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Identification of partial BAHD acyltransferases gene (CAAT) and its expression with the use of semi-quantitative and real time PCR in the tissues of citronella (*Cymbopogon winterianus*)

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Abstract: Citronella (*Cymbopogon winterianus*) is a medicinal and aromatic plant. Its medicinal value is due to presence of volatile esters citronellyl acetate and geranyl acetate, as constituents of essential oil in citronella is catalyzed by a single acyltransferase designated as CAAT that belongs to BAHD family of acyltransferases. In this study DFGWG motif was recorded which is highly conserved in BAHDs family. Two member genes (*CBL-AAT* and *CML-AAT*) of BAHD family of acyltransferases (AATs) specifically associated with secondary metabolism ester synthesis were identified. The interesting differential expression in different tissues/stages and response to treatments (high expression of *CBL-AAT* but not of *CML-AAT* in young leaf, suppression of *CBL-AAT* but over-expression of *CML-AAT* by mechanical injury, very substantial up-regulation of *CBL-AAT* in very young leaf but little/limited up-regulation of *CML-AAT*) of the two BAHDs suggested their differential physiological/metabolic role, hypothesized to be categoric as *CBL-AAT* for volatile esters (monoterpenoids and phenylropanoids) and *CML-AAT* for non-volatile secondary metabolites (anthocyanins etc.). The two genes, *CBL-AAT*, *CML-AAT* were identified and characterized from citronella leaf pertain to be the first report of any metabolic pathway gene from the *Cymbopogon* grasses in general and citronella (*Cymbopogon winterianus*) in particular.

Keywords: BADH, Alcohol acyltransferase, Conserve motif, Medicinal value, Semi-quantitative.

Introduction:

Citronella (*Cymbopogon winterianus*) is an aromatic grass of Poaceae family. The citronella is a good model for biochemical and molecular studies on volatile ester metabolism due to large accumulation of mono-terpenoids and their acetate esters in the leaves, stems and inflorescence of plant. Citronella accumulates considerable amount of geraniol, citronellol and corresponding acetate esters in the essential oil. Citronella plant has a high trade value in the cosmetics and perfumery market especially in the mosquitoes repelling creams and cakes [1].

Very few reports have been reported in these grasses pertaining to mono terpenoid and/or ester metabolism *e.g.*in *Cymbopogon martinii* [2]. Nonetheless, identification of alcohol acyltransferase (AAT) gene which is a member of BAHD super-family which involved in the ester biosynthesis has remained untouched so far. The expression of AAT gene in the citronella has pertained to down-stream pathway of acylation.

Esterification of volatile alcohols is often one of the major terminal metabolic transformation reactions in secondary metabolism. It turns a volatile alcoholic molecule (monoterpenoids, sesqueterpenoids, benzenoids, or phenylpropanoids) into a form of ester that possesses higher volatility. This metabolic transformation also brings about a modification in inherent odour property of the molecule [3]. It may also lead to altered polarity, chemical stability and biological activity [4]. Esters contribute significantly to the volatile emission from fruits during ripening [5], from flowers during pollination [6] and also from vegetative tissues/leaves, either constitutively or post injury. Recently, esterified volatile secondary metabolites have been reported from roots as well [7]. BAHD-AATS participating in the metabolic pathways related to the production of plant drugs/prodrugs like taxol, vindoline, morphine and ajmaline are examples of members producing non-volatile esters that serve as intermediary metabolites in their respective biosynthetic pathways [8].

BAHD super-family of proteins is characteristic in having two conserved motifs HXXXD and DFGWG, and identified by the property of wide substrate acceptability [9]. The catalytic reaction involves transfer an acyl moiety from a putative high energy donor such as acetyl coenzyme A or any acyl coenzyme A to the hydroxyl of an acceptor.

Till date cDNA sequences of volatile ester related BAHD-AATs that have been cloned and their enzymatic products characterized from fruits include banana, and strawberry, melon [10] and apple [11] etc. Representative examples from flowers include benzyl alcohol acetyltransferase (BEAT) and benzyl alcohol benzoyltransferase (BEBT) from *Clarkia breweri* and rose geraniol/citronellol AAT [12].

Tissue developmental stages (ontogeny) and environment have a profound effect on essential oil content and quality [13]. This effect is mediated by modulations in the expression of pathway genes particularly those related to regulatory steps of the pathway. Accordingly, the pathway may be considered in two sections- upstream steps represented by isoprenogebesis for monoterpenes (DOXP pathway) and downstream step represented by monoterpenol synthesis

and conjugation. The important catalytic enzymes of regulatory significance for DOXP pathway are DXS and DXR while those for the downstream pathway are monoterpenol synthases and acylation catalyzing AATs. Following paragraphs give a brief account (as case examples) of modulations in the levels of transcripts of these genes as a function of tissue ontogeny and environment.

In the literature it has been reported that one of the *Arabidopsis* AAT involved in the production of (Z)-3-hexen-1-yl acetate was highly facile to respond to mechanical damage. Similarly, the expression of the apple *AAT* gene (*MdAAT2*) was shown to be modulated by methyl jasmonate (MeJA), salicylic acid and ethylene after long-term cold storage [14]. MeJA and salicylic acid (SA) have been identified as vital cellular regulators that mediate diverse defense responses against biotic and abiotic stresses [15]. Sequence analysis of an apple *AAT* promoter has demonstrated that CAAT, CCAAT elements and several MYB transcription factor binding sites (CNGTT motif, AACCA, and CTAACCA elements) existed in the promoter region of MdAAT2 [16].

Thus, the studies on developmental and environmental effect on expression of volatile monoterpenoids biosynthetic genes are mostly related to floral tissues (fruits and flowers). There are very limited studies on vegetal tissues in this regard and almost negligible on vegetal tissues synthesizing abundant volatiles as essential oils. *AAT* gene of aromatic grasses have not been identified, cloned and characterized from any of the aromatic grasses including citronella. In view of the absence of knowledge on citronella with respect to biochemical aspects of dynamics of changes in essential oil composition with season and development, pathway enzymes and genes, this investigation was planned to generate knowledge on the plant with respect to certain domains of essential oil biosynthesis and modulation.

