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Isolation and characterization of germacrene A synthases gene in *Citrus unshiu* Marc

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ABSTRACT

CuSTS1 cDNA clone was isolated from Satsuma mandarin (*Citrus unshiu* Marc.) and functionally characterized. Genomic clone corresponding to *CuSTS1* consists of 7 exons and 6 introns which is typical structure of angiosperm sesquiterpene synthase genes. Their predicted proteins possess a general feature of plant sesquiterpene synthases and RR motif and DDXXD motifs were conserved. Phylogenetic tree analysis showed that the molecular evolutions of citrus germacrene synthase might occur according to enzymatic function before the divergence of *Citrus* species, in contrast to citrus monoterpene synthase genes. Functionally analysis indicated that *CuSTS1* protein produces mainly germacrene A from farnesyl diphosphate (FPP) in accompany with side product.

In Satsuma mandarin, transcription of *CuSTS1* were comparatively abundant in flowers and young fruit at 60 days after flowering (DAF), and peel at 120 DAF. The transcription was decreasing toward fruit maturing. In young fruit at 120 DAF, *CuSTS1* is induced by methyl jasmonic acid, salicylic acid and ethylene treatments, suggesting it would be involved in plant defense response.

1. Introduction

Monoterpenes and sesquiterpenes represent the C_{10} and C_{15} terpene classes and they play biological and ecological roles for the communication between plant–insect, plant–pathogen, and pollinator attraction (Pichersky and Gershenzon, 2002). *Citrus species* contain various volatile terpenoids of hemiterpenes, monoterpenes, and sesquiterpenes, including their derivatives, such as alcohols, esters, and acetates, and their components (Sawamura, 2000; Vekiari et al., 2002). In the volatile flavor components of Satsuma mandarin, 116 volatile components were identified by GC–MS analysis and limonene (82.8–89.2%) was the most abundant component, followed by γ-terpinene (4.2–5.1%) and myrcene (1.7%), while β-caryophyllene was the principal sesquiterpene hydrocarbon (Choi, 2004). This volatile data implicates that the existence of non-characterized monoterpene and sesquiterpene synthase genes that corresponds with numerous monoterpenes and sesquiterpenes in oil cells are expected. Recently, many cDNAs encoding monoterpene synthase genes and sesquiterpenes genes have been cloned and their gene expression and chemical function were characterized in citrus. For example, Lüecker et al., 2002 and Shimada et al. (2004, 2005) characterized limonene synthase and r-terpinene synthase genes, while Sharon-Asa et al. (2003) and Maruyama et al. (2001) characterized valencene and β-farnesene synthase genes. These monoterpene synthases and sesquiterpene synthases have common structural features of tandem arginine (RR) motif and DDXXD motif and most of them produce specific main product in accompany with significant amounts of by-products.

Sesquiterpenes are widely distributed in the plant kingdom derived from farnesyl diphosphate (FPP) and many biological activities of plant sesquiterpenes have been reported such as antifungal and anti-bacterial, attractive activity, anti-feedant activity to the insects, and phytoalexins (Picman, 1986; Dicke et al., 1990). In citrus, positive correlation between monoterpenes other than limonene and sesquiterpene content of the oils and the pathogen fungi inhibition was observed (Caccioni et al., 1998). This implicates that biological activity of limonene and sesquiterpenes would be directly concerning to their content in peel oils. In order to elucidate and utilize these biological activities through metabolic engineering of volatile terpenoids, it is important to isolate and characterize sesquiterpene synthase genes corresponding to numerous sesquiterpenes in oils of citrus peel. In this report, *CuSTS1* from Satsuma mandarin (*Citrus unshiu* MARC) was newly isolated and identified as a germacrene synthase A. The expression

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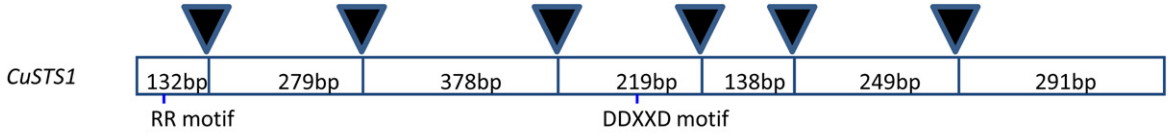
A)



B)

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(E)-beta-farnesene synthase   1:MKDMSI--PLLAAVSSSTEETVRPIADFHPTLWGNHFLKSAADVETIDAATQ-EQHAALK 57
CuSTS1                        1:MSLE-VSAPPAKVIQNAGKDSTRRSANYHPSIWGDHFLQYTCDTQETDDGSNVKHL-ELK 58
Valencene synthase            1:MS---------SG------E-TFRPTADFHPSLWRNHFLKGASDFKTVDHTATQERHEALK 45
                                    ..(RR motif)
(E)-beta-farnesene synthase  58:QEVRRMITTTANKLAQKLHMIDAVQRLGVAYHFEKEIEDELGKVSHDLD-SD------D- 109
CuSTS1                       59:KEIRRMLKADNKPSCT-LQLIDAIQRLGVSYHFESEIDEILGKMHKAYQDSDLCDNENDE 117
Valencene synthase           46:EEVRRMITDAEDKPVQKLRLIDEVQRLGVAYHFEKEIGDAIQKLCPIYIDSNRA----D- 100

