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ORIGINAL ARTICLE

Expression of polyamine biosynthesis genes during parthenocarpic fruit development in *Citrus clementina*

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Abstract Polyamines have been attributed a general role in fruit development in several plants like pea and tomato. To investigate the involvement of these compounds in parthenocarpic fruit development in *Citrus clementina*, we have isolated three genes encoding aminopropyl transferases in this species: *CcSPDS*, *CcSPM1* and *CcACL5*. The unambiguous identity of the proteins encoded by these genes was confirmed by phylogenetic analysis and by heterologous expression in yeast mutants deficient in aminopropyl transferase activity. The expression of these genes in *C. clementina* is not restricted to ovaries and fruits, but it is also detectable all throughout the plant. More importantly, gibberellin-induced parthenocarpic fruit set caused a decrease in *CcSPDS* expression in ovaries, paralleled by a decrease in spermidine; while the expression of *CcSPM1* and *CcACL5* was basically unaffected, resulting in the maintenance of spermine concentration during early fruit development. In addition, the variation in putrescine content was paralleled by changes in the expression of one of the two putative *CcODC* paralogs.

Keywords Citrus · Fruit development · Parthenocarpy · Polyamines

Abbreviations

PAs	Polyamines
Put	Putrescine
Spd	Spermidine
Spm	Spermine
Syn	Synefrine
Tspm	Thermospermine
Tyr	Tyramine
M-Tyr	Methyl-tyramine

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Introduction

Polyamines (PAs) are small polycationic molecules which have been attributed roles in multiple processes related to plant growth and stress resistance. In particular, PAs are viewed as modulators of plant development (Galston 1983). Accordingly, high levels of PAs are usually found in actively growing tissues suggesting that they are growth limiting factors (Smith 1985; Bagni and Torrigiani 1992). They also change in response to colder temperatures and during pathogenesis (Bellés et al. 1991; Kasukabe et al. 2004), suggesting a generic role in stress resistance. The most common PAs in the plant kingdom are the aliphatic amines putrescine (Put), spermidine (Spd), and spermine (Spm). Several other amines are particularly abundant in a species-specific manner, such as synephrine, tyramine, and methyl-tyramine in Citrus (Wheaton and Stewart 1970). Recently, thermospermine (Tspm), first found in prokaryotes (Oshima 1979), has also been suggested to be present in plants as it is the product of the in vitro activity of ACL5 (Knott et al. 2007).

In plants, Put can be synthesized from arginine or ornithine via arginine and ornithine decarboxylase (ADC and ODC), respectively, and changes in both enzyme activities generally seem to regulate changes in Put levels (Perez-Amador and Carbonell 1995; Alabadí et al. 1996). Spd and Spm are synthesized by the subsequent addition

of an aminopropyl moiety, derived from decarboxylated *S*-adenosylmethionine (dcSAM), to Put or Spd, respectively. These reactions are catalyzed by Spd synthase (SPDS) and Spm synthase (SPMS), and regulation of their activity correlates with the actual concentration of the corresponding PAs in different organs and developmental situations (Alabadí and Carbonell 1999; Alcázar et al. 2006; Carbonell and Blázquez 2009). An additional asymmetrical tetraamine, Tspm, can also be synthesized through the activity of an alternative Spd aminopropyltransferase, Tspm synthase (tSPMS), which is present only in plants and several prokaryotic genera (Minguet et al. 2008).

Aminopropyltransferase genes have been isolated from several plant species (Minguet et al. 2008). In *Arabidopsis thaliana*, two genes have been demonstrated biochemically and genetically to encode SPDS (Panicot et al. 2002; Imai et al. 2004), SPMS is encoded by one gene (Hanzawa et al. 2002; Panicot et al. 2002), and there is an additional gene, *ACL5*, which encodes another Spd aminopropyltransferase with tSPMS activity (Hanzawa et al. 2000; Knott et al. 2007; Kakehi et al. 2008).

Several lines of evidence support the involvement of PAs in fruit development. On the one hand, Spm concentration in pea and tomato ovaries is associated with their fate: it accumulates in unpollinated ovaries preceding senescence, while this increase can be reverted by treatments that trigger fruit development, either fertilization or gibberellin (GA) application (Carbonell and Navarro 1989; Alabadí et al. 1996). On the other hand, the function of ODC and ADC in fruit development seems to be associated with cell division and expansion (Fraga et al. 2004; Acosta et al. 2005). Exogenous supply of Spd overcomes the growth restriction caused by a deficiency in GA biosynthesis in tomato fruits (Fos et al. 2003). Finally, during ripening, a drop in the concentration of all PAs has been reported in a number of species, such as tomato (Saftner and Baldi 1990), apple (Biasi et al. 1988), and pepper (Saftner and Baldi 1990) among others.

In Citrus, seedless fruits are obtained from naturally occurring parthenocarpic varieties, or by exogenous application of GAs (El-Otmani et al. 2000). In fact, endogenous GA concentration has been correlated with parthenocarpic ability (Talón et al. 1990a, b, 1992), and although changes in the content of Put, Spd and Spm have been found during early fruit development (Nathan et al. 1984; Kushad et al. 1990), it is not clear whether this variation is causally connected to fruit set or fruit growth, since the examination of PA content in parthenocarpic and non-parthenocarpic Citrus varieties did not render any significant difference (Arias et al. 2005).

To get a deeper insight into the mechanism that regulates PA content during fruit set, we have isolated the genes encoding the enzymes that catalyze the last steps in PA biosynthesis, *SPDS*, *SPMS*, and *tSPMS*. We have found that, in citrus, SPDS, SPMS, and tSPMS are encoded each by a single gene, homologous to *SPDS*, *SPM1,* and *ACL5,* respectively, in other plant species. Furthermore, we have studied the variation in their expression level during early fruit set, in comparison with the changes observed in the corresponding PA levels.

