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Expression of a functional jasmonic acid carboxyl methyltransferase is negatively correlated with strawberry fruit development☆

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ABSTRACT

The volatile metabolite methyl jasmonate (MeJA) plays an important role in intra- and interplant communication and is involved in diverse biological processes. In this study, we report the cloning and functional characterization of a *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (*JMT*) from *Fragaria vesca* and *Fragaria* × *ananassa*. Biochemical assays and comprehensive transcript analyses showed that *JMT* has been erroneously annotated as gene fusion with a carboxyl methyltransferase (*CMT*) (gene15184) in the first published genome sequence of *F. vesca*. Recombinant FvJMT catalyzed the formation of MeJA with K_M value of 22.3 μM while FvCMT and the fusion protein were almost inactive. Activity of JMT with benzoic acid and salicylic acid as substrates was less than 1.5% of that with JA. Leucine at position 245, an amino acid missing in other JMT sequences is essential for activity of FvJMT. In accordance with MeJA levels, *JMT* transcript levels decreased steadily during strawberry fruit ripening, as did the expression levels of JA biosynthesis and regulatory genes. It appears that *CMT* has originated by a recent duplication of *JMT* and lost its enzymatic activity toward JA. In the newest version of the strawberry genome sequence (June 2014) *CMT* and *JMT* are annotated as separate genes in accordance with differential temporal and spatial expression patterns of both genes in Fragaria *sp*. In conclusion, MeJA, the inactive derivative of JA, is probably involved in early steps of fruit development by modulating the levels of the active plant hormone JA.

Introduction

The garden strawberry (*Fragaria* × *ananassa*) is cultivated worldwide for its attractive fruit. It is a hybrid species that originated approx. 250 years ago by an accidental cross between *Fragaria virginiana* with hermaphrodite flowers and tasty fruit, and *Fragaria chiloensis* with large fruit. *F.* × *ananassa* has a complex genome harboring eight sets of chromosomes ($2n = 8x = 56$) derived from at least four different diploid ancestors that makes functional analysis challenging. The diploid woodland strawberry (*Fragaria vesca*; $2n = 2x = 14$) has been chosen as a reference genomic system for Rosaceae in general and for *Fragaria* in particular because it has a rather small genome of approx. 240 Mb, is amenable to genetic manipulation and shares substantial sequence identity with *F.* × *ananassa* (Shulaev et al., 2011).

Unlike other Rosaceae family crops the strawberry is considered to be non-climacteric because the flesh does not ripen in response to the plant hormone ethylene but instead shows a lack of increased respiration as the fruit change color, texture and flavor. Besides, inhibitors of ethylene biosynthesis and of ethylene action fail to retard the onset of ripening (Manning, 1994). Nevertheless, strawberry can produce ethylene, although in limited amounts (Trainotti et al., 2005). However, indole acetic acid has a key role in strawberry fruit development (Veluthambi et al., 1985; Manning, 1994). The early growth of the receptacle depends upon a continuous supply

☆ Main conclusion: While jasmonic acid methyltransferase contributes to the inactivation of jasmonic acid during early stages of fruit development a second carboxyl methyltransferase probably functions in late stages of fruit ripening.

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of auxin from the seeds while the decline in auxin delivery during achene maturation triggers ripening. It has also been shown that abscisic acid (ABA) is a signal molecule that promotes strawberry fruit ripening as silencing of a key gene to ABA biosynthesis (9-*cis*-epoxycarotenoid dioxygenase gene) and of a putative ABA receptor gene resulted in uncolored fruits due to impaired anthocyanin biosynthesis (Jia et al., 2011).

Jasmonates are a class of plant hormone that regulate many aspects of plant development and growth including fruit ripening, root growth, production of viable pollen, and senescence (Avanci et al., 2010; Cheong and Choi, 2003). Jasmonic acid (JA) and its derivatives are cyclopentanone derivatives which are synthesized in the octadecanoid pathway from α-linolenic acid by a series of enzymes, starting with an oxygenation catalyzed by lipoxygenase (LOX). The primary product is then converted to 12-oxo-phytodienoic acid by allene oxide synthase (AOS) and allene oxide cyclase (AOC; Sasaki et al., 2001; Delker et al., 2006). AOS is the first specific enzyme and the major control point of JA biosynthesis (Krombrink, 2012; Wasternack and Hause, 2013). JA is finally produced through reduction by 12-oxo-phytodienoic acid reductase (OPR) and three steps of β-oxidation.

Currently, it is known that JAs-dependent responses require ubiquitin-mediated protein degradation (Devoto et al., 2002). Thus, it is assumed that COI1 (homologous to coronatine insensitive 1, an F-box protein part of the E3 ubiquitin ligase SCF complex) is a crucial factor in jasmonate signaling (Memelink, 2009) as COI1 controls the jasmonate-dependent degradation of one or more repressors of jasmonate responses. The latter belong to the JAZ protein family (Jasmonic Acid ZIM domain; Memelink, 2009). JAZ proteins are members of the larger TIFY family (due to the most conserved amino acid pattern TIF[F/Y]XG; Vanholme et al., 2007), which also includes PEAPOD proteins (PPD1 and PPD2), as well as TIFY8 protein (Vanholme et al., 2007; Pauwels et al., 2010).

