Roles of Ethylene Production and Ethylene Receptor Expression in Regulating Apple Fruitlet Abscission[1][OPEN]

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Apple (Malus × domestica) is increasingly being considered an interesting model species for studying early fruit development, during which an extremely relevant phenomenon, fruitlet abscission, may occur as a response to both endogenous and/or exogenous cues. Several studies were carried out shedding light on the main physiological and molecular events leading to the selective release of lateral fruitlets within a corymb, either occurring naturally or as a result of a thinning treatment. Several studies pointed out a clear association between a rise of ethylene biosynthetic levels in the fruitlet and its tendency to abscise. A direct mechanistic link, however, has not yet been established between this gaseous hormone and the generation of the abscission signal within the fruit. In this work, the role of ethylene during the very early stages of abscission induction was investigated in fruitlet populations with different abscission potentials due either to the natural correlative inhibitions determining the so-called physiological fruit drop or to a well-tested thinning treatment performed with the cytokinin benzyladenine. A crucial role was ascribed to the ratio between the ethylene produced by the cortex and the expression of ethylene receptor genes in the seed. This ratio would determine the final probability to abscise. A working model has been proposed consistent with the differential distribution of four receptor transcripts within the seed, which resembles a spatially progressive cell-specific immune-like mechanism evolved by apple to protect the embryo from harmful ethylene.

Vegetative and reproductive organs that are no longer needed or that are infected, damaged, or senescent may shed from the main plant body following a specific sequence of highly regulated events, known as abscission (González-Carranza et al., 1998; Taylor and Whitelaw, 2001; Estornell et al., 2013). This process relies upon a complex regulatory network, activated by the abscising organ, that leads to the activation of the abscission zones (AZs; Addicott, 1982; Zanchin et al., 1995; del Campillo and Bennett, 1996; Taylor and Whitelaw, 2001; González-Carranza et al., 2002, 2007; Lashbrook and Cai, 2008).

Fruit trees have set up a developmental strategy aimed at controlling fruit load according to nutrient availability, thus making efficient use of resources. This strategy is achieved through the so-called physiological drop or June drop, involving the abscission of young developing fruits mainly due to a correlative dominance effect of adjacent fruit and/or nearby shoots (Bangerth, 2000). This process differs from senescence-driven abscission, which consists of a developmentally programmed process occurring at or after ripening. The correlative effect is mainly transduced in nutritional terms (i.e. sugar starvation), thus generating intraorgan metabolic rearrangements and signals leading to AZ activation. The currently accepted model for correlatively driven abscission implies that auxin, produced by the subtending organ and transported through the AZ, can reduce its sensitivity to ethylene and delay its activation. Once the auxin flow through the AZ decreases or its transport is depolarized, the AZ becomes sensitive to ethylene and is activated (Dhanalakshmi et al., 2003; Blanusa et al., 2005; Meir et al., 2006, 2010). This downstream model, however, describes only the events occurring when abscission is induced, whereas it does not tell anything about how and why the auxin flow changes (i.e. the origin of the abscission signal).

Within this context, apple (Malus × domestica) was revealed to be a good model system to study the generation of the abscission signal in young

developing fruits (Botton et al., 2011; Eccher et al., 2013, 2014), as it develops corymbs with a clear gradient of correlative dominance related to the position and size of the fruit. This dominance is naturally responsible for the physiological fruit drop, which, however, is not able to guarantee high fruit quality and, on the other hand, a suitable return to flowering in the following season. Fortunately, this dominance can be magnified by means of chemical treatments, thus inducing a significantly higher rate of fruitlet abscission (Greene et al., 1992; Bangerth, 2000). Benzyladenine (BA) is a widely known chemical thinner (Bangerth, 2000; Buban, 2000) that can induce abscission in a controlled, inducible, and selective way through the enhancement of correlative inhibitions (Dal Cin et al., 2005, 2007, 2009a, 2009b; Botton et al., 2011). As a result, a model experiment with BA can provide different populations of fruitlets with clearly predictable abscission potentials: (1) small lateral fruitlets that abscise spontaneously even upon the thinning treatment (L1); (2) big lateral fruitlets that would naturally persist (L3); (3) big lateral fruitlets that abscise upon the BA treatment (LB3); and (4) big central fruitlets that would persist (C3) also upon the thinning treatment (CB3). This experimental system allowed light to be shed on the signaling pathways mediating the induction of apple fruitlet abscission, which can be summarized in a hypothetical model describing the cortex as the primary sensor of the nutritional stress occurring within the tree. In this tissue, the molecular mechanisms linking nutrient starvation to hormone signaling are consequently activated, mainly involving abscisic acid (Eccher et al., 2013) and ethylene, whose levels peak at 2 and 4 to 5 d after abscission induction, respectively. The seeds seem to perceive the situation at a later stage and with a less pronounced transcriptional and metabolic reprogramming, leading to their abortion (Botton et al., 2011). Failure of embryo development is the critical irreversible step of abscission induction (Goldschmidt and Koch, 1996; Yuan and Greene, 2000), which is somehow triggered by the primary reaction of the cortex. Since the seed is the primary source of auxin within the fruit, its abortion decreases the supply of this hormone to the AZ, leading to its activation according to the same model proposed for the leaf by Sakamoto et al. (2008).

Despite its robustness, confirmed by physiological, metabolic, and transcriptional data, this model still lacks a critical point: how is the reaction of the seed triggered? In other words, how does the cortex communicate the critical situation to the seed? Although the involvement of the gaseous hormone ethylene in apple fruitlet abscission has already been pointed out and discussed in several studies (Dal Cin et al., 2005, 2007; Botton et al., 2011), a direct mechanistic link with the physiology of this process is still missing. In this study, the relationship between ethylene and fruitlet shedding is revisited under a new perspective, pointing out a role for ethylene perception in the seed

tissues as a possible main factor involved in transducing the signal generated in the cortex. Experimental data also give some important indications about the molecular and cellular mechanisms that most likely determine the final destiny of the fruit, discriminating fruitlets destined to persist from those designated to abscise.

RESULTS

The Physiological Link between Ethylene and Apple Fruitlet Abscission

Ethylene biosynthesis was measured in the cortex of two fruitlet classes: (1) lateral fruitlets (control and BA treated) that abscise only upon a successful thinning treatment; and (2) central fruitlets (control and BA treated) that persist even upon the treatment (Fig. 1). These measurements were carried out during both a successful experiment (i.e. the level of fruit drop in treated trees was significantly higher than in controls; year 2008) and an unsuccessful one (year 2009). The former data have already been partially reported and discussed by Botton et al. (2011). However, additional information is given herein and discussed under a new perspective. Concerning the latter experiment, despite the fact that it was carried out according to the usual standards in terms of time of BA application (mean cross diameter of central fruitlets equal to 13 mm; Botton et al., 2011), the fruit drop of treated trees did not differ significantly from the controls (Supplemental Fig. S1). When the treatment succeeded, both lateral and central fruitlets that were treated with BA (LB3 and CB3) displayed, already at 16 days after petal fall (DAPF), an enhanced ethylene biosynthesis, significantly higher than that found in control samples. LB3 fruitlets, however, produced approximately 5-fold the amount of ethylene synthesized by the centrals, and, while in the centrals, ethylene biosynthesis continued to increase up to 17 DAPF and then decreased to the same levels of the control, in the laterals, it remained constant up to 17 DAPF and started to increase only thereafter, reaching its maximum peak at 21 DAPF. During the whole experimental period, all the control fruitlets showed almost stable ethylene emissions.