Materials and methods

Plant material

Five-year-old trees of *Citrus clementina* Hort. Ex Tanaka grafted onto Carrizo Citrange hybrid rootstock (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* Blanco) growing with drop irrigation in orchards located in Moncada (Valencia, Spain) were used. Terminal flowers between anthesis and 1 week before anthesis were emasculated (i.e. petals and stamens were removed). Fruit set was induced by dipping for 10 s emasculated flowers in a 500-μM gibberellic acid (GA_3) solution with 0.2% Tween-20 as wetting agent. Fruits were harvested at the times indicated in the figures, between anthesis and 21 days after anthesis, when fruit set was evaluated. Sampled ovaries and fruits were immediately frozen with liquid nitrogen and stored at −80°C until analysis.

Quantification of polyamine content

Extraction and quantification of PAs were performed on samples homogenized in 0.2 M perchloric acid, dansylation, and detection by HPLC as previously described (Arias et al. 2005), using 1,6-diaminohexane as internal standard (Carbonell and Navarro 1989). This method does not distinguish between Spm and Tspm, so both aliphatic tetraamines are identified simultaneously.

Cloning of polyamine genes

To isolate the *C. clementina* genes encoding SPDS, SPMS, and tSPMS activities, protein sequences from different plant species were aligned using CLUSTALX (Thompson et al. 1997), and degenerate oligonucleotides were designed targeted to conserved regions (Table 1). The following pairs were used as primers on PCR reactions using a mixture of cDNAs prepared from *C. clementina* ovaries at anthesis and 1, 2, and 3 weeks after treatment with 500 μM GA_3: SPDSdg1 and SPDSdg2 for *CcSPDS*, SPMSdg1 and SPDSdg2 for *CcSPM1*, and ACL5dg1 and ACL5dg2 for *CcACL5* (Table 1). The identity of the amplified fragments was confirmed by sequencing. The full-length cDNA clones corresponding to the three genes were isolated by

Table 1 Oligonucleotides used in this study

	Sequence (5′–3′)[a]
SPDdg1	CCTGGNTGGTTYTCNGARATNAGYCC
SPDdg2	CWCCRTCWCCTCCWCCAATNACCARNAC
ACL5dg1	GAYCTCAARTGGTCNTTYGC
ACL5dg2	TCACCWCCTCCCATDATRAA
SPMdg1	TAYTTYAAYAAYCCNATGTGG
SPD-qRT-f	CCAATTGGTCCTGCACAAGA
SPD-qRT-r	CCCCTGGACGAAGAGCTTTA
ACL5-qRT-f	TTGTGTCACCAGAATCCCAAAA
ACL5-qRT-r	TTCCCTCGCCGCAGAAC
SPM-qRT-f	TCAGCTGCCTTTGCATTGC
SPM-qRT-r	TTGGAGTTGGAGAATCACCAAGT
TUB-qRT-f	TTGTTTGAAACAACTTTTCGACG
TUB-qRT-r	AAAACGAGCACATGCAAACG
ADC-qRT-f	CCATGCCTGGGCCGTCTTGT
ADC-qRT-r	GCAAGGCTGCTAGCTAAAGC
ODC1-qRT-f	GAATCTTGCTGGTGTTAG
ODC1-qRT-r	CAGCATTTCCGTGCTTTTGACT
ODC2-qRT-f	GGTAACCTGCGATGAA GCA
ODC2-qRT-r	CGTATAGGCACCCATTTCG
SAMDC1-qRT-f	GCCTGACCAAGGCCCAGCT
SAMDC1-qRT-r	CAGGATGGGTGGGATTGA
SAMDC2-qRT-f	CGGCTACAGCATTGAAGA
SAMDC2-qRT-r	CTACTACTCTACGCTATCG
SAMDC3-qRT-f	TGCTTGCTTGCTTCAAACC
SAMDC3-qRT-r	CACGATTTCAAGATTGACCT
SAMDC4-qRT-f	GGAGTGAATCTAGCTTGTTCA
SAMDC4-qRT-r	CAACTTCGAATGAAGAGTGC

[a] In degenerate positions, N: (A, T, C, G); Y: (C, T); R: (A, G); W (A, T)

plaque-screening of the OF1 and OF3 cDNA libraries constructed from *C. clementina* ovaries (Forment et al. 2005).

A search of several available Citrus databases, including Citrus Functional Genomic Project (http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/), provided us with the sequences of the *CcADC*, *CcODC,* and *CcSAMDC* genes used in this study. The actual sequences used to design primers for expression analysis were *CcADC* (aCL1233), *CcODC-I* (aC31206E10), *CcODC-II* (aC02010H01), *CcSAMDC-I* (aCL3contig3), *CcSAMDC-II* (aCL3229contig1), CcSAMDC-III (aCL368contig1), and *CcSAMDC-IV* (ortholog of *AtBUD2*) (aC31206C08).

