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MADS Box Transcript Amount Is Affected by Ethylene during Abscission

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ABSTRACT

Thinning of young fruit is an important agronomical practice to ensure the maximum economic production. This practice is based on the control of the natural self thinning process occurring during fruit development. At the early stages of fruit development (fruitlet), the vegetative part of the tree is competing with the reproductive part of the tree and within the fruit clusters the different fruitlets are competing with each other. As a result the least fit organ abscises, Ethylene and auxin play a central role in this event but the role of ethylene is not thoroughly understood because in other systems abscission occurs partly with ethylene independent processes. We have followed the early development of fruitlets and studied the transcription patterns of MADS-box and ethylene related transcripts. Furthermore, we verified that ethylene has an effect on the expression of some ethylene related and MADS box genes. We propose that the ethylene burst during abscission induction is similar to a stage 2 ethylene system and it is related to fruitlet growth by affecting transcript amount of MADS-boxes which modulate seed development and cortex growth.

Keywords: *Apple, MADS-Box, Ethylene, Fruit, Ripening*

1. Introduction

Despite technical advances, growing fruit trees is nowadays still a very labour and time consuming activity [1]. The fact that these plants are grown over several years makes agronomic practices performed one year affecting both the same year production and the following year's yield. The case of apple (*Malus × domestica* L. Borkh) is a typical example of this situation because the number of fruits in one year affects both quality and biennial bearing [2,3]. The fruit load is an issue which has been dealt with for countless years with several approaches. The most important approach has been the fruit thinning performed by hand throughout the first stages of young fruit development (fruitlet) [4]. Nevertheless, the use of bioregulators to increase the natural tendency of the trees to shed surplus fruit has taken over by many growers [5]. In an attempt to further decrease the production costs for the growers, a genetic approach which would exploit natural variation of the germplasm is of primary importance [6]. In order to optimize the discovery of traits interesting for a specific fruit load, which would give the best economic value, the study of the abscission physiology in the different cultivars is a must [7,8].

Abscission is a common self-mechanism the plant adopts to get rid of specific organs [9]. Fruitlet abscission in apple (*Malus × domestica* L. Borkh) occurs in a period of competition between reproductive and vegetative parts and among fruitlets [7]. This competition is due to several factors [8,10,11]. Hormones play a central role in modulating abscission as can be proven by the fact that bioregulators are widely used to thin excess fruitlet load [5].

The studies performed on this species pointed out that auxin, ethylene and their interaction are of primary importance [12,13]. Briefly, it was previously found that a massive increase in *MdACO*1 transcript amount was concomitant with an increase in elements involved in the signal transduction pathway. Furthermore, the variation in transcript amount followed the increase in ethylene evolution [12]. In Arabidopsis, tomato, rice and several other species these genes are present as multigene families whose members may have specific functions depending on the tissue and/or developmental stage [14]. The situation in apple ripening is similar to the other plants [15]. Nevertheless, neither the involvement of ethylene nor the function of the specific elements during abscission is completely understood.

Ethylene is a hormone with a wide range of functions [16]. In apple abscission induction the increase in ethylene evolution corresponded to a decrease in growth rate

[8]. During abscission induction (the first weeks after full bloom) the stage of cell division is progressively taken over by cell expansion [17-19]. The flower pollination and the subsequent ovule fertilization bring about deep modification of the ontogenetic program of the organ [20]. It has been found that MADS-box proteins play a crucial role in flower and fruit development [21-25]. Although the main studies on fruit have been performed on tomato, which is a berry, there are reports indicating that the mechanisms are conserved, at least partially, in other fruit such as banana [26], grape berry [27-29], peach [30] cucumber [31] and apple [17,32-35]. MADS-boxes have also been found involved in vegetative processes [36-39] with good conservation of activity [40,41]. In this manuscript the role of ethylene during abscission is further investigated. Furthermore, the relationship between ethylene and the level of the transcripts of some MADS box genes is assessed.