The situation was substantially different when the thinning treatment did not succeed. Within the first 3 d after the BA treatment, both LB3 and CB3 did not show any increased ethylene production with respect to the controls. Ethylene biosynthesis was significantly enhanced in the treated laterals only at 20 DAPF, while in the centrals the treatment was able to stimulate the emissions of this hormone transiently and only at 18 DAPF. It is noteworthy that the untreated laterals at 15 DAPF displayed very high ethylene emissions (comparable to those achieved by the treated laterals at 21 DAPF in the successful experiment), with a decreasing trend throughout the experiment.
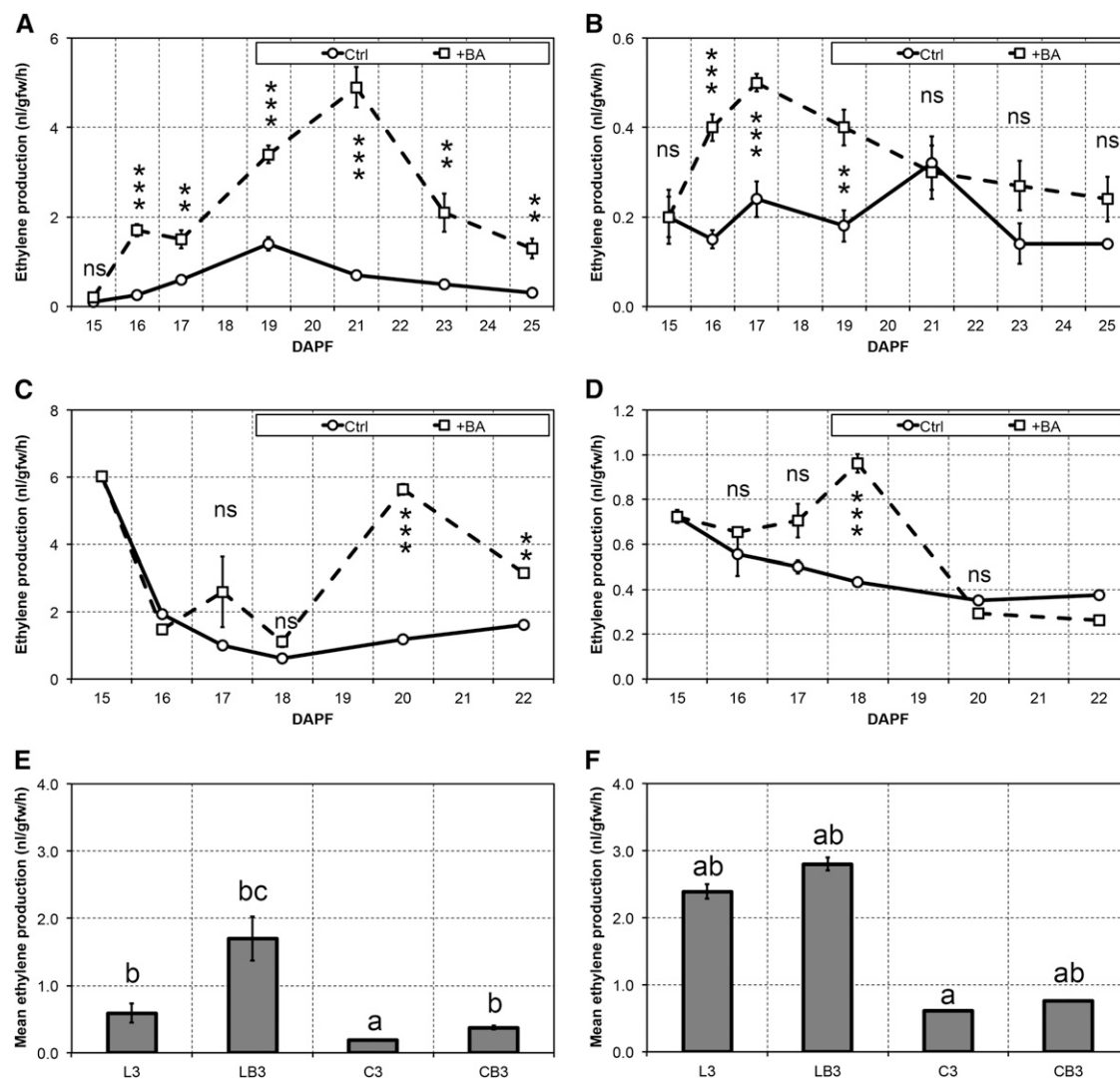
Figure 1. A to D, Ethylene production in big lateral (left) and big central (right) fruitlets measured in untreated control (Ctrl; circles, continuous lines) and BA-treated (+BA; squares, dashed lines) samples in a successful (A and B) and in an unsuccessful (C and D) experiment. Statistically significant differences are indicated: ***, $P < 0.001$; and **, $P < 0.01$. Error bars represent SE ($n = 4$). ns, Nonsignificant. E and F, Mean ethylene production as measured during abscission induction in a successful (E) and in an unsuccessful (F) trial. Error bars represent SE ($n = 16$ in E), whereas letters indicate significant differences as identified by the Waller-Duncan test ($P < 0.05$).

Considering the average amount of ethylene emitted by the different samples during the abscission induction period (i.e. within 3–4 d after the treatment), it is worthy to note that, when the thinning treatment was successful (Fig. 1E), an approximately 3-fold increase of mean ethylene emission ($P \leq 0.05$) was observed in the BA-treated laterals that will abscise. Similarly, BA-treated centrals of the same experiment displayed a highly significant ($P \leq 0.01$) 2-fold increase, although maintaining lower levels of ethylene production than the persisting laterals (i.e. the controls), consistent with their destiny. These differences, both in terms of entity and statistical significance, were not observed in the failed experiment (Fig. 1F). In particular, L3 and LB3

fruitlets showed similar mean ethylene emissions during the induction period, while the CB3 fruitlets displayed a significant increase of the biosynthetic rate of this hormone (approximately +25%), although not comparable to that observed in the previous experiment (approximately +95%).

Variations of the Ethylene-Related Transcriptome during Abscission Induction

Ninety-four genes encoding different elements of ethylene biosynthesis, perception, and signal transduction were identified using a BLASTP-validated

Hidden Markov Models approach carried out on the apple genome version 1.0 (see Supplemental Tables S1 and S2). Detailed descriptions of both the rationale on which gene selection was based and the identification pipeline are given as Supplemental Text S1 and Supplemental Figures S2 to S11.

The amount of gene transcripts was then measured by quantitative PCR (qPCR) in the cortex and seeds excised from lateral and central fruitlets sampled during the abscission induction (15–19 DAPF) in the successful experiment (year 2008). Genes that were not expressed and those with either unreliable expression levels among the replicates or too close to the detection limit were discarded from the following analyses, giving a final list of 64 targets (Supplemental Table S3). Principal component analysis (PCA) was performed separately for cortex and seed on a selection of the most variable ones (52 genes), chosen according to their coefficient of variation among the samples, in order to avoid the background noise.

