Early Induction of Apple Fruitlet Abscission Is Characterized by an Increase of Both Isoprene Emission and Abscisic Acid Content$^{1,2}$[W][OA]

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Apple (Malus domestica) fruitlet abscission represents an interesting model system to study the early phases of the shedding process, during which major transcriptomic changes and metabolic rearrangements occur within the fruit. In apple, the drop of fruits at different positions within the cluster can be selectively magnified through chemical thinnings, such as benzyladenine and metamitron, acting as abscission enhancers. In this study, different abscission potentials were obtained within the apple fruitlet population by means of the above-mentioned thinnings. A metabolomic study was conducted on the volatile organic compounds emitted by abscising fruitlets, allowing for identification of isoprene as an early marker of abscission induction. A strong correlation was also observed between isoprene production and abscisic acid (ABA) levels in the fruit cortex, which were shown to increase in abscising fruitlets with respect to nonabscising ones. Transcriptomic evidence indicated that abscission-related ABA is biologically active, and its increased biosynthesis is associated with the induction of a specific ABA-responsive 9-cis-epoxycarotenoid dioxygenase gene. According to a hypothetical model, ABA may transiently cooperate with other hormones and secondary messengers in the generation of an intrafruit signal leading to the downstream activation of the abscission zone. The shedding process therefore appears to be triggered by multiple interdependent pathways, whose fine regulation, exerted within a very short temporal window by both endogenous and exogenous factors, determines the final destiny of the fruitlets.

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2 This article is dedicated to the memory of our friend and colleague Angelo Ramina, who recently passed away, leaving a gap that cannot be filled.

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BA was recently used to shed light on the signaling pathways mediating the induction of apple fruitlet abscission. A hypothetical model for this process was proposed based upon both transcriptomic and metabolic data, indicating a strong link between abscission induction and the nutritional stress occurring within the tree, which was magnified by the BA treatment (Botton et al., 2011). This physiological condition is primarily perceived at the fruitlet cortex, in which the molecular mechanisms linking the sugar starvation to abscisic acid (ABA) signaling are consequently activated. In fact, the ABA-related transcriptome shows significant variations at 2 d after the BA treatment. A down-regulation of 14-3-3 genes, likely involved in ABA-ethylene cross talk and in responses to sugar starvation (Lancien and Roberts, 2006), was observed in the abscising fruitlets (AFs), consistent with the following burst of ethylene occurring 4 d after BA spraying. Moreover, a gene encoding an AMP-activated protein kinase, similar to an Arabidopsis (Arabidopsis thaliana) ABA-induced Sucrose Nonfermenting1-related kinase involved in ABA-sugar cross talk (Jossier et al., 2009), was up-regulated in the AFs. A gene similar to AtMPK11, which is known to be induced by ABA and putatively involved in both ethylene-ABA cross talk and modulation of ABA signaling (Xin et al., 2003), was also up-regulated. This entire transcriptomic context is placed within a physiological framework that includes a burst of reactive oxygen species (ROS) generated during the early inductive phases immediately after the perception and signaling of the sugar starvation status. These findings are also supported by several ROS-related transcriptomic signatures, such as the NADPH oxidase gene, which is highly similar to the Arabidopsis Respiratory Burst Oxidase Homolog D involved in ROS production, and the gene coding for a class III peroxidase with various possible roles (Cosio and Dunand, 2009), both up-regulated in the cortex of AFs. Interesting data also concern auxin, gibberellin, and cytokinin metabolic pathways. In this context, it is noteworthy that hormone-related transcriptomic changes measured in the cortex resemble those claimed to be responsible for the negative feedback regulation occurring before pollination and fertilization and preventing fruit set in tomato (Solanum lycopersicum; Vriezen et al., 2008). Consistent with this, ABA and ethylene signaling pathways are strongly up-regulated concurrently with a specific down-regulation of gibberellin signaling in the fruits induced to abscise.

The availability of physiological parameters marking the earliest steps of abscission currently relies solely upon the measurement of ethylene emission, which increases within 3 to 4 d after the induction of the process (Dal Cin et al., 2005). Transcriptomic evidence indicates that significant changes have already occurred in the fruit cortex at 2 d after the inductive treatment (Botton et al., 2011), raising the possibility of identifying earlier physiological markers of fruitlet abscission potential (AP). As mentioned above, apple fruitlet drop is the result of a nutritional stress paralleled by ROS production, which triggers downstream signaling pathways leading to the activation of abscission executors. Despite this critical situation, which is irreversible in the experimental conditions adopted by Botton et al. (2011), the tree activates specific homeostatic mechanisms, among which the detoxification systems against the ROS may represent an interesting starting point for the identification of new physiological markers of fruitlet abscission.

Although research on plant antioxidants has mainly focused on nonvolatile compounds, the involvement of certain volatiles of the isoprenoid family in the protection against oxidative and other abiotic stresses has also been observed. In plants, the common C5 backbone molecules of isoprenoids may derive from two different metabolic pathways: the cytosolic mevalonic acid pathway or the plastidic 2-C-methyl-d-erythritol 4-P (MEP) pathway (Lichtenthaler et al., 1997; Rohmer, 1999a, 1999b, 2003; Lichtenthaler, 2000; Karl et al., 2002). Volatile isoprenoids are generally lipophilic, and among this numerous family of compounds, only monoterpenes, sesquiterpenes, and some diterpenes are able to volatilize at common biological temperatures (Dudareva et al., 2006). Of particular interest is isoprene, a hemiterpene that is known to behave as an antioxidant (Loreto et al., 2001; Loreto and Velikova, 2001; Calafiandra et al., 2008) and as a thylakoid membrane stabilizer that reduces the formation of ROS (Velikova et al., 2012), and whose indirect role in cooperating with nitric oxide (NO) and other reactive species in the installation of secondary signaling has also been suggested (Vickers et al., 2009a). In addition to acting as antioxidants, volatile isoprenoids are involved in a wide variety of plant responses to exogenous stimuli, conferring protection against high temperatures (Sharkey and Yeh, 2001; Sharkey et al., 2008; Rasulov et al., 2010), excessive light radiation (Loreto and Sharkey, 1990; Loreto et al., 2006), drought (Brilli et al., 2007; Michelozzi et al., 2011; Nogués et al., 2012), salt stress (Loreto and Delfino, 2000; Bertognolet et al., 2011), herbivores (Laithawornkitkul et al., 2008), and mechanical wounding (Brilli et al., 2011, 2012), and also playing relevant roles in tritrophic interactions (Loivamäki et al., 2008).

To assess a possible role for isoprene in apple fruitlet abscission induction, as well as identify new volatile markers and shed light on the metabolic and physiological pathways in which they may be involved, an experimental approach was adopted similar to that of Botton et al. (2011). Fruitlet populations with different APs were obtained from either untreated trees or trees sprayed with one of two different types of thinning chemicals, i.e. BA and the herbicide metamitron (MET), both of which magnify fruit drop, but through different indirect mechanisms. MET, a triazine herbicide, is widely used for pre- or postemergence broad-leaved weed control in sugar beet (Beta vulgaris). While BA acts by stimulating shoot growth and lateral bud outgrowth (Bubán, 2000), the mode of action of MET is through the blocking of electron transfer between the
primary and secondary quinones of PSII by binding secondary quinones and accepting electrons from primary quinones (Abbaspoor et al., 2006). Photosynthetic electron transport is consequently interrupted, leading to the concurrent inhibition of ATP production and carbon fixation. This induces nutrient starvation in actively growing organs such as young fruits; the effect is therefore similar to that of BA. Control and treated fruitlets of two genotypes were collected and subjected to an untargeted analysis performed by means of proton-transfer-reaction mass spectrometer (PTR-MS; Lindinger et al., 1998). This approach allowed their volatile organic compound (VOC) emissions to be measured and linked both to the corresponding AP and the sequence of events occurring at the molecular and metabolic levels. A set of candidate markers was identified, among which isoprene showed the highest statistical score. A relationship between the emission of this volatile and ABA content in the fruit cortex was also observed, with interesting implications related to their biological activities.

