]CYCLOHEXYL}OXY)SILANE	15.827
Glucose oxime hexakis(trimethylsilyl)	16.165
2-O-Glycerol-.alpha.-d-galactopyranoside, hexa-TMS	17.684
Squalene	17.995

Uridine, 2',3',5'-tris-O-(trimethylsilyl)	18.804
ANDROSTANE, SILANE DERIV.	19.035
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	20.268
Cholest-5-en-3-ol (3.beta.)-, carbonochloridate	25.289
Tocopherol-.beta.-tms-derivative	26.885
STIGMAST-5-EN-3-OL, (3.BETA.)	27.546
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)	27.624
Stigmast-5-en-3-ol, oleate	27.891
9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta.,23E)	28.064
SILANE, [[(3.BETA.)-CHOLEST-5-EN-3-YL]OXY]TRIMETHYL-	28.401
9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)	28.55
6,7-DIHYDROXYCOUMARIN-.BETA.-D-GLUCOPYRANOSIDE, PENTA-TMS	28.868
Campesterol tms	29.219
TETRAHYDRODAMMARADIENOL	29.23
Stigmasterol trimethylsilyl ether	29.439
XYLOPYRANOSIDE, METHYL 2,3,4-TRIS-O-(TRIMETHYLSILYL)-, .ALPHA.-D-	29.667
Betulin	29.767
beta.-Sitosterol trimethylsilyl ether	29.875
Silane, (1,1-dimethylethyl)dimethyl(octacosyloxy)	30.044
TMS ETHER OF 2-MONOOLEGLYCEROL	31.02
14,17-Nor-3,21-dioxo-.beta.-amyrin, 17,18-didehydro-3-dehydroxy	31.733
SOLANESOL	32.355
SUGAR	
d-Ribose, 2,3,4,5-tetrakis-O-(trimethylsilyl)-, O-methyloxime	11.451
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.21
2,3,4,6-Tetra-O-trimethylsilyl-1-[1,7-dicarba-closo-dodecaboran-(12)-1-yl]-D-glucopyranose	13.256
D-FRUCTOSE, O-METHYLOXIM, PENTAKIS-O-(TRIMETHYLSILYL)	13.582
D-Fructose, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, O-methyloxime	13.688
D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)	14.661
1,2,3,4,6-PENTAKIS-O-(TRIMETHYLSILYL)HEXOPYRANOSE	15.132
D-Turanose, heptakis(trimethylsilyl)	21.007
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	21.389
D-Turanose, heptakis(trimethylsilyl)	22.162
D-Turanose, heptakis(trimethylsilyl)	22.352

D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)	22.989
D-Turanose, heptakis(trimethylsilyl)	23.303
D-Turanose, heptakis(trimethylsilyl)	23.511
Melibiose, octakis(trimethylsilyl)	27.349
D-Glucose, 4-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-galactopyranosyl]-2,3,5,6-tetrakis-O-(trimethylsilyl)-	28.697
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	30.592
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	30.685
alpha.-D-Glucopyranoside, 1,3,4,6-tetrakis-O-(trimethylsilyl)-.beta.-D-fructofuranosyl 2,3,4,6-tetrakis-O-(trimethylsilyl)	30.789
D-Fructose, 3-O-[2,3,4,6-tetrakis-O-(trimethylsilyl)-.alpha.-D-glucopyranosyl]-1,4,5,6-tetrakis-O-(trimethylsilyl)	31.883
AMINO ACID	
L-Leucine-2TMS	6.63
Serine tritms	7.305
N,O,O-Tris(trimethylsilyl)-L-threonine	7.687
l-Aspartic acid, bis(trimethylsilyl) ester	8.144
l-Norvaline, n-butoxycarbonyl-, butyl ester	8.349
Valylvaline, N,N'-dimethyl-n-propoxycarbonyl-, butyl ester	8.35
L-ASPARTIC ACID, N-(TRIMETHYLSILYL)-, BIS(TRIMETHYLSILYL) ESTER	9.456
Phenylalanine-2TMS	9.81
L-Asparagine, N2-trimethylsilyl-, trimethylsilyl ester	10.41
D,L-Alanine, N-(tert-butyldimethylsilyl)-N-methyl-, tert-butyldimethylsilyl ester	10.561
Glutamine, tris(trimethylsilyl)	10.65
L-Asparagine, N,N2-bis(trimethylsilyl)-, trimethylsilyl ester	11.291
L-Lysine-4TMS	11.88
L-Valine, N-(trimethylsilyl)-, trimethylsilyl ester	12.858
L-Methionine-2TMS	22.611
SUGAR ALCOHOL	
Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)	11.643
Ribitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)-	11.776
Xylitol, 1,2,3,4,5-pentakis-O-(trimethylsilyl)	11.783
l-(-)-Arabitol, pentakis(trimethylsilyl) ether	11.977
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)	12.154
D-GLUCITOL, 1,2,3,4,5,6-HEXAKIS-O-(TRIMETHYLSILYL)-	14.131
Myo-Inositol, 1,2,3,4,5,6-hexakis-O-(trimethylsilyl)-	15.672
Myo-Inositol, 1,3,4,5,6-pentakis-O-(trimethylsilyl)-, bis(trimethylsilyl)	18.575

phosphate	
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	30.401
Per-O-trimethylsilyl-(3-O-.alpha.-d-mannopyranosyl-4-O-.beta.-d-glucopyranosyl-d-glucitol)	31.03