MATERIALS AND METHODS

The various oligonucleotides/primers used in the gene cloning and expression studies were designed manually or with using appropriate bio-informatics tools and approaches with focus on alignment and conserved motifs. The software's used included among others ClustalW2, primer 3, Oligo-analyzer of integrated DNA technology (IDT), Primer Express etc. Sequences of the target genes available in public domain databases like NCBI were collected, aligned and used for designing the degenerate primers. Primers were got synthesized from Integrated DNA Technology (IDT), India. The primers used for PCR amplification are tabulated (Table 1) below.

Table 1. List of primers used in the study of genes expression.

Primer name	Sequences (5'-3')	Size	Purpose
AATNF2a	GTITTYTAYTAYCCIYTNGCMGG	23 mer	*AAT* degenerate forward primer
AATRP3	NGSICGICCCCANCCRAARTC	21 mer	*AAT* degenerate reverse primer
ANCBATF	TCAATTACTTTAGCTGTGGTGGG	23 mer	*CBL-AAT* forward primer for semi-quantitative and real time study
ANCBATR	CGTGTTGGACGATCTAAATATGGC	24 mer	*CBL-AAT* reverse primer for semi-quantitative and real time study
ANCMATF	CGCACCTGAAGTGTAGTGGACTC	23 mer	*CML-AAT* forward primer for semi-quantitative and real time study
ANC6MATR3	CAAACACAAACCTCTTCACGACAG C	25 mer	*CML-AAT* reverse primer for semi-quantitative and real time study

Total RNA from citronella tissues including roots was isolated with TRI reagent (Sigma) [17]. In brief, 100 mg of tissue was ground to a fine powder in liquid N_2 and homogenized in 1ml of TRI reagent (guanidinium thiocyanate and phenol). The cell lysate was properly mixed by inverting or gently vortexing and incubated at room temperature for 10 min to permit the complete dissociation of nucleoprotein complexes. 200 µl of chloroform was added into cell lysate, mix gently and incubated at room temperature for 10 minutes. Thereafter, the samples were centrifuged at 12,000 x g for 20 min at 4ºC. Following centrifugation, the mixture got separated into lower red phenol-chloroform phase, an interphase, and colorless upper aqueous phase containing RNA. Aqueous phase was transferred carefully into fresh micro centrifuge tube and equal volume of isopropyl alcohol was added to precipitate RNA. The samples were incubated at room temperature for 10 minutes and centrifuged 12, 000 x g for 20 minutes at 4ºC. The supernatant was discarded and RNA was obtained as sediment. RNA pellet was washed with 500 µl of 75% ethanol by gentle vortexing followed by centrifugation at 7,500 x g for 5 min at 4 ^{o}C.

The washing procedure was repeated and the RNA pellet was air dried for 5-10 min. The RNA pellet was dissolved in 50µl RNase free sterile Milli-Q water. RNA concentration was determined by Nanodrop spectrophotometer as absorbance at 260 nm and RNA purity was determined by measuring the absorption ratio at 260/ 280 nm. RNA integrity was assessed by 0.8% agarose gel electrophoresis in 0.5X TAE [18].

First strand cDNA was synthesized from total RNA by RT-PCR using oligo dT as anchored primer. RevertAid™ first strand cDNA synthesis kit (Fermentas) with following of manufacturer's instructions. The reaction mixer was carried out in a Master cycler (Eppendorf) programmed as: 94 °C for 2 min for initial denaturation, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1.5 min. After completion of 30 cycles the final extension for 7 min was provided at 72 °C. The primary PCR reaction product (mix) was used for Semi-quantitaive PCR. The obtained DNA were resolved with agarose gel electrophoresis. The purified PCR product was ligated (cloned) into pJET1.2/blunt vector (Fermentas) following manufacturer's instructions. Vector: Insert = 100: (100 X size of insert/ size of vector) [19].

Chemically competent *E. coli* (DH5α cells) were prepared as described by Ausubel et al. (2003) with few modifications. For transformation**,** the stored competent cells were thawed on ice. 5 µl of the ligation mix was added to 100 µl of competent cells and the mixture was kept on ice for 30 min. To screen the recombinant colonies, colony PCR was performed. To prepare the template, individual colonies were picked by sterile toothpicks and cells were suspended in 5 µl of autoclaved Milli Q water. Plasmid from positive recombinant colonies was isolated following alkaline lysis miniprep method as described by Ausubel et al. (1994) with few modifications. To ascertain the quality, plasmid preparation was electrophoresed on 1.2% agarose gel. 10 µg of total plasmid was outsourced for sequencing by vector primers pJETF and pJETR [20].

From the sequence obtained, vector sequence was removed by Vecscreen tool hosted at NCBI site (http://www.ncbi.nlm.nih.gov) and these sequences were used to search homology at nucleotide and protein level in NCBI database using BLASTn and BLASTx algorithms. Semi-quantitative gene expression analysis was performed essentially as per the method describe by [21].

Quantitative real time-PCR (qRT-PCR), the gene specific primers were also used for quantitative profiling of the genes expression through real time PCR (RT-PCR) approach. The cDNAs was synthesized using 5 µg of total RNA from different test tissues (very young leaves, young leaves, mature leaves, senescent leaves, stem and roots) were used as a template for RT-PCR. All the cDNAs were diluted 10 fold by adding Milli Q water and used as working templates. The reactions were carried out on StepOne^{TM} real time PCR system with 48-well block module (Applied Biosystems). Relative quantification of gene transcript abundance was performed by comparative $\Delta\Delta$ C_T method (where relative quantity or RQ = $2^{-\Delta\Delta CT}$) [22].

Agarose gels (DNA/RNA) were documented with Syngene (G box) UV trans-illuminator system.

RESULTS AND DISCUSSIONS

Expression of genes encoding alcohol acyltransferase, in the different tissues of *C. winterianus* and their modulations were performed. Cloning of *C. winterianus* gene encoding BAHD family alcohol acyltransferases, and modulation of their expression as a function of developmental stages exposure to physiological/environmental signal molecules and mechanical injury (wounding).

