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Setting up of molecular tools for studying abscission in apple (*Malus* x *domestica*)

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Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

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Abstract

Abscission is a natural self-regulatory mechanism whereby fruit trees shed part of the fruitlets, and is an important agricultural event from the farmer's point of view because it directly affects the final size and quality of the commodity. In spite of this self-regulatory mechanism, fruit trees set too many fruitlets negatively affecting not only the final quality, but also the returning bloom. To avoid these effects, farmers perform blossom or fruitlet thinning to adjust crop load and ensure a satisfactory fruit quality at harvest for commercial purposes. Chemical thinning is nowadays a common orchard practice in some apple cultivars but its effects on fruit trees depend on environmental factors and genotypes. A widely employed chemical thinner, the carbaryl (Sevin), has been withdrawn from the market and replaced with benzyladenine (BA), a cytokinin that has a milder effect both on the tree and human health.

At molecular level, abscission is a coordinated event under the control of auxin and ethylene. Both hormones play their role at the abscission zone (AZ) level. Auxin flux through the AZ target cell layers inhibits the onset of shedding whereas ethylene causes the up-regulation of degrading cell wall enzymes causing the cell separation and the fruitlet shedding. Nevertheless, no information is available about the signalling that causes the decreasing auxin flow and the gain in ethylene sensitivity in AZ.

In *Arabidopsis thaliana*, the isolation of mutants with defects in floral organ abscission allowed the identification and characterization of genes involved in AZ differentiation and/or the signalling pathways. A model has been proposed by characterizing genes encoding receptors and ligands involved in the transfer of the shedding signal.

In apple, a model to explain the fruitlet physiological drop is still missing as well as the isolation and characterization of genes involved in abscission. Although most of the researchers focused their attention mainly on the effect of chemicals on fruit quality and returning bloom, few of them considered also the dynamics of the transcriptomic changes occurring as consequence of the abscission induction. A working hypothesis taking into account the carbohydrate status within the fruitlet population and between fruitlets and shoots has been proposed.

The recent development of massive gene approaches, based on microarray technologies, allowed to deeply investigate many fundamental biological process from a transcriptomic

point of view. A recent work carried out in tomato investigated the genes involved in auxin homeostasis, as a consequence of IAA depletion during flower abscission induction. The model proposed represents a powerful platform for further analyzing the putative regulatory abscission-related genes involved in the gaining of ethylene sensitivity of the AZ.

In the present dissertation, two different approaches have been adopted to investigate the early phases of apple fruitlet abscission: 1) A transcriptomic approach to isolate genes involved in the early steps of abscission, and 2) the characterization of volatile organic compounds (VOCs) emitted during abscission induction.

1) Apple fruitlet abscission was induced using BA as thinner. Fruitlets differing in size and position within the cluster were collected over a 4-day time-course, after BA treatment, and their gene expression profiles were analyzed, separately in cortex and seeds, by means of a newly released 30K oligonucleotide microarray. The analysis of the transcriptomic profiles of abscising and non-abscising fruitlets was tested for statistical association with abscission potential to identify molecular signatures strictly related to fruit destiny. Reactive oxygen species (ROS) and carbohydrates (glucose, fructose, sucrose, sorbitol, and starch) were also measured. A hypothetical model for apple fruitlet abscission was obtained by putting together available transcriptomic and metabolomic data. According to this model, BA treatment would establish a nutritional stress within the tree that is primarily perceived by the fruitlet cortex whose growth is blocked by resembling the ovary growth inhibition found in other species. In weaker fruits, this stress is soon visible also at the seed level, likely transduced via ROS/sugar and hormones signalling crosstalks, and followed by a block of embryogenesis and the consequent activation of the abscission zone.

