

Functional Analysis in Leaf Trichomes Cell ESTs in *Mentha piperta* Using by Computational Approaches

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Abstract: *Mentha piperta* is a herbaceous perennial plant of the family Lamiaceae. Essence produced in shoot and stored in trichomes of leaves surface. Currently, using in-silico approach through mining expressed sequences tags [EST] have become an effectively for developing molecular analysis *in vitro*. In this research, we decided to use the ESTs data in the Library of *M. piperta*, a good image for genome surgery in medicinal plant. Results showed that 355contig and 442 singletons determined from 1676 trichomes leaves ESTs. Gene ontolgy revealed 12 functional groups from primary metabolism to secondary metabolism. In addition to with compotational approaches several miRNA predicted in trichomes leaves ESTs that can be used for regulation of gene transcription. Additionally, simple sequence repeats [SSRs] were also identified contributing to genes in menthol biosynthesis pathway, primary metabolism, response to stress, cell wall formation, response to stress, cell signaling dependent to calcium.

Key words: Analysis • Functional • *Mentha piperta* • Trichomes

INTRODUCTION

Perennial herbaceous plants; mint, one of the most widely used traditional medicinal plants. Its leaves are elliptical, cross, sharp-toothed with trichomes [1]. This plant is native to Europe, but is now cultivated in most temperate areas of the world as well as in most parts of Iran, especially in the region of the Alborz, North, Northeast and spread elsewhere. Fresh leaves of *M. piperta* with tannin, a bitter substance and 2.5% essence. Essence produced in shoot and stored in trichomes of leaves surface. The active ingredient is menthol in *M. piperta* that there are 30-70% menthol free and ester form. Menthol (Fig. 1) is certainly the best known of the monoterpenes, both as a pure compound employed as an ingredient and as a component of the essential oils from the genus *Mentha* of the Lamiaceae [2].

Menthol and related monoterpenes are representatives of the smallest members [C10] of the very large class of terpenoid [isoprenoid] natural products now numbering in excess of 40, 000 defined structures [3], [4], [5].

Menthol Biosynthesis: The eight-step pathway to menthol from primary metabolism (Fig. 2). The first step, monoterpenes to be derived from the plastidial methyl erythritol phosphate [MEP] pathway for the supply of the universal C5 isoprenoid precursors isopentenyl diphosphate [IPP] and dimethylallyl diphosphate [DMAPP]. IPP and DMAPP by a specific prenyltransferase Combined together for production of universal monoterpene precursor geranyl diphosphate. after cyclization of geranyl diphosphate produced cyclic precursor parental, limonene. hydroxylation at C3 and a series of four redox transformations [one oxidative, three reductive] with an intervening isomerization follow. This sequence of reactions generates a chiral center at C1 [p-menthane skeleton numbering], inverts the chiral center at C4 in two steps and introduces and then inverts in two steps, the chiral center at C3 to yield menthol [6, 7].

Sites of Essential Oil Production and Storage: The essential oil of peppermint and other *Mentha* species is produced and accumulated specifically in the peltate

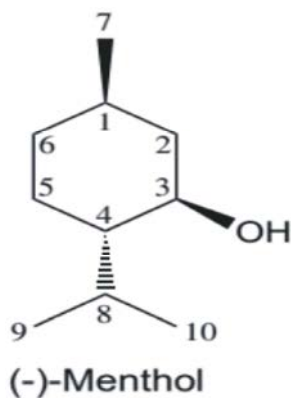


Fig. 1: Menthol chemical structure as existing medicinal ingredient Lamiaceae family plants