2. Materials and Methods

2.1. Plant Material

Experiments were performed as previously reported on eight-year old apple trees (cv Golden Delicious/M9) *Malus × domestica* bearing two fruitlet populations characterized by different abscission potential. These populations which were named abscising fruitlet (AF) and non abscising fruitlet (NAF) were obtained as previously described [42]. Apple trees display a natural tendency to shed lateral fruitlets while maintaining central ones. Nevertheless, this phenomena is amply variable and in order to maximize and homogenize the starting material the abscising fruitlet population (AF) was obtained from lateral fruitlets born on trees sprayed with benzylaminopurine (BA). The product was applied at 200 ppm (commercial form "Brancher-Dirado") when the average fruit diameter was 10 - 12 mm (17 d after petals fall, APF). In this period abscission induction of the shedding process occurs [43] It is in this time window that abscission which can be stimulated by thinners with the greatest efficiency [4,5]. A population with almost non abscission, named non-abscising fruitlets (NAF), was generated by removing all the laterals from the cluster at petal fall and leaving, exclusively, the central flower freely pollinated at bloom with compatible pollen (cv. Stark Red). Seed, cortex, peduncle (the central 15 mm) and abscission zone (AZ) of each population were collected from fruitlets at 0, 3, 5, 7 days after BA was applied to the AF, frozen in liquid nitrogen, and stored at –80˚C for molecular analysis. Fruitlet diameter was measured with a caliber and statistical analysis was performed with a 2 way ANOVA of the GRAPH PAD PRISM software. Parameters studied were population, time and time/population interaction. Threshold was 5%.

2.2. Ethylene Experiment

For the ethylene experiment, entire apple fruitlet clusters at 15 DAPF were treated with propylene (1000 $\mu L \cdot L^{-1}$) or 1-MCP (1 $\mu L \cdot L^{-1}$) or left untreated (control) as described in [44]. At the end of the treatment fruitlet clusters were retrieved and divided into central and lateral. Tissues (abscission zone, peduncle, cortex and seed) were collected at the beginning of the experiment (T0) and after 24 hours for molecular analysis.

2.3. Molecular Biology Studies

RNA extraction and expression analyses were as previously described [45,46]. Degenerative primers (**Table 1**) were designed as previously described [47]. The expression analysis was repeated at different number of cycles but to make the results easily understandable and comparable both between the two different populations: NAF and AF and among organs (seed, cortex, peduncle and AZ) only significant differences were presented and commented (**Tables 2** and **3**). Primers for expression analyses

Table 1. Degenerate primer sequences.

gene	ACCN	Dir	seq	T
ETR55	DQ 84545860	F	CAAGARGTTGGGWTGATGAAG	50
		R	ACTKGCCATCATHGCATTCT	
CTR2	DQ 845459	F	GGMATGGMWCWGAKGTTGCTGT	55
		R	CAAATCACGTGGAATCTCAAG	
EIN2	DQ 845461	F	ATCSCTKTGGKCYARRCARCC	55
		R	AYKCCCTGWAGRCGRTTGAGA	
EIN2 3p	DQ 845461	F	CTTTATGAAGCTGAAACTAGAGAGAT	68

The gene name, the accession number (ACCN), the sequences (seq) of the primer forward (F) and reverse (R) (dir) 5's→3' as well as the temperature in ˚C used in the annealing step of the amplification are presented.

Table 2. Primer sequences for ethylene specific genes.

gene	ACCN	Dir	Primer F
ACO2	DQ439790	F	CGGCACCTTCCTTCCTCA
		R	GATGACAATGGAGTGGTGCA
ACO4	CN907149	F	CTTGATGCCGTTCAGACAGA
		R	CCAAGATTCTCACACAACAAGC
ETR5	DQ845458	F	GTTCTTCCGGTTGCAGAT
		R	AGCCAGTTTCTCCCTCATTA
EIN2	DQ845461	F	GCTACAAGCGTAGATTATCC
		R	CAGAAGATGAGCTGTTTTCC
CTR2	DQ845459	F	TACACGTCCTCCAAACCT
		R	ACAATCGTCGGTGTACTG
UBI	DQ438989	F	CATCCCCCCAGACCAGCAGA
		R	ACCACGGAGACGCAACACCAA
rib 18s		F	GTTACTTTTAGGACTCCGCC
		R	TTCCTTTAAGTTTCAGCCTTG

The gene name, the accession number (ACCN), the sequences (seq) of the primer forward (F) and reverse (R) (dir) 5's→3' used in the amplification are presented.