Pyrimidinediones is another member of this class which has strong non-nucleoside inhibitors of the HIV-1 reverse transcriptase (RT). A range of pyrimidinediones with significantly enhanced antiviral efficacy and array of action with significantly reduced cellular cytotoxicity have also been developed [432]. Tocopherol and its different derivatives were other important metabolites significantly found in the tuber metabolome of wild type and transgenic tubers. Tocopherols are the member of vitamin E compounds (more precisely, various methylated phenols), present naturally in different plants. The nutritional advantages of vitamin E (d-alpha-tocopherol) and its significance as a daily part of the human diet have been well documented. Interestingly, two variants of tocopherol were present in the tuber metabolome (**Fig. 6.3 A-C**; **Table 6.1-6.3**). Uridine, a nucleoside was omnipresent across the wild type and transgenic lines. It contains a uracil attached to a ribose ring (known as a ribofuranose) via a β-N_1-glycosidic bond. Uridine plays a major role in linking the glycolysis pathway with that of galactose and the conversion of galactose to glucose. Additionally, antidepressant-like effects of uridine have also been established [433].

The prominence of sugar and sugar alcohol in wild type and transgenic lines amongst other metabolites was expected. However, sugars were the third major class in wild type (13%) as well as in COE (15%) and TOE (15%) lines. Glucose, fructose, mannose, ribose, turanose and their different variants were present in the tuber metabolome. These are natural sugar derivatives and their different isoforms or variants were present at different retention time across the wild type and the transgenic events (**Fig. 6.3 A-C**; **Table 6.1-6.3**). Moreover, these metabolites were the integral part of various basic metabolism of the cellular system. Considering their preponderance, amino acids lie in between the sugar and sugar alcohol and the fourth largest group of the tuber metabolome. Unlike secondary metabolites, amino acids are imperative for the maintenance of several basic metabolisms and found to be in a considerable amount in wild type as well as in the transgenic events. However, the concentration of the amino acids was considerably higher in the transgenic lines when compared to the wild type (**Fig. 5.8 A-M; Chapter 5**). Besides the usual amino acids, valylvaline, a derivative of valine was also obtained. Furthermore, the smallest class of tuber metabolome encompasses sugar alcohols. Sugar alcohols are a type of carbohydrates called "polyols" and are used as subdued calorie sweetener, and often in combination with high intensity artificial sweeteners to counter the low sweetness. These sugar substitutes offer fairly low calories than table sugar (sucrose), primarily due to its low

absorption and may even have a small laxative effect. Xylitol, glucitol, myo-inositol and arabitol were the sugar alcohols found to be present in the tuber metabolome.

6.3.3.2 Comparative metabolome profiling

6.3.3.2.1 Allocation of metabolites in wild type and transgenic events

Metabolite profiling of wild type (cv. OFSP-6) and two independent transgenic tubers (one each from COE and TOE lines) was performed by GC-MS. Metabolite profiling revealed several commonalities across the transgenic lines with that of wild type. Out of 154 metabolites identified in wild type, 140 were found to be non-redundant (**Table 6.1)**. However, 138 and 137 metabolites were non-redundant out of 150 and 135 in COE and TOE lines, respectively (**Table 6.2 and 6.3)**. Furthermore, comparing all the non-redundant metabolites, 117 were found to be universal across the wild type and transgenic events whereas, 9 metabolites were exclusively identified in transgenic lines. Interestingly, 8 and 1 metabolites were common in COE and TOE lines, respectively when compared with the wild type cv. OFSP-6 (**Fig. 6.4 A**). Nonetheless, 14 metabolites were unique to wild type, 5 to COE and 10 to TOE lines. The comparative analysis of differential metabolites suggested that there are 9 metabolites common in both the transgenic events (**Fig. 6.4 B**). However, most of them were the derivatives of metabolites already identified in OFSP-6. Moreover, none of the metabolites identified had any reports regarding toxicity or allergenicity and they are found to be the derivatives of natural compounds (**Table 6.4**).

6.3.3.2.2 Differential display of metabolites

A detailed evaluation pertaining to the disparity between the tuber metabolome of wild type (cv. OFSP-6) and transgenic events (COE and TOE lines) revealed the differential display of few metabolites. The metabolites exclusively present in the wild type were predominated by organic acids, sugar and sugar alcohols. Glycine was the only amino acid present exclusively in wild type. However, L-Norvaline, was found to be present distinctively in COE (RT-8.348) and TOE (RT-8.349) lines. L-Norvaline, a non-proteinogenic metabolite is an analog of the branched chain amino acid valine and it has been anticipated that L-Norvaline hampers the arginase enzyme activity and thus elevating arginine concentrations. Arginine is known to be converted into nitric oxide (NO) which is a crucial regulator and mediator in various physiological and pathophysiological events. Higher NO production has been implicated in quite a few cytostatic