Partial *AAT* genes were isolated from citronella leaf using degenerate primers (with internal conserved motifs as targets to design the degenerate primers) primed PCR using leaf tissue cDNA (prepared from total RNA) as template. The two predominant PCR products of expected size (900 bp and 750 bp) were obtained (Fig. 2). The PCR products on cloning sequencing and that on cloning and sequencing followed by BLASTx analysis of the nucleotide sequences revealed to resemble BAHD family acyltransferases. Their identity was as BAHD family alcohol acyltransferase (*AAT*). One of them (Fig. 3) showed closest sequence homology to a benzoyltransferase of the BAHD family whilst the other one was nearest in sequence similarity with a malonyltransferase of the family. The gene sequences were confirmed by designing the gene fragment specific primers and re-sequencing the PCR product for each of the BAHD-*AATs* of citronella leaf. These citronella *AATs* were referred to as citronella benzoyltransferase like *AAT* (*CBL-AAT*) and citronella malonyltransferase like *AAT* (*CML-AAT*).

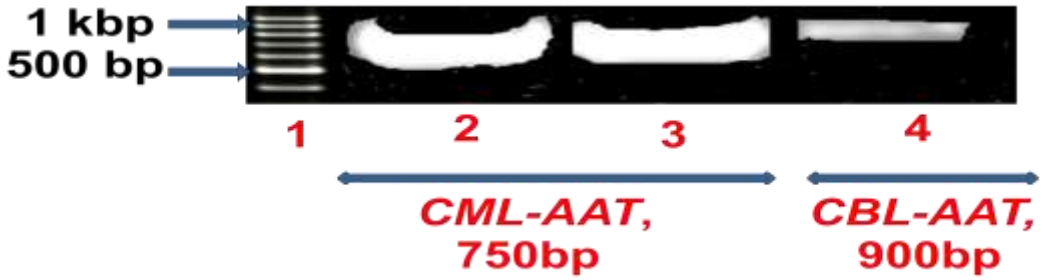

Fig.1, BAHD-AAT gene conserved motif specific degenerate primers based PCR amplification products obtained on using leaf cDNA as template. The PCR products and co-run molecular weight marker mixture were resolved on 1.2 % agarose gel. Lane 1, 1 kbp DNA size ladder (1 kbp); Lanes 2 and 3, amplified fragments of citronella leaf BAHD-AAT, *CML-AAT*; Lane 4, amplified fragments of citronella leaf BAHD-AAT, *CBL-AAT*.

Citronella benzoyltransferase like *AAT* (*CBL-AAT*), the partial cDNA (906 bp) sequence of *CBL-AAT*, its translated protein sequence and the multiple sequence alignment of amino acid sequence of the partial *CBL-AAT* fragment with those of several other BAHD-AATs are presented in Fig. 1.

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GTGTTTTATTATCCGCTAGCCGGACAAGTTAAAGATGGAGTCTTTGACTGTAATGACCGTGGTGCGATCT
TCATTGAAACTCAAGTAGTTGTAGATGAGATATCCAAGTTTGTTCAAAAAAGAGACAATAGTCTGTTTAA
GCAGCTATTGCCACAGGGGTAATATGCAAGAAATACTACTTATAGTAAAGCAAACCTAGTTCTGCAGGTC
AATTACTTTAGCTGTGGTGGGATAGCAATGACTGTTTGCTATAATCATATTCTTGTCGACGGAGCATCTG
CAGCTTACTTTGTCAAAAATTGGGCAGAAATTGCACATGGCGTCAATGATATAAAGGATGTGCTTGTTGA
TTATTCCTCCATCTGTATTCCACACGATGCCTATGCATTAATAAGAAGTCTTTACGAGCAAAATCGTTGG
GATTATAGTCCAACTGAACTTGTAGGAAAACATTTTATGTTTGATGGTGGAAAGATAGCTACTCTAAGAG
AAAAAATTGGTAGTGGGCCATATTTAGATCGTCCAACACGTTTTGAAGCTATAGCCGCACTTATTTCAGG
TGCGCTGATAAAGAATGCAGATAGGGGAACACATGAATTTGCTGTTAAACAAATAGCAGCAGCAATTGCT
ATCAATATGCGAAATATAGTGAATCCGTTATTGCCACCACAGAGTCTTGGAGCTATGGTGACGACAGCAT
TGCAAAATTGCCGATTAATGAAGAAATTGACTACAATTATTTAGCTGCAGAAGTTCACAAAGCTGTCAGA
ATGTGGTTGATCGGTATAAAAGAACATTCATGTTGGTGGTGCAATTGAAAGACTTGCAAACAGTATGGTG
ATTCATTTTTCTGATGTTTCTAAGATGCCATTTTACGAAGCCGATTTCGGGTGGGGCCGCGCCATC
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Fig. 2, Partial cDNA sequence of citronella benzoyltransferase like AAT (*CBL-AAT*) isolated and cloned from citronella (*C. winterianus*) leaf.

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YARNTTYSKANLVLQVNYFSCGGIAMTVCYNHILVDGASAAYFVKNWAEIAHGVNDIKDVLVDY
SSICIPHDAYALIRSLYEQNRWDYSPTELVGKHFMFDGGKIATLREKIGSGPYLDRPTRFEAIA
ALISGALIKNADRGTHEFAVKQIAAAIAINMRNIVNPLLPPQSLGAMVTTALQNCRLMKKLTTI
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Fig. 3, Amino acid sequence of the translated protein of the sequence of partial cDNA of citronella benzoyltransferase like AAT (*CBL-AAT*).