RESULTS

Fruit Drop Dynamics

Natural and chemically induced fruit drop dynamics were followed until their conclusion in two commercial apple genotypes, cv Golden Delicious and Red Chief, which are characterized by significant differences in their natural AP and sensitivity to thinning chemicals. While the former usually shows a high fruit set as well as a consequently high AP, and can be easily thinned with BA, the latter normally displays lower fruit set and AP, and is insensitive to this chemical. In preliminary experiments performed on cv Red Chief, MET was shown to be an effective thinner in this genotype (A. Dorigoni, personal communication), as observed in other apple cultivars (Dorigoni and Lezzer, 2007). This experimental scheme is suitable for the purposes of this study because it provides fruitlet populations with diverse genetic backgrounds and APs, obtained either from the natural availability or through different chemical treatments. Experiments were performed in 2008, 2009, and 2011. Data collected in 2008, which were largely confirmed in 2009 and 2011 (data not shown), are presented and discussed below for the cv Golden Delicious, because they are the most representative, and in the supplementary material for the cv Red Chief (Supplemental Fig. S1).

In control trees of cv Golden Delicious, fruit drop showed a biphasic dynamic (Fig. 1). The first peak of natural abscission occurred at 30 d after petal fall (DAPF), and the second occurred at 33 DAPF. A progressive decrease was observed thereafter, with almost null values measured at 44 (five fruits per tree) and 46 (zero fruits per tree) DAPF. At the end of the experiment, the total fruit drop was 163 fruits per tree (Fig. 1; inset).

Both BA and MET treatments magnified the natural AP, although with profound differences depending on the chemical. BA and MET increased total fruitlet abscission by about 66% and 91%, respectively. The application of BA resulted in the amplification of both the drop peaks and brought the first one forward by 1 d (at 29 DAPF). After the second peak, the trend of fruit drop in BA-treated trees overlapped that of the control. The MET treatment amplified only the first peak, but the amplification was much higher than in the BA treatment. As for the BA treatment, the first peak was also brought forward by 1 d relative to the control. After the second peak, fruit drop in MET-treated trees decreased rapidly to almost zero by 40 DAPF.

APs and Fruit Categorization

Preliminary experiments were conducted with several thinning chemicals on both cv Golden Delicious and Red Chief to determine the AP of the fruitlets in relation to their size and position within clusters characterized by a clear hierarchy (Supplemental Table S1; Botton et al., 2011). The information obtained from these experiments, which were performed over several years prior to this study (Botton et al., 2011; Angeli et al., 2002), indicates that the position of the fruit within the cluster is an important determinant of the hierarchy between competing fruits and, consequently, of their tendency to abscise. The central fruit develops earlier, as it originates from an earlier flowering event, and naturally exerts a correlative dominance over the lateral fruits, making the latter weaker sinks and more prone to abscise (Bangerth, 2000). A hierarchy also exists between lateral fruitlets. In fact, those deriving
from earlier blooming flowers (L3 fruitlets) reach a bigger size, have a stronger sink activity and a lower AP, and exert a correlative dominance over the smaller ones growing further down the inflorescence, which ultimately display higher APs (L2 and L1). In the absence of external perturbations, the central (C) and the biggest lateral (L3) fruits persist on the tree, while L2 and L1 are shed, although with a higher probability for the latter. A correlative reproductive dominance therefore naturally exists in apple clusters, starting from the central fruit toward the basal lateral ones, and this dominance is paralleled by an opposed increasing gradient of AP. As a consequence, fruit size and position within the cluster, being strongly correlated with the capacity to attract assimilates (Bangerth, 2000), may be considered reliable parameters for predicting the natural fruitlet’s AP, at least in well-hierarchized clusters.

BA action is mainly exerted by enhancing shoot growth and branching (Dal Cin et al., 2007) and therefore exacerbating the correlative competition between fruits. In cv Golden Delicious, BA provokes the abscission of an increased number of big lateral fruits, while leaving the L2 and L1 classes unaffected, both of which are already destined to be shed (Angeli et al., 2002). Treatments with MET usually display a stronger thinning effect than BA, causing not only a complete drop of the laterals of all classes, but also an increased AP of small central fruitlets of intact, but nonhierarchized, corymbs (data not shown). These central fruitlets are not able to compete with laterals and thus drop.

To assess the chemical thinning effect on the central fruitlets in the absence of competition with the laterals, a subpopulation was obtained by removing from the cluster all lateral flowers at full bloom, as described by Dal Cin et al. (2005, 2009a, 2009b), and leaving only the hand-pollinated central one. In cv Golden Delicious, the natural AP of these central fruitlets ranges from 0% to 10%, according to their size class (Supplemental Table S1). After treatment with BA or MET, AP increased up to 40% and 60%, respectively, in the small centrals, whereas larger fruits (size classes 2 and 3) were unaffected.

The same chemical treatments on cv Red Chief gave different results, as reported in Supplemental Table S1.

### VOC Emissions

VOC emissions were recorded by mass spectrometry in all fruitlet samples in 2008 and 2009 up to 8 and 7 d after the treatments (23 and 22 DAPF) in cv Golden Delicious and Red Chief, respectively. To identify possible associations between the fruitlets’ AP and volatile emissions, a statistical score (Z) was calculated for correlation between each measured mass-to-charge ratio (m/z) and fruit destiny. Statistical tests were conducted by ranking samples into two classes: AFs were those with AP greater than 50% and nonabscising fruitlets were those with AP less than 50%. This ranking was chosen to perform a raw selection of the most interesting VOCs. For cv Golden Delicious, m/z 69 was shown to have the highest Z score (26.03), followed by the protonated masses 75, 71, 43, and 61 (all with Z scores above 10), with all others showing lower statistical scores (Table I). The cv Red Chief statistics are reported in Supplemental Table S2. Given that the protonated mass 69 in plants has been unambiguously identified as isoprene (Barta and Loreto, 2006; Cinega et al., 2009; Behnke et al., 2010; Rasulov et al., 2010), whereas the other masses with high Z scores have not always been precisely identified, attention was focused on isoprene. However, because the emission of this volatile compound by apple fruitlets has not yet been reported, specific investigations were conducted by means of gas chromatography-mass spectrometry with a flame ionization detector, and additional measurements were performed by PTR-MS on ionization spectra as a function of collision energy to confirm the identity of m/z 69. Taken as a whole, the results of these assessments confirmed that m/z 69 is due to the contribution of protonated isoprene and not to fragments of other volatiles (further details are provided in Supplemental Fig. S2).

Isoprene emissions directly correlated with the AP not only when statistical analyses were performed on all fruit samples, but also separately for each time

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**Table I. Associations between VOC emissions and fruit destiny in cv Golden Delicious**

<table>
<thead>
<tr>
<th>Candidate/s</th>
<th>m/z</th>
<th>Z Score</th>
<th>Association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>33</td>
<td>3.98</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Acetic acid, acetate esters, propanol, hexanol</td>
<td>43</td>
<td>15.19</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>45</td>
<td>2.1</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Cluster methanol plus water</td>
<td>51</td>
<td>7.38</td>
<td>High in AFs</td>
</tr>
<tr>
<td>(E)-2-hexenal (i), butanol, propanol, ethyl propionate</td>
<td>57</td>
<td>9.07</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Acetic acid, ethyl acetate (f)</td>
<td>61</td>
<td>10.24</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Dimethyl sulfide</td>
<td>63</td>
<td>7.75</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Cluster ethanol plus water</td>
<td>65</td>
<td>3.55</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Pentanal, octanal, nonanal, 1-octen-3-ol, isoprene, methylbutanal</td>
<td>69</td>
<td>26.03</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Alcohol fragment (2-pentanol, octanal), 3-methyl-1-butanol, ethyl acetate</td>
<td>71</td>
<td>18.8</td>
<td>High in AFs</td>
</tr>
<tr>
<td>Methyl acetate, ethyl propionate</td>
<td>75</td>
<td>19.75</td>
<td>High in AFs</td>
</tr>
</tbody>
</table>

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**Z** scores were calculated by ranking fruitlets in two categories (AFs and nonabscising fruitlets). Only associations with Z scores greater than or equal to 2 are reported, sorted by increasing mass. f, Fragments.
point (Table II). In the latter case, the highest correlations (as high as 0.81388 [$P = 0.00004$]) were observed in cv Golden Delicious at 15 DAPF. Moreover, isoprene emissions were inversely correlated with fruit size (Table II) in all cases.