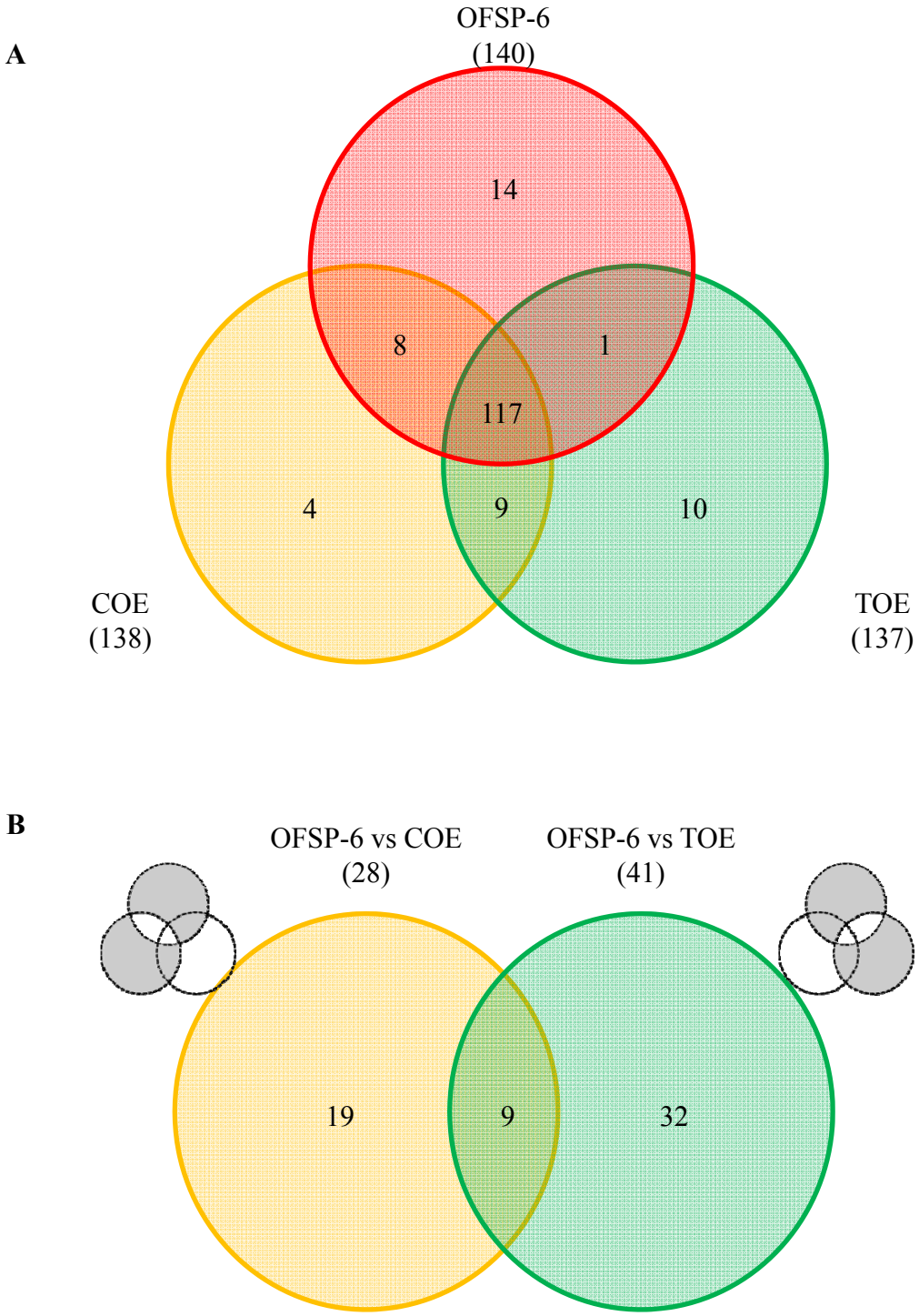

Fig. 6.4. Allocation of metabolites in wild type and transgenic events. Common and exclusive metabolites across the wild type (cv. OFSP-6) and transgenic lines are shown in Venn diagram (**A & B**). The area in panel B indicates the region of shaded area of circles alongside indicating the differential display of metabolites. The areas in the diagram are not proportional to the number of metabolites in the groups.

Table 6.4. Differential display of metabolites

OFSP-6		COE		TOE	
Name	**RT**	**Name**	**RT**	**Name**	**RT**
Glycine	6.471				
Butanedioic acid, bis(trimethylsilyl) ester	6.565	Butanedioic acid, [(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	11.417		
		1,2-Ethanediol, monoacetate	7.008	1,2-Ethanediol, monoacetate	7.008
		l-Norvaline, n-butoxycarbonyl-, butyl ester	8.348	l-Norvaline, n-butoxycarbonyl-, butyl ester	8.349
		PENTITOL, 3-DESOXY-TETRAKIS-O-(TRIMETHYLSILYL)-	10.776	PENTITOL, 3-DESOXY-TETRAKIS-O-(TRIMETHYLSILYL)	10.777
2,3,4,5-Tetrahydroxypentanoic acid-1,4-lactone, tris(trimethylsilyl)-	10.776				
		3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	10.919	3,8-Dioxa-2,9-disiladecane, 2,2,9,9-tetramethyl-5,6-bis[(trimethylsilyl)oxy]	10.61
				Amine, N,N,N-tris((trimethylsilyloxy)ethyl)	10.717
		Pentonic acid, 3-deoxy-2,4,5-tris-O-(trimethylsilyl)-, trimethylsilyl ester	11.058		
D-Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone	11.541	D-Arabinonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone	11.533		
				ERYTHRITOL-1-D1, TETRAKIS-O-(TRIMETHYLSILYL)	11.542