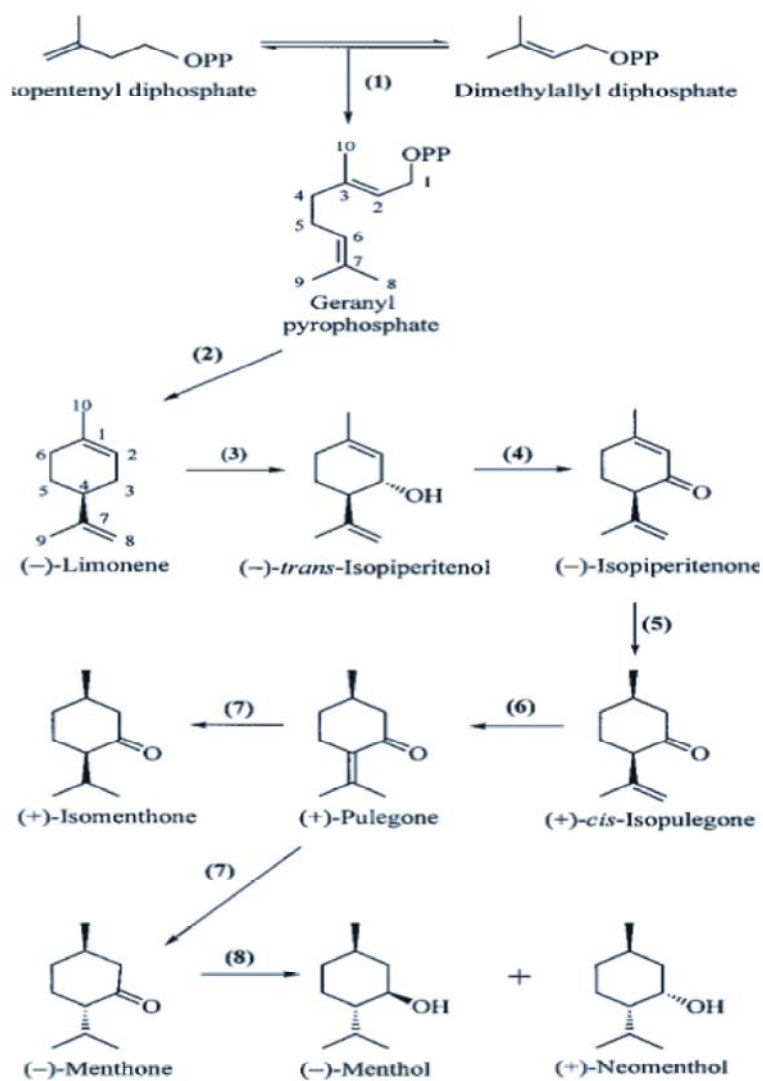


Fig. 2: Pathway of menthol biosynthesis in the secretory gland cells of peppermint. The enzymatic steps are catalyzed by, (1) geranyl diphosphate synthase (GPPS), (2) limonene synthase (LS), (3) limonene- 3-hydroxylase (4) trans-isopiperitenol dehydrogenase (iPD), (5) isopiperitenone reductase (iPR), (6) cis-isopulegone isomerase (iPI), (7) pulegone reductase (PR), (8) menthone reductase (MR)

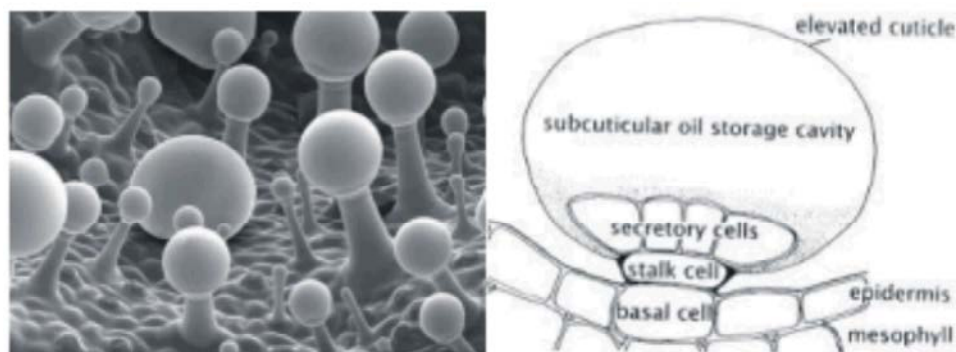


Fig. 3: Schematic diagram of the secretory cell in *Mentha* species. peppermint peltate glandular trichome showed the subcuticular oil storage cavity (SC), secretory cells (S), stalk cell (ST) and basal cell

glandular trichomes found on the aerial parts of the plant [8, 9]. These epidermal oil glands (Fig. 3) are composed of eight, radially distributed secretory cells [in which the essential oils are synthesized] that sit upon a single stalk cell and a basal cell that is embedded in the surface. The secretory cells are surmounted by a shared subcuticular cavity into which the oil is secreted and stored.