In control cv Golden Delicious fruits not treated with chemical thinners, when the experiment began (at 15 DAPF), isoprene emissions (Fig. 2A) were the highest for the class most likely to abscise (L1) and the lowest for the classes least likely to abscise (C2 and C3). Big and medium-sized lateral (L3 and L2) and small central (C1) fruitlet classes showed intermediate emissions, significantly higher in C1 than in L2 and L3.

By pooling AFs and nonabscising fruitlets data, statistically higher isoprene emissions can be observed in the former class at all time points except for 15 DAPF (Fig. 2B). Considering these data as a whole, the behavior of cv Golden Delicious was better defined in terms of isoprene emission, with a higher statistical significance and a clearer kinetics than cv Red Chief throughout the experiment. Therefore, because the AP of the different fruit classes as well as the drop dynamics of cv Golden Delicious had already been characterized in previous studies (Botton et al., 2011), further studies were done only on this variety. Investigations focused on the abscission induction phase, which had been previously determined to occur within 2 d of the thinning treatment (i.e. at 17 DAPF; Botton et al., 2011).

Considering the single fruit classes separately (Fig. 3), a general decreasing trend of isoprene emission can be observed throughout the experiment in all samples and treatments, as already observed in the pooled data. In small laterals, the BA treatment caused a transient drop in isoprene emission relative to the control fruits at 16 DAPF, whereas MET spraying resulted in an increase in isoprene emission at 17 DAPF, which was maintained up to 21 DAPF. MET treatment had the same effect on medium and big laterals, but the increase occurred 1 d earlier than in the small ones, and the pattern was less clear. The BA treatment significantly increased isoprene emission only in big lateral fruitlets at 17 DAPF. Overall, BA showed no clear pattern. In the centrals, the only significant difference in isoprene emission was provoked by MET, which repressed isoprene production at 17 DAPF in small central fruitlets. In the other cases, no significant difference was observed, especially in the big central fruitlets in which isoprene emission of treated samples almost exactly matched those of the control. cv Red Chief data of single fruit classes are reported in Supplemental Figure S3.

### ABA Content

Previous studies conducted in isoprene-emitting species by Barta and Loreto (2006) identified a direct association between isoprene emission and foliar ABA, the latter being most likely generated through a direct synthetic route closely dependent on the activity of the

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**Table II. Correlations between isoprene emission, AP, and fruit size in cv Golden Delicious**

Pearson correlation coefficients were calculated along with their $P$ level of statistical significance, considering either all samples together or distinctly at each time point. Correlations were considered significant with $P \leq 5\times 10^{-2}$.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Isoprene versus AP</th>
<th>Isoprene versus Fruit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pearson $P$</td>
<td>Pearson</td>
</tr>
<tr>
<td>All</td>
<td>-0.79475</td>
<td>0E+00</td>
</tr>
<tr>
<td>15 DAPF</td>
<td>0.79122</td>
<td>6E-02</td>
</tr>
<tr>
<td>16 DAPF</td>
<td>0.81388</td>
<td>4E-05</td>
</tr>
<tr>
<td>17 DAPF</td>
<td>0.78084</td>
<td>1E-04</td>
</tr>
<tr>
<td>19 DAPF</td>
<td>0.79628</td>
<td>1E-04</td>
</tr>
<tr>
<td>21 DAPF</td>
<td>0.80061</td>
<td>7E-05</td>
</tr>
<tr>
<td>23 DAPF</td>
<td>0.78398</td>
<td>1E-03</td>
</tr>
</tbody>
</table>

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**Figure 2.** A. Isoprene emission in untreated fruitlet samples of cv Golden Delicious at the beginning of the experiments (15 DAPF). Bars represent $sd (n = 5)$, whereas letters indicate significant differences as determined by Waller-Duncan test ($P < 0.05$). B. Isoprene emission obtained by pooling data from abscising (squares and dashed line) and nonabscising (circles and continuous line) fruitlets of cv Golden Delicious (as classified in Supplemental Table S1), including both BA- and MET-treated samples. Statistically significant differences as determined by Student’s $t$ test are also indicated (three asterisks at $P \leq 0.005$; two asterisks at $P \leq 0.005$; one asterisk at $P \leq 0.05$). Bars represent $sd (n = 9$ in cv Golden Delicious). Data were collected in 2008. ns, Nonsignificant; L1, small lateral fruitlets; L2, medium lateral fruitlets; L3, big lateral fruitlets; C1, small central fruitlets; C2, medium central fruitlets; C3, big central fruitlets.
MEP pathway. Moreover, a key role of ABA signaling and its cross talk with other hormones in the early phases of apple fruitlet abscission induction was recently suggested based upon transcriptomic data (Botton et al., 2011). ABA content was therefore measured in the cortex of the same cv Golden Delicious fruitlet samples taken at 15, 17, and 19 DAPF and used by Botton et al. (2011) for global transcriptomic analyses (i.e. only fruitlets of 2008 treated with BA, along with their controls). Attention was focused on three fruitlet classes characterized by strictly diverse behavior in terms of AP in relation to the thinning treatment: (1) the L1 class, destined to abscise regardless of the BA treatment; (2) the L3 class, destined to abscise or persist with or without BA treatment, respectively; and (3) the C3 class, destined to persist regardless of the BA treatment. At the beginning of experiments (15 DAPF), ABA levels were significantly higher in L1 than in the other classes (Fig. 4A). A time course analysis of the individual fruitlet classes characterized by strictly diverse behavior in terms of AP in relation to the thinning treatment: (1) the L1 class, destined to abscise regardless of the BA treatment; (2) the L3 class, destined to abscise or persist with or without BA treatment, respectively; and (3) the C3 class, destined to persist regardless of the BA treatment. At the beginning of experiments (15 DAPF), ABA levels were significantly higher in L1 than in the other classes (Fig. 4A). A time course analysis of the individual fruitlet classes characterized by strictly diverse behavior in terms of AP in relation to the thinning treatment: (1) the L1 class, destined to abscise regardless of the BA treatment; (2) the L3 class, destined to abscise or persist with or without BA treatment, respectively; and (3) the C3 class, destined to persist regardless of the BA treatment.

Isoprene and ABA measurements on fruitlet samples at different time points were used to assess possible correlations, as observed by Barta and Loreto (2006). A statistically significant correlation was calculated between isoprene emission and ABA content, as high as 0.92 (Pearson coefficient; $P = 0.0000014$) when all fruitlets were included (Fig. 5A). However, when fruitlets were split into single classes, correlations were high only in L1 and C3, with statistical significance only in the latter (Fig. 5, C–E). ABA content was also directly associated with the AP, although this association was statistically significant only when calculated on all samples (Fig. 5B).