1-Dodecanol, 3,7,11-trimethyl	11.751	1-Dodecanol, 3,7,11-trimethyl	11.754		
Isododecyl alcohol, trimethysilyl derivative	11.768				
Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)	12.08	Xylonic acid, 2,3,5-tris-O-(trimethylsilyl)-, .gamma.-lactone, D-	12.067		
({4,5-BIS[(TRIMETHYLSILYL)OXY]TETRAHYDRO-3-FURANYL}OXY)(TRIMETHYL)SILANE	12.783				
				n-Tridecanoic acid, trimethylsilyl ester	12.867
		2,3,4,6-Tetra-O-trimethylsilyl-1-[1,7-dicarba-closo-dodecaboran-(12)-1-yl]-D-glucopyranose	13.254	2,3,4,6-Tetra-O-trimethylsilyl-1-[1,7-dicarba-closo-dodecaboran-(12)-1-yl]-D-glucopyranose	13.256
d-Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, o-methyloxyme, (1Z)	13.767				
				Glucose oxime hexakis(trimethylsilyl)	14.019
				Acetamide, N-[2-[5-methoxy-1-(trimethylsilyl)-1H-indol-3-yl]ethyl]-N-(trimethylsilyl)	14.109
(1E,3Z)-1-PHENYL-3-(TRIMETHYLSILYLOXY)-1,3-PENTADIENE	14.114				
		D-Glycero-L-manno-Heptonic acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)	14.122		
				D-Glycero-D-gulo-Heptonic	14.237

				acid, 2,3,5,6,7-pentakis-O-(trimethylsilyl)-, .gamma.-lactone	
				D-Mannopyranose, 1,2,3,4,6-pentakis-O-(trimethylsilyl)	14.661
		D-Galactose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)	14.661		
		PALMITELAIDIC ACID 1TMS	14.817		
				Arabino-hexaric acid, 3-deoxy-2,4,5-tris-O-(trimethylsilyl)-, bis(trimethylsilyl) ester	15.903
2,4'-Bis(trimethylsilyloxy)diphenylmethane	16.111	2,4'-Bis(trimethylsilyloxy)diphenylmethane	16.113		
cis-15-Tetracosenoic acid, trimethylsilyl ester	17.533				
				2-O-Glycerol-.alpha.-d-galactopyranoside, hexa-TMS	17.684
		Isopimaric acid TMS	17.907	Isopimaric acid TMS	17.908
				Squalene	17.995
1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl	17.917				
1,5,9-Decatriene, 2,3,5,8-tetramethyl-	17.925	1,5,9-DECATRIEN, 2,3,5,8-TETRAMETHYL	17.925		
Uridine, 2',3',5'-tris-O-(trimethylsilyl)-	18.804			Uridine, 2',3',5'-tris-O-(trimethylsilyl)	18.804
		ANDROSTANE, SILANE DERIV.	19.033	ANDROSTANE, SILANE DERIV.	19.035
10-Undecenoic acid, tert-	20.				

butyldimethylsilyl ester	797				
4.alpha.,5-Cyclo-A-homo-5.alpha.-cholestan-6-one	28.55				
		9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)-	28.55	9,19-Cycloergost-24(28)-en-3-ol, 4,14-dimethyl-, acetate, (3.beta.,4.alpha.,5.alpha.)	28.55
Pentacosanoic acid, tert-butyldimethylsilyl ester	28.577				
GLUCOPYRANOSIDE-6,6-D2, METHYL-TETRAKIS-O-(TRIMETHYLSILYL)-	28.726	GLUCOPYRANOSIDE-6,6-D2, METHYL-TETRAKIS-O-(TRIMETHYLSILYL)	28.721		
		6,7-DIHYDROXYCOUMARIN-.BETA.-D-GLUCOPYRANOSIDE, PENTA-TMS	28.87	6,7-DIHYDROXYCOUMARIN-.BETA.-D-GLUCOPYRANOSIDE, PENTA-TMS	28.868
alpha.-Tocopherol (vitamin E), trimethysilyl derivative	28.313	alpha.-Tocopherol (vitamin E), trimethysilyl derivative	28.311		
10,12-Tricosadiynoic acid, trimethylsilyl ester	29.1				
		Thymol-.beta.-d-glucopyranoside, tetrakis(O-trimethylsilyl)-	30.592		
1,2,2,3,4,4,5,5,6,6-Decamethylhexasilinane-1,3-diol	30.266				
METHYL 3-TRIMETHYLSILYLOXYTETRADECANOATE	32.05				

and cytotoxic actions mediated by macrophages, including the host tumoricidal and antimicrobial effects [434]. Moreover, there are significant secondary consequences of reduction in the availability of arginine due to its catabolism by arginase, including reductions in the NO synthesis (and increased superoxide production by NO synthase when arginine levels are sufficiently low) [435]. L- Norvaline is also known to be a natural component of antifungal peptides produced by some bacteria and plants to combat fungal pathogens [436, 437]. Another important metabolite unique to the transgenic lines was isopimaric acid [in COE (RT-17.907) and in TOE lines (RT-17.908)]. Isopimaric acid, extracted from the immature cones of *Pinus nigra*, illustrated its activities against MDR and MRSA strains of *S. aureus* which are becoming progressively more resistant to antibiotics [438]. Another metabolite exclusively found in COE and TOE lines was 6,7-dihydroxycoumarin (also known as aesculetin, esculetin, and cichorigenin) and androstane. Derivative of coumarin 6,7-dihydroxycoumarin, is a natural lactone that derives from the intramolecular cyclization of a cinnamic acid derivative with pleiotropic biological activities and can exert their chemopreventive and anti-tumor activities [439]. Additionally, a remarkable antibacterial activity against a vast range of pathogenic bacteria is also reported for this compound [440]. Androstane found only in COE (RT-19.033) and TOE (RT-19.035) line is a steroid reportedly documented in few plant extracts, putatively known for its antidiabetic activity [441].