The quantification and identification of biological molecules combined with insilico analysis led to will be produce a lots of useful information for researchers in experimental researches. For a gene to be functional, it must be transcribed, processed and translated into a functional protein. Expression analyses of genes are important part of gene function studies. Currently, using in-silico approach through mining expressed sequences tags [EST] have become an effectively for developing molecular analysis *in vitro*. Molecular markers play an important role in many aspects of plant breeding, such as identification of the genes responsible for desirable traits. Molecular markers have been widely used to map important genes and assist with the breeding of plant. Compared with other types of molecular markers. Based on the original sequences used to identify simple repeats EST-SSRs are derived from expressed sequences, which are more evolutionary conserved than noncoding sequences; therefore, EST-SSR markers have a relatively high transferability [10]. Transcript pattern characterization, prediction miRNAs with their successful prediction from sequence data using by homology-based models [11]. In this research, we decided to use the ESTs data in the Library of *M. piperta*, for metabolomic, gene regulation research and identifying molecular markers [EST-SSR] because of the economic value of the materials stored in the leaf trichomes.

MATERIALS AND METHODS

Sequence Source of *M. Piperta*: 1676 EST sequences of the library *M. piperta* was received of the database NCBI [www.ncbi.nlm.nih.gov].

Assembly ESTs Sequences from Trichomes Glandular *M. piperta*: Remove vector sequences, duplicates and organelles [mitochondria and chloroplasts] and assembled the sequences obtained, using the online service Egassembler. Vector sequence in order to identify pollution and their length and quality of service using by bioinformatics Egassembler [www.egassembler.hgc.jp]. After removal of vector sequences, repetitive sequences, chloroplast and mitochondrial sequences were removed [12].

Gene Ontology and BlastX Search Sequences for Identify Hits of *M. piperta*: Searching BlastX for all unigene [contig and Singleton] with the maximum E-Value NCBI sequences. BLASTX results were used to determine the functional groups. The Gene Ontology project provides an ontology of defined terms representing gene product properties. The ontology covers three domains: cellular component, molecular function, biological process. Gene ontology for ESTs from trichomes glandular determine with uniport server addressed www.uniport.org.

Important Proteins That Detected in Gene Ontology of Trichomes Glandular *M. piperta*: Using by results of gene ontology for EST from trichomes glandular *M. piperta*, ESTs that encoded enzymes in menthol biosynthesis detected (Table 1).

Prediction of miRNAs for Est Sequences from Trichomes Glandular *M. piperta* with Modeling in *Arabidopsis Thaliana* and *Salvia Scalaria*: For these prediction used all

Table 1: Results of Remove vector sequences, duplicates and organelles (mitochondria and chloroplasts) and assembled with Egassembler

Total EST number	Total length of the genome covered(bp)	Total number Unigene	Number of contig	Number of singlone
1676	428918bp	797	355	442

contig and singletons sequence. Results predicted with online service addressed <http://plantgrn.noble.org> (Xinbin Dai and Patrick X. Zhao, 2011).

Frequency and Distribution of EST-SSRs Found in dbEST Sequences: Potential SSRs [simple sequence repeats] markers were detected using the tool of SSRIT [Simple Sequence Repeat Identification Tool] online with online service addressed <http://archive.gramene.org/db/markers/ssrtool>. The minimum repeat unit was defined as five for dinucleotides, tri-nucleotides and four for tetra-, penta- and hexa-nucleotides. To predict the position of SSRs with respect to coding regions.