**Effect of ABA Treatments on Fruit Drop and Isoprene Emission**

The relationship between ABA, isoprene, and fruit drop was examined more closely by applying ABA to apple trees. The effects of ABA treatments at different concentrations on fruit drop and isoprene emission were assessed in cv Golden Delicious trees at phenological stages that are suitable for chemical thinning. Total fruit drop was significantly affected only by the ABA treatment at 100 µL L$^{-1}$ (about +25% in both 2011 and 2012; Supplemental Fig. S4). Based on the daily
measurements of shed fruitlets, it was deduced that the increased abscission was due mainly to the shedding of the L3 class (data not shown). It is worth noting that fruit drop of control trees was higher than usual (for a comparison see the inset of Fig. 1), most likely because of particularly favorable climatic conditions during flowering, which resulted in a high fruit set. According to the data provided by the meteorological station located in the experimental orchards, maximum temperatures measured during the full bloom of cv Golden Delicious were from 1.3°C to 3.5°C (in 2011) and from 1.8°C to 3.8°C (in 2012) higher than the average of the previous decade (i.e. 2000–2010). Because pollination and fertilization in apple are particularly sensitive to temperature (Yoder et al., 2009), these conditions may have determined a higher fruit load and, therefore, a higher natural fruitlet abscission. Consequently, total fruitlet drop in treated trees may have been partially biased, not in absolute terms, but with respect to the control (i.e. a reduced magnification).

The higher AP shown by the control trees was also accompanied by higher isoprene emission of the fruitlets of all classes (Fig. 6) than in previous experiments (see Fig. 3 for a comparison). During the whole experimental
period (15–21 DAPF), the minimum and maximum temperatures did not differ significantly from the average of the same period in the previous decade (data not shown). The ABA treatment at 100 μL L⁻¹, in addition to yielding a statistically significant increase in fruit drop, also significantly affected isoprene emission in L1 and L3 classes (Fig. 6). In the former fruitlets treated with ABA, isoprene emission was significantly repressed at 16 DAPF relative to the control, whereas it was enhanced at the same time point in the latter (P ≤ 0.05). No significant effect of the treatment was observed on isoprene emission in the C3 class.

Fruit drop and isoprene emission data collected in 2011 (also shown in Supplemental Fig. S5) were largely confirmed in 2012, except for the ABA-treated C3 class, which did not show any variation in isoprene emission in the second year (data not shown).

**Gene Expression Analyses**

To gain a global view of the possible transcriptional regulation mechanisms associated with the increase of both isoprene emission and ABA content in the fruitlet cortex, attention was focused on the secondary metabolism by using a double approach based upon enrichment analysis and MapMan analysis (see Supplementary Material). Microarray expression data produced by Botton et al. (2011) and archived in the ArrayExpress database (accession no. A-MEXP-1852) were used for this, although the probe set adopted did not guarantee a total coverage of the apple transcriptome. Both analyses showed that during abscission induction in L3 fruitlets, some pathways were preferentially activated (Supplemental Fig. S6) and the corresponding genes were overrepresented among those differentially expressed (Supplemental Table S5): terpenoids (isoprenoids), phenylpropanoids, and carotenoids. Considering the general indications given by these partial transcriptomic analyses, attention was then focused on the MEP pathway feeding both isoprene and ABA biosynthetic routes and on the final steps of ABA biosynthesis. Therefore, genes encoding key enzymes (either rate-limiting or catalyzing relevant steps), such as 1-deoxy-D-xylulose 5-P synthase (DXS), zeaxanthin epoxidase (ZEP), 9-cis-epoxycarotenoid-dioxygenase (NCED), abscisic aldehyde oxidase (AAO), and molybdenum cofactor sulfurase (MoCo), were identified by sequence homology in the apple genome sequence (Velasco et al., 2010; currently available at http://genomics.research.iasma.it/), as described in the Supplementary Materials. The quantitative PCR expression profiles were also examined in L1, L3, and C3 fruitlets, untreated or treated with either BA or ABA. In addition, the expression of genes encoding elements of ABA metabolism, including ABA-8'-hydroxylase and β-glucosidase, were also considered based on the microarray data published by Botton et al. (2011). A summary is shown in Figure 7 including only the genes with detectable expression levels in the cortex.

**Figure 6.** Isoprene emission in single fruit classes of cv Golden Delicious in 2011. Small lateral (A), big lateral (B), and big central (C) fruitlets of control (circles and continuous line) and ABA-treated (100 μL L⁻¹; squares and dashed line) trees are shown. Bars represent SD (n = 3). Statistically significant differences as determined by Student’s t test are indicated with one asterisk (P ≤ 0.05).

DXS has been shown to be a rate-limiting enzyme for plastidic isoprenoid biosynthesis in plants (Estévez et al., 2001), and a likely transcriptional regulation of the step catalyzed by DXS was suggested, at least for carotenoids biosynthesis during tomato fruit ripening (Lois et al., 2000). For this reason, the expression of the corresponding gene/s in apple fruitlets may be an important physiological indicator of the regulatory mechanisms.
triggered during the early phases of abscission induction to support both isoprene and ABA biosynthesis. Four candidate DXS genes were identified in apple, three of which belonging to class II (MDP0000253802, MDP0000253952, and MDP0000793656) and one to class I (MDP0000798878), according to the classification of Walter et al. (2002) and Zhang et al. (2009; see the phylogenetic analysis in Supplemental Fig. S7). Expression of MDP0000253802 was substantially unaffected by BA in all fruit classes and time points and also by ABA at abscission-inducing concentration (100 μL L⁻¹; Fig. 7). MDP0000253952 transcription was strongly up-regulated in BA-treated C3 fruitlets at both time points, whereas in L1 and L3, it was

Figure 7. Simplified version of the isoprenoid biosynthetic pathways and expression patterns, assessed in small lateral, big lateral, and big central fruitlets sampled in 2011, of genes encoding key (either rate-limiting or relevant) enzymes of the MEP/DOXP pathway and ABA biosynthesis (ZEP, NCED, AAO, and MoCo) and metabolism (ABA-8'-hydroxylase and β-glucosidase). Different colors mark each gene/enzyme in both the heat maps and the pathways. Gene expression was assessed at different time points (expressed as DAPF, according to the specific experiment) following treatments with either BA (+BA) or ABA at 100 μL L⁻¹ (+ABA100). Genes are indicated with the identifications of the publicly available apple genome sequence (http://genomics.research.iasma.it/), and their expression is reported in the heat map as a log ratio of quantitative PCR mean normalized values (treated versus control at the same time point) with conventional color codes. Expression patterns of ABA-8'-hydroxylase and β-glucosidase were extracted from the microarray data of Botton et al. (2011). The asterisk indicates a ZEP gene whose expression actually refers to two transcripts (see main text). L1, Small lateral fruitlets; L3, big lateral fruitlets; C3, big central fruitlets; GA3P, D-Glyceraldehyde 3-P; PEP, phosphoenolpyruvate; PYR, pyruvate; Ac-CoA, acetyl-CoA; HMG-CoA, 3-hydroxy-3-methyl glutaryl-CoA; MVA, mevalonic acid; DOXP, 1-deoxy-D-xylulose 5-phosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; ABA-GE/GS, 1-abcisyl-β-D-glucopyranoside/1'-O-ABA-β-D-glucopyranoside; PA, phasic acid; DPA, dihydrophaseic acid; 8’OH-ABA, ABA-8’-hydroxylase; BG, β-glucosidase.
inhibited by the thinning treatment, especially in the former class. ABA did not affect MDP0000253952 transcript accumulation, except for a slight up-regulation at 18 DAPF in C3 fruitlets. MDP0000793656 was down-regulated at 17 DAPF and up-regulated at 19 DAPF in BA-treated fruitlets of all three classes. In L3 treated fruitlets, the up-regulation at 19 DAPF, when abscission induction is already underway (Botton et al., 2011), was surprisingly strong (as high as $10^{11}$-fold). ABA affected the expression of this gene in a way very similar to MDP0000253952. Instead, an up-regulation of MDP0000798878 was observed in L1 fruitlets treated with either chemical. Finally, in L3 and C3 classes, MDP0000798878 was down-regulated by BA at both time points, whereas ABA did not affect its transcript levels.