Palmitelaidic acid (9-*trans*-Hexadecenoic acid) was a metabolite present only in the metabolome of COE line whereas, squalene was restricted to TOE line. Palmitelaidic acid has been suggested to have favorable effects on human health, including decreased adiposity. While its impacts on cholesterol levels are poorly studied, palmitelaidic acid may have very diverse effects from those of palmitoleic acid on lipid metabolism and mobilization [442]. However, another isomer of it is present across the wild type and transgenic lines as well. Alternatively, squalene, an isoprenoid compound structurally analogous to β-carotene, is an intermediary metabolite in the biogenesis of cholesterol. In humans, about 60% of dietary squalene is engrossed and transported in serum usually in association with very low density lipoproteins and is disseminated ubiquitously in tissues, with the maximum concentration in skin, where it is one of the key components of skin exterior lipids. It is less susceptible to peroxidation and appears to function in the skin as a quencher of reactive oxygen species (ROS), protecting human skin exterior from lipid peroxidation due to exposure to UV and other sources of ionizing radiation.

Supplementation of squalene to mice has been shown to increase the cellular and nonspecific immune functions in a dose-dependent manner. Squalene can also act as a "sink" for extremely lipophilic xenobiotics. While it is a nonpolar compound, it has an elevated affinity for un-ionized drugs. In animals, supplementation of the diet with squalene can decrease the cholesterol and triglyceride levels. In humans, addition of squalene might be useful to potentiate the impacts of some drugs responsible for reducing cholesterol level. The most important therapeutic use of squalene presently in the adjunctive therapy lies in different types of cancers [443].

6.3.3.3 Evaluation of transgene mediated effect on metabolites

The absolute quantification was performed using ribitol as internal standard selecting at least one metabolite from each class other than amino acids, found prominently across the metabolome of wild type and transgenic tubers. The rationale behind this investigation was to analyze the effect(s) of increased content of protein reflected also at the level of amino acids in transgenic tubers. We focused onto examine the level and extent of change(s) in the acquisition of metabolites in transgenic tubers pertaining to transgene introduction, azelaic acid, ferulic acid, oleic acid, and ribonic acid (organic acid), stigmast-5-en-3-ol, tocopherol (other classes), glucose (sugar) and glucitol (sugar alcohol). This analysis revealed no significant changes in the metabolites particularly ribonic acid, stigmast-5-en-3-ol, tocopherol, glucose and glucitol (**Fig. 6.5 A-E**). These findings further substantiated the observation at the level of primary metabolites. Additionally, a comparable level of glucose in wild type (cv. OFSP-6) and transgenic events was in agreement with the level of total carbohydrates (**Fig. 5.9 A; Chapter 5**). However, level of azelaic and ferulic acid increased considerably in transgenic lines (**Fig. 6.5 F and G**). Higher level of ferulic acid substantiated the higher TPC content in transgenic lines.

6.4 Discussion

Plants generate a massive collection of biochemically different compounds. Plant metabolites are not simply vital for plants themselves and their interactions with the environment but also offer indispensable sources for humans as resources of nutrition, energy and medicine. Additionally, secondary metabolites such as phenolics and flavonoids have crucial roles in chemical defenses against biotic and abiotic stresses, and flavonoids also confer health-promoting effects against chronic diseases and certain cancers in humans. The immense