RESULTS AND DISCUSSION

The analysis was performed on the sequence of trichomes leaf *M. piperta* in the family Lamiaceae, the results were obtained in Table 1, according was introduced to the total number of EST [1676 EST], 355 contig and 442 Singleton and a total unigene 797. searching BlastX for all unigene [contig and Singleton] with the maximum E-Value NCBI sequences. Proteins grouped in transportation and binding proteins for ions and other small molecules, RNA processing, polymerizing, splicing and binding proteins and enzymes, cell replication, histones, cyclins and allied kinases, cytoskeleton and membrane proteins, protein synthesis co-factors, ribosomal proteins, intermediary synthesis and catabolism enzymes, stress response, detoxification and cell defense proteins, protein degradation and processing, proteases, transportation and binding proteins for proteins and other macromolecules, apoptosis-related proteins, signaling receptors and signaling ligands, transcription factors and other gene regulatory proteins, sequence-specific DNA-binding proteins, proteins involved in gene regulation, chromatin proteins other than with regulatory function. Functional group for these protein and ratio importance in total function (Fig. 4).

Gene ontology for ESTs from trichomes glandular determined protein from trichomes glandulars that some important protein in menthol biosynthesis pathway showed in Table 2. geranyl diphosphate; the mint geranyl diphosphate synthase included large and small subunits, that catalyzes the transfer of an isoprenoid [farnesyl or

geranylgeranyl] usually on cysteine residues. Limonene synthase; belongs to the terpene synthase family. The Asp-Asp-Xaa-Xaa-Asp/Glu [DDXXD/E] motif at 342 – 346 is important for the catalytic activity, presumably through binding to Mg^{2+} . In addition to this motif 487, 491 and 495 residues realated to Mg^{2+} binding. Limonene-3-hydroxylase is a cytochrome P450 oxygenase that is responsible for the hydroxylation at C-3 position of [4S]-limonene to produce [-]-trans-isopiperitenol. Isopiperitenol dehydrogenase; This enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donor with NAD^+ or $NADP^+$ as acceptor. The systematic name of this enzyme class is [-]-trans-isopiperitenol: NAD^+ oxidoreductase. This enzyme participates in monoterpenoid biosynthesis. Isopiperitenone reductase; Monoterpene synthase that catalyzes the specific reduction of the 1₂-double bond of [-]-isopiperitenone to produce [+] -cis-isopulegone. The two binding sites were important in enzymes: Binding site for substrate and Nucleotide binding, 182 and 10-33 residues. Compositional bias in enzymes is 187 – 190 residues by poly-Leu rsidues. pulegone reductase; monoterpene synthase that catalyzes the specific reduction of the 4, 8-double bond of [+] -pulegone to produce both [-]-menthone and [+] -isomenthone in a 70:30 ratio. Unable to utilize either [-]-isopiperitenone or [+] -cis-isopulegone, or to catalyze the reverse reaction with [-]-menthone or [+] -isomenthone. Has an absolute requirement for NADPH. Binding site, 189, 205, 229, 331 and Nucleotide binding 163 – 166, 251 – 257, 281 – 283. Menthol reductase is most active in late gland development, the corresponding gene was acquired by related molecular means from transcripts isolated from a mature population of oil gland secretory cells. The deduced amino acid sequences of these two reductases share 73% identity, provide no apparent subcellular targeting information and predict inclusion in the short-chain dehydrogenase/reductase family of enzymes. Menthofuran synthase is a heme-thiolate protein [P-450]. Menthofuran provide an unusual example of a small molecule acting to down-regulate [at the mRNA level] a downstream step of an extended biosynthetic pathway. Menthofuran as a regulator of pulegone reductase are direct or involve other intermediaries, or whether these effects are exerted at the levels of *pr* transcription or translation, or *pr* message stability.