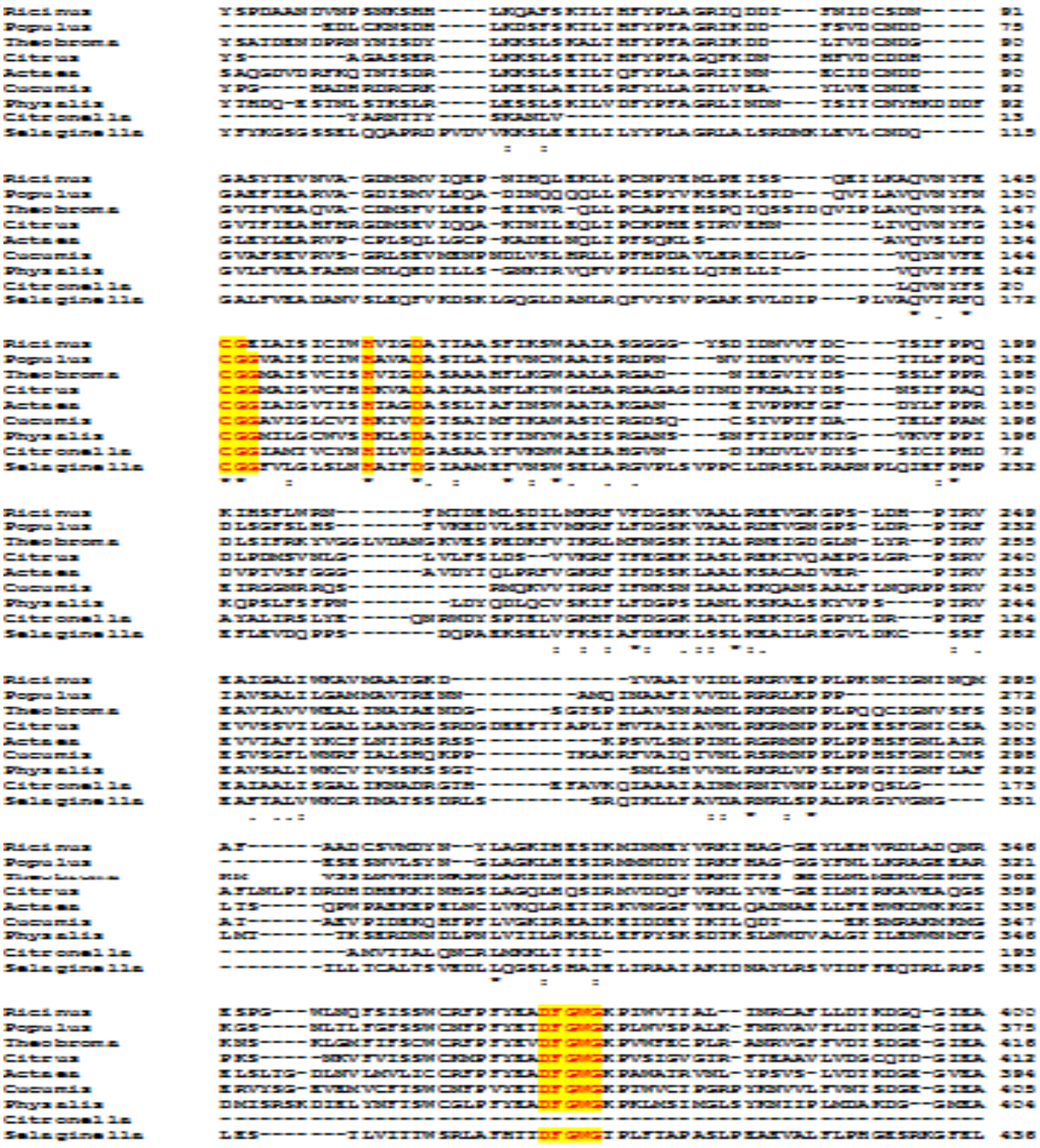

Fig. 4, Multiple sequence alignment (MSA) of the citronella benzoyltransferase like AAT (*CBL-AAT*) partial protein sequence with sequences of other known plants BAHD-AAT protein. Sequence alignment was performed using the CLUSTALW 1.8 program. The source DXR sequences are indicated on the left and the number/position of amino acid residues on the right. Conserved motifs of the protein are highlighted in red in yellow background.

Multiple sequences alignment results have revealed presence of HXXXD motif in the *CBL-AAT*. HXXXD is the most conserved motif of BAHD-AATs that is critical for the catalytic action of the enzyme [23]. In addition, a short conserved motif CGG typically present in BAHDs on N-terminal side in close vicinity to HXXXD motif was also located in the *CBL-AAT* [24]. The DFGWG motif or its homologue could not be localized in the *CBL-AAT* as the protein partial sequence fell short to reach the locale of this motif present towards C-terminal of this BAHD-AAT. The HXXXD of *CBL-AAT* was defined as HILVD. This is different from HKLYD form of HXXXD observed in the partial proteomics of citronellol acetyl coenzyme A acetyltransferase (CAAT) isolated and biochemically characterized from citronella leaf in this study. Therefore, it appeared to be another member amongst the BAHDs of the plant.

Citronella malonyltransferase like AAT (*CML-AAT*), the partial cDNA (750 bp) sequence of *CML-AAT*, its translated protein sequence and the multiple sequence alignment of amino acid sequence of the partial *CML-AAT* fragment with those of several other BAHD-AATs are presented,

AGCCAATTATCCGATTTGTCGGTTCAGACCACGCACCTGGAGTGTAGTGGACTCGCAATC
GCAATTGCGTTTCGCCATCGGATATTCGACCATCAATGGGGACGTTGGTCGCCGCCTGGT
CGAATGCTAGCAATCCCACGATCCAATACGGCTACATTATATCCCATCTTCAACTCCGAG
TCGCTGCTTCCCGCTAAAATTCAAAATGGAAGTCCTTATTCCCAAGTTGTACGCGAGACT
AGTCCAGCTGTCGTGAAGAGGTTTGTGTTTGACAAGGAAGCCATAGGCCGCCTTAGATCC
AAGTTAAGGCAAGGGATGATGTCGAGGGTGCGTGTGGTGTCTGCCTTTATAGCCAAGGCC
TTGATCAGGGCGGACGGGGCCAAGCAAGGTGGAGCGGCGAGACGTTGCTTTATCCTGCAA
TTGATTAACATGCGAGAGAGAACAATCCCACCTATGGCGAAGCATGCATGTGGAAACCTT
GTGATACGGTCGTGCACGGAGGCTAAGGACATTGGATTTCAGGAGCTGGCTGATCTGATG
AGCGAGGACGTTGACAGATCCATTTCCAAATGCAGTGAAATACTGTCTCTAGACGAGGAT
GGGCTTAACAAACTCATGGTGGAGTCGTTGATGGGGACAGTGACAGCATCACTGAGTGGT
GAAGCTAACCCCGTGTGGTTTACCGATTGGAGTAAGTTTGGATTCTACGGAGTCGATTTT
GGTTGGGGCCGCGCCATCTTG

Fig. 5, Partial cDNA sequence of citronella leaf malonyltransferase (*CML-AAT*) cloned from the leaf tissue of the aroma oil plant.