Table 3. primer sequences for MADS box specific genes.

GENE	ACCN	D	seq
MADS5	ABG85297	F	TTGAGTACGCCACAGATTCAT
		R	ATTCAAAGGTCCAGTTACCC
MADS7	CAA04323	F	CCCTCATCTTTTTATCC
		R	GGTTAAGATCAGAGGTAGACA
MADS9	CAA04920	F	TGGCAATATATAAACTGCATG
		R	AGCAAGAAATAAGTTCGCAA
MADS10	CAA04324	F	GTACAAGGCATGTTCAGAT
		R	GCTTCTCCACCCTCGATCA
MADS11	CAA04325	F	GGTATGAGCAAAACCCTTGA
		R	GATCTTCACCAAGCAAATGC
MADS12	CAC86183	F	AGCAGTGGATGACAATGATTC
		R	TCAAATGGCGAAAGCATC
MADS14	CAC80857	F	GCAGCAGCAAACACATATGAT
		R	ATTGGACTCCAAGATCACAG
MADS15	CAC80858	F	CCACGATCAGATTTCTCTTCA
		R	GACAAGAAATCCCCTTCCAT
PIST	AJ291491	F	GGCACTGGAAGATGAGAATAA
		R	AAGGCAAAAGGTATCTGCTG

The gene name, the accession number (ACCN), the sequence (seq) of the primers 5'→3' and their direction D (F is forward and R is reverse) used in the amplification are presented.

were designed with the Gene fisher program http://bibi-serv.techfak.uni-bielefeld.de/genefisher2/ [48] Similarity research was performed at the ncbi website http://blast.ncbi.nlm.nih.gov/Blast.cgi with the blastp algorithm [49]. Phylogenetic analysis was performed with th MEGA4 program [50].

3. Results

3.1. Isolation of Ethylene Related Transcripts

The fragments isolated in this study encode for an ETR5, a CTR2 and an EIN2. *MdETR*5 is 663bp (DQ845460) and encodes a fragment of 211aa. The blast analysis indicated a high level of similarity to proteins from several species, among which an ETR5 from *Malus* and *Pyrus,* and Neverripe and ETR4 from tomato (**Table 4**). *Md*-CTR2 (DQ845459) is 614bp and encodes a fragment of 193 aa. The bioinformatic analysis indicated close similarity to EDR1 of Arabidopsis thaliana and TCTR2 of tomato (**Table 5**). *MdEIN*2 (DQ845461) is 1158bp long and encodes for a partial protein of 302aa, which is highly similar to EIN2 of Arabidopsis and Petunia (**Table 6**).

Table 4. Level of similarity between *MdETR*5 isolated in this study and other elements present in the database.

Annotation	ACCNN.	identity	E-value
ethylene receptor 5 [*Malus × domestica*]	ABI58287	99%	5e-101
putative ethylene receptor [*Pyrus communis*]	AAL66193	98%	1e-99
unnamed protein product [*Vitis vinifera*]	CAO65024	80%	6e-78
ethylene receptor [*Persea americana*]	ABY76321	75%	9e-72
hypothetical protein [*Vitis vinifera*]	CAN66907	77%	2e-71
ethylene receptor neverripe [*Lycopersicon esculentum*].	AAU34077	72%	4e-70
ethylene receptor homolog (ETR4)	AAD31396	70%	3e-68

The annotation retrieved by similarity search, the accession number (ACCN), identity and E-values are reported.

Table 5. Level of similarity between *MdCTR*2 isolated in this study and other elements present in the database.