The PCA scores of the cortex based upon the first two principal components (Fig. 2) revealed that, upon abscission induction, the ethylene-related transcriptome of big lateral fruitlets (LB3) followed a pathway that substantially overlapped that of the small laterals undergoing natural abscission (L1). Abscising fruitlets showed an increase of both principal components from 15 to 16 DAPF, followed by a decrease of PC1 concurrent with a further increase of PC2 up to 17 DAPF. Then, from 17 to 19 DAPF, PC1 continued to decrease, whereas PC2 stayed almost stable. All the persisting fruitlets, including the BA-treated centrals, were closely grouped and did not show any significant and/or continuous and/or treatment-dependent variation of their ethylene-related transcripts. The first two principal components accounted for 48.7% of the total variance, with 35.7% explained by PC1 and 13% by PC2. An increase up to 60% of total variance could be achieved by including PC3, at the expense of the overall readability of the chart and without giving any additional information. Examination of the loadings (Fig. 3) indicated that the differences explained by the PCA were due to equal contributions of genes belonging to any of the three categories (i.e. biosynthesis, perception, and signal transduction). Moreover, the tendency to abscise was largely accompanied by a

Figure 2. PCA of ethylene-related genes. Gene expression was measured by qPCR in the following samples: L10 to L13 (small lateral fruitlets at 15–17 and 19 DAPF); L30 to L33 (big lateral fruitlets at 15–17 and 19 DAPF); LB31 to LB33 (BA-treated big lateral fruitlets at 16, 17, and 19 DAPF); C30 to C33 (big central fruitlets at 15–17 and 19 DAPF); and CB31 to CB33 (BA-treated big central fruitlets at 16, 17, and 19 DAPF). A, PCA scores plot of the first two principal components (PC1 and PC2) explaining 48.7% of the total variance in the cortex. Arrows indicate the path followed by naturally abscising fruitlets (na) and those abscising because of the BA treatment (+ba). B, PCA scores plot of the first two principal components explaining 58.3% of the total variance in the seed. Arrows indicate the early reaction of the ethylene-related transcriptome in the naturally abscising fruitlets and in those treated with BA.
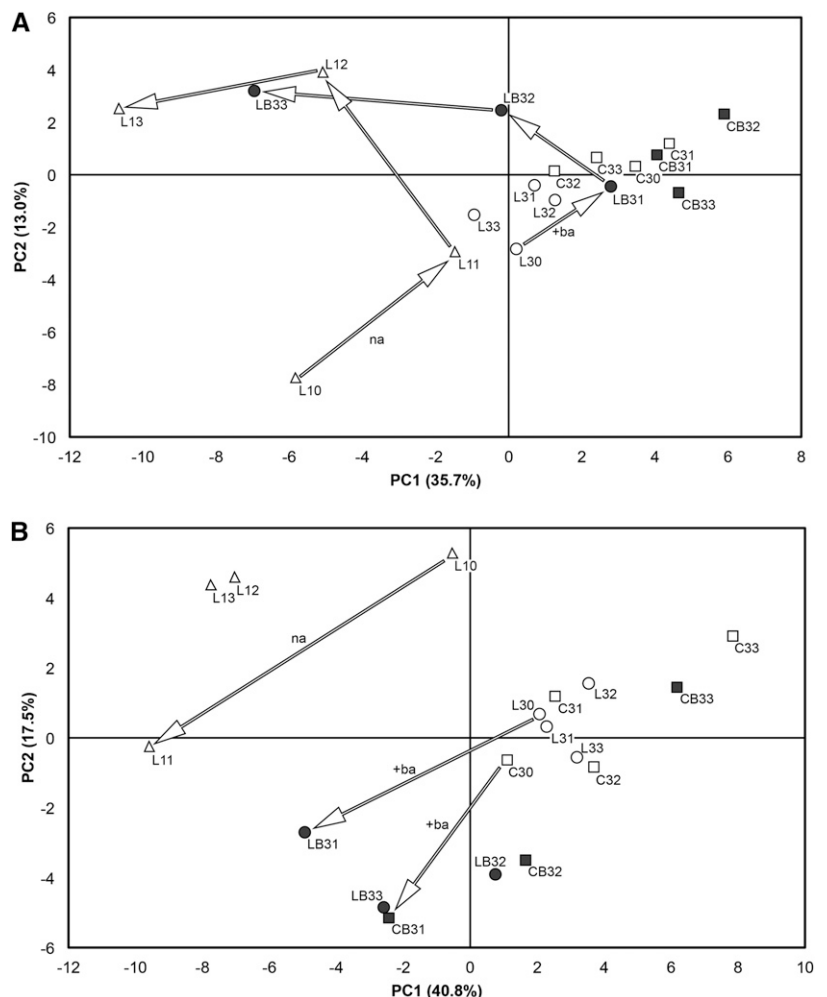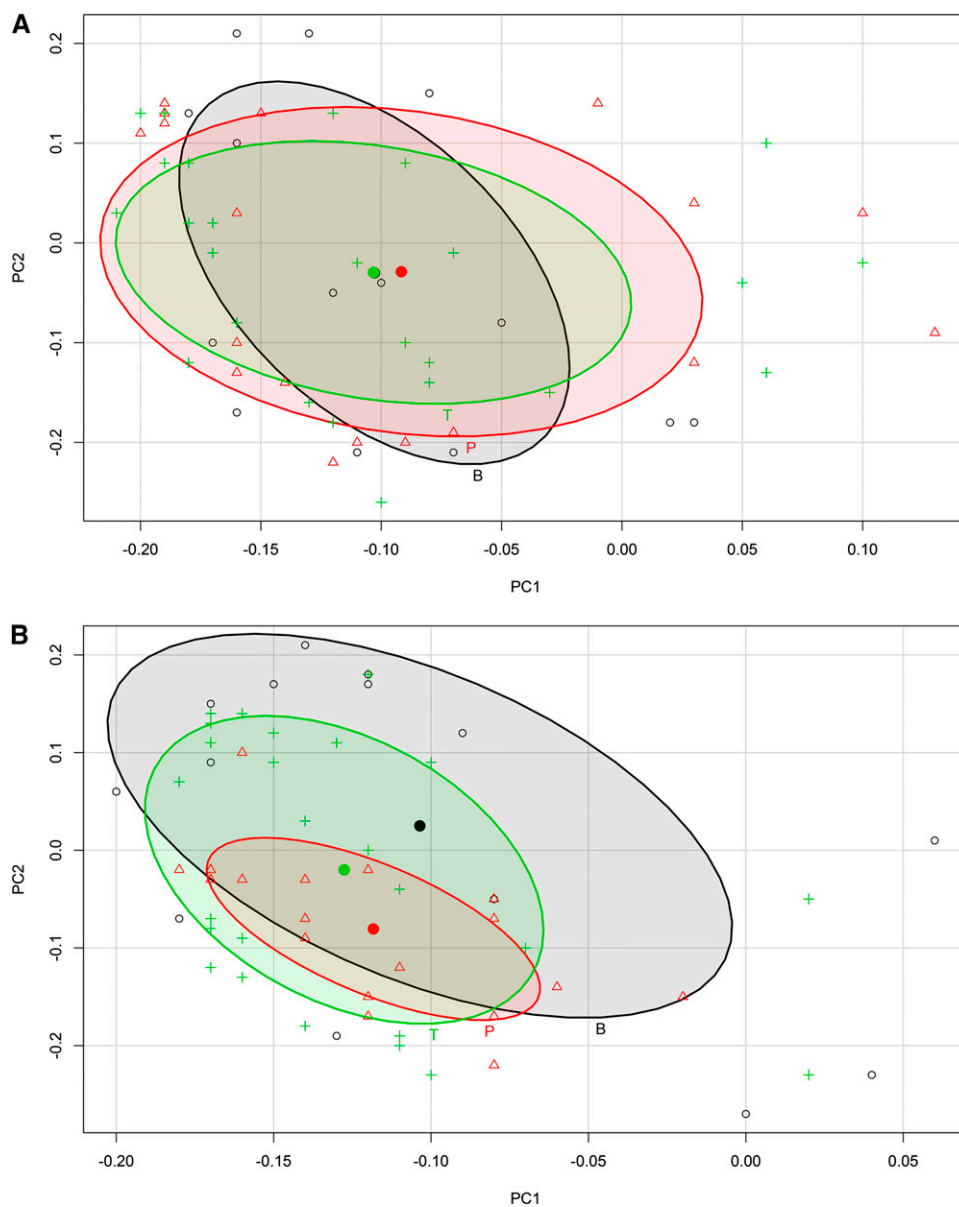
Figure 3. PCA loadings plot. The loadings of cortex (A) and seed (B) are indexed according to three categories: ethylene biosynthesis (B; black circles), perception (P; red triangles), and signal transduction (T; green crosses). Data ellipses and their centers are also shown with a confidence interval of 50%. The colors are the same as for the loading symbols described above.