SQLSDLSVQTTHLECSGLAIAIAFRHRIFDHQWGRWSPPGRMLAIPRSNTATLYPIF
NSESLLPAKIQNGSPYSQVVRETSPAVVKRFVFDKEAIGRLRSKLRQGMMSRVRV
VSAFIAKALIRADGAKQGGAARRCFILQLINMRERTIPPMAKHACGNLVIRSCTEA
KDIGFQELADLMSEDVDRSISKCSEILSLDEDGLNKLMVESLMGTVTASLSGEAN
PVWFTDWSKFGFYGVDFGWGRAIL

Fig. 6, Translated amino acid sequence of *CML-AAT* gene fragment (750 bp) cloned from citronella leaf. Conserved motifs HXXXD, CSG in place of CCG and DFGWG are highlighted in the Figure.

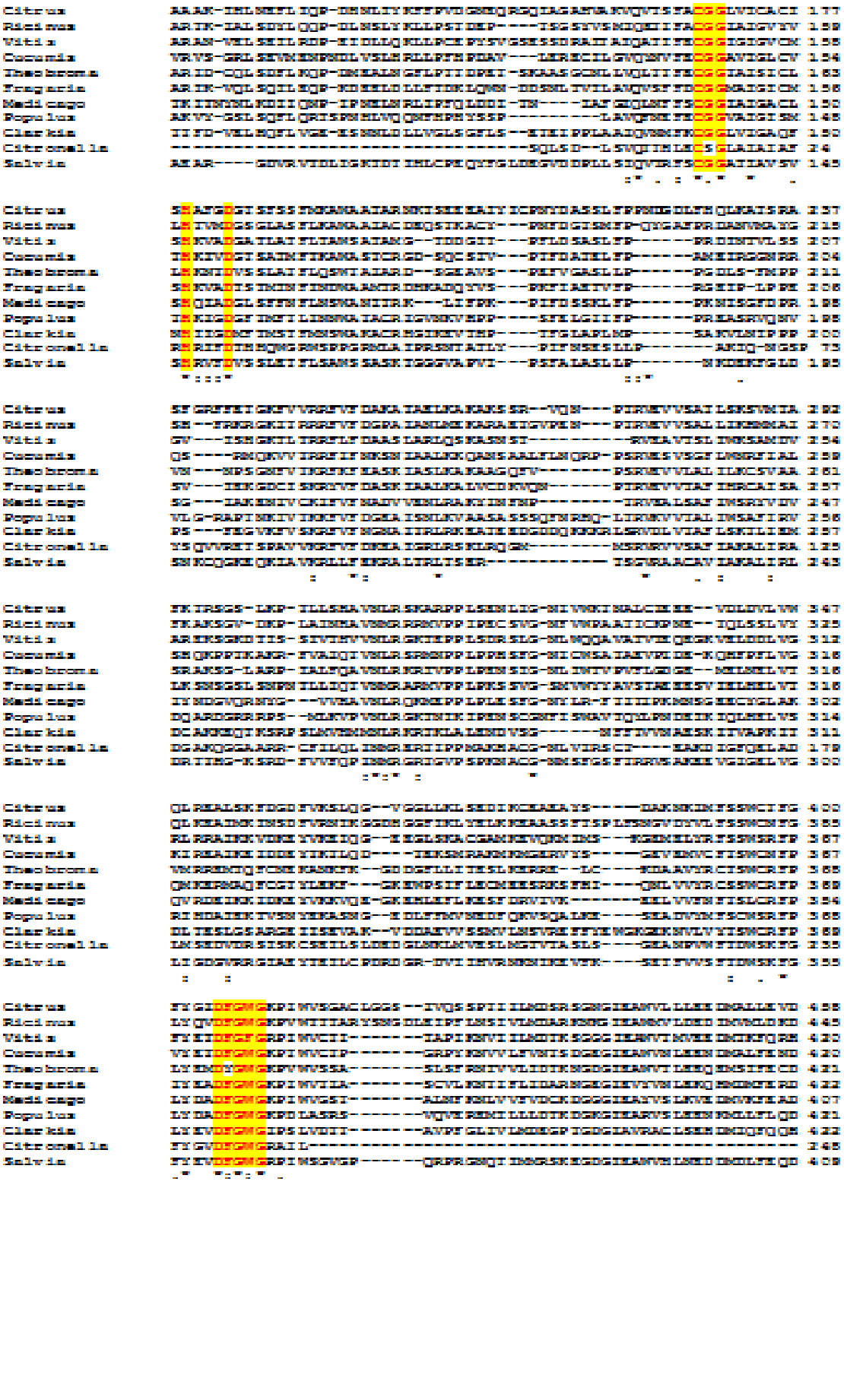

Fig. 7, Multiple sequence alignment (MSA) of citronella *CML-AAT* protein amino acid sequence with amino acid sequence of other BAHD-AATs of different substrate specificities. Conserved residues (HXXXD, CSG as a CGG and DFGWG) among the acyltransferases are shadowed in yellow and red colour. Asterisks indicate the positions of the conserved amino acids at active site/other regions of the plants BAHDs.

This Sub-section of results and discussion on dynamics of the key gene for downstream metabolic steps in the biogenesis of volatiles as a function of nature of tissue, leaf tissue ontogeny, exposure to salicylic acid, ethylene, mechanical injury etc. These sets of genes include BAHD-AATs (*CBL-AAT* and *CML-AAT*) as representatives of secondary metabolite ester synthesis pathway. The transcript abundance was estimated both semi-quantitatively (by PCR and agarose gel electrophoresis using gene specific primers) as well as quantitatively (through real-time PCR, qRT-PCT). The results are aimed to give an impression of regulatory factors of extrinsic and extrinsic nature in the relevant process of secondary metabolism of the plant.