Concerning ABA biosynthesis, four candidate ZEP genes were identified (Supplemental Fig. S8), among which one did not show detectable levels of expression in the cortex (MDP0000128205). Two of the remaining three genes (MDP0000319667 and MDP0000740968) were not distinguishable in quantitative PCR expression assays because of a high nucleotide sequence identity. Therefore, the pair of primers used in quantitative PCR actually measured the expression of both genes. Taken as a whole, only a few slight variations were observed in the expression levels of the candidate ZEP genes in all fruitlet classes, time points, and treatments.

Regarding the NCED-encoding genes, whose related proteins are known key regulators of ABA levels (Schwartz et al., 2003), five candidates were identified (MDP0000228070, MDP0000246006, MDP0000386460, MDP0000813805, and MDP0000929213) according to the phylogenetic analysis (Supplemental Fig. S9), all of which expressed at the fruitlet cortex level. While MDP0000228070 and MDP0000813805 correspond to the already-identified MdNCED1 (AB593328) and MdNCED2 (AB593329; Kondo et al., 2012) that are very similar to the peach (Prunus persica) PpNCED1, the remaining three genes represent new candidates. In particular, while MDP0000246006 was very close to MDP0000228070, MDP0000386460 and MDP0000929213 clustered close to peach PpNCED2. It is worth noting that in this case the number of NCED genes reflected that found in Arabidopsis (Tan et al., 2003). The BA treatment showed an overall negative effect on the expression of NCED genes in the L1 class, with the only exception of MDP0000228070 at 19 DAPF (slight up-regulation). This inhibitory effect was even more evident and broad in C3 fruitlets, in which, at 17 DAPF, MDP0000228070 was down-regulated by $10^{10}$-fold. In L3 fruitlets, MDP0000228070 and MDP0000386460 were up-regulated by BA at 17 DAPF and down-regulated thereafter, whereas MDP0000246006 and MDP0000929213 were down-regulated at 17 DAPF and up-regulated 2 d later. Finally, expression of MDP0000813805 was slightly repressed by the thinner at 19 DAPF. The transcription of MDP0000228070 was induced by 8-fold concomitantly with the increase in ABA levels observed at 17 DAPF and then followed by a marked down-regulation (more than 50-fold). With the ABA treatment, expression of NCED genes at 16 DAPF was in most cases repressed. At 17 DAPF, transcription of MDP0000228070 and MDP0000246006 was stimulated in L1 and L3, while it remained unaffected in C3. The transcripts of MDP0000386460, MDP0000813805, and MDP0000929213 were mostly down-regulated at the same date, with the exception of MDP0000813805, which was unaffected by the hormone. At 18 DAPF, MDP0000929213 was slightly up-regulated in L1 fruitlets, whereas the other four NCEDs were unaffected. In the L3 class, expression patterns were similar to those observed in L1, except for MDP0000228070, which was still up-regulated in the ABA-treated fruitlets. Lastly, expression of all NCED genes in the C3 class was strongly down-regulated.

Among the seven candidate AAO genes (Supplemental Fig. S10), only four (MDP0000138495, MDP0000158054, MDP0000237624, and MDP0000664537) were expressed in the fruit cortex at detectable levels. Among these, MDP0000138495 and MDP0000158054 were induced by the BA treatment, although with different patterns in L1 and L3 classes. While MDP0000138495 was steadily up-regulated in the former class at both 17 and 19 DAPF, in the latter, its expression was induced only at the second time point. Transcription of MDP0000158054 was always up-regulated in L1 fruitlets, reaching 8-fold at 19 DAPF. However, in L3, this gene followed a different pattern, being down-regulated at 17 DAPF and up-regulated 2 d later. In C3, the expression of AAO genes was substantially unaffected. The effects of the ABA treatment on the transcriptional regulation of these four candidate AAOs were quite erratic. The transcription of MDP0000138495 was repressed in L1 samples, strongly down-regulated in L3 fruitlets at 16 and 18 DAPF, and slightly up-regulated at the intermediate time point. Its expression in the C3 class was very similar to that observed in L3. MDP0000158054 and MDP0000237624 showed similar expression patterns in L1 and L3 fruitlets treated with ABA. In L1, both were repressed at 16 DAPF, stimulated at 17 DAPF, and then unaffected at 18 DAPF. In L3 fruitlets, both were unaffected at 16 DAPF and up- and down-regulated at 17 and 18 DAPF, respectively. In C3, they showed different patterns of expression, with MDP0000158054 being slightly up-regulated at 17 DAPF and substantially repressed at both other dates, and MDP0000237624 increasingly down-regulated from 16 to 18 DAPF. Finally, MDP0000664537 showed an increasing up-regulation, especially at 18 DAPF, from L1 to L3 and C3.

Only one MoCo candidate (MDP0000272554) was found in the apple genome. Its expression was substantially unaffected in all fruit samples, except for a weak down-regulation at 17 DAPF in BA-treated L3 fruitlets.

Concerning the genes encoding elements of ABA metabolism and deconjugation (ABA-8'-hydroxylase and β-glucosidase, respectively), no variation was found in their expression patterns in either L1 or L3 classes treated with BA. Instead, a broad up-regulation.
was observed in C3 fruitlets at 17 DAPF for all the ABA-8'-hydroxylase genes, whereas the β-glucosidase transcripts were down-regulated at 19 DAPF.

**DISCUSSION**

The apple fruitlet cluster represents a unique model system for studying correlatively driven abscission, and the availability of chemicals that can selectively induce fruit shedding makes it possible to set up controlled field experiments aimed at magnifying the natural physiological drop to obtain fruitlet populations with clearly predictable and different APs. Botton et al. (2011) took advantage of these features to conduct a massive transcriptomic analysis of abscission induction and suggested a hypothetical model describing the early transcriptional changes associated with the process at both the fruit cortex and seed level. This model may be validated and also have wider future applications in other species to identify possible overlapping and/or specificities with respect to apple. To further explore the early regulatory events leading to or associated with fruitlet abscission in apple, an untargeted analysis of emission of VOCs in the abscising versus nonabscising fruitlets was performed on two apple cultivars with differing responsiveness to thinning treatments. Both BA and MET thinning chemicals were applied on the two genotypes to identify abscission-related metabolic signatures independently of the genotype or chemical being used. Fruitlet populations with clearly distinguishable APs were obtained by adopting a well-tested strategy (Dal Cin et al., 2005, 2007, 2009a, 2009b; Botton et al., 2011), consisting of using the thinning chemicals to synchronize abscission of lateral fruits versus persistence of central fruits within clusters. The choice of using MET was made to enhance and synchronize abscission in the difficult-to-thin spur cv Red Chief. Most analyses were focused on the temporal window (within a maximum of 4 d after treatment) during which the first tentative reaction of the fruits to the abscission-inducing stress occurs, along with a likely metabolic rearrangement, both of which are highly regulated and coordinated, presumably by ABA (Botton et al., 2011).

Our results showed that both BA and MET simulated similar VOC emissions in both varieties, in particular isoprene (Table I; Supplemental Table S2). Moreover, statistical analyses identified a strong correlation between emission of isoprene by the fruitlets and their AP, either natural or chemically enhanced (Table II; Supplemental Table S3). As well as being emitted at higher levels by AFs taken as a whole (Fig. 2), isoprene emission was even higher in naturally AFs (i.e. L1; Fig. 3A). The diagnostic value of isoprene was shown to be very high in both genotypes, although the most specific emission profile was observed in cv Golden Delicious, which was therefore chosen as a model cultivar. This model genotype also showed a better resolution of the two fruitlet drop peaks, along with a clear completion of the shedding process within the target physiological temporal window.