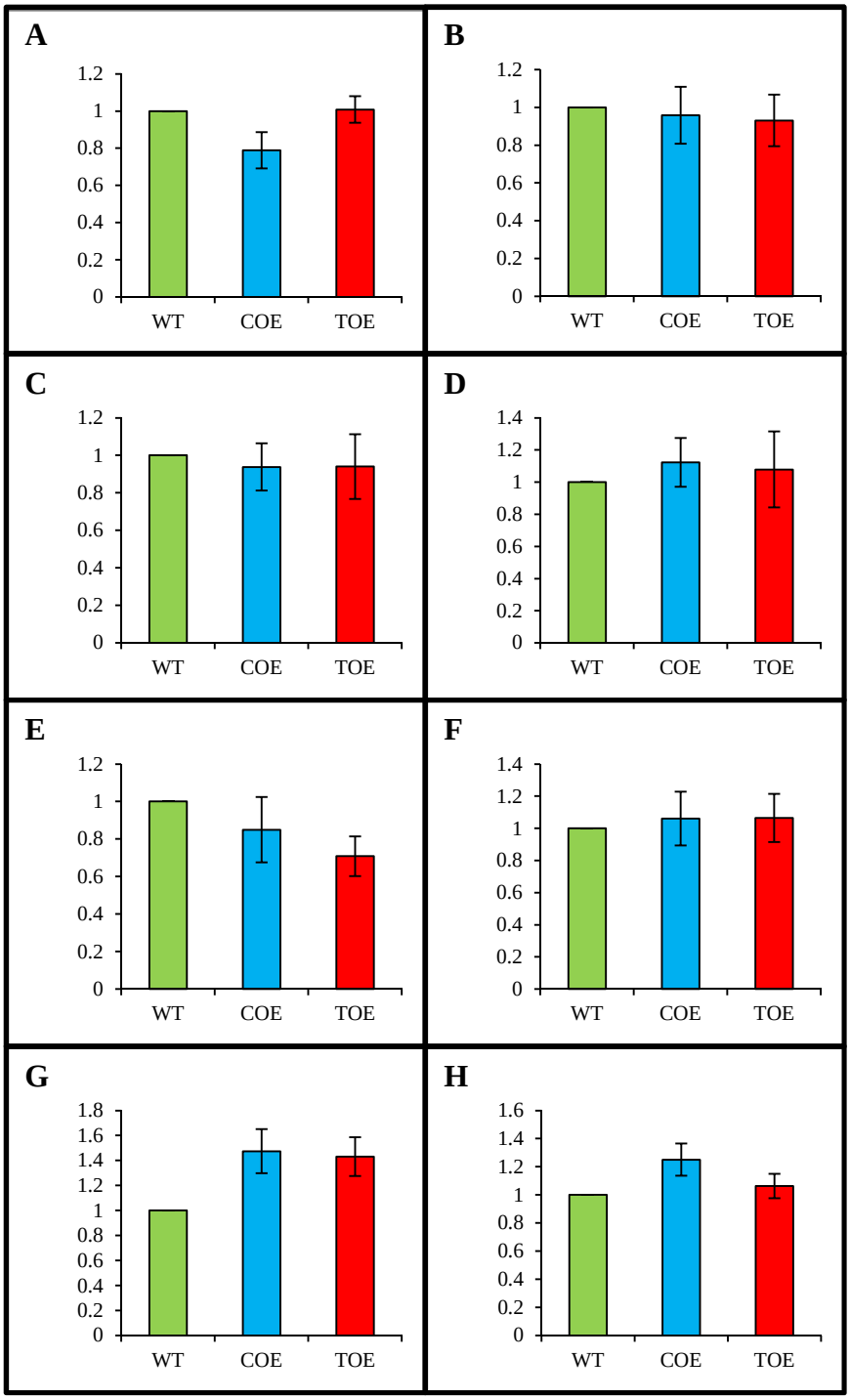

Fig. 6.5. Evaluation of transgene mediated effect on metabolites. Comparison of levels of different metabolites in mature AmA1 tubers with those in tubers of wild-type (A) Ribonic acid, (B) Stigmast-5-en-3-ol, (C) Tocopherol, (D) D-Glucose, (E) D-Glucitol, (F) Oleic acid, (G) Ferulic acid and (H) Azelaic acid. Data are normalized to the mean response calculated of each replicate taking ribitol as internal standard. Y axis represents the value of relative response ratio.

quantitative as well as qualitative diversities in metabolites have made plants the perfect models to scrutinize the biosynthetic pathways and regulation of metabolites. Unraveling the genetic basis of natural variation of the metabolome is indispensable for the excellence, reliability and sustainability of the world's food supply [417]. Metabolomics attempts to answer the issue of the effect(s) gene or gene product and their interactions with the environment. Connecting functional metabolomic knowledge to mRNA and protein expression data makes it feasible to envisage the functional genomic repertoire of an organism. Metabolomics is becoming an extensively used technology to assess global metabolite levels. With respect to the functional genomics, the non-targeted profiling of metabolites in biological samples is at present considered as a possible complementation to protein and transcript profiling technologies. Transgenic or mutant plants in combination with metabolomics provide an excellent means to look at changes in metabolic networks through the specific perturbation of a gene of interest and *vice-versa* [444]. In addition, a modification in the gene structure either via mutagenic or transgenic approach may or may not be reflected at the level of metabolome. Therefore, metabolomics may possibly be used in the evaluations of substantial equivalence of the transformants with that of their wild type counterparts. Furthermore, metabolomic study will be of enormous theoretical significance for understanding metabolic responses in more detail [410, 445]. To examine the transgene mediated changes in metabolome of transgenic tubers, a comprehensive analysis of metabolites was performed.

Detailed analyses revealed contrasting trends at the level of phytophenols and carotenoids. The TPC content was significantly higher in transgenic lines and to some extent followed the trend of the expression of transgene, *AmA1*. Considering both the wild type varieties, TPC was found to be lower in OFSP-6 in comparison to WFSP-WT whereas, it has a higher TFC content. Intriguingly, TFC content was remarkably higher in COE as well as in the TOE lines. The TAC content of transgenic events was found to be higher than that of its wild type counterpart cv. OFSP-6. However, elevation in TCC content was found to be restricted to higher transgene expressing lines. Nonetheless, TAC and TCC were remarkably higher in OFSP-6 when compared with WFSP-WT (**Fig. 6.1**). A higher TCC was expected in cv. OFSP as the intensity of orange color is attributed to the carotenoid content of an individual cultivar [45, 415]. Higher contents of anthocyanin and carotenoids in transgenic lines substantiated its higher ‘a’ and ‘b’ color space values (**Fig. 5.5; Chapter 5**). Orange-fleshed cultivars are known for

their high β-carotene content, yet high genetic variation has been observed in different cultivars [11, 45, 47, 414]. The higher acquisition of carotenoids in cv. OFSP is likely to be a value added signature of this cultivar. The phytophenols and carotenoids, often vary across the genotypes and are thought to be associated with genetic factors, which play a vital role in the formation of secondary metabolites [7]. **Padda and Picha** (**2007**) have earlier reported a higher phenolic content in small-sized roots of potato than that of larger-sized roots. The decrease in phenolic content with the development of tubers in root crops has been attributed to a dilution effect resulting from tuber bulking [446]. Polyphenols are synthesized from the phenylalanine produced by the shikimic acid pathway, and flavonoids are produced through a bifurcation of this pathway. Therefore, increase in overall amino acid content including phenylalanine in particular (**Fig. 5.7 and 5.8**) may be anticipated as the increased flux of TPC and TFC. A different analysis by **Dao et al. (2010)** also revealed that the accumulation of phytophenols and flavonoids in *CHS* transformed Arabidopsis was a consequence of increased amino acids in the transgenic plants [447]. These findings were in agreement with the higher expression of *CHS*, a key flavonoid pathway gene. Additionally, other early pathway (*CHI*) as well as late pathway genes (*DFR, ANS* and *UFGT*), were also up-regulated in transgenic lines when compared with the wild type counterparts cv. OFSP-6 (**Fig. 6.2**). Nonetheless, early pathway as well as late pathway genes were comparatively higher in cv. OFSP-6 than that of WFSP-WT. However, *F3H* transcript was slightly induced in WFSP as well as in those transgenic lines where TFC content was comparatively lower. This may be attributed due to the concerted feedback or feedback regulation of the gene product(s) [448-450]. In recent years, a mounting interest in producing food crops with increased levels of flavonoids necessitated the need to combine targeted metabolomic and transcriptomic approaches that might possibly help in determining gene-metabolite relationships. **Carvalho, et al. (2010)** showed a strong correlation between the expressions of genes in flavonoid pathway [451]. Furthermore, anthocyanin was found to be strongly associated with *DFR* gene. The downregulation of *DFR* gene has been shown to inhibit the accumulation of anthocyanin and proanthocyanidin, but increase flavonol influx [452].