2) Fruitlets of two cultivars (Golden Delicious and Red Chief) with different abscission potentials treated with two different thinning agents (BA and metamitron) were analyzed by means of proton transfer reaction mass spectrometer (PTR-MS) over a 10-day time-course looking for volatile organic compounds (VOCs) differentially emitted. Results pointed out that isoprene is more abundantly emitted by the abscising fruitlets in both cultivars. A relationship between isoprene emission and ABA content in the fruit cortex was also pointed out, along with a specific activation of the corresponding biosynthetic genes. A delayed transcriptional activation of a biosynthetic gene involved in a key step of methyl-erythritol phosphate (MEP) pathway, supplying the precursors for both isoprene and ABA

biosynthesis, was also shown. According to the main findings, a role for isoprene as a ROS-detoxifying mechanism mediated and transcriptionally controlled by ABA may be hypothesized.

Future perspectives will be focused on all the research lines herein adopted. The biological function of the genes identified by means of the transcriptomic approach will be studied at the cellular level, with *in situ* hybridization, and their expression pattern further validated. Attention will be focused in particular on transcription factors and other regulatory elements involved in hormonal cross-talk. The abscission-related VOCs (*i.e.* isoprene) will be validated to assess the possible applications in fruit load prediction systems, in order to fine-tune the use of thinning chemicals according to a more-environment-friendly agriculture.

Riassunto

L'abscissione è un meccanismo auto-regolativo per cui gli alberi da frutto rilasciano naturalmente parte dei frutticini ed è un evento agronomico importante dal punto di vista del produttore perché incide sulla qualità del raccolto a maturazione. Nonostante questo meccanismo auto-regolativo, gli alberi da frutto trattengono troppi frutticini, influendo negativamente non solo sulla dimensione e la qualità finale dei frutti, ma anche sulla fioritura nell'anno successivo. Per evitare questi effetti negativi, i coltivatori utilizzano comunemente diradanti chimici che agiscono su fiori o frutticini, allo scopo di regolare il carico iniziale ed ottenere così una qualità della frutta corrispondente alle esigenze di mercato. In melo il diradamento chimico è una pratica comune la cui efficacia sugli alberi da frutto è dipendente, purtroppo, da fattori ambientali e dai diversi genotipi. Un diradante chimico ampiamente utilizzato, il carbaryl (Sevin), è stato ritirato dal mercato e parzialmente sostituito con la benziladenina (BA), una citochinina che ha un effetto più contenuto sull'albero e sulla salute umana.

A livello molecolare, l'abscissione è un processo coordinato principalmente dall'auxina e dall'etilene. Entrambi questi ormoni svolgono il loro ruolo a livello della zona di abscissione (AZ). Il flusso continuo dell'auxina attraverso la AZ inibisce il processo di abscissione, mentre l'etilene induce una regolazione positiva degli enzimi degradanti la parete cellulare provocando la separazione delle cellule della AZ e la caduta dei frutticini. Le informazioni riguardanti il segnale che causa la diminuzione del flusso di auxina e l'aumento nella sensibilità all'etilene nella AZ sono tuttavia ancora parziali e piuttosto carenti. In *Arabidopsis thaliana* l'isolamento di mutanti con difetti nel processo di abscissione dei fiori ha permesso l'individuazione di geni coinvolti o nel processo di differenziazione della AZ o nella via di trasduzione del segnale. Per quanto riguarda la cascola fisiologica dei frutticini in melo, le informazioni riguardanti il segnale che genera l'evento abscissione sono tuttora carenti e le collezioni di geni legati a tale fenomeno sono ancora molto parziali. La maggior parte delle ricerche, infatti, si è concentrata principalmente sullo studio dell'effetto di prodotti chimici sulla qualità della frutta e sulla fioritura, mentre solamente pochi studi hanno considerato la dinamica dei cambiamenti trascrizionali conseguente all'induzione dell'abscissione. Sulla base dei dati disponibili, è stata proposta l'ipotesi che considera lo stato nutrizionale all'interno della popolazione di frutticini e fra i fruitticini ed i germogli

vegetativi come segnale necessario per l'attivazione dell'abscissione. Recentemente, lo sviluppo di approcci trascrittomici di carattere massale, basato sulle tecnologie microarray, ha consentito di studiare in maniera più approfondita questo processo biologico. Un recente studio effettuato in pomodoro ha permesso di studiare geni coinvolti nel mantenimento dell'omeostasi dell'auxina a livello di AZ in seguito ad una diminuzione del flusso della stessa durante l'induzione dell'abscissione nel fiore. Il modello proposto rappresenta un punto di partenza molto rilevante per identificare altri geni coinvolti nella regolazione dell'abscissione e nella sensibilizzazione dell'AZ all'etilene.