Yeast complementation studies

To express the *C. clementina* polyamine biosynthesis enzymes in yeast, two sets of plasmids for yeast expression were constructed, based on YEpACT (Rández-Gil et al. 1995) and pAN10 (Navas et al. 1993). The *Eco*RI–*Xho*I fragment from the full-length cDNA clones for *CcSPDS*, *CcSPM1,* and *CsACL5* were cloned into the filled-in *Hin*dIII restriction site in YEpACT (*ACT1* promoter, *LEU2* marker) and pAN10 (*ADH1* promoter, *URA3* marker). YEpACT-derived plasmids were transformed into yeast strain YN158 [*MATalpha his6 leu2 ura3-52 spe3*::*URA3*; kindly provided by N. Hamasaki-Katagiri (Hamasaki-Katagiri et al. 1997) and H. Tabor (National Institutes of Health, Bethesda, MD, USA)] using the lithium acetate transformation method (Gietz and Sugino 1988). Transformants were selected in minimal medium [2% Suc and 0.7% yeast nitrogen base without amino acids (Difco)] supplemented with His (30 mg/L) and Spd (100 μM; free of other polyamines) at 30°C. An isogenic wild-type yeast strain, 2602 (*MATalpha his6 leu2 ura3-52 SPE3*), was used as a control (Hamasaki-Katagiri et al. 1997). For the complementation of *spe4* mutants, pAN10-derived plasmids were transformed into Y504 (*MATalpha his6 leu2 ura3-52 spe4*::*LEU2*) (Hamasaki-Katagiri et al. 1998).

Gene expression analyses

Total RNA extraction and cDNA synthesis for quantitative PCR were carried out as previously described (Forment et al. 2005). Quantitative real-time PCR (qRT-PCR) was done with a Perkin Elmer thermocycler using specific oligonucleotides for each gene (Table 1), and normalized by the expression of a tubulin gene (Gimeno et al. 2009).

For Northern-blot analyses, total RNA (10 μg) was analyzed by electrophoresis on 6% formaldehyde/1.2% agarose gels and blotted onto nylon membranes Nytran Plus (Schleicher and Schuell, Keene, NH, USA). DNA probes were labeled with [α-^{32}P]dCTP by the Ready-to-Go DNA-labeling kit (Amersham Biosciences, Barcelona, Spain), and purified with probe purification columns QuickSpin (Roche, Madrid, Spain). The RNA blots were hybridized as previously described (Church and Gilbert 1984), using ^{32}P-labeled probes. The ethidium bromide staining of the RNA gel was used as a control for equal loading of all lanes. Blots were stripped between hybridizations in 0.1% SDS at 90–95°C for 1 h. Quantification of hybridization signals was achieved using a FujiBass (Fujifilm, Dusseldorf, Germany).

Results

Variation of polyamine content during fruit set in *Citrus clementina*

To know if PAs have a role in fruit set in clementine mandarin, we analyzed PA content in ovaries of *C. clementina* at anthesis and at short-time intervals after the induction of

Table 2 Polyamine content in ovaries at anthesis

	Year 1		Year 2	
	µg/g FW	SD	µg/g FW	SD
Putrescine	32.7	4.5	42.1	3.4
Spermidine	40.9	1.6	51.4	0.6
Spermine	8.7	0.9	15.8	0.6
Synephrine	1,350.5	93.4	2,503.7	281.5
Tyramine	3,115.2	144.6	5,117.7	225.9
Methyl-tyramine	246.9	10.2	438.5	40.5

FW fresh weight, *SD* standard deviation of three replicates

fruit development with GA_3 treatments (see "Materials and methods"). As expected, the most abundant PAs in Citrus ovaries were Put, Spd, Spm, synephrine, tyramine, and methyl-tyramine (Table 2). However, the relative variations in the content of each compound during fruit set displayed a rather different profile (Fig. 1). While spermine content hardly changed during the first 3 weeks of fruit development after anthesis, synephrine and methyl-tyramine experienced a gradual increase in the content, spermidine and tyramine decreased around 50%, and the content of putrescine suffered a transient drop during the first 7 or 15 days after anthesis before the level was re-established (depending on the year that the experiment was carried out).

Interestingly, the decrease in Spd and tyramine might not be related to fruit set, since the same drop in the content of these two polyamines was also found in ovaries that had not accomplished fruit development 21 days after the treatment and showed early senescence symptoms (Fig. 1a, c). On the contrary, the content of Put, Spm, synephrine, and methyl-tyramine was much lower in senescent ovaries than in young fruits 21 days after anthesis (Fig. 1a, c).

Identification of aminopropyl-transferase genes from *Citrus clementina*

To find out if the variation of PA concentration during fruit set is caused by equivalent changes in the transcription of the genes encoding aminopropyltransferases, we decided to isolate these genes from *C. clementina*. Using a molecular strategy based on PCR with degenerated primers (see "Materials and methods"), we were able to isolate three cDNAs (*CcSPDS*, *CcSPM1,* and *CcACL5*) that displayed a very high sequence identity to the *SPDS*, *SPMS,* and *ACL5* genes from Arabidopsis, and even from a monocot like rice (Fig. 2). In particular, *Cc*SPDS which was 346 aminoacids long, showed 78, 76, and 77% identity with *At*SPDS1, *At*SPDS2, and *Os*SPDS, respectively; the 355-aminoacid-

