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Biochemical characteristics of a novel vegetative tissue geraniol acetyltransferase from a monoterpene oil grass (Palmarosa, *Cymbopogon martinii* var. *Motia*) leaf☆

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ABSTRACT

Plants synthesize volatile alcohol esters on environmental insult or as metabolic induction during flower/fruit development. However, essential oil plants constitutively produce them as the oil constituents. Their synthesis is catalyzed by BAHD family enzymes called alcohol acyltransferases (AATs). However, no AAT has been characterized from plant foliage synthesizing acyclic monoterpenoids containing essential oils. Therefore, we have purified and biochemically characterized a geraniol: acetyl coenzyme A acetyltransferase (GAAT) from Palmarosa aroma grass (*Cymbopogon martinii*) leaf. MALDI-assisted proteomic study of the 43 kDa monomeric enzyme revealed its sequence motif novelties e.g. relaxed conservation at Phe and Trp in DFGWG'. This suggests permissiveness of variations in the conserved motif without loss of catalytic ability. Also, some new conserved/semi-conserved motifs of AATs were recognized. The GAAT k_{cat}/K_m values (300–700 M^{-1} s^{-1}) were low (a generic characteristic for secondary metabolism enzyme) but higher than those of some floral AATs. Wide substrate acceptability for catalyzing acetylation of diverse primary alcohols (chain of $\geq C_6$) implied its catalytic description as a 'primary aliphatic alcohol acetyltransferase'. It signifies metabolic ability to deliver diverse aroma esters, should the acceptor alcohols be available *in planta*. To our knowledge, this is the first report of detailed kinetics of a vegetal monoterpenol acyltransferase.

1. Introduction

Plants are known to synthesize a myriad of secondary metabolites or specialized phytochemicals. Their current count is ~200,000. 'Aromatic' compounds constitute an important group of secondary metabolites and mainly comprise of terpenoids, phenylpropanoids, indoles and sulfur compounds. Their property of volatility is used by the producer plant for efficient and specific communications with other individuals/organisms for attraction, co-operation, deterrence or defense [1,2]. Biogeneration of the aroma compounds involves diverse metabolic frameworks that have evolved for functional specificity at the levels of plant species, organs, tissues and cells, ontogeny, environment etc. Aroma alcohols pertaining to monoterpenoids and phenylpropanoids form the largest group of such compounds. Their individual fragrance is further specialized through metabolic transformation into esters of characteristic organoleptic notes [2]. For example, they contribute to the quality of aroma emitted from fruits during ripening [1,7] and flowers after bud opening [8]. In vegetative tissues, they are either constitutively synthesized and sequestered as in essential oil plants or are produced only on external stimulus (mechanical injury, herbivory, high irradiance) as a defense response in most of the plants [10,11]. The significant contribution of esters to 'aroma signatures' of plants or tissues rendered investigation of their biosynthesis as an attractive domain of secondary metabolism.

Production of volatiles and their esters can be visualized in diverse biogenic perspectives like (i) producer tissue-specificity [3] like floral (flowers and fruits) and vegetative (leaf, stem, root), (ii) inductive (produced under environmental cues like herbivory, damage, high irradiance etc.) or constitutive biosynthesis, (iii) ontogeny integrated biogenesis (like tissue developmental stage specificity such as opening of flower buds, onset of fruit ripening). Moreover, the volatiles may be synthesized and sequestered in surface oil glands (secretory trichomes) like epidermal secretory trichomes of mints [4] or 'deep-buried' parenchymatous oil cells [5] as in leaves of aromatic grasses (*Cymbopogon* species) and roots of vetiver (*Chrysopogon zizanioides*).

Esterification of alcohols is a major conjugative step in the terminal phase of biosynthetic pathway of volatiles. It leads to enhanced volatility, modified olfactory properties, altered polarity as well as biological activities [6]. It is catalyzed by alcohol

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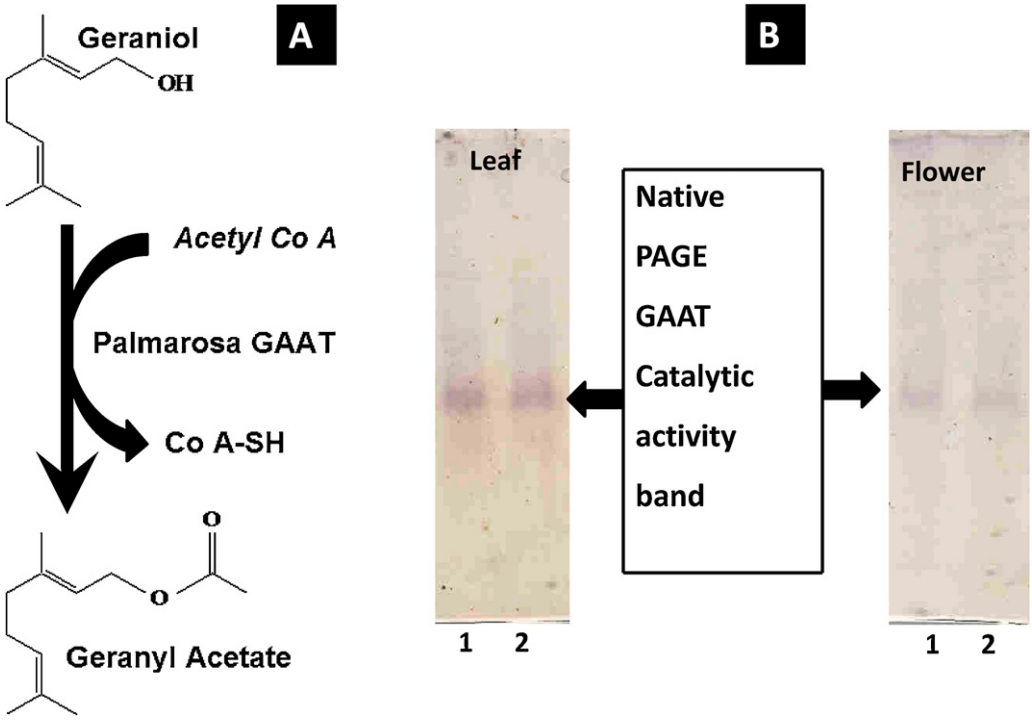