JA levels increase rapidly in response to biotic and abiotic stresses such as wounding, insect attack, pathogen infection, and UV damage (Shan et al., 2007). Thus, the concentration of JA in plants varies as a function of tissue and cell type, developmental stage, and in response to several different environmental stimuli. High levels of JA are also found in flowers and pericarp tissues of developing reproductive structures (Wasternack et al., 2013). The function of JA accumulation in flower and fruit is unclear. It has been shown that JA can modulate fruit carotenoid composition and expression of genes encoding seed and vegetative storage proteins most likely through activation of ethylene forming enzymes (Czapski and Saniewski, 1992). In plants, JA is further catabolized to JA methyl ester (MeJA), *cis*-jasmone, cucurbic acid, 12-hydroxy-JA, JA-O-glucose-ester, and other conjugates (Delker et al., 2006; Sasaki et al., 2001). This raises the question whether JA and its metabolites exhibit separate biological activity or the derivatives are simply inactive metabolites.

MeJA has been identified in a limited number of fruit (Meyer et al., 1984). It is formed by jasmonic acid carboxyl methyltransferase (JMT) and probably acts as a cellular regulator of the levels of physiologically active JA, thus mediating diverse developmental processes (Seo et al., 2001; Wu et al., 2008). Considerable levels have been found in lemon peels (75 μg in the peel of one lemon; Nishida and Acree, 1984) but highest amounts occurred in immature fruits. In green, unripe strawberry fruit, levels of 38.3 $\mu g\,kg^{-1}$ JA (cv. *Senga sengana*) and 277.5 $\mu g\,kg^{-1}$ MeJA (cv. Kent) have been quantified (Gansser et al., 1997; Mukkun and Singh, 2009). Since the concentration of MeJA steadily decreased during fruit development to about 3.3 $\mu g\,kg^{-1}$ (Gansser et al., 1997) it was concluded that endogenous MeJA contributes to the initiation and modulation of developmental processes through adaptation of JA levels and could be related with cell division and growth at the beginning of fruit development.

Methyl esters of naturally occurring carboxylic acids serve important functions in plants. In addition to contribute to the flavor of fruits, they play a role as inactive derivatives of phytohormones, and can act as signals which activate disease resistance (Wu et al., 2008; Schieberle and Hoffmann, 1997; Avanci et al., 2010; Shulaev et al., 1997). Several enzymes, mainly from *A. thaliana*, have been functionally characterized that catalyze the transfer of methyl groups form S-adenosyl-L-methionine to low molecular weight acids (Ross et al., 1999; Murfitt et al., 2000; Seo et al., 2001; Zubieta et al., 2003; Qin et al., 2005; Yang et al., 2006; Zhao et al., 2007, 2009, 2013; Hippauf et al., 2010). Here, we describe the cloning of a full-length carboxyl methyltransferase gene from wild strawberry fruit (*FvJMT*) by a degenerate primer approach and the functional characterization of the encoded protein. Once the genome sequence of *F. vesca* became available (Shulaev et al., 2011) we recognized that *FvJMT* is fused to a second carboxyl methyltransferase gene without any interjacent stop codon. Thus, the major aim of the study was the elucidation of the relevance of the gene fusion by detailed gene expression analyses rather than the role of JA for fruit development. The newest version of the strawberry genome sequence (June 2014) confirm the outcome of the following experiments that *FvJMT* and the second carboxyl methyltransferase gene are two single genes and not fused to each other.

Materials and methods

Plant material and reagents

Fragaria vesca and *Fragaria* × *ananassa* (cv Elsanta) plants were grown in the greenhouses of the group Biotechnology of Natural Products in Freising, Germany whereas *Fragaria* × *ananassa* (cv. Camarosa) plants were cultivated in open fields in Malaga, Spain. All chemicals and solvents were obtained from Sigma, Fluka and Aldrich (all Taufkirchen, Germany), Carl Roth (Karlsruhe, Germany). Jasmonic acid, benzoic acid, salicylic acid was purchased from Sigma Aldrich (Steinheim, Germany). [Methyl-^{14}C]-S-adenosyl-L-methionine (SAM, 50 mCi/mmol; sulfuric acid pH 2.0/ethanol 9:1; 0.1 mCi/ml) was obtained from Biotrend, Köln, Germany. DNA modifying enzymes were obtained from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Frankfurt am Main, Germany). Primer synthesis and sequencing were performed by Microsynth AG (Switzerland). For isolation of DNA fragments from agarose gels the QIAEX® II Gel Extraction Kit (Promega, Mannheim, Germany) was used, whereas plasmid DNA was prepared with the Wizard® Plus SV Miniprep DNA Purification System (Promega, Mannheim, Germany).

Cloning of jasmonic acid carboxyl methyltransferase

Jasmonic acid carboxyl methyltransferase (*FvJMT*) full length coding sequence was generated by 3′- and 5′-RACE (Life technologies, Darmstadt, Germany), and specific end to end primers (JMT-fwEcoRI; JMT-revNotI; Table 1) were used, which enabled the amplification of the final cDNA clone via proof-reading RT-PCR. Carboxyl methyltransferase (*FvCMT*) was cloned with specific end to end primer (CMT-fwEcoRI, CMT-revNotI; Table 1). Furthermore, *FvCMT* and *FvJMT* were modified via end to end primer (CMT-fwEcoRI/CMT-revNcoI, JMT-fwNcoI/JMT-revNotI) with *NcoI* restrictions site for ligation to generate the fusion sequence (*FvFusion*). The PCR product was subcloned into the TA cloning system pGEM-T (pGEM®-T Vector System, Promega).

Molecular procedures

Total RNA was extracted from different plant tissues (root, stem, leaf, flowers, small green, green white, white, turning and red fruits)