Citrus R&T (e-ISSN 2236-3122)
Citrus Research & Technology, v. 38, n. 1, 2017
http://dx.doi.org/10.4322/crt.ICC084

Artigo/Article

Expression analysis of genes coding for pectolytic enzymes and a Mads-Box putatively involved in the maturation process of sweet orange cultivars

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SUMMARY

Since *Citrus sinensis* (L.) Osbeck cultivars have originated from bud sports, they show variability in terms of firmness, that is fruit turgidity and peel integrity. Common and navel orange fruits are generally characterized by high firmness and prolonged shelf life, while blood oranges usually produce softer fruits. The reduction of fruit firmness during maturation is due to pectolytic enzymes and the phenomenon is under the control of regulatory genes. In the present paper the expression analysis of pectinesterase/pectin methylesterase (PECS/PME) and polygalacturonase (PG) and the gene sequencing and transcription analysis of CsMADSSEP3-like genes is showed. Gene expression was performed on the flesh of early and late, common and blood sweet orange cultivars from mature to senescent stage. Among PECS/PME, only PECS2 expression increased during the maturation, similarly to the softness trend. The same behavior was also observed for the PG gene. An increased expression of CsMADSSEP3-like from mature to senescent period, more relevant in early cultivars than in late ones, was also presented, suggesting a probable involvement of this gene in the citrus fruit maturation. Moreover, considerations on CsMADSSEP3-like putative alternative transcripts on various tissues were also demonstrated. A coordinated activity of more genes controlling the complex mechanism of maturation could be the reason for the difference between early and late cultivars.

Index terms: *Citrus sinensis*, cell-wall genes, real time PCR, firmness, maturity, fruit ripening.

Análise de expressão de codificação de genes para enzimas pectolíticas e Mads-Box envolvidas no processo de maturação de variedades de laranja doce

RESUMO

Uma vez que as variedades de *Citrus sinensis* (L.) Osbeck se originaram de gemas, elas mostram variabilidade em termos de firmeza, ou seja, turgência de frutos e integridade da casca. As frutas de laranja comum e de umbigo, geralmente são caracterizadas por alta firmeza e vida útil prolongada, enquanto as laranjas sanguínea normalmente produzem frutos menos firmes. A redução da firmeza dos frutos durante a maturação é devida a enzimas pectolíticas e o fenômeno está sob controle de genes reguladores. No presente trabalho, mostra-se a análise de expressão da pectinesterase/pectina

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metilesterase (PECS/PME) e poligalacturonase (PG) e a sequência de genes e a análise da transcrição dos genes semelhantes a CsMADSSEP3. A expressão do gene foi realizada na polpa dos frutos das variedades de laranjeira precoce, tardia, comum e sanguínea, desde o estádio maduro até o senescente. Entre PECS/PME, apenas a expressão de PECS2 aumentou durante a maturação, de forma semelhante à tendência de suavidade. O mesmo comportamento também foi observado para o gene PG. Foi também apresentada uma maior expressão de CsMADSSEP3 do período maduro para o senescente, mais relevante nas cultivares precoces do que nas tardias, sugerindo um provável envolvimento desse gene na maturação dos citros. Além disso, também foram demonstradas considerações sobre transcritos putativos alternativos CsMADSSEP3 em vários tecidos. Uma atividade coordenada de mais genes que controlam o mecanismo complexo de maturação pode ser o motivo da diferença entre as variedades precoce e tardia.

Termos de indexação: *Citrus sinensis*, genes de parede celular, PCR em tempo real, firmeza, maturação, amadurecimento dos frutos.

INTRODUCTION

Fruit ripening is a highly genetically programmed and irreversible phenomenon that involves physiological and biochemical events, as metabolisms concerning sugar, citric acid, cell-wall, pigments and flavour. There are also organoleptic changes, leading to the development of a soft, edible fruit (Prasanna et al., 2007). These events are correlated with increased susceptibility to physical damage and diseases, influencing several commercial traits, as shelf-life and transport capability and, as consequence, the consumer choice. Texture/structure changes during ripening of fruits and vegetables involve the dismantling of multiple polysaccharide networks by diverse families of cell-wall-modifying proteins, including enzymes for pectin and cellulose catabolism (Wu et al., 2014b).