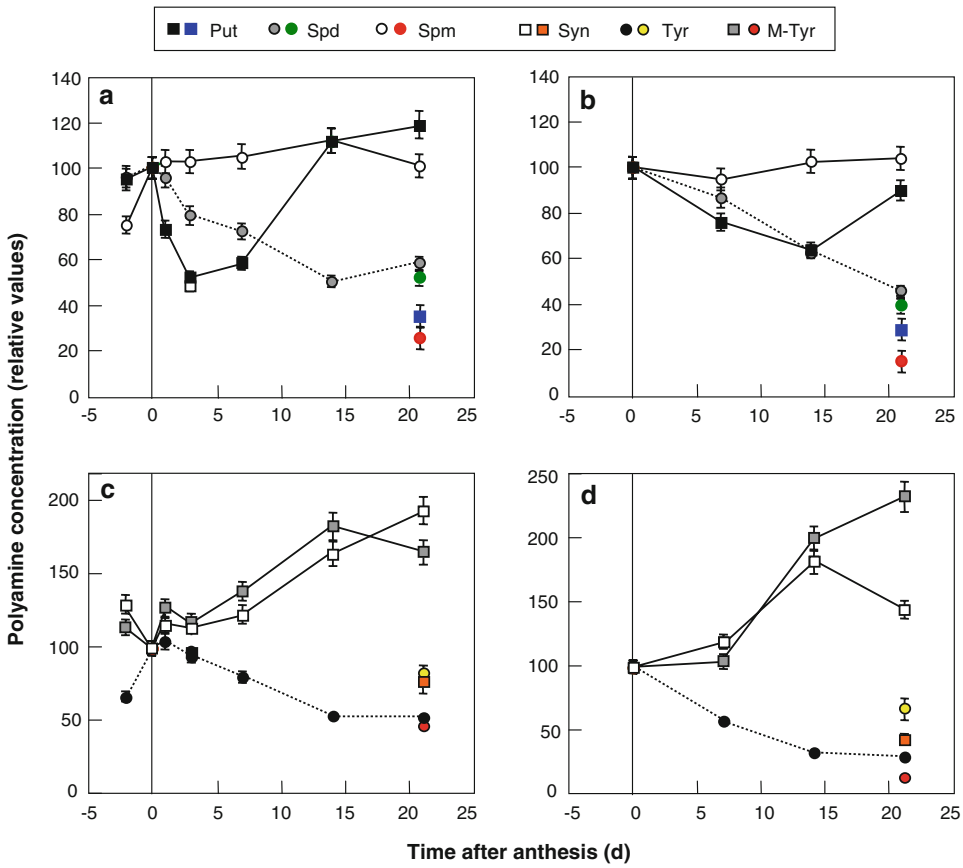

Fig. 1 Variation of the content of polyamines during GA-induced fructification in two consecutive years: 2003 (**a**, **c**) and 2004 (**b**, **d**). Values are normalized as 100% at anthesis (see Table 2 for the actual contents). Ovaries were treated with GA_3 at anthesis and harvested as described in "Materials and methods". **a**, **b** *Blue*, *green*, and *red dots* represent Put, Spd, and Spm content in senescent ovaries. **c**, **d** *Orange*, *yellow*, and *red dots* represent Syn, Tyr, and M-Tyr, respectively, in senescent ovaries