Citronella benzoyltransferase (BAHD-AAT) like protein (*CBL-AAT*) gene:

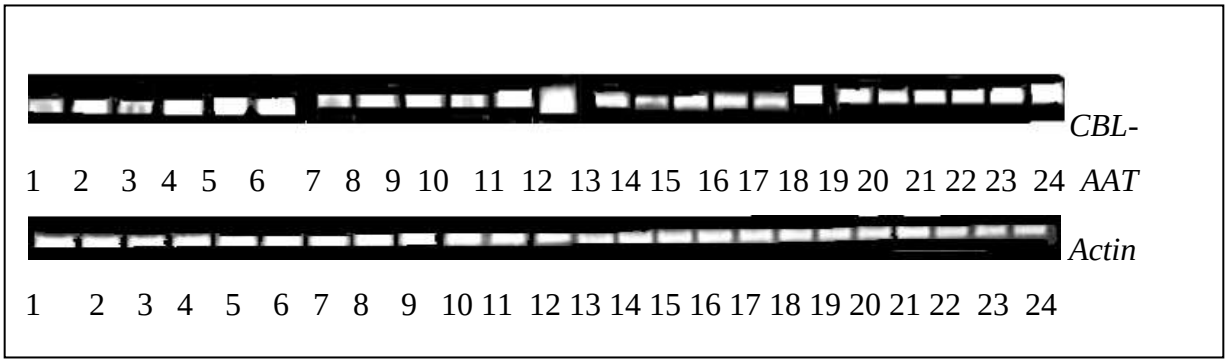

Fig. 8, Pattern of relative expression of *CBL-AAT* gene as analyzed by semi-quantitative PCR in different tissues/tissue stage and under different treatments (SA, mechanical injury and ethylene) in citronella (upper panel). Actin was used as an internal control (lower panel). 1, Control very young leaf; 2, control young leaf; 3, control mature leaf; 4, control old (senescent) leaf; 5, control stem (culm); 6, control root; 7, mechanical injury treated very young leaf; 8, mechanical injury treated young leaf; 9, mechanical injury treated mature leaf; 10, mechanical injury treated old (senescent) leaf; 11, mechanical injury treated stem (culm); 12, mechanical injury treated root; 13, ethylene treated very young leaf; 14, ethylene treated young leaf; 15, ethylene treated mature leaf; 16, ethylene treated old (senescent) leaf; 17, ethylene treated stem (culm); 18, ethylene treated root; 19, salicylic acid treated very young leaf; 20, salicylic acid treated young leaf; 21, salicylic acid treated mature leaf; 22, salicylic acid treated old (senescent) leaf; 23, salicylic acid treated stem (culm) and 24, salicylic acid treated root.

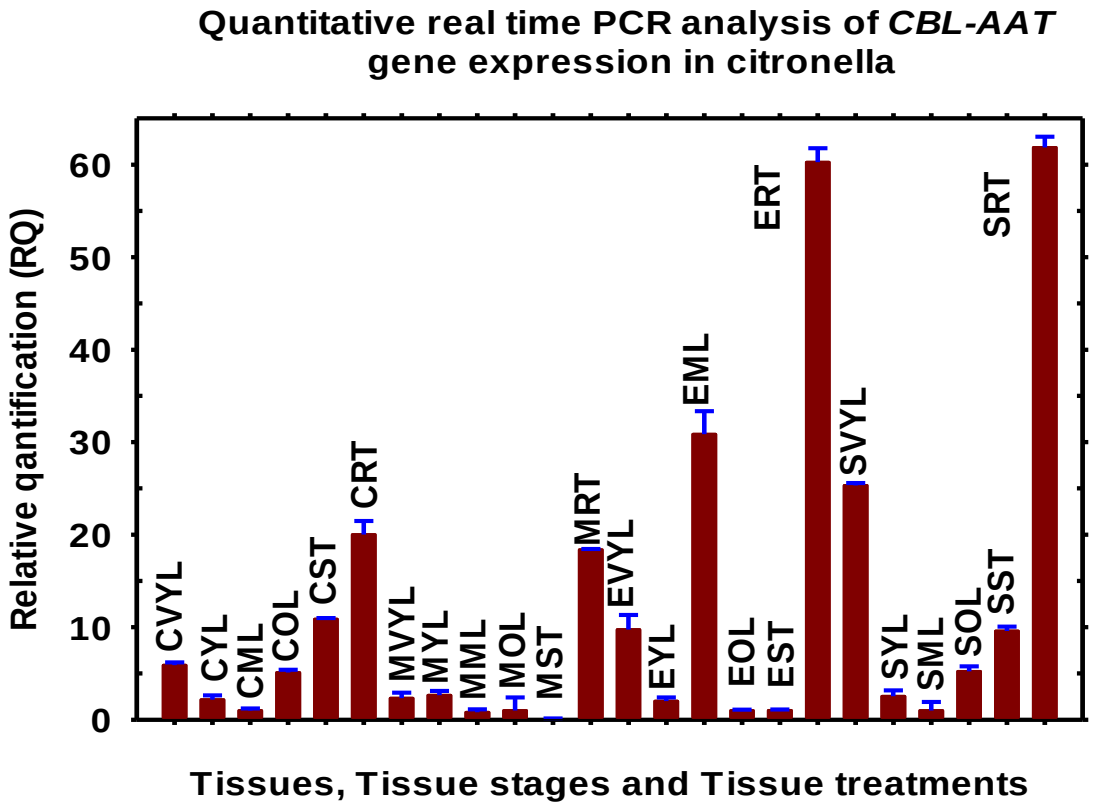

Fig. 9, Quantitative (qRT-PCR based) pattern of expression of benzoyltransferase (*CBL-AAT*) gene in the different tissues, different stages of leaf development of citronella and under mentioned treatments. CVYL, control very young leaf; CYL, control young leaf; CML, control mature leaf; COL, control old (senescent) leaf; CST, control stem (culm); CRT, control root; MVYL, mechanical injury treated very young leaf; MYL, mechanical injury treated young leaf; MML, mechanical injury treated mature leaf; MOL, mechanical injury treated old (senescent) leaf; MST, mechanical injury treated stem (culm); MRT, mechanical injury treated root; EVYL, ethylene treated very young leaf; CYL, ethylene treated young leaf; CML, ethylene treated mature leaf; COL, ethylene treated old (senescent) leaf; CST, ethylene treated stem (culm); CRT, ethylene treated root; SVYL, salicylic acid treated very young leaf; CYL, salicylic acid treated young leaf; CML, salicylic acid treated mature leaf; COL, salicylic acid treated old (senescent) leaf; CST, salicylic acid treated stem (culm) and CRT, salicylic acid treated root.