A statistically significant inverse correlation was also observed between the volatile emission and fruit size, thus indicating that isoprene release may naturally decrease during fruitlet development. When isoprene emission and total chlorophylls content data of different untreated fruit classes (i.e. fruits at different developmental stages) are plotted into a unique time course according to their cross diameter (Fig. 8), the following becomes evident: (1) The emission rate of isoprene naturally decreases throughout early fruit development (at least within the range 6–21 mm of cross diameter), concurrently with the progressive decrease of fruit photosynthetic capacity occurring at these developmental stages (photosynthetic capacity is highly correlated with total chlorophylls content in cv Golden Delicious; Fleancu, 2007); (2) its levels are not dependent upon the intracluster competition, as the central fruitlets of pruned clusters (i.e. with no intracluster competition) also showed decreasing trends from C1 to C3; and (3) developmentally programmed isoprene emissions can be perturbed by exogenous factors, such as the thinning treatments, to different extents and in different directions according to the stage of development of the fruit. These data further support the idea that L3 fruitlets are both metabolically and developmentally more sensitive than the other fruit classes to alterations in the nutritional status generated by the thinning treatments. This higher sensitivity may be due to the fact that cell division is still fully proceeding in L3 fruitlets, whereas it may have just ceased in the centrals. During such a delicate developmental stage in which the final fruit size is defined, fruitlets may be particularly sensitive to nutritional

![Figure 8. Relationship between fruit cross diameter (i.e. fruit developmental stage) and total chlorophylls (chlorophyll a + chlorophyll b; white circle) or isoprene emission (gray square) as measured in small lateral, big lateral, small central, and big central untreated fruitlets. The Pearson correlation coefficients and their P level of significance are also shown. L1, Small lateral fruitlets; L3, big lateral fruitlets; C1, small central fruitlets; C3, big central fruitlets.](image-url)
stresses more severe than those imposed endogenously.

Isoprenoid compounds are thought to play a pivotal role in abiotic stress tolerance, according to the mechanistic view of a single biochemical mechanism for multiple physiological stressors (Vickers et al., 2009a). It is demonstrated here that the isoprenoid pathway was activated and isoprene emission increased during the induction of abscission in apple fruitlets. Isoprene may thus represent a potential marker to predict the fruit destiny early. It is not surprising that isoprene is highly emitted by organs, such as the AFs, suffering shortage of assimilates. In fact, isoprenoid emissions have often been found to be sustained when carbon supply becomes scarce under stress conditions or even in the presence of null photosynthetic rates (Monson and Fall, 1989; Loreto and Sharkey, 1990; Sharkey and Loreto, 1993; Loreto and Delfino, 2000; Brilli et al., 2007). This would mean that the function of isoprene might be considered vital (or at least relevant) for the plant under stress. In particular, a role for isoprene in altering the oxidative status of plants under stress has been proposed. Because the oxidative balance of AFs was disrupted by high levels of ROS (Botton et al., 2011), it may be speculated that isoprene could act directly to contrast ROS accumulating in the apple fruit cortex, as recently found in other species (Loreto et al., 2001; Loreto and Velikova, 2001; Affek and Yakir, 2002; Velikova et al., 2004) and confirmed in transgenic tobacco (Nicotiana tabacum) plants carrying an Isoprene Synthase gene extracted from poplar (Populus spp.; Vickers et al., 2009b). Therefore, the fruit stimulated to abscise may exploit isoprene emissions to recover a noncytotoxic oxidative status by means of a nonenzymatic ROS-scavenging system. This early reaction would fall within the homeostatic mechanisms set up by the fruits during abscission induction, as recently hypothesized (Botton et al., 2011). In this specific case, however, ROS levels did not decrease as a consequence of increased isoprene emission, probably because abscission induction was at this point irreversible. Isoprene, in addition to acting directly as an antioxidant, may also be involved in specific signaling pathways leading to a broader cellular response to abiotic stresses. Cooperating with NO, isoprene was shown to reduce oxidative damage in leaves (Velikova et al., 2008), and this effect is likely to be dependent upon interactions occurring at the signaling level rather than direct scavenging reactions (Vickers et al., 2009a). The involvement of NO in the signal cascade that initiates the hypersensitive response occurring in plant-pathogen interactions (Delledonne et al., 1998) and its cooperation with isoprene may suggest that a number of other processes are also likely to be affected, such as the mitogen-activated protein kinase cascade and the elicitation of jasmonate and salicylate signaling pathways typically involved in the hypersensitive response (Vickers et al., 2009a). Transcriptomic analyses performed by Botton et al. (2011) indicate that the above-cited processes were triggered early (at 17 DAPF) during abscission induction, especially concerning the up-regulation of genes coding for a jasmonate-induced protein, a mitogen-activated protein kinase, and a mitogen-activated protein kinase kinase. For the latter two genes, a role in the cross talk with ethylene signaling, which is activated downstream (Dal Cin et al., 2005), may be hypothesized, along with an interaction with NO signaling, as previously pointed out (Ederli et al., 2006; Mur et al., 2008).

Short-term changes in isoprene emission are primarily determined by availability of the isoprene synthase sub- strate dimethylallyl pyrophosphate (Falbel and Sharkey, 2005; Nogués et al., 2006; Rodriguez-Concepción, 2006; Rasulov et al., 2009; Rasulov et al., 2010; Vickers et al., 2010), which is synthesized by the MEP pathway. Because DXS was proven to catalyze a rate-limiting step of the MEP pathway (Estévez et al., 2001) and given that this limitation may partially rely upon a transcriptional regulation as shown in tomato (Lois et al., 2000), the up-regulation of DXS genes may finally result in an increased availability of the chloroplastic pool of dimethylallyl pyrophosphate. This will, in turn, feed not only isoprene biosynthesis (Falbel and Sharkey, 2005) but also the other downstream pathways that rely upon the same substrate, including ABA biosynthesis via carotenoids (Fig. 7). The BA thinning treatment was shown to up-regulate the expression of three different DXS according to the fruit class, and thus developmental stage, considered. Among these three genes, MDP0000793656 was up-regulated only in AFS of the L1 and L3 classes. This up-regulation occurred at 19 DAPF and was preceded in both cases by an increased emission of isoprene (at 17 DAPF), as measured in BA- treated fruitlets. Nevertheless, this difference was statistically significant only in L3 fruitlets, which also showed a more sustained ABA production than the untreated control at the same time point. It may therefore be hypothesized that the dramatic up-regulation of the apple candidate DXS gene may have been required to provide an adequate amount of substrate to sustain the high biosynthetic rates of both compounds. It is also worthy of note that MDP0000793656 putatively encodes a class II DXS, whose function is more related to the secondary metabolism rather than a housekeeping role (Walter et al., 2002). In addition, because MDP0000793656 was shown to be substantially unaffected by the ABA treatment, a positive feedback control exerted by the hormone on DXS transcription may be unlikely.