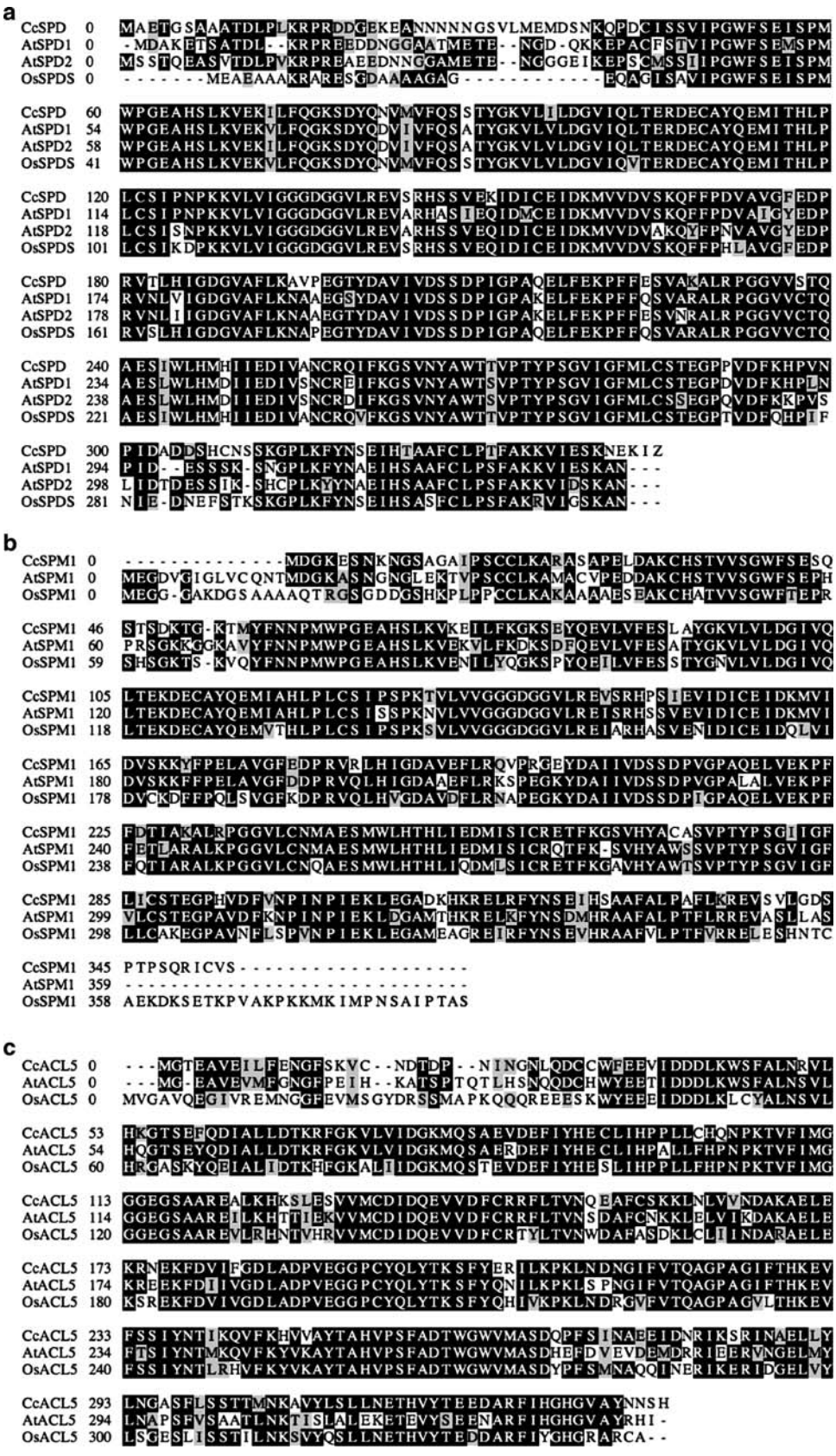

Fig. 2 Alignments of protein sequences corresponding to CcSPDS (**a**), CcSPM1 (**b**), and CcACL5 (**c**). Citrus, Arabidopsis and rice full-length sequences were aligned with CLUSTALX (Thompson et al. 1997) and the results displayed with BOXSHADE. *Black boxes* represent identical amino acids, while *gray color* is applied to chemically related residues

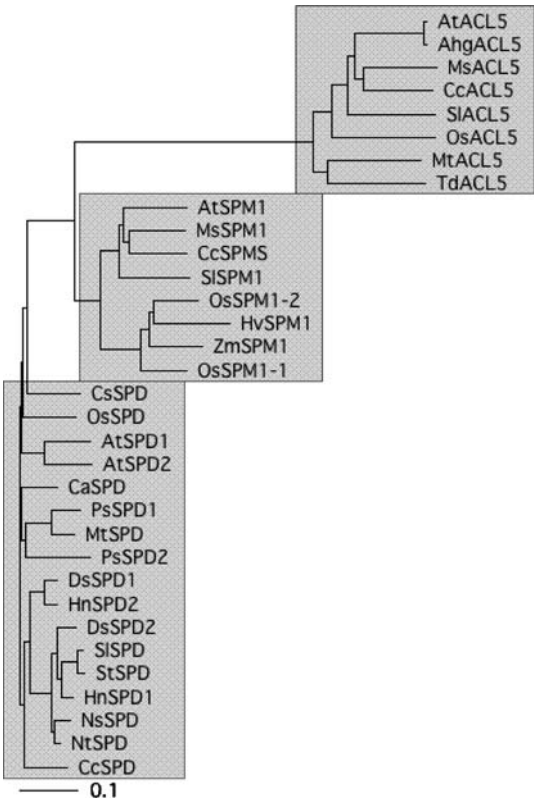

Fig. 3 Dendrogram of aminopropyltransferase genes. All available sequences from various plant sources were aligned using CLUSTALX, and a tree was constructed using the Neighbor Joining method. Ah, *Arabidopsis halleri*; At, *Arabidopsis thaliana*; Ca, *Coffea arabica*; Cc, *Citrus clementina*; Cs, *Cucumis sativus*; Ds, *Datura stramonium*; Hv, *Hordeum vulgare*; Hn, *Hyosciamus niger*; Ms, *Malus sylvestris*; Mt, *Medicago truncatula*; Ns, *Nicotiana sylvestris*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; Ps, *Pisum sativum*; Sl, *Solanum lycopersicum*; St, *Solanum tuberosum*; Td, *Taxodium distichum*; Zm, *Zea mays*