To investigate the pattern of expression of *CBL-AAT* gene and its modulation by external signals in *C. winterianus*, the real times as well as semi-quantitative PCR approaches were used using gene specific primers. The relative pattern of the gene expression is presented in Fig. 2 and Fig. 3. The results on relative level of expression of *CBL-AAT* revealed that the gene had a pattern of root>stem (culm)>leaf. Among the different stages of leaf development, it was maximal in very young leaf and minimal in mature leaf (Fig. 4). Its expression in leaf was not up-regulated at any

of its developmental stages by mechanical injury. However, its response to ethylene treatment, it was profoundly up-regulated in mature leaf and root (~3 fold increment) (Fig. 5). Further, the results on effect of salicylic acid treatment exhibited an interesting pattern- Salicylic acid treatment up-regulated the expression of the benzoyltransferase in very young leaf and root tissues (~3 fold increment) whilst it had no or negligible effect on the expression of the gene in the leaf tissues at other stages of development and stem (culm). Several fruit tissues have been shown to exhibit a very profound up-regulatory impact of ethylene treatment on expression of *AATs* [25]. In contrast to ethylene, effect, fruits have been shown to undergo a down regulation of the *AATs* on salicylic acid treatment. Whilst leaves of non-aromatic model plants like *Arabidopsis thaliana* (that emit volatiles only transiently under certain environmental insults like herbivory, high photo-irradiance etc.) are known to over express *AAT* transcripts, albeit as a slow rise response that peak during 3 to 6 h post-treatment [26]. A benzoyltransferase of *C. brew* has also shown to be expressed as an induction phenomenon after the damage to foliage [27].

Citronella malonyltransferase (BAHD-AAT) like protein (*CML-AAT*) gene, relative level of expression of the *CML-AAT* was *a priori*, assessed by semi-quantitative PCR analysis using gene specific primers as presented in Fig. 6. The level of expression of the malonyltransferase gene was observed to be down-regulated on mechanical injury to the tissue (Fig. 7) whereas the ethylene treated mature leaf and root were found to have over-expression of the *CML-AAT* gene. The gene expression was suppressed in all tissues at all the stages on treatment with salicylic acid.

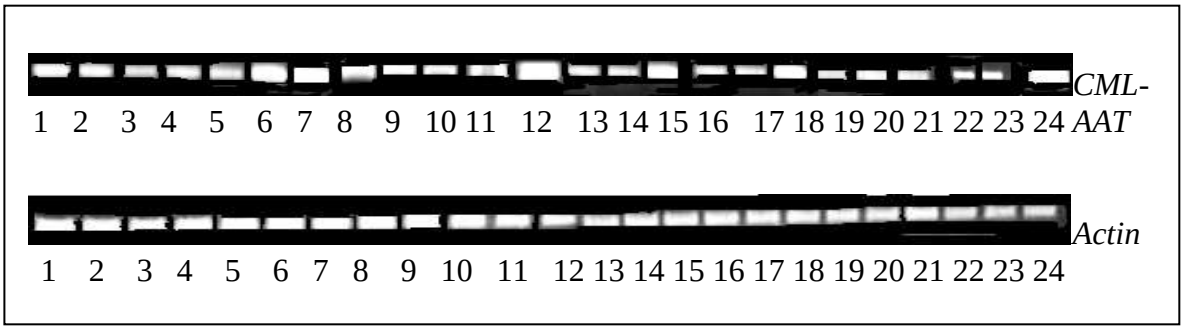

Fig. 9, Semi-quantitative expression analysis of *CML-AAT* gene of citronella in the different tissues, leaf developmental stages and in response to different elicitor treatments with defense signals such as salicylic acid, ethylene and on mechanical wounding (upper panel). Actin was used as an internal control (lower panel). 1, Control very young leaf; 2, control young leaf; 3, control mature leaf; 4, control old (senescent) leaf; 5, control stem (culm); 6, control root; 7, mechanical injury treated very young leaf; 8, mechanical injury treated young leaf; 9, mechanical injury treated mature leaf; 10, mechanical injury treated

old (senescent) leaf; 11, mechanical injury treated stem (culm); 12, mechanical injury treated root; 13, ethylene treated very young leaf; 14, ethylene treated young leaf; 15, ethylene treated mature leaf; 16, ethylene treated old (senescent) leaf; 17, ethylene treated stem (culm); 18, ethylene treated root; 19, salicylic acid treated very young leaf; 20, salicylic acid treated young leaf; 21, salicylic acid treated mature leaf; 22, salicylic acid treated old (senescent) leaf; 23, salicylic acid treated stem (culm) and 24, salicylic acid treated root.