Annotation	Acc. N	identity	E-value
CTR2 protein kinase [Rosa hybrid cultivar]	AAK30005	94%	3e-110
unnamed protein product [*Vitis vinifera*]	CAO42985	93%	5e-109
mitogen-activated protein kinase [*Medicago sativa*]	ABD76389	92%	3e-107
enhanced disease resistance 1 [*Arabidopsis thaliana*]	ABR45968	88%	2e-103
enhanced disease resistance 1 [*Arabidopsis lyrata*]	ABR45984	88%	2e-103
TCTR2 protein [*Solanum lycopersicum*]	CAA06334	88%	2e-103
hypothetical protein OsJ_009144 [*Oryza sativa* (japonica cultivar-group)]	EAZ25661	86%	6e-102

The annotation retrieved by similarity search, the accession number (ACCN), identity and E-values are reported.

Table 6. Level of similarity between *MdEIN*2 isolated in this study and other elements present in the database.

Annotation	Acc. N	identity	E-value
ethylene signaling protein [Prunus persica]	ABC94581	85%	2e-146
hypothetical protein [Vitis vinifera]	CAN66374	72%	2e-121
EIN2 (ETHYLENE INSENSITIVE 2); transporter [Arabidopsis thaliana]	NP_195948	69%	4e-117
EIN2 [Petunia × hybrida]	AAR08678	71%	1e-114
sickle [Medicago truncatula]	ACD84889	68%	4e-112
ethylene-insensitive 2 [Lycopersicon esculentum]	AAZ95507	71%	3e-111
ethylene insensitive 2 [Zea mays]	AAR25570	55%	4e-78

The annotation retrieved by similarity search, the accession number (ACCN), identity and E-values are reported.

3.2. Expression of Ethylene Related Genes during Abscission

The overall results indicate the genes *MdACO*2, *MdETR*5 and *MdCTR*2 are expressed in all the tissues at similar level; whereas *MdACO*4 and *MdEIN*2 are mainly expressed in peduncle and AZ (**Figure 1**). *MdACO*4 transcripts in seed decreased in both AF and NAF although in NAF the decline was less severe. In cortex the expression was constant in AF whereas it peaked in NAF at day 3 then plunged. In peduncle expression was constant in AF whereas it declined in NAF. In AZ expression remained constant. *MdETR*5 expression in seed first increased in both AF and NAF then declined but in NAF the decline was less pronounced. In cortex expression was constant in AF whereas a decline was monitored in NAF. In peduncle transcripts increased in both populations then decreased in NAF while remaining constant in AF. In AZ expression was high and constant in both AF and NAF. *MdEIN*2 transcript level in seed paralleled the one of *MdETR*5. In cortex the expression was at its lowest and further declined in both AF and NAF. In peduncle and AZ expression did not vary. *MdCTR*2 transcript level was constant in AF whereas it decreased in NAF. In cortex expression slightly increased in AF whereas it steadily declined in NAF. In peduncle and AZ transcript level did not change.

3.3. Fruitlet Growth

It was previously found that fruitlet diameter increased even in the days preceding abscission [8]. In this study we confirmed this discovery and found that fruitlet diameters significantly increased in both populations but in NAF the increase was more pronounced than in AF (**Figure 2**).

## 3.4. Bioinformatic Analysis of the MADS-Box in *Malus × domestica*

The databases present in the world wide web were searched for MADS-box genes of different species: *Arabidopsis*

thaliana, *Solanum lycopersicum*, *Populus tremula* and *Malus domestica*. The results obtained by the blast P algorithm (data not shown) and by the phylogenetic analysis were similar (**Figure 3**). The sequences of apple
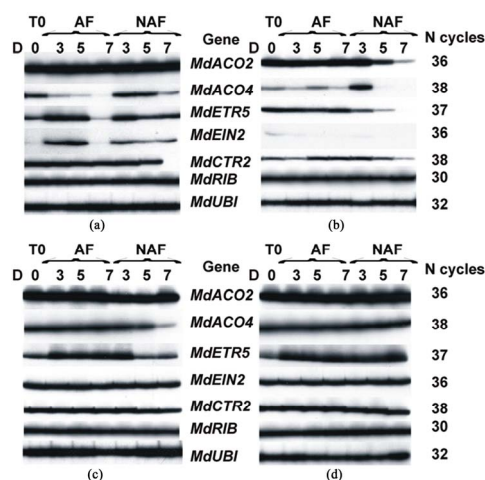
Expression analysis on the two populations studied: abscising fruitlets (AF) and non abscising fruitlets (NAF) at different days: 3, 5 and 7. T0 means the beginning of the experiment. Tissue Fruitlet parts are: seed (a), cortex (b), peduncle (c) and AZ (d) of different genes. The number of cycles (N cycles) is reported.