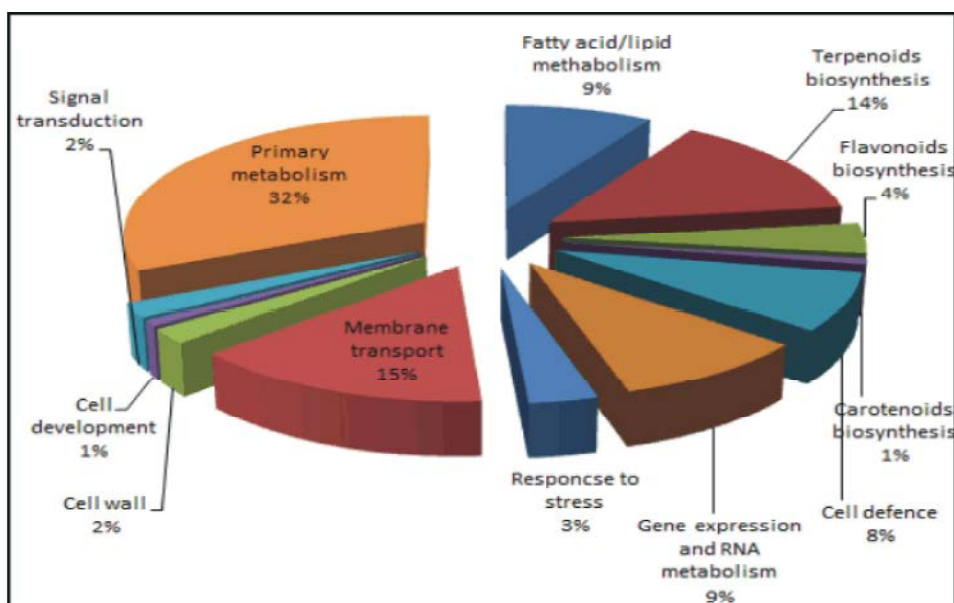


Fig. 4: functional group determined for ESTs from trichomes glandular.

Table 2: Contigs or Singletons detected for Enzymes in menthol biosynthesis pathway in glandular thricomes *M. piperta*.

Enzymes in menthol biosynthesis pathway	Contigs or singletons related to Enzyme
Geranyl diphosphate synthase	Contigs (7-93-100-268-320), singletons(107)
Limonene synthase	Contigs(12-25-33-82-132-149-171), singletons(388)
Limonene-3-hydroxylase	Contigs(72-105-107-150-174-311), singletons(110-125-303-351)
Trans-isopiperitenol dehydrogenase	Contigs (64-339), singletons(298)
Isopiperitenone reductase	Contigs (6-27-34-189-229), singletons(51)
Cis-isopulegone isomerase	Not detected in my analysis.
Pulegone reductase	Contigs(18, 122, 331)
neoMenthole reductase	Singletons(272)
Menthofuran synthase	Contigs (11-36-350), singletons(103-360-409)

These findings have important application in the production of commercial mint oils of high quality and they may have broad implications for the control of natural products biosynthetic pathways. miRNA detected in ESTs from trichomes *M. piperta* with modeling in *Arabidopsis thaliana* as shown in Table 3.

Several hundred miRNAs have been identified in plants by computational and experimental approaches. But so far the species has not been reported either in the laboratory or computational identification of miRNA in *M. piperta* species. Predicted small RNA mediated gene regulation in process that related to regulation of energy cell, amino acid biosynthesis in the light period, transport methyl group, Ubiquitin conjugation pathway, autophagosomes formation, Transcription factor involved in photomorphogenesis in the light, response to phytohormone such as response to gibberellin, jasmonic acid, salicylic acid, ethylene, abscisic acid [ABA] and auxin [IAA], Involved

in epidermal cell fate specification, signal transduction process, protection of cell from superoxide radicals. These prediction to gain insight into miRNAs and regulatory functions important in trichomes cell using computational approach. EST-SSR markers identified for EST trichomes leaf as shown in Table 4 results for identifying of EST-SSR showed that important genes in menthol biosynthesis pathway such as isopiperitenone reductase, menthofuran synthase, limonene synthase, limonene hydroxylase had several EST-SSR markers. Being of these markers help to breeder for fit selection in *Mentha* genus. Addition to other identified markers for genes that played roles in primary metabolism, response to stress, cell wall formation, response to stress, signaling. Further functional analyses on genes identified from the ESTs of *M. piperta* will provide more information about the molecular mechanism that is involved in the evolution and development of trichomes cell.