These results may also provide some interesting indications about the balance of metabolic fluxes feeding isoprene biosynthesis. The majority of the carbon incorporated in this molecule was proven to come from photosynthesis in several species (Delwiche and Sharkey, 1993; Affek and Yakir, 2002; Karl et al., 2002; Loreto et al., 2004; Schnitzler et al., 2004; Falbel and Sharkey, 2005; Trowbridge et al., 2012), although additional sources either chloroplastic, such as starch degradation (Karl et al., 2002), or extrachloroplastic, such as transported carbohydrates (Kreuwieser et al., 2013).
2002; Schnitzler et al., 2004), or CO₂ coming from mitochondrial respiration (Anderson et al., 1998), may also contribute. In abscising apple fruitlets, isoprene biosynthesis was shown to be sustained even with photosynthesis inhibition, which is known to occur soon after MET treatments in apple and persist up to 5 d after the chemical application (McArtney et al., 2012). Moreover, the nutritional stress caused by both BA and MET indirectly reduces the already low assimilate availability to the sinks (i.e. the fruitlets), suggesting that a contribution to isoprene biosynthesis from transported carbohydrates may be ruled out. Taking into account that Botton et al. (2011) observed an increase of Suc and a decrease of starch concentration in the cortex of AFs, it may be hypothesized that isoprene biosynthesis in these fruits is supported by both starch degradation and respiration (following Suc breakdown). Specific investigations will be focused on these aspects to shed light on which metabolisms are more active in feeding isoprene biosynthesis in apple fruitlets undergoing a nutritional stress, such as that caused by the thinning treatments.

The determination of ABA levels in the cortex allowed the identification of a significant correlation between the hormone content and isoprene emission (Fig. 5). Increased levels of the hormone observed in BA-treated L3 fruitlets (Fig. 4C) concurrently with the up-regulation of the ABA-responsive *MdNCED1* gene (MDP0000228070; Fig. 7) suggests an activation of the indirect biosynthetic pathway of the hormone. Moreover, taking into account that the same gene was found to be up-regulated during naphthylacetic acid- and shading-induced apple fruitlet abscission (Zhu et al., 2011), its specific role in fruit shedding may be hypothesized. It is also interesting that both BA and ABA treatments caused a strong and broad down-regulation of all the *NCED* genes in C3 fruitlets. A strong developmental control of these genes may therefore also be hypothesized. Expression data concerning genes involved in ABA metabolism (*ABA-8′-hydroxylase*) and deconjugation (*β-glucosidase*) indicate that the increase of the hormone in BA-treated L3 fruitlets may not be imputed to either a block of the metabolism or a stimulation of deconjugation. However, because the presence of two different ABA pools

![Figure 9. Hypothetical model explaining the involvement of ABA within the regulatory network that leads to abscission induction. Fruit shedding, already naturally triggerable because of interorgan, intercluster, and intracluster competition for assimilates, can be magnified indirectly by means of thinning treatments (i.e. BA or MET), which cause a nutritional stress, thus enhancing the competition for assimilates between vegetative (i.e. shoots and buds) and reproductive sinks (i.e. fruitlets). This condition is perceived by the sentinel fruit cortex, in which a primary sugar signaling compatible with abscission induction is triggered. This signaling would start the shedding process by means of a multiple network of interactions between hormones (mainly ABA and ethylene) and other signaling molecules (i.e. the ROS). During abscission induction, the production of both isoprene and ABA appears to be coregulated, with the former possibly involved in detoxification from ROS and in the activation of secondary signaling pathways (cross talk with ethylene). Exogenous ABA treatments are able to magnify fruitlet abscission but unable to affect isoprene emission, thus positioning the involvement of ABA right upstream of abscission induction and not as a side effect of this process. The abscission signal is then transmitted to the abscission zone, most likely through the seeds, whose viability is finally compromised due to a developmental block caused by assimilate shortage. exABA, Exogenous ABA; AZ, abscission zone.](https://www.plantphysiol.org/doi/abs/10.1104/pp.113.217517)
has been speculated in plants (Barta and Loreto, 2006), it cannot be ruled out that a contribution to the hormone increase may derive from a direct biosynthetic pathway, which still remains elusive in higher plants. As far as ABA metabolism and deconjugation are concerned, it is interesting that in C3 fruitlets, the related genes are regulated by the BA treatment in a way that would facilitate fruit development by keeping the concentration of the hormone at low levels (Fig. 7).

The pivotal role of ABA in abscission has been widely studied and debated in different species (Guinn and Brummett, 1988; Yuan and Huang, 1988; Talon et al., 1990; Sagee and Erner, 1991; Gómez-Cadenas et al., 2000), especially concerning its cross talk with ethylene. More specifically, Talon et al. (1997) found a close relationship between ABA concentration, ethylene biosynthesis, and abscission in citrus fruit, hypothesizing a specific stimulatory effect of ABA on 1-aminoacyclopropane-1-carboxylic acid synthase transcription. These findings have recently been confirmed on the basis of the activation of ABA-specific transcriptional signatures, showing that several ABA-signaling genes (e.g. those encoding WRKY, bZIP, MYC/MYB, and APETALA2/Ethylene Response Factor transcription factors and mitogen-activated protein kinases) are activated during the early phases of abscission induction in apple (Botton et al., 2011). Moreover, the overall transcriptional activation of ABA target genes following abscission induction has been proven by HORMONOMETER analysis (Supplemental Fig. S11). Briefly, this bioinformatics tool allows, in terms of correlation (or anticorrelation), the similarity (or dissimilarity) to be described between a query transcriptional response and a transcriptional response typically assessed with a certain hormone treatment as defined by known hormone indexes in Arabidopsis. For a detailed description of the tool, see Volodarsky et al. (2009) and recent applications in peach (Bonghi et al., 2011) and grape (Vitis vinifera; Ziliozzo et al., 2012). Microarray data produced by Botton et al. (2011) were used as input for this analysis (see Supplementary Material for further details), indicating that ABA-related transcriptional indexes were active at both 17 and 19 DAPF. Taken together, these data would indicate that the increased levels of the hormone were in fact perceived and thus have a signaling value.

CONCLUSION

A hypothetical model originally developed by Botton et al. (2011) was extended on the basis of the main findings described herein to explain the regulatory action exerted by ABA in apple cortex during the early phases of abscission induction and the putative role of isoprene in the process (Fig. 9). The induction of the shedding process at the cortex level is orchestrated by a multiple network of interactions between hormones (mainly ABA and ethylene) and other signaling molecules (i.e. the ROS). During abscission induction, the production of both isoprene and ABA appears to be temporally coordinated, with the former possibly involved in a tentative detoxification from ROS and in the activation of still-unknown secondary signaling pathways. Gene expression data indicate that the stable levels of ABA maintained in fruitlets induced to abscise may be due to a stimulation of its biosynthesis and that the hormone activates its signal transduction pathways. Exogenous ABA treatments can induce fruitlet abscission in the L3 class but are unable to consistently affect isoprene emission in the same fruits. Based upon these observations, the involvement of ABA may be upstream abscission induction (i.e. downstream isoprene) and not a side effect of this process, at least during the early inductive phase. The involvement of ABA, isoprene, and ROS described by this model, however, is just one piece of the abscission puzzle and reconfirms the seed-protecting role played by the cortex (as a sentinel) during fruit shedding. Further investigations should focus on the role of seeds, at which level the master regulators of abscission induction are thought to be triggered by transducing the nutritional stress perceived by the cortex into the abscission signal that activates the abscission zone.

As a concluding comment, future applications involving the measurement of isoprene emission at the field level may be useful to predict the fruit load. Isoprene is an important parameter from an environmental point of view, and for this reason, its levels have already been monitored with different systems, including satellite observations (Xu et al., 2002). However, the resolution of these imaging systems is not sufficient to provide precise data at the orchard level. A high-resolution dedicated system is therefore being developed to test its efficacy in predicting fruit load and, consequently, allow thinning treatments to be tuned according to a modern environmentally friendly precision agriculture.

MATERIALS AND METHODS

Plant Material and Treatments

Experiments were conducted over four different years on apple (Malus domestica) trees of cv Golden Delicious/M9 and Red Chief/M26 trained with standard horticultural practices at the experimental farm Mase Part of the Istituto Agrario di San Michele all’Adige, Edmund Mach Foundation. Thinning experiments were done in 2008, 2009, and 2011, and ABA treatments were done in 2011 and 2012.