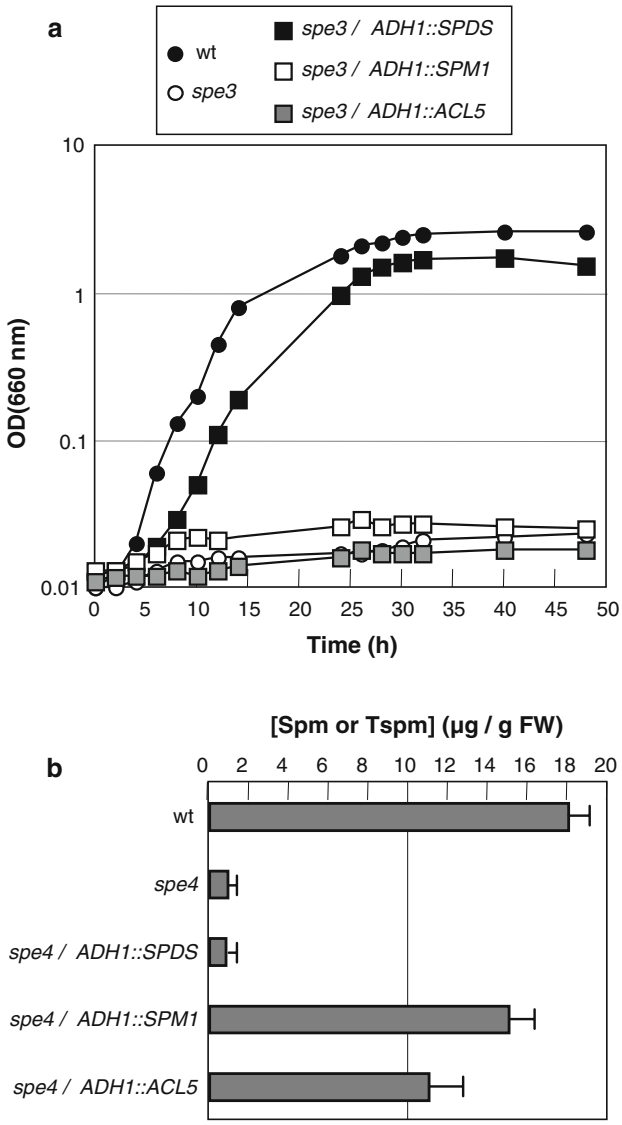

Fig. 4 Complementation of yeast polyamine mutants by expression of *C. clementina* polyamine genes. **a** Growth curve of yeast cells in minimal medium depleted of polyamines. *S. cerevisiae* wild type, *spe3* mutant (deficient in SPDS activity), and *spe3* transformed with *CcSPDS*, *CsSPM1,* and *CcACL5* under the control of the constitutive yeast *ACT1* promoter, were selected in minimal medium containing Spd, and aliquots of the preinocculum were diluted into minimal medium without Spd. Growth was monitored by absorbance of the culture at 660 nm during the following 2 days. **b** Tetraamine content in yeast mutants devoid of SPMS activity. *S. cerevisiae* wild type, *spe4* mutant (deficient in SPMS activity), and *spe4* transformed with *CcSPDS*, *CsSPM1* and *CcACL5* under the control of the constitutive yeast *ADH1* promoter, were grown on minimal medium and cells harvested at $OD_{660\ nm}$ = 0.8. Polyamines were extracted as described in "Materials and methods" from 100 mg (yeast wet weight) samples, and tetraamines quantified by HPLC. The molecular nature of Spm and Tspm impairs their separation in this assay

long *Cc*SPM1 protein was 78 and 70% identical with *At*SPMS and *Os*SPMS, respectively; and *Cc*ACL5 contained 339 aminoacids and was 76 and 68% identical with *At*ACL5 and *Os*ACL5. As shown in Fig. 2, the identity at the amino acid level is maintained throughout the whole sequence for the three enzymes, including the residues that have been suggested to be essential for enzyme activity and substrate specificity, based on crystallographic analysis (Korolev et al. 2002) and protein modeling (Minguet et al. 2008).

Comparison of aminopropyl transferase genes from *C. clementine* with other known sequences from plants is shown in Fig. 3. *Cc*ACL5 and *Cc*SPM1 showed a high degree of similarity to those from *Malus silvestris,* while *Cc*SPDS showed a quite different distance with SPDs from other origins.

The *Saccharomyces cerevisiae spe3* mutant, deficient in SPDS, is unable to grow in a Spd-free medium, and transformation with *CcSPDS*—but not with *CcSPM1* or *CcACL5*—under the control of a yeast constitutive promoter could restore growth (Fig. 4a). On the other hand, the *S. cerevisiae spe4* mutant is perfectly viable, but unable to synthesize any tetraamine unless it is transformed with

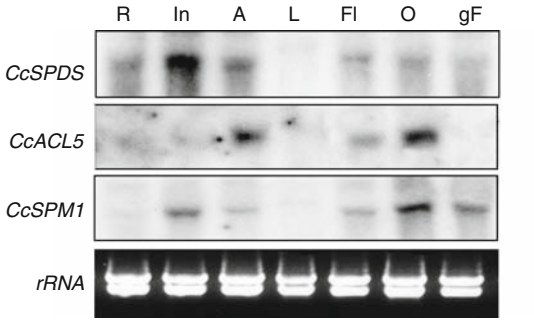

Fig. 5 Expression of polyamine biosynthesis genes in Citrus tissues. Total RNA was extracted as described in "Materials and methods". *CcSPDS*, *CcACL5,* and *CcSPM1* transcripts were analyzed by hybridization with the corresponding full-length cDNA probes as indicated. *R* roots, *In* internodes, *A* apices, *L* leaves; *Fl* flowers, *O* ovaries; *gF* 3-week-old developing fruits

the *CcSPM1* or *CcACL5* genes under the control of a yeast promoter (Fig. 4b).

Expression of polyamine biosynthesis genes in *Citrus clementina*

Given that the three genes were isolated from an ovary- and fruit-specific cDNA library, it was important to find out at which level they were expressed in other plant tissues. Contrary to what has been reported for Arabidopsis (Hanzawa et al. 2002), expression of *CcSPDS*, *CcSPM1,* and *CcACL5* was rather low or undetectable in roots and mature leaves (Fig. 5). Besides, *CcSPDS* and *CcSPM1* were expressed ubiquitously in all the other tissues examined, but preferentially in internodes and ovaries. In the case of *CcACL5*, maximal expression was detected in apices and ovaries.

To investigate whether the variation in Spd and Spm contents observed during fruit set (Fig. 1) was caused by equivalent changes in the expression of the genes encoding the corresponding biosynthetic enzymes, we analyzed by qRT-PCR the mRNA level of *CcSPDS*, *CcSPM1,* and *CcACL5* in ovaries that had been induced to produce fruits with GA_3 treatments at anthesis, as previously described (see "Materials and methods"). Expression of *CcACL5* did not exhibit any alteration during the first three weeks of fruit set (Fig. 6). On the other hand, expression of *CcSPDS* underwent a transient decrease during the first week, although it recovered the initial level three weeks after anthesis. More interestingly, expression of *CcSPM1* was upregulated almost twofold in the first weeks after the initiation of fruit development, and it was maintained at this level for the following 2 weeks. This behavior, also confirmed by northern analysis (Fig. 6c), contrasts with the lower expression level showed by 21-day-old senescent ovaries.