general increase of all the ethylene-related transcripts, as pointed out by the colocalization given by the loadings with respect to the samples as shown by the PCA scores.

A different behavior was displayed by the seeds, as shown by PCA scores of the first two principal components (Fig. 2). First, the overall variations in terms of gene expression differed between the samples undergoing natural abscission and those induced to shed by the BA treatment. In this case, moreover, the ethylene-related transcripts behaved similarly in the BA-treated laterals and centrals, although only during the early abscission induction. A clear differentiation was displayed thereafter, in that the CB3 at 19 DAPF returned to be consistently grouped with the nonabscising fruitlets, while the LB3 at the same time

point remained distant from the persisting samples, although not colocalizing with the naturally abscising ones (L1). Also in this case, the persisting fruitlets, including the BA-treated centrals at 19 DAPF, were closely grouped and did not show any significant continuous or treatment-dependent variation of their ethylene-related transcripts. The first two principal components accounted for 58.3% of the total variance, with 40.8% explained by PC1 and 17.5% by PC2. Also in this case, including PC3 would not significantly affect the final result but only increase the overall explained variance up to 66% of the total. The loadings plot (Fig. 3) indicated that the different behaviors shown by the seeds of the different fruitlet classes could be ascribed to diverse components of the ethylene-related transcriptome. On the one hand, the

variations observed in the seeds of the naturally abscising fruitlets were largely determined by genes encoding biosynthetic elements, while on the other hand, LB3 and CB3 fruitlets displayed variations of the expression of genes coding for ethylene perception components.

Expression of Ethylene Perception Genes

The overall analysis of the ethylene-related transcriptome in the seed pointed out that (1) BA-induced abscission may determine a different transcriptional reprogramming with respect to natural fruit shedding; (2) the BA treatment induced similar transcriptomic reactions, at least as far as the ethylene-related genes, in lateral and central fruitlets during early abscission induction; and (3) the common reactions in the two fruitlet classes, mainly due to ethylene perception elements, determined a different physiological outcome (i.e. the LB3 abscised, while the CB3 persisted).

Based upon these basic observations, attention was focused on seven genes encoding ethylene receptors: *Apple ETHYLENE RESPONSE SENSOR1* (*MdERS1*; MDP0000288468), *MdERS2* (MDP0000242413), *Apple ETHYLENE RESISTANT1* (*MdETR1*; MDP0000234442), *MdETR102* (MDP0000920189), *MdETR105* (MDP0000168345), *MdETR2* (MDP0000393617), and *MdETR5* (MDP0000231172), as well as on *Apple CONSTITUTIVE TRIPLE RESPONSE1* (*MdCTR1*; MDP0000230308). Among the receptor-coding genes, *MdETR1, MdETR5, MdETR102,* and *MdETR2*, in this order, showed the highest expression levels in the seeds (Fig. 4). Their expression patterns well represent the situation highlighted by the PCA. In particular, all four of these genes were overexpressed with respect to the control in the seeds of BA-treated fruitlets at 16 DAPF (i.e. 24 h after the thinning treatment). In the following days, they remained substantially up-regulated, although to lower extents, with the only exception of *MdETR5* in the centrals. It is noteworthy that this gene even reversed its expression, showing higher transcript levels in the control seeds than in the BA-treated ones. It is also worthy to note that, in LB3, the genes with lower expression levels also were in general up-regulated, including two *ERS* genes. Concerning *MdCTR1*, only a slight, but not significant, up-regulation was observed at 16 DAPF, higher in LB3 than in CB3; no

difference was pointed out thereafter between the two fruitlet classes.

In Situ Localization of *ETR1*, *ETR2*, *ETR5*, and *ETR102* Transcripts in the Seed

The seed encloses a series of complex structures, made up of different cell types of either maternal or filial origin. Therefore, a deep insight into the distribution within the different seed tissues and structures of the ethylene receptor transcripts may help in ascribing a functional meaning to the up-regulation of these genes during abscission induction. For this reason, in situ hybridizations were carried out with gene-specific probes (Supplemental Fig. S12) to detect the transcripts of *MdETR1*, *MdETR2*, *MdETR5*, and *MdETR102*.

Two main expression domains were pointed out by these analyses: (1) filial tissues (i.e. embryo and endosperm), and (2) maternal tissues (i.e. nucellus and inner integument). At the time when the experiments were carried out, the embryo was at the globular stage, while the endosperm was showing different degrees of cellularization, starting from the chalazal side toward the embryo. *MdETR1* transcripts were mainly localized at the level of the first domain with a very strong and unequivocal signal in the embryo and in most endosperm cells (Fig. 5). Moreover, a weak but distinguishable hybridization was detectable also in the nucellus cells. *MdETR2* showed a similar expression pattern (i.e. embryo and endosperm), although with weaker hybridization signal than *MdETR1*, consistent with its lower expression levels as detected by qPCR. In the different replicates, however, the extension of the signal of *MdETR2* hybridization in the endosperm seemed more restricted to the cells at the chalazal side (data not shown), which are those resulting from earlier events of cellularization. Taken as a whole, *MdETR1* and *MdETR2* transcripts mainly showed filial tissue localizations. On the other hand, *MdETR102* and *MdETR5* transcripts were mainly localized in maternal tissues (Fig. 6), although also in this case with a specific pattern. *MdETR102* signal was weakly detectable in the small and compact cells of the embryo and undetectable in the endosperm, whereas its transcripts were clearly visible in the nucellus cells, with a positive gradient toward the outer layers and a visible signal in

Figure 4. Heat map showing the expression (as a log ratio) of eight selected genes during abscission induction (15–17 and 19 DAPF) in the seeds of BA-treated fruitlets with respect to their control ones (LB3/L3, BA-treated versus control big laterals; CB3/C3, BA-treated versus control big centrals). Mean expression levels are also shown with a white-blue color scale separately for each fruitlet class. The gene name and identifier are reported.