Metabolomics is the study of all the metabolites of a biological sample. A number of investigative methods namely gas chromatography (GC), liquid chromatography (LC), mass spectrometry (MS) and nuclear magnetic resonances (NMR) have been used for metabolome profiling. However, none of these is competent to give a comprehensive vision of the complete

metabolome. The dynamic range of metabolites, polarity, volatility, and stability are in fact few limiting factors in acquiring a complete picture of all metabolites. A combination of the variety of methods might provide the most comprehensive view on the metabolome. Nevertheless, with every single analytical method a wealth of information about the metabolome of an organism can already be obtained under certain conditions. GC-MS has been considered as a powerful platform in metabolomics due to the ease of sample preparation, time spell for analysis and additionally a robust technique that allows the qualitative as well as the quantitative analysis of all metabolites [293, 410]. A detailed analysis of metabolites was performed with wild type (cv. OFSP-6) and two independent transgenic tubers (one each from COE and TOE lines). The purpose of the analysis was to examine the possibility of the changes in the status of metabolites in transgenic sweet potato. The transformation of AmA1 in sweet potato had a greater influence on the overall status of amino acids and protein in the transformants. However, most of the metabolites having no direct association with amino acids, yet there is a possibility that that an overall proteome rebalancing may affect the metabolite status.

A detailed comparative analysis of metabolites in transgenic events *vis-à-vis* to their wild type counterparts revealed no major change in the metabolome of transgenic tubers (**Table 6.1- and 6.3)**. Most of the metabolites eluted precisely at or around the same retention time across the wild type and transgenic lines indicating strong conservation of metabolites. Nutritional diversity among the cultivars is a distinguished and established fact, and are often observed in different transgenic lines, when compared with their wild type counterparts. Several studies on genetic diversity between closely related genotypes had focused on the similarities as well as the difference in the metabolites owing to genetic factors and/or the expression of transgene [298, 453, 454]. A significant difference in metabolites was also found in this study between the two closely related but contrasting wild type cultivars grown in identical conditions. Changes in the accumulation of different metabolites may be attributed to pleiotropic and/or multigenic effect(s). Several earlier reports related to the global transcriptome and metabolome analysis suggested no substantial differences between transgenic and wild types, when applied to pathway-engineered transgenic plants. Likewise, GC-MS analysis of fructan-producing transgenic potato tubers did not display any considerable alterations, except for metabolites directly associated to the introduced pathway [312]. To better evaluate the impact of transgene expression, evaluating the influence of natural genotypic differences and environmental factors

on multiparallel datasets is of paramount importance. To circumvent superfluous bias, the transgene in particular should not directly influence metabolic pathways in the target plants. A comparative analysis of wheat-flour metabolome by NMR resulting from field-grown transgenic wheat expressing high molecular weight glutenin and the analogous parental line revealed that, despite some differences in central free amino acid and sugar metabolism between transgenic and wild type cultivars, agroecological conditions had a greater impact on the dataset than the expression of transgene [316]. In addition, transcriptome and metabolome profiling of field grown transgenic barley lack induced differences but showed cultivar-specific variances [293].

It has been increasingly established via different analyses that the increase in the concentration of amino acids may lead to the increase in phytophenols as the formation of three aromatic amino acids; tryptophan, tyrosine and phenylalanine is via shikimic acid pathway [447, 455, 456]. Biosynthesis of the majority of phenolic compounds is by phenylalanine, even though hydrolyzable tannin is directly produced through the gallic acid in the shikimic acid pathway. The production of phenolic compounds is catalyzed by phenylalanine ammonia-lyase (PAL). Whereby, PAL is a key gateway enzyme in the secondary metabolic pathway leading to the synthesis of phenolic compounds. The increase in the concentration of ferulic acid in transgenic lines further corroborated these findings. Furthermore, phenylalanine, tyrosine and tryptophan are the primary metabolites which provide the precursors for several natural secondary products namely flavonoids, phenolic acids, coumarins, alkaloids, glucosinolates and cyanogenic glycosides [457]. Additionally, the majority of secondary metabolites with antioxidant properties including the various classes of phenolic compounds are synthesized via the shikimic acid pathway. Although malonic acid pathway is also involved in the biosynthesis of some of these compounds (flavonoids), its role is rather minor in higher plants [458]. Among phytochemicals possessing antioxidant capacity, phytophenols are one of the most significant groups [459]. Phenolic contents are affected by biotic stresses and abiotic stresses besides storage conditions, post-harvest treatments and the estimation methods. Alltogether these factors, besides the biosynthesis of phenolic antioxidant compounds, influence the final concentration of polyphenols in plant tissues. Phenylalanine ammonialyase (PAL), the key enzyme catalyzing the biosynthesis of phenolics from the aromatic amino acid phenylalanine, was found to be responsive to biotic and abiotic stresses. Therefore, the substrate of PAL *i.e.* phenylalanine is also responsible directly or indirectly for stress responses [460]. Accumulation of azelaic acid in