To contemplate the involvement of other known genes that encode enzymes participating in PA biosynthesis, we

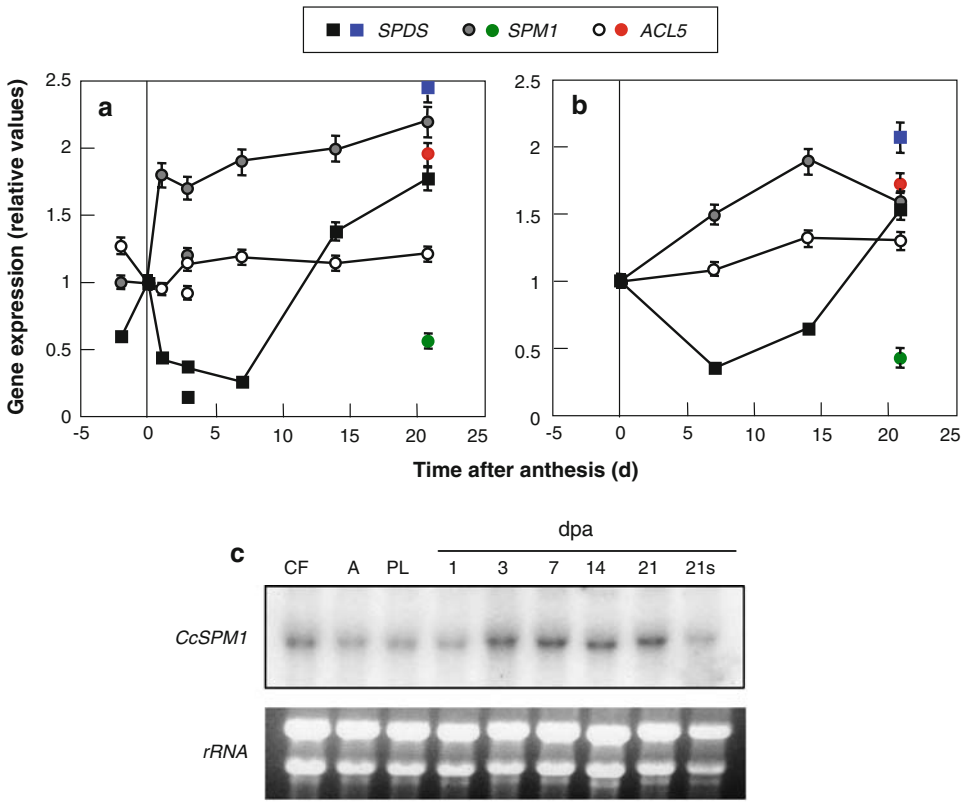

Fig. 6 Variation in the expression of aminopropyl transferase genes in Citrus during GA-induced fructification. **a**, **b** qRT-PCR analysis of the expression of *CcSPDS*, *CcSPM1,* and *CcACL5* in two consecutive years: 2003 (**a**), 2004 (**b**). *Colored* symbols represent the expression in 21-day-old senescent ovaries. **c** *CcSPM1* expression during GA-induced fructification, analyzed by northern blot. *CF* ovaries of closed flowers, *A* ovaries at anthesis, *PL* ovaries at petal loss, *dpa* days post anthesis, 21 s are 3-week old senescent ovaries (for comparison with ovaries undergoing fructification)

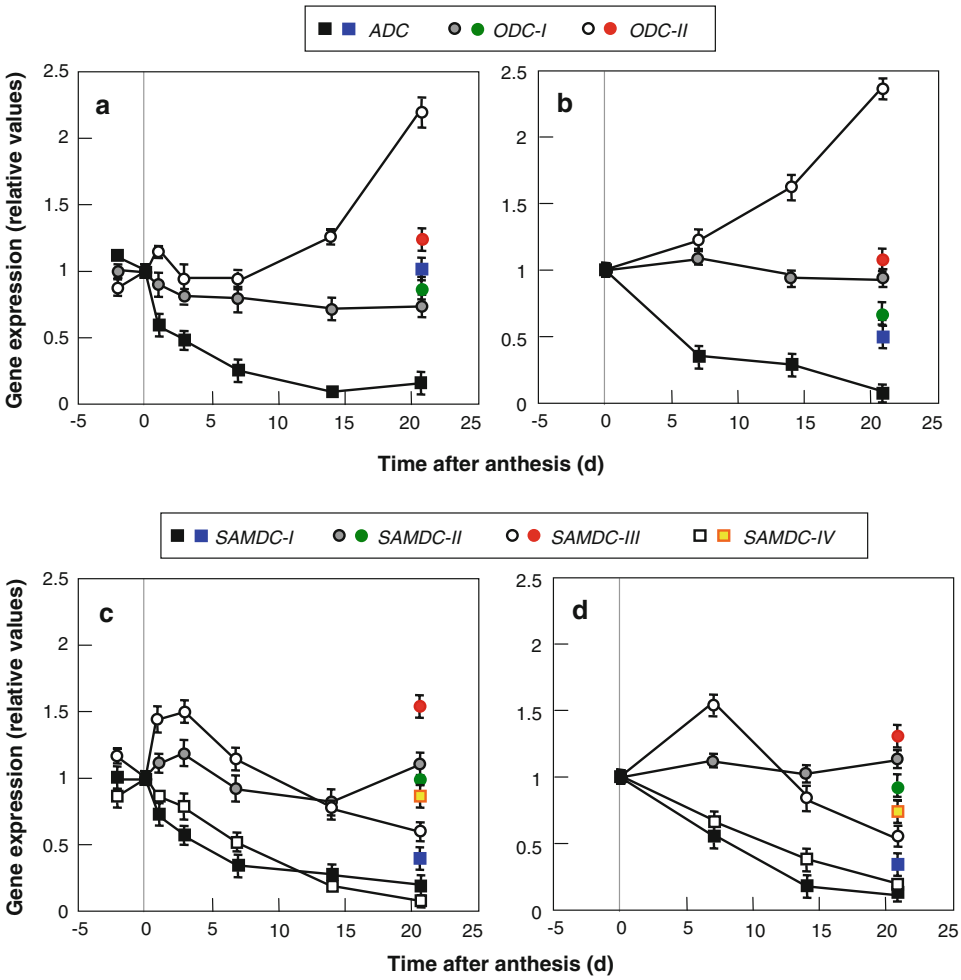

Fig. 7 Variation in the expression of decarboxylase genes in Citrus during GA-induced fructification. qRTPCR analysis of the expression of *CcADC*, *CcODC-I* and *CcODC-II* (**a**, **b**), or *CcSAMDC* genes (**c**, **d**) in two consecutive years: 2003 (**a**, **c**), 2004 (**b**, **d**). *Colored symbols* represent the expression in 21-day-old senescent ovaries