Hydrolytic enzymes involved in the degradation of the cell-wall include pectate lyases (PLs), polygalacturonases (PGs) and pectin methylesterases, called also pectinesterases (PMEs/PECSs) (Brummell & Harpster, 2001). Pectins are the most abundant class of macro-molecules of the cell-wall involved in regulating intercellular adhesion (Castillejo et al., 2004). During fruit softening, pectins typically undergo solubilization and depolymerization, contributing to wall loosening and disintegration (Fischer & Bennett, 1991).

The softness process was extensively studied through genetic and enzymatic approaches in various fruits and vegetables, as citrus (Christensen et al., 1998; Laratta et al., 2008; Nairn et al., 1998), kiwi (Soda et al., 1986), avocado (Zauberman & Schiffmann-Nadel, 1972), strawberry (Castillejo et al., 2004), pear (Fonseca et al., 2004), grape (Deytieux-Belleau et al., 2008), melon (Hadfield et al., 1998), papaya (Fabi et al., 2014), tomato (Eriksson et al., 2004), carrot (Markovic et al., 2002) and sweet pepper (Ahmed et al., 2011). These data showed a relationship between expression and fruit softening, even though none information is available about the link with anthocyanin pigmentation. Pectin acidification, performed by PECSs, is an obligatory step before further degradation by either PGs or PLs, and consequently, firmness loss and decrease in viscosity (Castaldo et al., 1997; Laratta et al., 1995).

In the transcription factor networks affecting fruit ripening, MADS box family serve as key regulatory genes (Gapper et al., 2013; Smaczniak et al., 2012). In plants, MADS-box genes regulate the development of different organs, such as flower, ovule, fruit, leaf and root (Ng & Yanofsky, 2001). Recently, thanks to the mandarin (*Citrus reticulata*) and sweet orange (*C. sinensis*) genomes (Gmitter et al., 2012; Xu et al., 2013; Wu et al., 2014a), a general characterization of MADS-box gene family was performed (Hou et al., 2014), even if none information on maturity process was advanced.

Firmness and maturity ratio are of extreme importance for breeders, because they are key parameters for consumer acceptance. In fact as firmness ensures a longer shelf-life, maturity ratio, determining a good balance between sugar and acidity content, characterizes the fruit taste. Based on these considerations, the intention of citrus breeding consists not only in the release of new accessions to protract the availability of fruits all the year, but also to extend shelf-life and to ensure a good maturity ratio.

The availability of numerous citrus mutations, which are different, among other things, for maturity period (early, medium, late), presence/absence of anthocyanin in the flesh and firmness, gives the opportunity to investigate on the possible association between fruit firmness and genes related to cell-wall metabolism and regulation. In the present paper, the expression analysis of three PECS isoforms/genes and a PG gene was performed on four *C. sinensis* (L.) Osbeck cultivars differing for fruit softness, maturity time and presence/absence of

anthocyanins during three stages following maturation (from mature to senescent).

The relation among physical resistance parameters, as index of firmness, the maturity ratio and gene expression were considered. Moreover, the expression pattern of a member of the MADS-box gene family and its possible alternative transcripts was studied, supposing the isolation of a gene putatively involved in the control of citrus maturity time.

MATERIAL AND METHODS

Common (Ovale), Navel (Newhall) and pigmented (Tapi and Meli Tarocco clones) sweet oranges cultivars were harvested at the experimental orchard of CREA - ACM, located at Palazzelli (Lentini – SR, Italy). Fruits of early (Newhall and Tapi) and late (Ovale and Meli) cultivars were collected at three stages of ripening (Early I = December 4th, 2007; Early II = January 17th, 2008; Early III = March 5*th*, 2008; Late I = March 5th, 2008; Late II = March 31th, 2008; Late III = May 6th, 2008). Juice from about 10 fruits of three different plants for each sample was collected and stored at -80°C until RNA extraction. Total acidity (TA) was determined by the titration of 5 ml of juice using an automatic titrator (Titrex Steroglass srl, Italy). Total soluble solids (TSS) were evaluated as described in Kimball (1999). Fruit firmness was determined by measuring penetration force using a penetrometer (Zwick/Roell DO-FBO.STS model 2002).