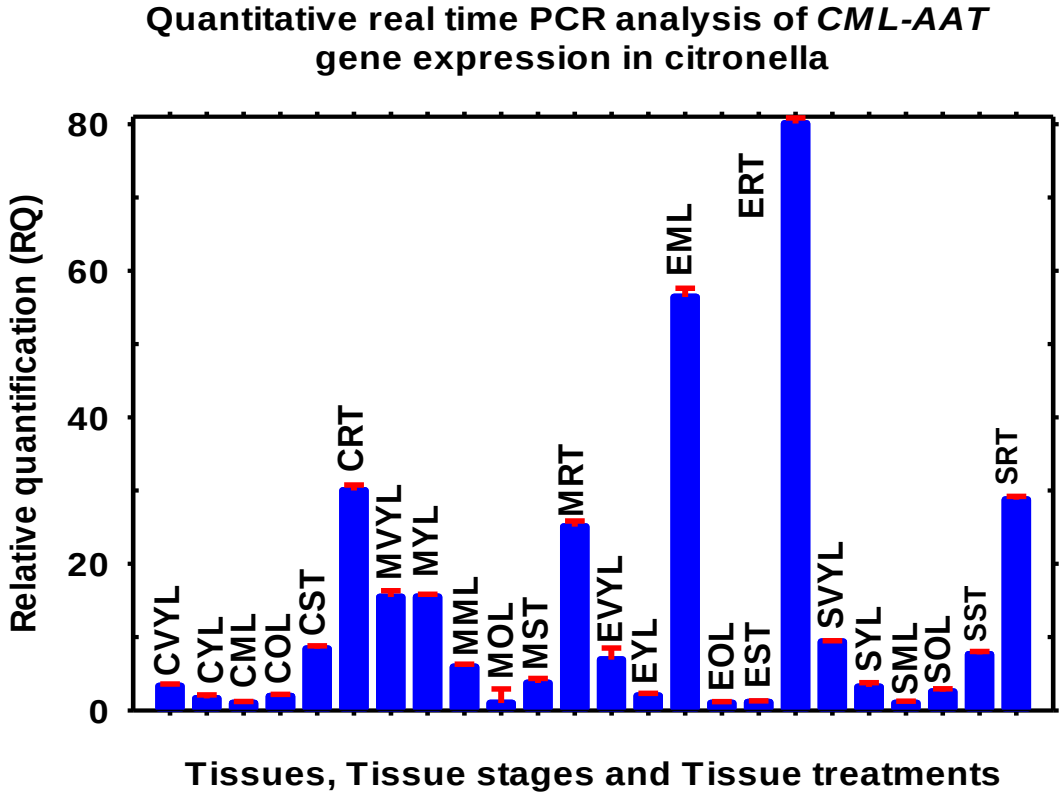

Fig. 10, Real time PCR (qRT-PCR) based quantitative analysis of transcript abundance of malonyltransferase (*CML-AAT*) gene citronella tissues, leaf developmental stages, and tissue exposure to salicylic acid, ethylene and mechanical injury. Error bars on the vertical bars represent standard error from three independent biological replicates. CVYL, control very young leaf; CYL, control young leaf; CML, control mature leaf; COL, control old (senescent) leaf; CST, control stem (culm); CRT, control root; MVYL, mechanical injury treated very young leaf; MYL, mechanical injury treated young leaf; MML, mechanical injury treated mature leaf; MOL, mechanical injury treated old (senescent) leaf; MST, mechanical injury treated stem (culm); MRT, mechanical injury treated root; EVYL, ethylene treated very young leaf; CYL, ethylene treated young leaf; CML, ethylene treated mature leaf; COL, ethylene treated old (senescent) leaf; CST, ethylene treated stem (culm); CRT, ethylene treated root; SVYL, salicylic acid treated very young leaf; CYL, salicylic acid treated young leaf; CML, salicylic acid treated mature leaf; COL, salicylic acid

treated old (senescent) leaf; CST, salicylic acid treated stem (culm) and CRT, salicylic acid treated root.

Comparison of the level of the malonyltransferase like protein (*CML-AAT*) transcripts in different tissues of the plant (leaf, culm, and root) revealed that transcripts of the gene were far more abundant in root tissue as compared to stem and leaf of the plant under normal conditions (Fig. 7). Amongst the leaf developmental stages, it was slightly higher in very young leaf as compared to other stages (Fig. 8). However, the variations were in too narrow range to emphasize any physiological significance of the phenomenon. Further, mechanical injury to very young leaf and young leaf had most profound inductive effect on expression of the gene in these tissues. The effect of mechanical injury in leaf tissues at other stages of development, and in root and stem was little or negligible (Fig. 9). The effect of ethylene treatment to these tissues was in contrast to that under mechanical injury i.e. expression of *CML-AAT* was profoundly up-regulated in mature leaf (~3 fold increment) and root (~2.5 fold increment), and down-regulated in stem (culm) while little effected in leaf at other stages of its development (Fig. 10). Salicylic acid treatment to the tissues led to hardly any change in transcript abundance of *CML-AAT* except a slight or marginal elevation in the expression of the gene in case of very young leaf [27].

Conclusion:

In conclusion, this Sub-section of the dissertation work dealing with studies on the focused secondary metabolism relevant gene isolation, their tissue/ontogeny-wide expression dynamics and modulation of their transcript levels has suggested that in case of monoterpenes aroma oil grass, citronella (*Cymbopogon winterianus*) (i) leaf, MEP pathway (upstream part of the biosynthetic process) regulatory genes like *DXS* and *DXR* as well as terminal steps (downstream part of the biosynthetic process) related genes such as *AATs* were most expressed at the very young stage, (ii) this pattern was in cohesion with the pattern of essential oil biogenesis/sequestration most actively associated with very young leaf stage, (iii) response of the pathway upstream genes (*DXS* and *DXR*) and downstream genes (*AATs*) to mechanical injury, ethylene and salicylic acid were qualitatively differential, (iv) the observed differential response of gene expression modulation was perceptible in terms of differential volatility/chemical properties of the products sequestered as first set (monoterpenes) and second set (acylated products) as well as their differential physiological significance, (v) certain interesting and characteristic differences of expression-response to above treatments were also observed even between the two *AATs* (*CML-AAT*) and *CBL-AAT*). They may mark certain specialized biochemical activities related a relevant in physiological process remaining un-suggestive at this stage. Future biochemical characterization of these as recombinant proteins and other molecular biological approaches may address such issues. This study and its results contribute substantially to generate knowledge around monoterpene metabolism (secondary metabolism) and help

understand physiological, biochemical and molecular aspects of biogenesis of essential oil and its monoterpene ester constituents in the aroma oil grass, citronella (*C. winterianus*).

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