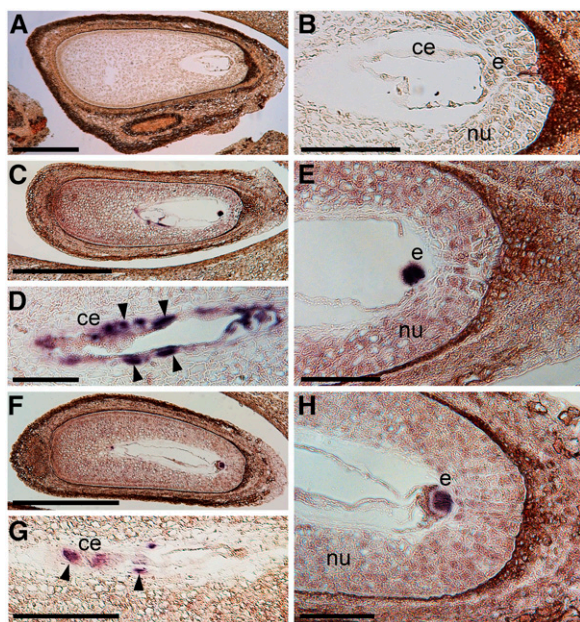|	LB3/L3					CB3/C3				
DAPF	15	16	17	19	Mean	15	16	17	19	Mean
MdERS1 (MDP0000288468)										
MdERS2 (MDP0000242413)										
MdETR1 (MDP0000234442)										
MdETR102 (MDP0000920189)										
MdETR105 (MDP0000168345)										
MdETR2 (MDP0000393617)										
MdETR5 (MDP0000231172)										
MdCTR1 (MDP0000230308)										

Figure 5. In situ hybridization analyses of *MdETR1* (C–E) and *MdETR2* (F–H) expression in apple seeds collected from persisting fruitlets during the abscission induction period (17 DAPF). Longitudinal sections of the seeds were hybridized with gene-specific antisense digoxigenin-labeled probes. A hybridization without probe was used as a negative control (A and B) in order to discern the specificity of the signal in terms of color and intensity, especially at the level of the embryo and endosperm. In the latter, arrowheads indicate the strongest signals. ce, Cellularized endosperm; e, embryo; nu, nucellus. Bars = 500 μm (A, C, and F), 200 μm (B and G), and 100 μm (D, E, and H).

the cells of the inner integument. Finally, *MdETR5* was mainly localized in the inner integument, with only weak signals in the nucellus and in filial tissues. Its localization in the inner integument was marked especially at the chalazal side, although it was visible also around the micropyle, where cells are less compact and thus may bias the signal detection.

DISCUSSION

The role of ethylene in different abscission processes has long been investigated in diverse plant model and crop species, although most of these studies were focused on the involvement of this hormone at the level of the AZ, where only the last steps of organ shedding take place (Patterson and Bleecker, 2004; Butenko et al., 2006; Meir et al., 2010; Bar-Dror et al., 2011; Sawicki et al., 2015). Taken together, the available information indicates two anatomical layers of ethylene functional involvement: (1) within the AZ, and (2) within the abscising organ. An organ may undergo shedding because of different cues, either developmentally programmed or as a response to particular physiological contexts, such as ongoing abiotic and/or biotic stress. However, although the context in which

abscission occurs may vary, it can be hypothesized that one or more signals/signal cascades always start from the abscising organ to finally communicate to the AZ that the process is induced.

As far as apple fruitlet abscission is concerned, recent studies were able to shed light, at least in part, on the molecular cascades occurring in the cortex and seed during the early inductive phases of the process upon a chemical thinning induction (Botton et al., 2011; Eccher et al., 2013). The current model describes the early reaction of the cortex of abscising fruitlets to abscission induction mainly in terms of (1) enhanced ethylene biosynthesis, (2) increased abscisic acid production, and (3) increased reactive oxygen species production. Then, later on, the seed would react in terms of (1) ethylene signaling, (2) reactive oxygen species signaling, (3) block of embryogenesis, and (4) a likely block of auxin biosynthesis. Consequently, the reduced supply of auxin to the AZ concurrent with a likely depolarization of its transport would enhance its sensitivity to ethylene and the consequent activation of cell wall-degrading enzymes (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001).

But how does the seed perceive the situation? Does the cortex communicate with the seed? If yes, how? Again, what determines the different responses observed in lateral versus central fruitlets?
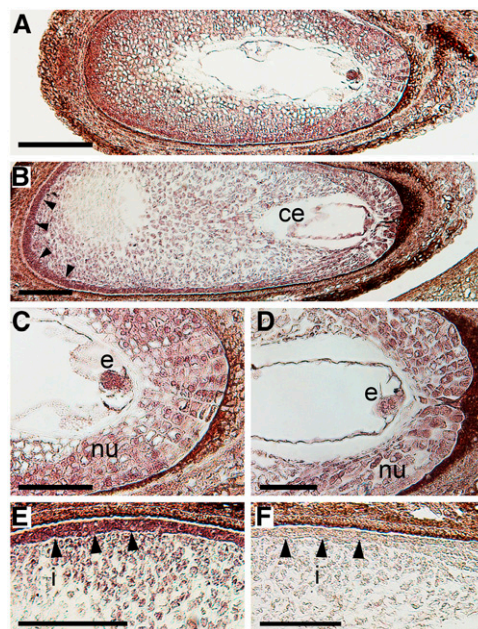

Figure 6. In situ hybridization analyses of *MdETR102* (A and C) and *MdETR5* (B, D, and E) expression in apple seeds collected from persisting fruitlets during the abscission induction period (17 DAPF). Longitudinal sections of the seeds were hybridized with gene-specific antisense digoxigenin-labeled probes. A hybridization without probe is shown as a negative control (F) in order to discern the specificity of the signal in terms of color and intensity. Arrowheads indicate the inner integument. ce, Cellularized endosperm; e, embryo; i, inner integument; nu, nucellus. Bars = 200 μm (A and B) and 100 μm (C–F).