transgenic lines indicated its possible role especially in biotic stress. A high concentration of polyphenolic compounds, such as phenolic acids and flavonoids, have been reported in amaranth grains which is the source of gene *AmA1*. Moreover, the optimization of germinating conditions resulted in enhanced phenolic acids and flavonoids in amaranth grain [461]. AmA1 contains two agglutinin domains (**Fig. 1.3; Chapter 1**). However, the biological function of agglutinin is still not well characterized particularly in plants but expression and characterization of two domains of agglutinin from *Pinellia ternate,* its overexpression in tobacco chloroplast, suggests its role in the tolerance against a varieties of pathogens [128, 129]. These further indicated the role of AmA1 especially in biotic stress presumably via increasing the amino acid concentration in the cellular pool in *Amaranthus* as well as in other host plant via increasing the concentration of phytophenols. A hypothetical model is proposed based on these findings (**Fig. 6.6**) wherein, AmA1 is directly contributing towards the increase in phenylalanine which is consequently directed to the shikimic acid pathway leading to the higher concentration of phenolic acids and flavonoids. A detailed analysis in this regard is needed to elucidate the underlying mechanisms which may unravel some new dimensions pertaining to the role of seed storage proteins.

Conclusively, it can be believed that AmA1 is directly affecting the overall protein and amino acid contents in transgenic sweet potato, which has indirectly influenced the accumulation of those metabolites which were associated with amino acids. Moreover, AmA1 introduction in sweet potato had no significant impact on global metabolome in transgenic tubers. However, it significantly increased the antioxidative activities by augmentation of phytophenols.

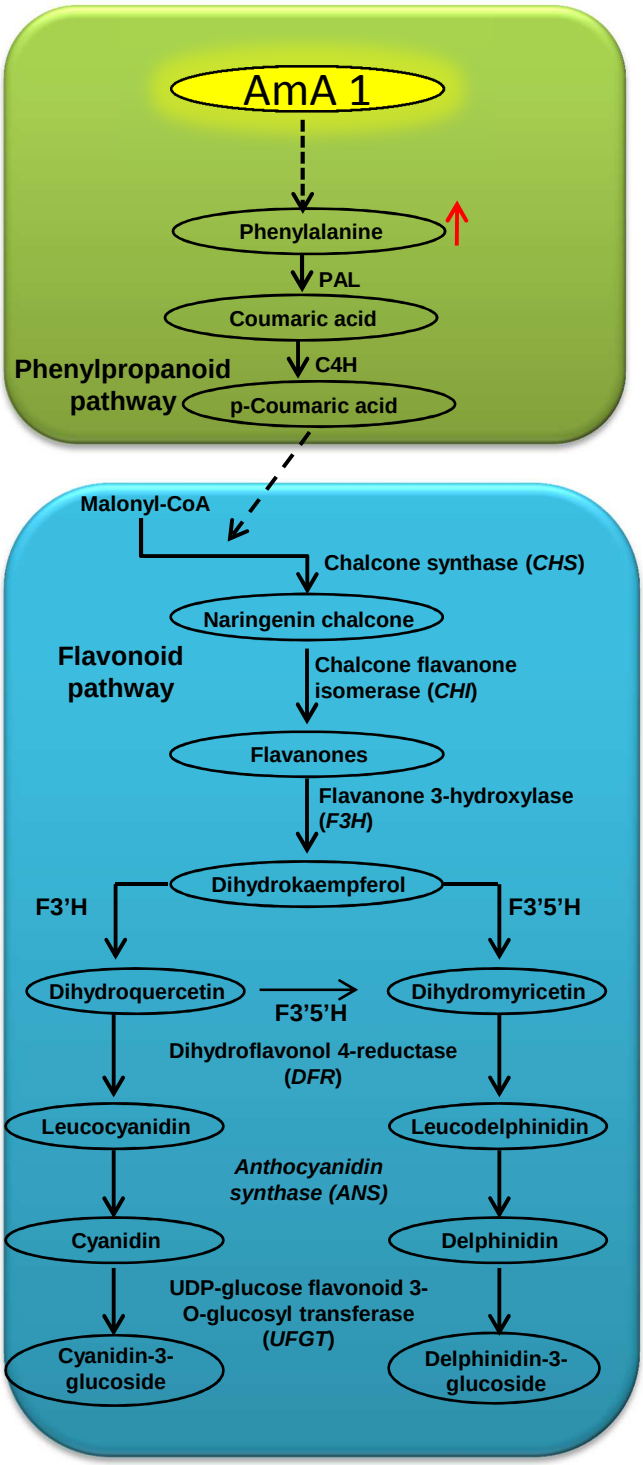

Fig. 6.6. A proposed hypothetical model depicting the role of AmA1 in transgenic tuber. The model depicts the possible role of AmA1 in the increased level of phytophenols in transgenic tubers. By modulating shikimic acid pathway via increasing the level of phenylalanine AmA1 is possibly leading to the higher concentration of phenolic acids and flavonoids.