A randomized block experimental design was adopted in all the experiments (four blocks, each including five trees, for each experimental thesis of each cultivar). Concerning the thinning experiments, trees of both cultivars were treated with MET sprayed at 350 μL L⁻¹ (commercial name, Brevis; Makhteshim Agan) or BA at 200 μL L⁻¹ (commercial name, Bracher Drirado; Agrimport), when fruits had an average size of 13 mm (about 15 DAPF). ABA treatments were performed in 2011 and 2012 on trees of cv Golden Delicious/M9 at 15 DAPF. ABA (commercial name, ProTone; Valent Biosciences) was sprayed at 30 (only in 2012), 100, 300, and 1,000 μL L⁻¹.

In all the experimental trials, fruit drop was monitored from 15 DAPF until it ended (47 DAPF in cv Golden Delicious and 49 DAPF in cv Red Chief) on one tree per block plus two additional trees (n = 6) by counting the fruits dropped onto nets previously spread on the ground under the tree. Only in 2008, fruit drop was monitored on pilot clusters separately for each fruit class.
as described by Botton et al. (2011). Fruits were collected at the same time of day (8 AM), counted, and weighed, and their cross diameters were measured. APs were assigned to each class of fruit of both cultivars as reported in Supplemental Table S1.

**PTR-MS Measurements**

VOCs emissions were measured with a PTR-MS on at least eight representative fruitlets for each class, which were harvested, immediately placed in a 100-mL glass bottle (Kavalar), topped with Teflon caps, and kept at a constant room temperature of 20°C throughout the measurement time. The headspace of the different samples was sent to the drift tube of the PTR-MS system through a gas inlet maintained at 20°C, with an air flux of 15 cm3 min−1 at standard temperature and pressure. Mass data were collected in a range from 20 to 200 atomic mass units (m/z) with a dwell time of 0.5 s mass−1 under drift tube conditions of 120 Townsend (where 1 Townsend = 10−17 V cm2 mol−1) as reported by Vezzaro et al. (2011). A representative spectrum for each sample was obtained by averaging the last five acquired spectra after having reached steady-state conditions. Blank subtraction was then carried out, and values were converted into nanoliters per hour per gram of fresh weight.

**Quantification of ABA and Total Chlorophylls**

Fifty milligrams of fruitlet cortex, previously frozen in liquid nitrogen and ground with a mortar, in 1.5 mL of water was boiled at 100°C for 5 min to prevent hydrolysis of endogenous ABA conjugates that would have caused an overestimation of free ABA (Loveys and Van Dijk, 1988). Samples were then extracted for 12 h in complete darkness at 4°C on a shaker. The extracts were centrifuged at 10,000g for 25 min as indicated by Barta and Loreto (2006), and the ABA content of a 1:10 dilution of each sample was then quantified in an ELISA using the Phytodetek-ABA kit (Agdia Inc.) according to the indications of the manufacturer. The monoclonal antibody raised against ABA was previously shown to have high specificity for 2-cis-(5′)-ABA and no significant cross-reactivity against 12 different structurally ABA-related compounds (Weiler, 1982). Total chlorophyll content was determined by methanol extraction and spectrophotometry as described by Mackinney (1941).

**Quantitative PCR Expression Analyses**

For quantitative PCR analyses, total RNA was extracted in 10 mL of extraction buffer from 0.60 g of cortex tissue following the method of Ruperti et al. (2001), with a few adaptations as described by Botton et al. (2008, 2011). Total RNA was quantified with the NanoDrop 2000c spectrophotometer (Thermo Scientific), and its integrity was checked by running 1 μg in a 1% (w/v) agarose gel stained with SYBR Safe (Life Technologies). For quantitative PCR analyses, total RNA was extracted in 10 mL of extraction buffer from 0.60 g of cortex tissue following the method of Ruperti et al. (2001), with a few adaptations as described by Botton et al. (2008, 2011). Total RNA was quantified with the NanoDrop 2000c spectrophotometer (Thermo Scientific), and its integrity was checked by running 1 μg in a 1% (w/v) agarose gel stained with SYBR Safe (Life Technologies). Total RNA was reverse transcribed 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 se-
quences of the primers for both the target and reference genes are reported in Supplemental Table S4. 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 Microsoft Excel spreadsheet. QGene designed by Simon (2003), using the modifications of the comparative threshold cycle method suggested by Pfaffl (2001). Gene expression values were normalized to three housekeeping genes [MDP0000375455, putatively encoding for a Leu-rich Repeat-protein kinase; MDP0000767855, encoding for a 3(1)-pyrrole-5-carboxylate dehydrogenase (GenBank accession no. ACL13350); and MUFBI (GenBank accession no. DQ430899), the same used by Dal Cin et al., 2005] chosen among nine candidates because of their stability of expression, as checked by means of the BestKeeper spreadsheet version 1.0 (Pfaffl et al., 2004). Expression levels were then reported as arbitrary units of mean normalized expression, calculated using equation 2 of the QGene spreadsheet.

**Statistical Analyses**

The Globaltest package version 4.14.4 (Goeman et al., 2004) of R software version 2.15.0 (http://www.r-project.org/) was used to test the overall volatile emissions for significant association with the AP of the samples analyzed. A Z score, calculated for each volatile, gives the strength of these associations.

All multiple comparison statistics were calculated with the R software. In detail, normality was verified with Shapiro-Wilk test, homoscedasticity was verified with Bartlett’s and/or nonparametric Levene’s test, and differences among samples were verified with either ANOVA (normality and homoge-
neous variances) or Welch’s one-way ANOVA (normality and nonhomoge-
neous variances) followed by post hoc t test or Waller-Duncan test, respectively, and with Kruskal-Wallis (nonnormality and homogeneous variances) or Friedman test (nonnormality and nonhomogeneous variances).

Pearson correlation coefficients and their significance, along with common Student’s t tests, were calculated with the StatPlusmac LE 2009 version 5.8.2.0 package (AnalystSoft, Inc.) for Microsoft Excel.

Sequence data from this article can be found either in the GenBank/EMBL data libraries or at the Fondazione Edmund Mach-Istituto Agrario di San Michele all’Adige website (http://www.genomics.research.iasma.it/).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Fruit drop dynamics in cv Red Chief.

**Supplemental Figure S2.** PTR-MS measurements showing ionization of isoprene fragments as a function of collision energy.

**Supplemental Figure S3.** Isoprene emission in control and BA- or MET-treated fruitlets of cv Red Chief.

**Supplemental Figure S4.** Effects of ABA treatments on total fruitlet drop.

**Supplemental Figure S5.** Effects of ABA treatments (three concentrations) on isoprene emissions.

**Supplemental Figure S6.** MapMan analysis of secondary metabolisms during abscission induction.

**Supplemental Figure S7.** Neighbor-joining phylogenetic tree of DXS from different plants.

**Supplemental Figure S8.** Neighbor-joining phylogenetic tree of ZEPs from different plants.

**Supplemental Figure S9.** Neighbor-joining phylogenetic tree of NCEDs from different plants.

**Supplemental Figure S10.** Neighbor-joining phylogenetic tree of AAPs from different plants.

**Supplemental Figure S11.** HORMONOMETER analysis.

**Supplemental Table S1.** Abscission potentials of apple fruitlets of cv Golden Delicious and Red Chief, with or without thinning treatments.

**Supplemental Table S2.** Associations between VOC emissions and fruits destiny in cv Red Chief.

**Supplemental Table S3.** Correlations between isoprene emission, abscis-
sion potential, and fruit size in cv Red Chief.

**Supplemental Table S4.** Primers used in quantitative PCR experiments given in 5′-3′ orientation.

**Supplemental Table S5.** Enrichment analysis.

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**LITERATURE CITED**


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Isoprene and Abscisic Acid during Abscission Induction