The relationship between ethylene and apple fruitlet abscission has long been discussed (Dennis, 2002), and the role of this hormone is still controversial. The data presented here, however, strengthen the direct relationship existing between ethylene and apple fruitlet abscission, although under a different perspective with respect to previous studies. First, an early (i.e. within 24 h from the thinning treatment) stimulation of ethylene biosynthesis (Fig. 1) seems a conditio sine qua non for abscission to occur. Actually, the importance of the first 24 h was already inferred (Botton et al., 2010), although indirectly and based upon limited evidence. Moreover, the peak of ethylene at 5 d after the thinning treatment in fruitlets from BA-treated trees was observed also in 2009 (i.e. the unsuccessful experiment), but in that case, it was neither preceded by an early increase of its biosynthesis nor followed by an enhanced abscission rate. In addition, the fact that the BA-treated central fruitlets that also will persist in the successful experiment do show an enhanced production of the hormone indicates that, although indispensable, this early increase itself is not sufficient for abscission to occur, as stated previously (Dennis, 1987), and that it is rather the degree of amplification of ethylene biosynthesis with respect to the basal levels at the time of the thinning treatment (Fig. 1, E and F) that most likely determines a successful abscission induction, as inferred previously (Botton et al., 2010). Summarizing, two strictly related conditions are required for a successful thinning with BA: (1) stable basal ethylene biosynthesis at the time of the treatment, and (2) its early stimulation upon the BA treatment. The strict relationship between these two conditions may depend upon an immediate availability of ethylene precursors and, thus, on the level of ethylene biosynthesis at the time of the treatment. In fact, when the thinning experiment did not succeed, both central and lateral fruitlets were already showing high ethylene emissions at 15 DAPF, leading us to hypothesize that an early increase of its production could not be achieved due to the limited availability of its precursors. Later on, a stimulation of the upstream biosynthetic steps may have allowed the increased ethylene production observed at 20 DAPF in lateral fruitlets.

Ethylene biosynthesis was shown previously to depend on temperature, especially at the level of 1-aminocyclopropane-1-carboxylate synthase activity, often with an optimum within the range of 20°C to 25°C (Burg and Thimann, 1959; Field, 1985; Biggs et al., 1988; Atta-Aly, 1992). Nevertheless, it is known to decline just after fruit set in several species (Vriezen et al., 2008; Wang et al., 2009; Martínez et al., 2013), most likely along with the availability of its immediate precursor 1-aminocyclopropane-1-carboxylate (A. Botton, unpublished data). According to the data provided by the meteorological station located in the experimental orchards, during the 5 d preceding the thinning treatment (10–14 DAPF), mean and maximum temperatures in 2009 were 3.3°C (11.2°C versus 14.5°C) and 6.2°C (15°C versus 21.2°C) lower than in 2008, respectively. Additionally, total rainfalls during the same period were 101.4 mm in 2009 versus 7.3 mm in 2008. Such conditions may have affected photosynthesis, thus causing an enhanced limitation of assimilates and a partial block/slowdown of fruit development. Since BA efficacy relies mainly upon an active competition for assimilates between central and lateral fruitlets and between fruitlets and shoots, the thinning treatment performed in nonoptimal conditions of fruit growth was ineffective. A possible physiological explanation for the high production of ethylene observed at 15 DAPF and the following progressive drop of its biosynthesis observed thereafter, when mean temperatures recovered to normal values (23.2°C), in untreated fruit of 2009 could be related to two main reasons: (1) the rise in mean and maximum temperatures that occurred from 14 to 15 to 18 DAPF allowed a rapid recovery of ethylene biosynthetic rates at 15 DAPF, due to a suddenly wide availability of precursors that were not consumed before, and (2) a greater receptor degradation rate, as observed recently by Shakeel et al. (2015) at elevated temperatures, which may have caused a higher ethylene sensitivity of the whole system at 15 DAPF (both in laterals and centrals), in turn causing a homeostatic reaction and a decreased ethylene biosynthesis. Within this context, the BA treatment, besides being performed under nonoptimal fruitlet growth conditions, could not induce an immediate rise of ethylene biosynthesis because of the shortage of precursors, whereas thereafter, from 18 to 20 DAPF, a transient burst of ethylene biosynthesis was allowed by their increased availability.

Expression analyses carried out on ethylene-related genes in the cortex indicate that a successful chemically induced abscission (i.e. in LB3) determined a series of transcriptional events closely similar to those occurring during natural abscission (i.e. in L1), whose synchronism was due to the perfect timing chosen for the thinning treatment (see "Materials and Methods"; Botton et al., 2011). The early burst of ethylene observed in LB3 was associated with an increase of both the principal components (Fig. 2A), which in turn was negatively correlated to most of the genes analyzed (Supplemental Table S4). This would mean that this burst, achieved within the first 24 h also in persisting central fruitlets, was not accompanied by a transcriptional activation of ethylene-related genes but rather was due to an enhanced activity of rate-limiting ethylene biosynthetic enzymes and a concurrent sufficient availability of precursors. A general down-regulation was observed for all three gene categories (biosynthesis, perception, and signal transduction) at 16 DAPF. Among the down-regulated genes, those encoding elements of ethylene perception (i.e. the receptors) may play a relevant role in determining the following events and responses. Fewer receptors means more sensitivity to the hormone (Chang et al., 1993; Cancel and Larsen, 2002; Kevany and Klee, 2007). Therefore, during this early inductive phase, ethylene produced

by the cortex most likely saturated the receptors (i.e. was perceived) and determined the response that was observed thereafter, including the transcriptional activation of ethylene-responsive genes and the strong increase of biosynthesis, with a peak at 21 DAPF, observed in the laterals.

In spite of the initial increase of ethylene production, the cortex of BA-treated central fruitlets did not show any significant transcriptional rearrangement of the considered genes, behaving similarly to untreated fruitlets (L3 and C3). This may be explained in part by observations carried out on the seeds, whose early response was exactly the opposite of that shown by the cortex. Indeed, as a general trend, the ethylene-related transcriptome of the seeds was up-regulated within the first 24 h, with BA-treated fruitlets, both laterals and centrals, showing closely similar variations. Despite these similarities, the final outcome is known to be completely divergent, as confirmed by the different PCA scores at 21 DAPF of the BA-treated laterals (LB33) and the BA-treated centrals (CB33), the latter

being positioned among the persisting fruitlets. The loading plots (Fig. 3) as well as the correlation coefficients (Supplemental Table S5) indicate that, while the cortex of abscising fruitlets reacted by coordinating the whole ethylene-related transcriptome (i.e. the ethylene was likely synthesized, perceived, and its response triggered), the seeds of treated fruits, especially the centrals, mainly reacted in terms of ethylene perception genes, without activating the large transcriptional response observed in the cortex. This would indicate that the seeds may have perceived the ethylene produced by the cortex, at higher levels in LB3 than in CB3, within the first 24 h and then responded by selectively up-regulating the receptor genes, more strongly in CB3 than in LB3, as shown by PCA and loading plots taken together. This would resemble a homeostatic reaction aimed at restoring the normal situation. The up-regulation of ethylene receptor genes (Fig. 4), in particular *MdETR1*, *MdETR5*, *MdETR102*, and *MdETR2*, was stronger and longer in seeds of laterals, while *MdETR5* was even down-regulated in
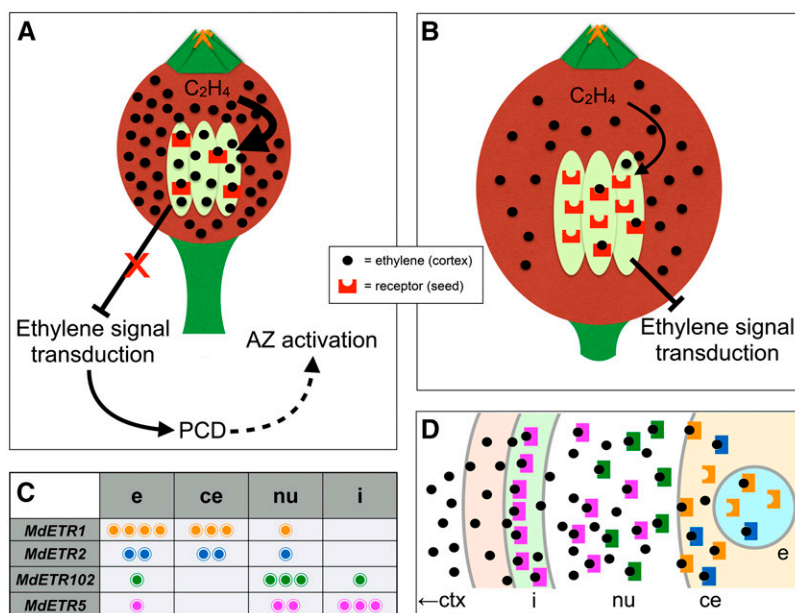