Figure 1. Expression analysis of genes involved in ethylene.
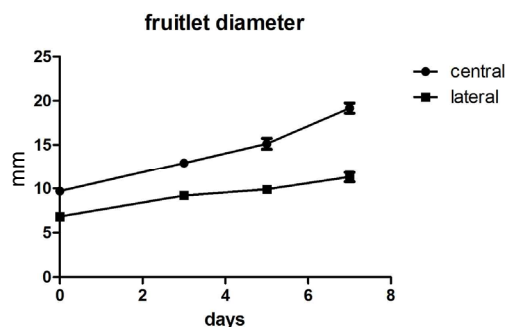
Fruitlet diameter of the cortex fruitlets expressed in mm. Cortex diameter of lateral fruitlets from AF is represented as square. Cortex diameter of fruitlets from NAF is presented as circles. Bars represent SE.

Figure 2. Fruitlet diameter.

MADS-box genes of different species: *Arabidopsis thaliana* (At), *Solanum lycopersicum* (Sl), *Populus tremula* (Pt) and *Malus domestica* (Md). Similar MADS box proteins are grouped together. The stars indicate the apple MADS box proteins. The accession number follows the name of the proteins.

Figure 3. Phylogenetic study of the MADS box proteins known and previously studied in apple.

already known are widespread among the several MADS-box clades [51,52]. The genes we found expressed clustered in the groups with previously identified genes with known phenotypes. Nevertheless, there was not clear horthologos. MADS5 resemble MC of tomato and CAL of Arabidopsis [53]. MADS7 resembles the tomato RIN and other Arabidopsis SEP genes [53]. MADS9 is in the SEP group and is similar to PHE of Arabidopsis. MADS10 is an AGL probably seedstick (STK) [54]. MADS11 is another AGL similar to TDR3 and 8 of tomato. MADS12

clusters with fruitful (FUL) [55]. MADS14 is an AGL similar to shatterproof (SHP1) [56]. MADS15 is an agamous in Arabidopsis (AG) and tomato (TAG1). PIST is a pistillata gene [57] and JNT is similar to tomato jointless [58] (**Figure 3**).

3.5. Expression of MADS Box Genes during Abscission

The overall expression analysis indicated that the most expressed genes were MADS5 and MADS10 in terms of

         AJPS

number of cycles used during analysis and intensity of the signal. Furthermore, the tissues displaying the main changes along abscission were the seed and the cortex (**Figure 4**). MADS5 transcripts were at a steady state in peduncle and AZ whereas the amount increased in NAF seed and decreased in AF cortex. MADS7 expression was specific of seed where it declined along the experiment in both populations but an abrupt increased was detected at day 7 in AF. MADS9 expression in seed declined along the experiment but in NAF it was delayed. In cortex expression increased in NAF. In peduncle and AZ expression was at a higher level than seed and cortex and unchanged along the experiment. MADS10 transcript level in seed decreased in AF while it remained unchanged in NAF. In cortex expression steadily declined in AF whereas it was a sudden in NAF. Similar situation was observed in peduncle. In AZ the expression was limited to NAF at day 3. MADS11 expression in seed decreased in AF whereas it remained constant in NAF. In cortex expression declined along the experiment in AF more gradually than in NAF. In peduncle transcript level remained at a steady level in AF whereas it declined in NAF. In AZ expression increased in both AF and NAF although earlier in the latter. MADS12 transcripts were detected only in seed at day 7. MADS14 expression was unchanged in seed and peduncle whereas in cortex a decrease was observed along the experiment more suddenly in NAF. In the AZ of AF the level suddenly decreased at day 7. MADS15 expression in seed declined along experiment mainly in AF. In cortex expression declined along experiment, more abruptly in NAF. No variation was observed in peduncle and AZ. *MdPIST* was detected only in seed of the NAF at day 3 and 7 and in peduncle of the NAF at day 7. Transcripts of JMD were detected only in peduncle and AZ and no difference or variation was observed (data not shown).