Sequence and phylogenetic analysis of PECS and PG genes

Nucleotide sequences of Citrus coding for 'pectinesterase' and 'pectin methylesterase' were retrieved from the NCBI database [PECS1.1 (U82973.1), PECS1.2 (U82974.1), PME (DQ458770.1), PECS2.1 (U82977.1) and thermostable PME4 (AY040711.1)]. The corresponding gene sequences were retrieved from Orange Genome Annotation Project (Center for Bioinformatics, 2017) (Cs1g16550 corresponds to PECS1.1, PECS1.2 and PME; Cs4g06630.1 corresponds to PME4) and Phytozome (2017); orange1.1g010441 corresponds to PECS2.1

A complete cDNA, encoding a *C. sinensis* PG, was identified in the Genbank database (EF185420.1) and corresponding to Cs1g12840 in the sweet orange genome (Center for Bioinformatics, 2017).

Total RNA extraction and expression analysis were conducted according to Butelli et al. (2012). The primer set is listed in Table 1.

The corresponding protein sequences of PECS/PME, as well as PG, of sweet orange were separately compared to other plant species (retrieved by NCBI database), aligned using a ClustalX2 software and the phylogenetic tree was deduced using MEGA6 software (Tamura et al., 2013).

5' RACE-PCR, sequences and phylogenetic analysis of CsMADSSEP3-like gene

The EST (EG358383) coding for a MADS-box gene isolated and described in Licciardello et al. (2008) was lengthen using the 5' RACE PCR amplification, to obtain the full length cDNA. Two gene-specific primers were designed (MADS-74 rev 5'-ATCGGCGTGCCTTAGCGTCATCGTCCTT-3' and MADS-197 rev_nested 5'-AAGGCGTGAAAGA AGGTATCGCCCTGAGGT-3') to follow the experimental procedures of the SMARTer RACE cDNA Amplification kit (Clontech). The PCR amplicons were cloned into pGEM-T Easy vector, sequenced, submitted on NCBI (CsMADSSEP3-like Accession number KR136378). For the phylogenetic analysis was used the same approach of PECS/PME and PG.

Moreover, the Gbrowser showed the existence of five alternative transcripts (Cs7g10980.1, Cs7g10980.2, Cs7g10980.3, Cs7g10980.4, Cs7g10980.5). Each translated sequence was aligned to CsMADSSEP3-like using the ClustalX2 software. The CsMADSSEP3-like oligos were drawed to specifically amplified each alternative transcript. The primer set is listed in Table 1. To discriminate the existence of MADS-box predicted alternative transcripts, PCR experiments were conducted on cDNA of leaves, ovaries and juice sacs. The amplification mixture was performed as previously described (Licciardello et al., 2014).

RESULTS AND DISCUSSION

Relation between firmness and ripening

The goal of the present paper consisted in the analysis and the clarification of genes coding for pectolytic enzymes, known to be involved in the fruit firmness/softness. In Citrus, two quality parameters strictly related to ripening are fruits firmness and maturity index, determined as ratio between TSS and TA. The harvesting period of a variety is linked

to maturity ratio, but also depend on the break-strength involved in the fruit drop and firmness. This association is important to have a global evaluation of ripening period of each variety. In our experiment (Table 2), Newhall reached an optimum maturity index already in the first sampling (10.7), while the lower TSS/TA of Tapi (early as Newhall) is typical of blood oranges (8.3) in comparison with navel ones (Starrantino et al., 1999). Also fruits of late cultivars (Ovale and Meli) were considered mature already in the first sampling (9.7 and 8.4, respectively). This is because the specific collocation into late group depends not only from maturity-ratio but also by the break-strength and firmness. The highest value of firmness is registered for Ovale (629.1 ± 28.1 Newton), which is known to be characterized by very hard fruits. Ovale is a chimeric mutation of Biondo comune (a typical not pigmented sweet orange) and in the past, it was an important late-ripening cultivar in Italy (La Rosa 2012).