Figure 7. Hypothetical model for the ethylene receptor-based defense mechanism adopted by the seeds of central fruitlets to prevent them from abscising. In the lateral fruitlets that were stimulated to shed by BA (A), the high amount of ethylene produced by the cortex can saturate the receptors and thus trigger the following signal transduction pathway leading to programmed cell death (PCD), seed abortion, and AZ activation. In the central fruitlets (B), instead, the proportion between ethylene biosynthesis and the expression of ethylene receptor genes is more in favor of the latter. Therefore, since the low levels of ethylene production stimulated by the thinning treatment cannot saturate the receptors, the signaling of this hormone remains blocked and abscission does not occur. Within the seed, four ethylene receptor genes are mainly expressed: *MdETR1*, *MdETR2*, *MdETR102*, and *MdETR5*. Their transcripts are differentially distributed within the seed's tissues, as shown in the table (C). ce, Cellularized endosperm; e, embryo; i, inner integument; nu, nucellus. These different expression domains (D) may lead one to think about a spatially progressive interception of ethylene by the different receptors at different cell layers. The ethylene produced by the cortex (ctx) also diffuses through the inner tissues toward the seeds, but once it enters the seed, it is first intercepted by the receptors being expressed at the level of the inner integument, then by those expressed in the nucellus cells, followed by those present in the cellularized endosperm, and, finally, by those expressed by the embryo. In this way, the amount of ethylene is progressively depleted, so that the receptors cannot be fully saturated, especially at the level of the embryo, and not leading to seed abortion such as in the lateral fruitlets.

the centrals already at 17 DAPF. Taken as a whole, these results indicate a different response of CB3 seeds with respect to LB3. Considering the final outcome, in the centrals the attempted homeostatic recovery was successful, while in the laterals it was not. This may be due to the lower sensitivity to ethylene achieved in seeds of central fruitlets by up-regulating the expression of its receptor genes. A similar mechanism was found to be involved in female cucumber (*Cucumis sativus*) flower development (Wang et al., 2010). In that case, the down-regulation of *ETR1* and the concurrent enhancement of ethylene sensitivity were shown to promote DNA damage specifically in primordial anthers, resulting in female flowers whose development was paralleled by a reduced sensitivity to the hormone caused by higher levels of its receptors. Nucleic acid degradation was also observed in the seeds of naturally abscising fruitlets, as indirectly shown by the progressively lower yields of RNA that could be purified from those samples already at 16 DAPF (data not shown).

According to this interpretation, ethylene produced by the cortex functions as an alarm signal, which is perceived by the seeds of both laterals and centrals but may lead to different outcomes according to the ratio between receptors and ethylene levels, which is higher in centrals than in laterals. The lower the amount of receptors, the higher the ethylene sensitivity (i.e. receptors are saturated; LB3) and, consequently, its downstream effects (i.e. seed abortion). On the contrary, the higher the amount of receptors, the lower the sensitivity to the hormone (CB3), and since the receptors are not fully saturated by the lower levels of ethylene produced by central fruitlets, the ethylene signaling remains blocked, the seed survives, and abscission does not occur (Fig. 7). Actually, from a mechanistic point of view, the ethylene perception is governed by a very complex interplay among ethylene, its receptors, and CTR1, given by differential kinetics and rates of transcription, protein turnover, interactions, and subcellular localization of the players involved. The article recently published by Shakeel et al. (2015) sheds light on most of these aspects and proposes a model for Arabidopsis (*Arabidopsis thaliana*) that partially overlaps with the findings presented here. Although the internal concentration of ethylene in the fruitlets was not assessed, the two physiological contexts of lateral and central fruitlets may be fairly positioned within the model of Shakeel et al. (2015) as follows. (1) The seeds of central fruitlets were perceiving, already before the thinning treatment, the low amount of basal ethylene produced by the cortex; such low levels were most likely counterbalanced in terms of sensitivity by suitable transcription rates of ethylene receptor genes and *CTR1* and a relatively low receptor protein turnover. Upon the BA treatment, these seeds immediately perceived the increased ethylene production, which transiently up-regulated the receptor genes, thus lowering ethylene sensitivity; once the ethylene wave decreased, the system recovered to the normal sensitivity. (2) The seeds of the laterals were closer to the one-way threshold, as they produced more ethylene and more receptors than the centrals, with a likely higher rate of receptor degradation; however, ethylene sensitivity was kept low enough to prevent a harmful response. The BA treatment leads to a strong up-regulation of receptors due to the high amounts of ethylene produced but also a likely high turnover of the respective proteins; consequently, ethylene sensitivity is enhanced and a response is achieved, leading to seed abortion.

The different expression domains of the four up-regulated ethylene receptor genes (Figs. 5 and 6) give some additional hints about the importance of this mechanism and explain, at least in part, the early down-regulation observed for *MdETR5* in seeds of CB3. First, it is worthy to note that not only the maternal tissues are involved in this reaction. The specific localization of *MdETR1* and *MdETR2* transcripts at the level of the embryo and endosperm, both filial tissues, further strengthens the indispensability of this defense mechanism, whose final aim is to protect the embryo from the potential damage caused by ethylene, at least at this stage of seed development. Embryos with altered expression levels of these genes may undergo abortion as a selective strategy. A higher level of protection is achieved by expressing specific ethylene receptors also in the outermost cell layers, especially *MdETR5*, localized in cells of the inner integument. Also, the gradient-like localization of *MdETR102* seems to resemble a progressive barrier toward ethylene, aimed at intercepting as much hormone as possible and as soon as possible, to prevent it from reaching the embryo (Fig. 7D). The success of this strategy is proved by the fact that, once the ethylene wave passed, *MdETR5* immediately returned to its expression levels. More importantly, the central fruitlets persist.