screened the available Citrus genomic databases and found one *ADC* gene encoding arginine decarboxylase, two *ODC* paralogs encoding ornithine decarboxylase, and four *SAMDC* genes encoding *S*-adenosylmethionine decarboxylase. As supposed, the expression of one of the *ODC* genes, *CcODC-II,* mirrored the increase in Put content in ovaries that were undergoing fruit development, compared with those that did not (Fig. 7a, b), while the expression of *CcADC* gene decreased in all cases. On the other hand, the expression of the most abundant *SAMDC* gene, *CcSAMDC-II*, did not vary during the first 21 days of fruit development (Fig. 7c, d), which suggests that dcSAM might not be limiting in this phase, and is in agreement with the results previously shown in apple trees (Hao et al. 2005).

Discussion

Three genes, *CcSPDS*, *CcSPM1,* and *CcACL5*, coding for the aminopropyl transferases that catalyze the synthesis of spermidine, spermine, and thermospermine, have been identified and cloned from *C. clementina*. Two pieces of evidence support the unambiguous identity of the encoded proteins as aminopropyl transferases. First, it has been shown that all aminopropyl transferases are evolutionarily related (Minguet et al. 2008) and the three *C. clementina* sequences clustered with the corresponding subgroups when they were analyzed together with the protein sequences from diverse plant species (Fig. 3), including the reported apple enzymes (Zhang et al. 2003; Kitashiba et al. 2005) and the sequences available in databases from several monocots. Second, complementation assays of yeast mutants impaired in SPDS or SPMS activities (Hamasaki-Katagiri et al. 1997, 1998) confirmed that the cDNAs isolated from *C. clementina* encode functional enzymes with the expected activities (Fig. 4).

The most relevant observation derived from this study is that the different changes in the transcript levels of the three aminopropyl transferases—transient decrease in *CcSPDS*, increase in *CcSPM1*, and no significant changes in *CcACL5* (Fig. 5)—could be correlated with the decrease of Spd and the relative maintenance of tetraamine levels (Spm + Tspm) (Fig. 1), suggesting that transcriptional regulation could indeed play a role in the control of PA concentration

during parthenocarpic growth, although this correlation is not always maintained in all physiological situations (Rambla et al. 2010). However, additional points of regulation might exist; for instance, the transient decrease in Put concentration preceding the Spd decrease suggests an influence of substrate availability for Spd biosynthesis. In this respect, it is important to note the existence of certain degree of coordination in the regulation of the expression of the different genes involved in PA biosynthesis, including at least *CcODC-II* and *CcSAMDC-II* (Fig. 7). Finally, although we have not considered the contribution of the expression of polyamine oxidase genes to final PA homeostasis in the context of fruit set, it cannot be excluded that they participate here as in other developmental and stress-related processes (Kamada-Nobusada et al. 2008; Rodríguez et al. 2009).

Given all the observed changes, it is difficult to discern whether it is the variation in Spd content or the maintenance of tetraamine levels the more determinant factor in fruit set and fruit development. Physiological studies in tomato have demonstrated a crucial role for Spm in fruit development, especially during the early stage of fruit development (Egea-Corines et al. 1993), so it is possible that *Cc*SPM1 plays an important role at the cell-division stage. In pea, all correlative evidence points to Spm having a decisive role in the establishment of fruit set *versus* ovary senescence (Carbonell and Navarro 1989). Similarly, the expression of *CcSPM1* and the content of Spm remain high and unaltered during the first weeks after fruit induction (Figs. 1, 5, 6), and decrease in senescent ovaries. However, the lack of genetic evidence prevents a clearcut conclusion. Furthermore, it is important to remark that other PAs like synephrine, tyramine, and methyl-tyramine, which are fairly abundant in Citrus ovaries, also experience variations in their concentration during fruit set, suggesting a regulatory role for these compounds in fruit development, which has not been elucidated yet. In fact, these precursors of alkaloid biosynthesis have been found in several other species, such as in barley roots and seeds (Mann et al. 1963), still without any attributed function, except a possible role in the response to wounding (Guillet and De Luca 2005).

Although the scope of this work was focused on the mechanism for regulation of PA concentration during fruit set, the isolation of *CcSPDS*, *CcSPM1,* and *CcACL5* genes from Citrus opens the possibility to study the function of PAs in other processes of interest for Citrus biology, as prompted by the observation that the expression of these genes, isolated from ovary-specific libraries, is not restricted to this organ and developing fruits (Fig. 5). Based on the phenotype of the corresponding knockout lines in Arabidopsis, two processes seem particularly worth of future analysis in this woody plant: the involvement of *Cc*SPM1 in drought- and salt-stress resistance (Yamaguchi et al. 2006, 2007), and the regulation of vascular development by *Cc*ACL5 (Muñiz et al. 2008; Vera-Sirera et al. 2010).

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