Sequence and expression analysis of PECS/PME isoforms in early and late sweet orange varieties during the ripening

From a genetic point of view, we considered six sequences of Citrus coding for PECS/PME. The phylogenetic tree, including also PECS/PME genes of other plants, known to be involved in the control of ripening, showed

Table 1. Characteristics of primers used in the expression analysis

Name	Sequence primer (5'-3')	Experiment	Lenght amplicon (bp)
PECS1(II)-Fw	ACAGCTGCTGTTGTTGGTGA	Real time PCR	69
PECS1(II)-Rev	TGAGGGGCCTGCTGTGTTTTGGA		
PECS2(I)-Fw	GATGGGATTGGCAAGACTA	Real time PCR	92
PECS2(I)-Rev	TCTCCAACAACAGCAACAGT		
PME4(I)-Fw	TGGAGATGGGAGGACTACTACAA	Real time PCR	89
PME4(I)-Rev	CCACAGCAACAGTTGCAGAGTTGA		
PG(II)-Fw	AAGTGATTGCACCTGCTGAAAG	Real time PCR	64
PG(II)-Rev	ACACCACGAGATGCACTGATG		
MADS.SSH 5'UTR Fw	GGAGAAGAAAATGGGAAGGGGTA	Real time PCR	93
MADS.SSH IIES Rev	CCTCCCTTGCGGACACATTCGGTT		
MADS IVES Fw	GGAGAAGAACTTGGCCCTCTAAA	Real time PCR	89
MADS_FL_RT_rev2	AGAAGGTATCGCCCTGAGGT		
MADS.5 VES Fw	CTGCAACATAAGTTGCTGAGCGAA	RT-PCR	268
MADS_FL_RT_rev2	AGAAGGTATCGCCCTGAGGT		
MADS_FL_RT.4_fw	GACCCTCAAACAAAGGTTGATGGAG	RT-PCR	367
MADS_FL_RT_rev2	AGAAGGTATCGCCCTGAGGT		408
MADS_FL-Fw1	TTTGCTGGTTTGCAGTTGAT	RT-PCR	243
MADS_FL_RT_rev2	AGAAGGTATCGCCCTGAGGT		282
EF-344.Fw	AAGCTGGTATCTCCAAGGATGGT	Real time PCR	72
EF-404Rev	CCAAGGGTGAAAGCAAGCAA		

Table 2. Characteristics of sweet orange fruits discussed in the manuscript regarding total anthocyanins, maturity ratio and firmness related to the three ripening stages. 'E' is for early, 'L' is for late

Variety	Total anthocyanins (mg L^{-1})			Maturity ratio (TSS/TA)			Firmness (Newton)		
	I	II	III	I	II	III	I	II	III
Newhall (E)	/	/	/	10.7	15.1	33.3	17.5 ± 9.0	30.0 ± 6.5	44.4 ± 3.4
Tapi (E)	2.7	50.2	58.6	8.3	9.4	13.8	22.5 ± 15.0	14.7 ± 5.8	23.5 ± 0.2
Ovale (L)	/	/	/	9.7	9.4	10.7	629.1 ± 28.1	50.7 ± 3.8	49.7 ± 1.4
Meli (L)	0.8	25.4	30.3	8.4	8.8	9.2	163.4 ± 42.6	16.7 ± 2.5	20.8 ± 2.6

the separation of the sequences into two main groups (Figure 1a).

As expected, PECS1 and PECS2 are genetically separated. It is consistent with the previous report in which isoforms one and two are considered genetically distant (Tiznado-Hernández et al., 2004). Isoform 1 (PECS1), together with the thermostable PME4, is included in the group II with PMEU1 of tomato and PE3 of strawberry, which function was reported to be involved on firmness or generally in maintaining of cell wall integrity throughout the fruit development (Micheli et al., 1998). The isoform 2 (PECS2) belong to the group I together with PE4 of strawberry, which expression increased during the ripening and notably involved in the softening of strawberry (Castillejo et al., 2004).

It is also known that PE4 is not specifically expressed only in the fruit tissues. In sweet orange the PECS2 flesh-specificity, ascertained after an evaluation on various tissues (leaves, ovary, albedo, peel and flesh on a typical pigmented and a common variety), was also evaluated, together with PECS1 and PME4 on Newhall, Tapi, Ovale and Meli cultivars in mature, edible and senescent fruits. PECS2 expression data showed a regular increasing along samplings in all cultivars, except for Ovale, which expression of sampling II was higher compared to the last one (Figure 2).

The increased trend of PECS2 is also a confirmation of data previously reported (Licciardello et al., 2008), even if no difference in terms of expression between pigmented (Tapi and Meli) and not pigmented (Newhall and Ovale) cultivars was observed. The only differences are related to high expression values of early cultivars, compared to lower values in late ones. Based on Real time PCR expression analysis, PECS1 increased during samplings in early cultivars, while the expression resulted down-regulated in late ones (Figure 2).