3.6. Ethylene Effect on Transcripts

The treatments with propylene and 1MCP indicated that *MdACO*2 is negatively affected by ethylene, whereas *MdERS*1, *MdETR*5, *MdCTR*2 and to some extent *MdEIN*2 expression is positively regulated by ethylene. Of all the MADS investigated, only MADS9 (in cortex) and MADS10 (in seed) showed to respond to the treatments, being both negatively regulated by ethylene (**Figure 5**).

4. Discussion

The genes isolated in this study encode for elements involved in ethylene perception and transduction [14]. Previous research showed that in apple like in other plants ethylene receptors are encoded by a multigene family [12] and *MdETR*5 is likely to be the same gene previously isolated [15]. *MdCTR*2 represents a new element isolated

in this species. It has been shown that CTR2 is similar to other CTR1 elements in both Arabidopsis [59] and tomato [60]. It has also been proposed that the member present in Arabidopsis is likely to be involved in defense mechanisms [61]. The data here presented indicate that CTR2 in apple may have a general role in ethylene signal transduction during apple development. Likewise, it has been demonstrated in tomato that this element may have
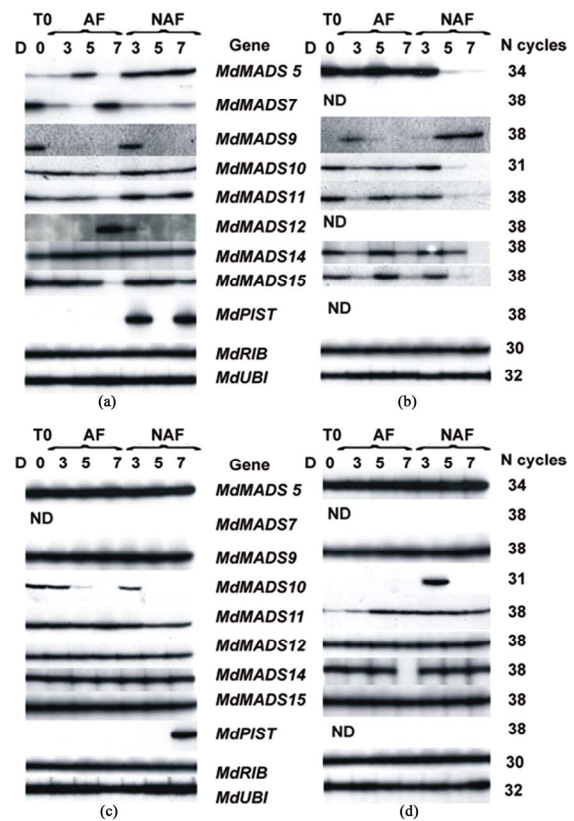
Expression analysis on the two populations studied: abscising fruitlets (AF) and non abscising fruitlets (NAF) at different days: 3, 5 and 7. T0 means the beginning of the experiment. Tissues Fruitlet parts are: seed (a), cortex (b), peduncle (c) and AZ (d) of different genes. The number of cycles (N cycles) is reported.

Figure 4. Expression analysis of MADS box genes.
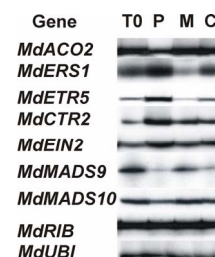
Expression analysis of the genes affected by propylene (P) or 1-MCP (M). The control (C) and the samples at the beginning (T0) of the experiment are also presented. Apart from MADS box 10 (MADS10, studied in seed) all the other expression analysis are from the cortex tissue.