(E)-beta-farnesene synthase 110:LYVVSLRFRLFRQQGVKISCDVFDKFKDDEGKFKESLINDIRGMLSLYEAAYLAIRGEDI 169
CuSTS1                      118:LYYISLHFRLLRQNGYKISADVFKKFKDRDGNFKTSLAKDVRGMLSLYEATHLGVHEEDI 177
Valencene synthase          101:LHTVSLHFRLLRQQGIKISCDVFEKFKDDEGRFKSSLINDVQGMLSLYEAAYMAVRGEHI 160

(E)-beta-farnesene synthase 170:LDEAIVFTTTHLKSVISISDHSHANSNLAEQIRHSLQIPLRKAAARLEARYFLDIYSRD- 228
CuSTS1                      178:LDEALAFTTSHLESIATHQIRSP----LVEQVKHALVQPIHRGLQRLEARQYIPIYQEES 233
Valencene synthase          161:LDEAIAFTTTHLKSLVAQD---HVTPKLAEQINHALYRPLRKTLPRLEARYFMSMINSTS 217

(E)-beta-farnesene synthase 229:D-LHDETLLKFAKLDFNILQAAHQKEASIMTRWWNDLGFPKKVPYARDRIIETYIWMLIG 287
CuSTS1                      234:PHNE--ALLTFAKLDFNKLQKLHQKELGDISRWWKELDFAHKLPFIRDRVAECYFWILGV 291
Valencene synthase          218:DHLCNKTLLNFAKLDFNILLELHKEELNELTKWWKDLDFTTKLPYARDRLVELYFWDLG- 276
                                                  .....(DDXXD motif)
(E)-beta-farnesene synthase 288:VSYEPNLAFGRIFASKVVCMITTIDDTFDAYGTFEELTLFTEAVTRWDIGLIDTLPEYMK 347
CuSTS1                      292:YFE-PQYSFARRILTKVISMTSVIDDIYDVYGKIEELELFTSAIERWDISAIDQLPEYMK 350
Valencene synthase          277:TYFEPQYAFGRKIMTQLNYILSIIDDTYDAYGTLEELSLFTEAVQRWNIEAVDMLPEYMK 336

(E)-beta-farnesene synthase 348:FIVKALLDIYREAEEELAKEGRSYGIPYAKQMMQELIILYFTEAKWLYKGYVPTFDEYKS 407
CuSTS1                      351:LCYRALLGVFSEAEKDLAPQGKSYRLYYAKEAMKNMVKNYFYEAKWCLQNYVPTVDEYMT 410
Valencene synthase          337:LIYRTLLDAFNEIEEDMAKQGRSHCVRYAKEENQKVIGAYSVQAKWFSEGYVPTIEEYMP 396

(E)-beta-farnesene synthase 408:VALRSIGLRTLAVA-SFVDLGDFIATKDNFECILKNAKSLKATETIGRLMDDIAGYKFEQ 466
CuSTS1                      411:VALVTSGSPMLSTTS-FVGMGDIVTKES-FEWLFSNPRFIRASSIVYRLMDDIVSHKFEQ 468
Valencene synthase          397:IALTSCAYTFV-ITNSFLGMGDFAT-KEVFEWISNNPKVVKAASVICRLMDDMQGHEFEQ 454

(E)-beta-farnesene synthase 467:KRGHNPSAVECYKNQHGVSEEEAVKELLL--EVANSWKDINEE-LLN-PTT-VPLPMLQR 521
CuSTS1                      469:SRGHVASSVECYIKQHGATEEEACNEFRKQ--VSNAWKDINEDCLR--PTVVPM-PLLMR 523
Valencene synthase          455:KRGHVASAIECYTKQHGVSKEEAIKM--FEEEVANAWKDINEE-LMMKP-TVVARPLLGT 510

(E)-beta-farnesene synthase 522:LLYFARSGHFIYDDGHDRYTHSLMMKRQVALLLTEPLAI-                    560
CuSTS1                      524:ILNLTRVIDVIYKYEDG-YTHSAVVLKDFVASLFINPVPI                    562
Valencene synthase          511:ILNLARAIDFIYKEDDG-YTHSYLIKDQIASVLGDHVPF-                    548
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Fig. 1. Exon–intron structure and deduced amino acid sequences of *CuSTS1*. The genomic clone of CuSTS1 consists of 7 exons and 6 introns. Citrus sesquiterpene synthases were aligned with the Genetyx-Win program. Accession number of each sesquiterpene synthase gene is as following, *CuSTS1*: AU186519, (E)-beta-farnescene synthase from *C. junos*: AAK54279, valencene synthase gene from *C. sinensis*: AAQ04608. The black background shading indicates identical amino acids. Dots are painted on the amino acids that were highly conserved between plant terpene synthases as RR motif and DDXXD motif.

of *CuSTS1* is induced by salicylic acid (SA), methyl jasmonate (MeJA) and ethylene and would be involved in plant defense response.

2. Materials and methods

2.1. Plant material and treatment

Satsuma mandarin (*Citrus unshiu* Marc.), cultivated at National Institute of Fruit Tree Science, Department of Citrus Research, Okitsu (Shizuoka, Japan) was used. Sample of flower, leaf, fruit tissues (juice sac and peel) at 60, 120 and 180 days after flowering (DAF) were collected and immediately frozen in liquid N_2 and then store at −80 °C for RNA isolation. Fruits at DAF 120 were treated by 100 μM salicylic acid, 100 μM methyl jasmonic acid and 10 ppm ethylene. The peels were excised from the fruits at 24 h after these treatments and immediately frozen in liquid nitrogen for RNA isolation. Genomic DNA was isolated from leaf by the method of Dellaporta et al. (1983). Total RNA was extracted by the methods of Ikoma et al. (1996).