Considering the decreasing trend of firmness observed during the maturation, the relation with PECS1 expression is different between early and late cultivars. Similarly, the expression of PME4 (Figure 2) increased specifically in early cultivars, while the trend is different in late ones (up in Meli, down in Ovale). Differences are not only evident

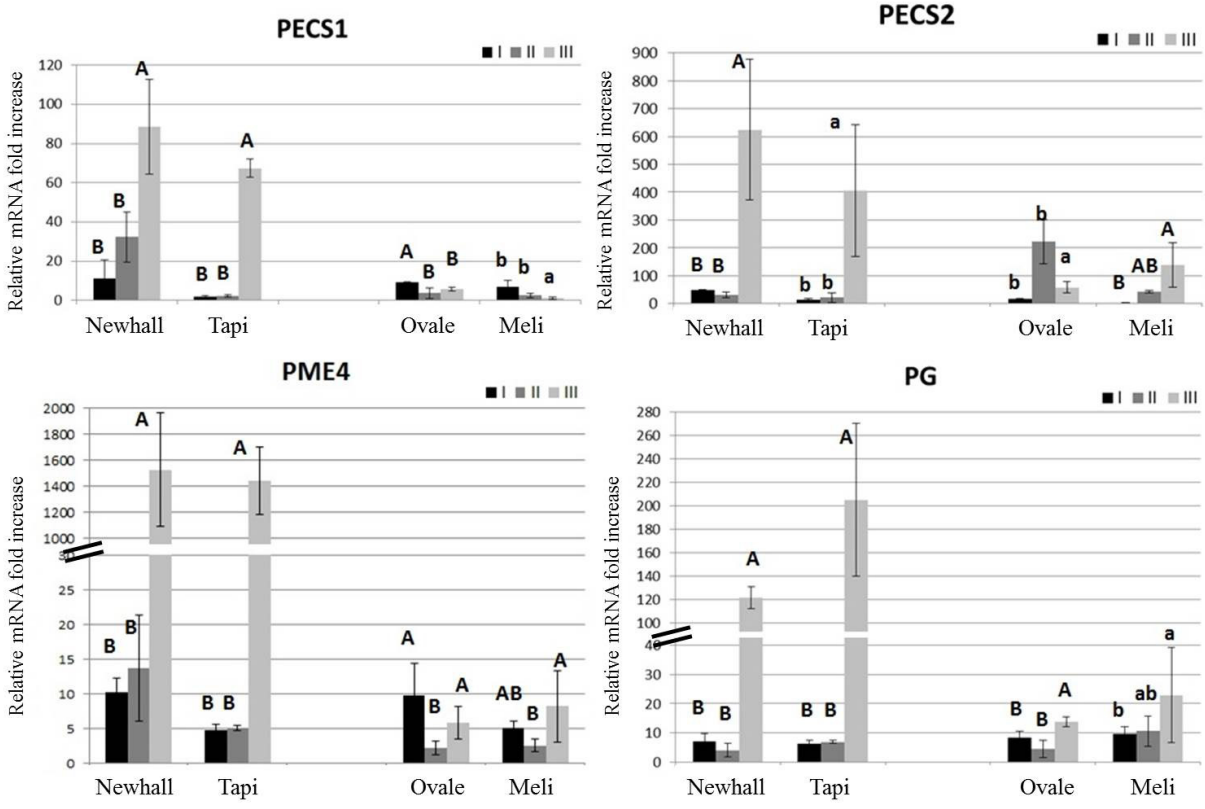

Figure 1. Relative expression profiles of PECS1, PECS2, PME4 and PG genes. Standard deviation is indicated as bars. I, II and III refers to mature, edible and senescent stages of sweet orange fruits. ANOVA and mean separation by Tukey's HSD test (capital letters for $p \leq 0.01$; small letters for $p \leq 0.05$).