CONCLUSION

The chemical nature of ethylene and its state allow it to work as the perfect mobile signal. Once again, this hormone was shown to take advantage of these features, functioning as an efficient way to exchange physiological information between different organs/tissues/cells. In this study, ethylene was shown to link the destiny of the whole fruit to that of the seed, by putting in communication the cortex with the seed, the maternal tissues with filial tissues (i.e. embryo and endosperm), thus determining a relevant developmental outcome, whether persistence or abscission. The destiny of the fruit may thus depend on the balance between ethylene biosynthesis in the cortex and the number of receptors in the seed, which in turn depends on the developmental phase resulting from the position of the fruitlet within the cluster. This occurs within a short temporal window, during which the nutritional stress, either natural or induced,

triggers the whole mechanism. At this point, it would be important to shed light on the regulatory background on which a certain developmental stage relies, at least within the thinning window, in order to integrate the proposed model into the overall model of fruit development. For example, the role of auxin and its synergism with ethylene would deserve more attention.

For the first time, to our knowledge, the differential localization of four ethylene receptor gene transcripts was reported in seed tissues within the context of a well-defined physiological process (i.e. apple fruitlet abscission). A working model has been proposed consistently with their differential distribution within the seed, which resembles a spatially progressive cell-specific immune-like mechanism evolved by apple to protect the embryo from harmful ethylene action. A validation of this model in other apple cultivars (with different self-thinning behaviors) and species is necessary in order to determine if it represents an apple-specific mechanism or a conserved strategy aimed at selecting the fruits having the highest probability to survive until the end of development. Further confirmations can also be obtained by quantifying the number of receptor proteins, although it requires a high number of starting samples (i.e. seeds), which are difficult to obtain especially from abscising fruitlets.

Once this model is fully validated and/or integrated with additional evidence, it may allow the setting up of species-specific and, hopefully, cultivar-specific thinning strategies able to finely tune fruit load at the desired level.

MATERIALS AND METHODS

Plant Material and Measurements

Experiments were conducted in 2008 and 2009 on 8-year-old apple (*Malus* × *domestica* 'Golden Delicious'/'M9') trees trained with standard horticultural practices at the experimental farm Maso Part (Mezzolombardo) of the Istituto Agrario San Michele all'Adige, Edmund Mach Foundation.

A randomized block design was adopted in all experiments, with four blocks, each including five trees, for each experimental thesis. Each block represented a biological replicate. Chemical thinning was performed by spraying BA at 200 μL L^{-1} (Brancher Dirado; Agrimport) when fruits had an average size of 13 mm (approximately 15 DAPF). Fruit drop was monitored daily up to the conclusion of June drop (i.e. the physiological fruitlet drop) to establish the effect and success of the treatment. A more detailed description of the experimental strategy can be found in Botton et al. (2011). Cortex and seeds of each biological replicate were collected from a suitable number of fruitlets (varying from 10 to 15, according to their size) of the following classes: L1, small lateral fruitlets undergoing naturally induced abscission; L3, big lateral fruitlets that would naturally persist; LB3, big lateral fruitlets from trees treated with BA that abscise; C3, big central fruitlets that would naturally persist; CB3, big central fruitlets from trees treated with BA that persist. All samples were immediately frozen in liquid nitrogen and stored at −80°C for later molecular analyses.

The laser-based photoacoustic detection of ethylene was carried out on four biological replicates using a previously described experimental device (Harren et al., 1990a, 1990b; Harren and Reuss, 1997) as reported by Dal Cin et al. (2005, 2007). A suitable number of fruitlets, varying from 10 to 15 (according to their size), were assessed for each biological replicate.

RNA Isolation and qPCR

For qPCR analyses, total RNA was extracted in 1 or 10 mL of extraction buffer from 0.02 g of seeds or 0.6 g of cortex following the method of Ruperti et al. (2001), with a few adaptations as described by Botton et al. (2009, 2011). Total RNA was quantified with the NanoDrop 2000c spectrophotometer (Thermo Scientific), and its integrity was checked by running 1 μg on a 1% (w/v) agarose gel stained with SYBR Safe (Life Technologies).

Complementary DNA was synthesized with the SuperScript VILO cDNA Synthesis Kit (Life Technologies) from 1 μg of DNA-free total RNA in a final volume of 40 μL, according to the instructions provided by the manufacturer. The reaction was performed in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems).

Real-time PCR relative quantification was performed in triplicate on two biological replicates, as described by Botton et al. (2011). The nucleotide sequences of the primers for both the target and reference genes are reported in Supplemental Table S3. Data were acquired, elaborated, and exported with the StepOne Software version 2.1 (Applied Biosystems), whereas all the final calculations were made with the automated Excel spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta cycle threshold method suggested by Pfaffl (2001). Gene expression values in the seed were normalized to *Md18S* (Dal Cin et al., 2005) as described by Botton et al. (2011), while those of the cortex were normalized to three housekeeping genes (*MDP0000375455*, putatively encoding for a Leu-rich repeat protein kinase; *MDP0000767855*, encoding for a Δ^1-pyrroline-5-carboxylate dehydrogenase, GenBank accession no. ACL13550; and *MdUBIQUITIN*, GenBank accession no. DQ438989, the same used by Dal Cin et al. [2005]) as described by Eccher et al. (2013). Expression levels were then reported as arbitrary units of mean normalized expression, calculated using Equation 2 of the Q-Gene spreadsheet.

Plant Fixation, Embedding, and in Situ Hybridization

Seeds were excised from persisting lateral fruitlets (sampled at 16 DAPF) along with the surrounding ovary tissues, fixed, and embedded as described by Canãs et al. (1994). Longitudinal sections of 7 μm were hybridized with sense *MdETR1*, *MdETR2*, *MdETR5*, and *MdETR102* RNA probes labeled with digoxigenin-11-UTP using T3 polymerase following the protocol of the manufacturer (Roche). Gene-specific probes were obtained by PCR carried out on fruitlet complementary DNA using the primers listed in Supplemental Table S6, designed on nonconserved coding regions of each gene (Supplemental Fig. S1). All in situ hybridization steps, with the exception of staining, were carried out using the Gene Paint suite accessories (Freedom EVO100; Tecan) as described by Begheldo et al. (2013). The signal was developed with detection buffer containing nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche) following the manufacturer's instructions. Sections were mounted in 50% (v/v) glycerol and observed with an Olympus BX50 microscope (Olympus) equipped with differential interference contrast optics. Images were captured with an MRc5 Axiocam color camera (Carl Zeiss) and processed with Photoshop (Adobe) for brightness/contrast corrections and normalization.

Statistical Analysis

Basic statistical analyses were performed with the StatPlus:mac LE.2009 version 5.8.3.7 package (AnalystSoft) for Microsoft Excel.

PCA and all multiple comparison statistics were calculated with R software version 3.1.1 (http://www.r-project.org/). Specifically, normality was verified with the Shapiro-Wilk test, homoscedasticity with both Bartlett and/or nonparametric Levene tests, differences among samples with either ANOVA (normality and homogenous variances) or Welch one-way ANOVA (normality and nonhomogenous variances) followed by posthoc LSD or Waller-Duncan test, respectively, and with Kruskal-Wallis (nonnormality and homogenous variances) or Friedman test (nonnormality and nonhomogenous variances).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Fruit drop dynamics in control and BA-treated trees in 2009 trials.

ACKNOWLEDGMENTS

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