Fig. 1. (A) Metabolic reaction of esterification of geraniol into geranyl acetate by Palmarosa geraniol: acetyl Co A acetyltransferase (GAAT); (B) Native polyacrylamide gel electrophoresis (PAGE) of crude enzyme extracts of Palmarosa *(Cymbopogon martinii* var. *Motia*) leaf and flower, and in situ staining for geraniol: acetyl Co A acetyltransferase (GAAT) activity. Lanes 1 and 2 represent samples run in duplicate.

acyltransferases (AATs), the BAHD family of enzymes and involves transfer of an acyl moiety from a donor (such as acetyl coenzyme A) to a hydroxyl group of the acceptor alcohol. Although about 50 plant AATs are known [12], however, the set of characterized BAHD-AATs is predominated by those related to metabolism of non-volatile secondary metabolites like indole- and isoquinoline alkaloids, anthocyanins, taxoids and other higher terpenoids [13–16]. AATs related to volatile ester biosynthesis known so far are relatively few, whereas the number of pairs of alcohol (substrate) and its ester (product) known to occur in plant essential oils is very high. Furthermore, volatile esters related AATs that have been characterized so far are restricted to floral tissues (fruits and flowers), except two of them i.e. an AAT catalyzing synthesis of injury-inducible green leaf volatile ester (hexanyl acetate) in *Arabidopsis thaliana* [17] and an AAT catalyzing menthyl acetate formation in leaves of *M. piperita*, an aromatic plant producing cyclic monoterpenoid rich essential oil [18].

Although, there is typically very low sequence identity among members of BAHD super family but they share some fully conserved motifs [19,20] viz. HXXXD and DFGWG. Data from strawberry and apple AATs [1,21,22] have indubitably revealed that substrate specificities of AATs need biochemical illustrations rather than sequence based inferences. Furthermore, observed huge differences in kinetics of some native versus recombinant AATs are emphatic to suggest (a) need for screening *a priori* new sets of volatile ester-containing plants for their AATs, and (b) parallel relevance of biochemical characteristics of native enzymes to infer the data nearer to *in planta* functional situation.

Aroma oil grasses (*Cymbopogon* species, Poaceae) are cultivated for their monoterpenoid essential oils. These foliage essential oils are mainly comprised of acyclic monoterpenoids [8,23–27]. Among the grasses, Palmarosa (*Cymbopogon martinii* var. *Motia*) is one of the most important aroma oil cash crops. Its foliage and inflorescence essential oil finds extensive significance in flavor, fragrance and perfumery industries owing to its rose-note. The hydro-distilled oil (about 0.6% on fresh mass basis) contains geraniol and its ester (geranyl acetate). This acyclic monoterpenol and its ester (Fig. 1A) account for >90% of the oil and occur in the relative proportion of about 9:1, respectively. Biogenesis of these volatiles is characteristic in being (i) constitutive, (ii) abundant and cellularly sequestered, and (iii) vegetative tissue localized. Thus, it forms a discrete case of monocot/grass foliage for investigation of its essential oil ester (geranyl acetate) biosynthesis related AAT. Therefore, we have investigated kinetic and proteomic characteristics of the geraniol: acetyl coenzyme A acetyltransferase (GAAT) from this aroma grass leaf.

2. Materials and methods

2.1. Plant material and chemicals

Palmarosa (*C. martinii*, var. *Motia*, cultivar PRC1) plants were raised at the experimental farm of Central Institute of Medicinal and Aromatic Plants, Lucknow (India) following standard agronomic practices. The young (~50% expanded) leaves were sampled in a liquid nitrogen container and stored at −80 °C until used. For AAT isozyme detection in inflorescence, the half opened florets of the plant were sampled. All chemicals, biochemicals, substrates, were from Sigma–Aldrich–Fluka. Chromatographic matrices were purchased from Pharmacia/GE Health Sciences. Electrophoresis chemicals, reagents and supplies were from Bio-Rad. ^{14}C-acetyl coenzyme A for the radiometric assays was procured from GE Health Sciences.

2.2. Enzyme extraction and purification

2.2.1. Extraction and $(NH_4)_2SO_4$ fractionation

All operations of extraction and purification were carried out at 0–4 °C, unless stated otherwise. Young leaves (400 g) of palmarosa were powdered in liquid nitrogen in the presence of 5%