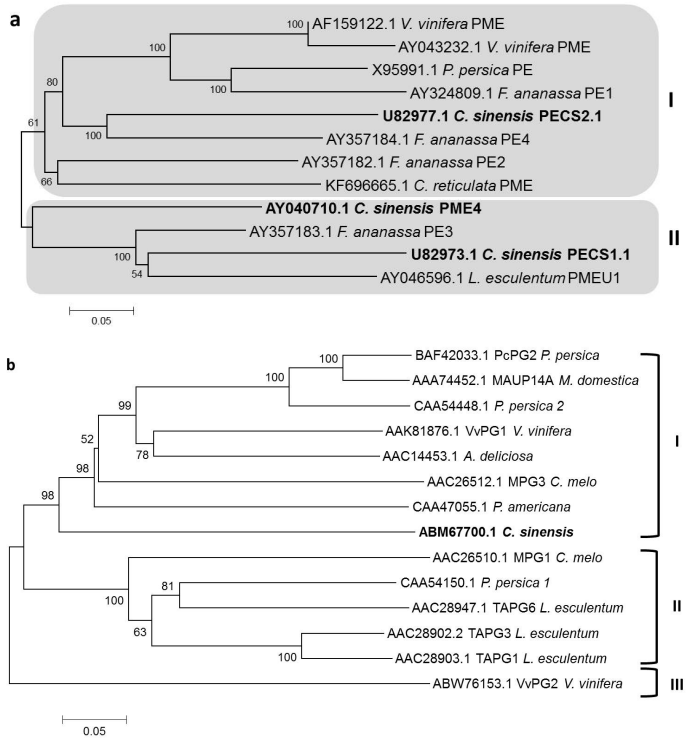

Figure 2. Phylogenetic tree deduced on amino acidic sequences of (a) PECS/PME and (b) PG *Citrus* and other plants. In bold sequences of citrus discussed in the manuscript. I, II and III are main groups on which sequences are separated.

in terms of genes/isoforms trend, but also considering the expression values within cultivars of ripening group, high in early and low in late cultivars, mostly in PECS1 and PME4. All data allowed to hypothesize that, among PECS/PME isoforms, the comparable increasing of PECS2 expression during the maturity time in early and late groups (with the partial exception of Ovale) suggested a putative involvement on ripening control of the gene in citrus fruits. The different behavior between early and late cultivars is difficult to connect to the only gene expression, considering the complexity of phenomenon controlling the maturation process.

As PECS, PG gene family, studied for its involvement in softening during ripening (Wang et al., 2000), was widely investigated in plant species (Atkinson et al., 2002). Phylogenetic tree (Figure 1b) including PGs isolated from tomato and other species, is divided into three main branches. The inclusion of citrus PG into the group I (together with PcPG2 of pyrus, MAUP14A of apple, VvPG1 of grape, MPG3 of muskmelon), supported the involvement in softening during ripening process (Wang et al., 2000). Considering the expression, data showed an increase of PG during maturation, more relevant in terms of expression level in early cultivars compared to late ones (Figure 2).

Similarly to PECS2, also PG expression profiles showed an interesting correlation with fruit firmness, hypothesizing its putative involvement in softness, in agreement with previous reports on other species (Errington et al., 1997; Fenwick et al., 1996; Rao & Paran, 2003).

Isolation and characterization of a MADS-box transcription factor

In the transcription factor networks affecting fruit ripening, MADS box family serve as central regulators of ripening. In the present paper we introduce a MADS-box cDNA, which was completed, cloned, sequenced and the full-length sequence was deposited in the Genbank database, named CsMADSSEP3-like (Accession number KR136378). The CsMADSSEP3-like consists on an ORF of 774 bp, a 5'-UTR (untranslated region) of 190 bp and a 3'-UTR (untranslated region) of 269 base pair. The predicted CsMADSSEP3-like protein, deduced

using the Expasy tool (http://web.expasy.org/translate/), consisted of 258 amino acids. The phylogenetic analysis, including MADS-box domain members of different subfamilies from climacteric and non-climateric fruits, allowed the clusterization of CsMADSSEP3-like into the SEP-like group, known to be involved in the control of fruit ripening (Figure 3).

The close relation among MADS-box sequences in tomato, pepper, citrus and banana justified the not separation between climateric and non-climateric fruits. Prior studies indicated that SEP genes played a central role in the developmental regulation of ripening in both climacteric and non-climacteric fruits (Seymour et al., 2011). According to our data, CsMADSSEP3-like could be considered an ortholog of AtSEP3, also strictly similar to MaMADS2 of apple, the latter highly expressed in fruit (Elitzur et al., 2010) and acting in the pulp of tomato before the increase in ethylene production similarly to SlMADS-RIN. The BlastN of CsMADSSEP3-like on the citrus genome (http://Citrus.hzau.edu.cn/orange/), corresponding to Cs7g10980, showed the possible existence of five alternative variants.