Figure 5. Expression analysis of genes affected by ethylene.

a wider involvement [62] (Lin *et al*., 2008). The EIN2 in this study is almost identical to the one previously isolated [15] and the expression pattern of this gene and of the others genes involved in ethylene biology indicate that the hormone may be involved in mechanisms of development [63], cell differentiation and programmed cell death like in maize kernel [64]. Nonetheless, these elements have not been previously studied during abscission induction. As previously found [12] the organs in which the changes are most evident during abscission induction are the seed and the cortex. Analogously to previous findings, the peduncle also showed late variation in *MdETR*5 transcript amount. These results confirm that seed and cortex are likely to control the changes in ethylene biosynthesis and transduction previously described [12]. The results throughout abscission and in the ethylene experiment suggest that *MdACO*2, *MdETR*5 and *MdCTR*2 are either directly or secondarily controlled by ethylene. It has been suggested that in apple, ethylene could affect fruit development in the late stages of fruit maturation [65]. Immature apple abscission occurs during the first stages of fruit development in which cell division leads the way to fruitlet growth by cell enlargement [17]. Ethylene is not to be considered related to the processes occurring immediately after ovule fertilization [20] because 2 weeks after pollination seeds are already in advanced development. It has been found that ethylene is present at basal level in NAF whereas an increase is present in AF [12]. Ethylene at this stage may have a broad importance in development of the different tissues [16]. The expression pattern in seed of *MdERS*1, *MdETR*5 and *MdEIN*2; higher in NAF than AF followed by a sudden decrease may be seen as a delay in development and a total block by day 7. This hypothesis is supported by the drop in both *MdAHS* and *MdPIN*1 transcripts previously reported [10,13] and recently revisited in [66]. *MdCTR*2 transcript level on the other hand may be mainly related to the transduction of the high ethylene level during the first stages of abscission induction [12,8]. This situation may be compared to a system II-like ethylene production where increases in ethylene determine a positive feedback as previously reported in citrus [67]. The effect of ethylene on fruitlet growth and development previously suggested [8] and here confirmed is likely connected to auxin [13]. The results of the *MADSBOX* transcripts indicate these genes are in general expressed in both reproductive and vegetative parts but could be recruited for different functions as previously found [36]. Nevertheless, there are some specific domains for *MdMADS*7 and *MdPIST* in seed and *MdMADS*12 in peduncle and AZ. The results concerning *MdMADS*12 in seed were obtained by overexposure of the films and its physiological relevance outside the organs where the horthologs are generally expressed, cannot be inferred without excessive speculation. Our study was not meant to elucidate whether the MADS box isolated in apple have exactly the same physiological function of the genes closely similar in other species. Indeed, as previously found in banana [26] the horthologous genes may not have completely conserved function across species. This statement is especially valid for MADS box with a SEP motif [68]. On the other hand, the function of MADS box proteins with the AG and PIST motives are more conserved across species probably because they have undergone a limited number of duplication [69]. The fact that MdPIST transcripts were found only in seed at day 3, at day 7 transcripts were detected only by overexposure of the film, and the expression analysis in published literature [57] indicate a specific action of MdPIST during normal ovule development. MdPIST expression may be a result of a signal activating specific changes during ovule development. The results here presented also indicate that throughout abscission induction there are variations in the transcript amount of *MADS-box* genes: especially *MdMADS*5. *MdMADS*9, *MdMADS*10, MADS15 and *MdPIST*. The low transcript amount of *MdMADS*10 and *MdPIST* in AF seeds compared to NAF seeds are likely due to a total block or reduced development of the ovule and are the most interesting findings. The decrease or absence of these proteins may reduce development and make the fruitlet sensitive to competition with other fruitlets and with the vegetative part of the plant leading to abscission. Another possibility is that these genes are just related to senescence processes occurring during abscission. A further interesting possibility is that the downregulation of some MADS-box may actually lead to abscission as verified in tomato [70] and that ethylene affects seed development besides other processes such as auxin production and transport and senescence [10,13].

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