Table 3: miRNA detected in ESTs from trichomes *M.piperta* with modeling in *Arabidopsis thaliana*

miRNA detected	Unigenes	Gene regulates with miRNA	Biological process regulated with genes
ath-miR159c	Singleton 175	adenylate kinase	Enhanced cell growth and increased amino acid biosynthesis in the light period.
ath-miR163	Singleton 320	cytochrome c	oxidation-reduction process in Electron transport.
ath-miR414	Singleton 78	flavonoid 4'-O-methyltransferase (OMT4)	Methyltransferase.
ath-miR414	Singleton 441	flavonoid 4'-O-methyltransferase (OMT4)	Methyltransferase.
ath-miR5021	Singleton 19	E3 ubiquitin-protein ligase RING1-like	Ubl conjugation pathway.
ath-miR5628	Singleton 259	phosphoenolpyruvate/phosphate translocator 2	Sugar transport.
ath-miR5641	Singleton 275	autophagy-related protein 8c	Ubiquitin-like modifier involved in cytoplasm to vacuole transport (Cvt) vesicles and autophagosomes formation. May mediate the delivery of the vesicles and autophagosomes to the vacuole via the microtubule cytoskeleton.
ath-miR858a	Singleton 416	transcription factor WER(MYB21)	Transcription factor involved in photomorphogenesis in the light. May act downstream of the light receptor network and directly affects transcription of light-induced genes. In darkness, its probable degradation prevent the activation of light-induced genes. Required to activate expression of PAL. Acts redundantly with MYB24 and MYB57 to control stamen filament elongation in the late developed flowers. Contributes with MYB24 to induction of MYB108 by jasmonate. Repressed at the transcript levels by DELLA proteins
ath-miR858a	Singleton 377	transcription repressor MYB6	Transcription regulation, response to gibberellin, jasmonic acid, salicylic acid and Induction By ethylene, asbsciscic acid(ABA), auxin (IAA).
ath-miR858b	Singleton 416	transcription factor WER(MYB21)	Transcription activator, when associated with BHLH2/EGL3/MYC146 or BHLH12/MYC1. Involved in epidermal cell fate specification. Together with GL3 or BHLH2, promotes the formation of non-hair developing cells (trichoblasts) Regulates stomata spatial, Binds to the WER-binding sites (WBS) promoter regions and activates the transcription of target genes such as GL2 and of CPC and Repressed by CPC in hair cells.
ath-miR858b	Singleton 377	transcription repressor MYB6	Transcription regulation, response to gibberellin, jasmonic acid, salicylic acid and Induction By ethylene, asbsciscic acid(ABA), auxin (IAA)
ath-miR861-5p	Singleton 398	Zinc finger MYND domain-containing protein	May be involved as a regulatory molecule in signaling.
ath-miR398a	Contig15	superoxide dismutase (SOD5)	Superoxide dismutases serve to convert damaging superoxide radicals, a key form of ROS, to less damaging hydrogen peroxide that can be converted into water by catalase action, Leads to sensitivity to hydrogen peroxide when cells were grown in nutrient-limited conditions
ath-miR398b	Contig15	superoxide dismutase (SOD5)	Superoxide dismutases serve to convert damaging superoxide radicals, a key form of ROS, to less damaging hydrogen peroxide that can be converted into water by catalase action, Leads to sensitivity to hydrogen peroxide when cells were grown in nutrient-limited conditions
ath-miR398c	Contig15	superoxide dismutase (SOD5)	Superoxide dismutases serve to convert damaging superoxide radicals, a key form of ROS, to less damaging hydrogen peroxide that can be converted into water by catalase action, Leads to sensitivity to hydrogen peroxide when cells were grown in nutrient-limited conditions
ath-miR414	Contig292	glutaredoxin	glutaredoxin are involved in flower development and Salicylic acid signalling.
ath-miR414	Contig102	flavonoid 4'-O-methyltransferase (OMT4)	Methyltransferase
ath-miR5021	Contig14	YABBY-like transcription factor PROLONGATA (PROL)	Control of growth and cell identity.
ath-miR5021	Contig261	flavonoid 3'-O-methyltransferase (OMT3)	Methyltransferase.
ath-miR5021	Contig303	acyl carrier protein 1,	Carrier of the growing fatty acid chain in fatty acid biosynthesis, fatty acid biosynthetic process.
ath-miR835-3p	Contig255	1-aminocyclopropane 1-carboxylate oxidase homolog 4-like	Enzyme involved in theAlkene biosynthesis, ethylene biosynthesis. May promote stem elongation by maximizing the extensibility cells, possibly by activating ethylene biosynthesis, in response to very-long-chain fatty acids.