To investigate on the probable involvement of CsMADSSEP3-like in the citrus maturation process and to evaluate the possible existence of correspondent Cs7g10980 variants, qualitative expression analysis was performed on leaves, ovary and juice sacs of a typical common orange. RT-PCR expression analysis, performed using forward and reverse primers strategically located to specifically amplify each putative alternative transcript,

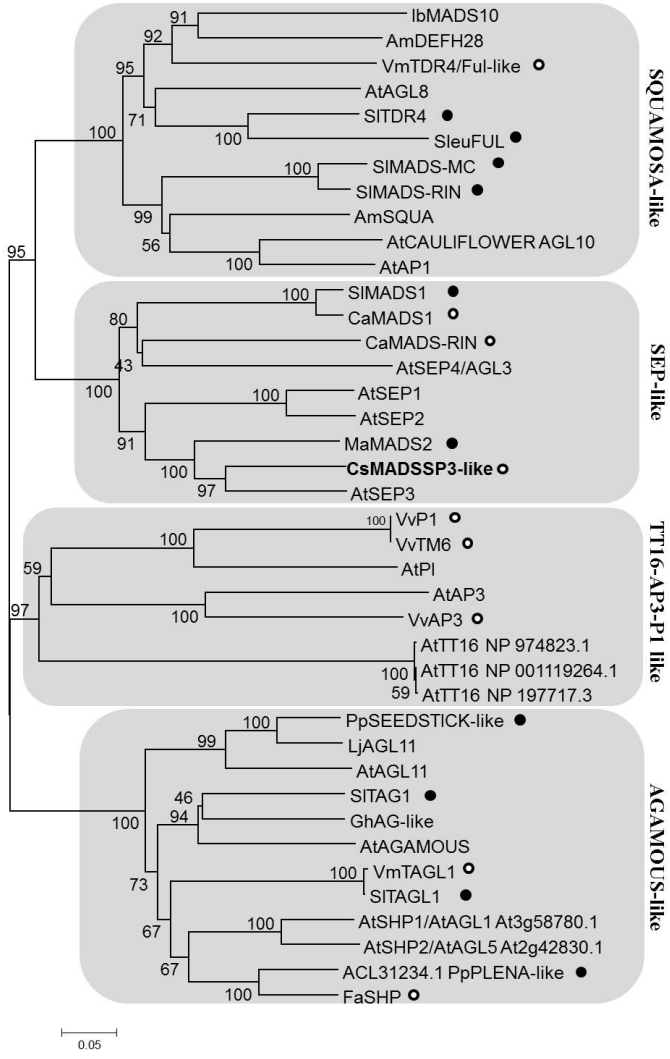

Figure 3. Phylogenetic tree of various eudicot plants which is known to control the ripening process. Black circles indicated climacteric fruits, white circles non-climateric ones. In bold the CsMADSSEP3-like, object of the present paper.

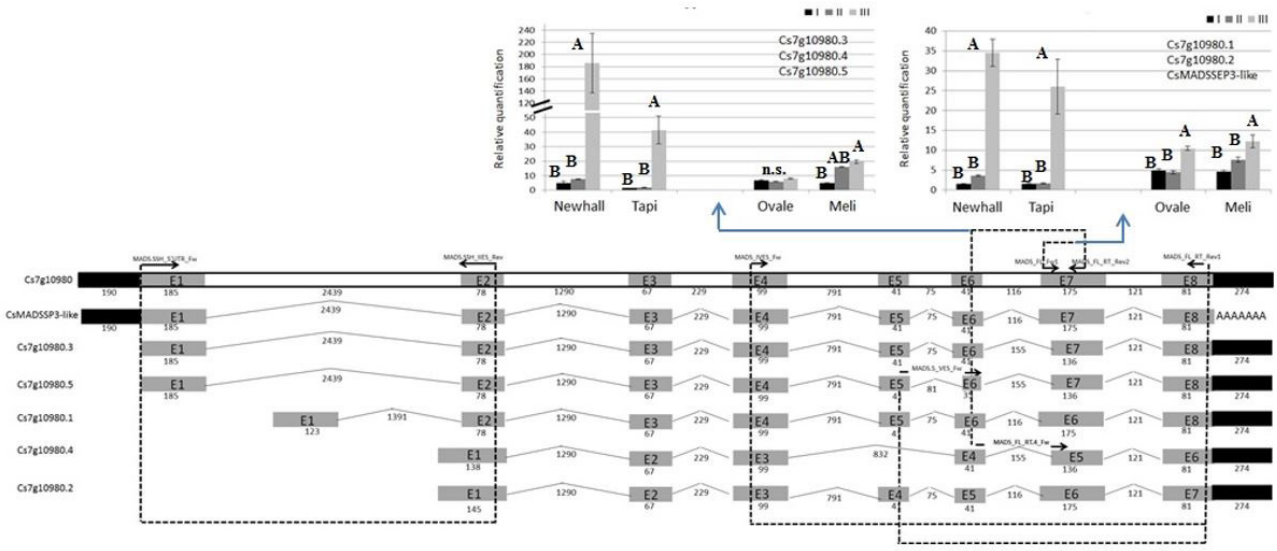

Figure 4. Schematic view of intron/exon structure and expression analysis of CsMADSSEP3-like and Cs7g10980 alternative transcripts. Genomic region and corresponding transcripts are showed. In black bars 5' and 3' UTR are indicated. Exon sequences (E) are indicated as grey bars and numbered from 1 to 8 basing on the number of exons recognized in the sequence. Intron sequences are indicated with open boxes. Exons and introns length are also indicated as base pairs. Transcripts names are shown on the left. Forward and reverse primers are indicated with black arrows. Quantitative relative expression profiles of CsMADSSEP3-like and Cs7g10980 alternative transcripts during the maturation are showed in correspondence of relative region. Bars indicate standard deviation. I, II and III refers to mature, edible and senescent stages of sweet orange fruits. ANOVA and mean separation by Tukey's HSD test (capital letters for $p \leq 0.01$; small letters for $p \leq 0.05$). Bars indicate standard deviation.

confirmed the amplification of each one in evaluated tissues, with the exception of leaves (data not shown). The assimilation of expression pattern in ovary and juice suggested a possible role in the fruit physiology, but it is necessary to be further investigated. Ascertained the expression on juice, to evaluate the probable involvement of CsMADSSEP3-like and alternative transcripts in the maturation process, we used oligos located between the sixth and seventh exon because assuming all possible transcripts (Figure 4).

Real time data showed an increased expression of both transcripts along samplings (Figure 4). In particular the expression values of the region corresponding to Cs7g10980.3, Cs7g10980.4 and Cs7g10980.5 resulted higher in early compared to late cultivars, and also compared to the region correspond to Cs7g10980.1, Cs7g10980.2 and CsMADSSEP3-like. The increased expression from mature to senescent sampling reinforces the hypothesis of a putative involvement of CsMADSSEP3-like in the regulation of ripening. Of course higher values of expression of alternative transcripts Cs7g10980.3, Cs7g10980.4 and Cs7g10980.5 encourage about a possible major involvement of these alternative forms compared to Cs7g10980.1, Cs7g10980.2 and CsMADSSEP3-like itself.

CONCLUSIONS

In conclusion, this is the first time in which molecular studies on gene putatively involved in the maturity were performed on various early and late citrus cultivars during the maturation process. The no homogeneous expression level between blood and common oranges, early and late one, reinforces the concept based on the complexity of citrus maturation process, as previously reported (Aharoni, 1968; Eaks, 1970).

These preliminary data have to be considered a starting point and need further information to determine the precise physiological and biological roles controlling the maturation of citrus cultivars at different ripening time. Understanding the mechanisms that regulate fruit maturation can help to improve fruit quality. The identification of ripening-related

MADS box proteins opens opportunities for the study of ripening regulation. In the future further in-depth analysis, such as genome information, germplasm evaluation and functional analysis, could be used to better monitor and study the maturation mechanism, in order to optimize the production of cultivars during all the calendar of ripening.

ACKNOWLEDGEMENTS

Authors are grateful to Marco Caruso for the critical writing assistance and Maria Allegra who performed statistical analysis. Research carried out within the project "Implementation of the FAO International Treaty on Plant Genetic Resources for Food and Agriculture" of the Ministry of Agriculture, Alimentation and Forestry Policies.

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Received: February 15, 2017
Accepted: October 28, 2017