Table 4: EST-SSRs found in contigs from ESTs sequences from *M. piperta*

Sequence	EST-SSR		Gene coded with contigs
	Motif	Rep	
Contig 6, 27, 189, 229	ag	4	isopiperitenone reductase
Contig34	ag	4	
Contig34	at	4	
Contig10-1	ag	6	protein PXR1
Contig10-2	ag	6	
Contig10-3	cag	4	
Contig11, 36	gcg	4	menthofuran synthase
Contig36, 350	ag	5	
Contig20-1	ct	6	membrane steroid-binding protein 2-like
Contig20-2	gag	4	
Contig23-1	tc	6	glyceraldehyde-3-phosphate dehydrogenase
Contig23-2	tc	4	
Contig25-1	gaa	4	limonene synthase
Contig132-1	gat	4	
Contig31-1	at	4	E3 ubiquitin-protein ligase RHF2A
Contig314-1	ct	4	
Contig314-2	ctg	4	
Contig37, 166	tc	5	Ferredoxin
Contig37, 166	ct	4	
Contig166-2	ca	5	
Contig54-1	ag	4	vascular protein 8
Contig54-2	ta	4	
Contig67-1	tc	4	cobalamine-independent methionine synthase
Contig299-1	tc	5	
Contig299-2	ag	4	
Contig71-1	ga	4	thioredoxin H-type
Contig71-2	ag	4	
Contig79-1	ca	5	phosphatidylglycerol/phosphatidylinositol transfer protein
Contig79-2	ct	6	
Contig89-1	ct	8	fructose-bisphosphate aldolase 3
Contig89-2	ca	4	glucose-6-phosphate dehydrogenase 5
Contig98-1	ga	5	glucose-6-phosphate dehydrogenase 5
Contig98-2	ta	4	
Contig103-1	at	4	ubiquitin-conjugating enzyme
Contig346-1	ga	4	
Contig105-1	at	4	limonene hydroxylase
Contig311-1	at	4	
Contig154-1	tc	4	aspartic protease in guard cell
Contig154-2	ca	4	
Contig154-3	ct	5	
Contig154-4	ct	4	
Contig154-5	tc	4	
Contig154-1	tc	4	2-alkenal reductase (NADP(+)-dependent)-like
Contig133-1	ac	4	2-alkenal reductase (NADP(+)-dependent)-like
Contig133-2	ct	4	
Contig219-1	ag	4	dehydrin 13 (Dhn13) gene
Contig219-2	ag	4	
Contig219-3	aga	4	
Contig219-4	cag	4	
Contig196-1	at	5	polygalacturonase non-catalytic subunit
Contig196-2	at	5	
Contig196-3	at	4	

Table 4: Continued

Contig143, 349	ga	4	6-phosphogluconate dehydrogenase
Contig349-1	ct	4	
Contig216-1	tg	4	RD22-like protein (rd22)
Contig216-2	at	4	
Contig235-1	tc	4	ran-binding protein 1 homolog c-like
Contig235-2	ag	4	
Contig235-3	ct	4	
Contig236-1	at	5	phytochrome-associated serine/threonine-protein phosphatase
Contig236-2	at	4	
Contig242-1	tc	4	protein FMP32
Contig242-2	at	4	
Contig247-1	tc	9	CBL-interacting serine/threonine-protein kinase 25
Contig247-2	ca	8	
Contig274-1	ta	4	gamma-terpinene synthase (TPS4)
Contig274-2	ta	4	
Contig327-1	ag	4	Rac-GTP binding protein
Contig327-2	gt	4	

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