In questa tesi sono stati impiegati due differenti approcci per studiare l'abscissione in melo: 1) Un approccio massale trascrittomico per isolare i geni strettamente coinvolti nelle prime fasi induttive dell'abscissione e 2) lo studio di composti organici volatili (VOCs) emessi durante l'induzione dell'abscissione.

1) L'abscissione di frutticini di melo è stata indotta usando la BA come agente diradante. Frutticini differenti per dimensione e posizione all'interno del corimbo sono stati raccolti entro i quattro giorni dal trattamento. L'espressione genica è stata analizzata per mezzo di un vetrino 30K recentemente sviluppato. L'analisi dei profili trascrizionali dei frutticini cascolanti e non cascolanti è stata esaminata allo scopo di identificare marcatori molecolari associati al destino del frutto. Il livello di specie reattive dell'ossigeno (ROS) e di alcuni carboidrati (glucosio, fruttosio, saccarosio, sorbitolo e amido) è stato misurato nella cortex degli stessi campioni. Un modello ipotetico per l'abscissione di frutticini di melo è stato ottenuto unendo i dati trascrittomici e metabolomici disponibili. Secondo questo modello, il trattamento con la BA amplificherebbe lo stress nutrizionale già in atto all'interno dell'albero, il quale viene percepito soprattutto dalla cortex di frutticini il cui sviluppo viene quindi bloccato. Nei frutti più deboli, questo stress viene quindi percepito a livello del seme. La traduzione di questo stress avviene probabilmente attraverso il *crosstalk* tra ROS, zuccheri e ormoni ed è seguito da un blocco dell'embriogenesi e dall'attivazione della AZ.

2) Frutticini di due diverse cultivar (Golden Delicious e Red Chief) con differente potenziale di abscissione trattati con due differenti diradanti chimici (BA e metamitron) sono stati analizzati per mezzo del PTR-MS (proton transfer reaction mass-spectrometer), entro i dieci giorni dal trattamento, allo scopo di identificare composti organici volatili (VOCs) associati all'abscissione. I risultati hanno evidenziato che i frutticini con potenziale

di abscissione maggiore in entrambe le cultivar emettono più isoprene rispetto ai frutti persistenti. E' stata inoltre evidenziata una correlazione significativa tra emissioni di isoprene e contenuto di ABA della cortex, parallelamente all'attivazione specifica dei rispettivi geni biosintetici. Successivamente avviene l'attivazione ritardata dei geni coinvolti nei passaggi chiave della via del metileritritolo fosfato (MEP), che fornisce i precursori per la biosintesi sia del volatile che dell'ormone. Secondo questi risultati, si può ipotizzare per l'isoprene un ruolo di detossificatore di ROS, la cui attivazione è mediata e controllata a livello trascrizionale dall'ABA.

Le prospettive future di questa ricerca saranno focalizzate su tutte le linee di ricerca finora perseguite. La funzione biologica dei geni identificati tramite l'approccio trascrittomico sarà ulteriormente studiata a livello cellulare, tramite ibridazioni *in situ*, e i loro profili di espressione genica saranno ulteriormente validati. Particolare attenzione sarà prestata ai fattori di trascrizione e agli altri elementi regolativi coinvolti nel cross-talk ormonale. Per quanto attiene i composti volatili (isoprene) saranno validati i risultati finora ottenuti allo scopo di verificare possibili applicazioni pratiche in sistemi previsionali che consentano di predire il livello di carica fruttifera e, quindi, di dosare i trattamenti diradanti nell'ottica di un'agricoltura sostenibile.

Chapter 1 – General introduction

Introduction

Many fruit species bear an abundance of flowers, which produce a surplus of fruits that the tree is unable to support. In anticipation of this, the major fruit species developed an immature fruit (fruitlet) physiological drop as a self-regulatory mechanism. This process is at least in part a consequence of the competition among fruits and between fruits and shoots for carbon assimilates. Fruit drop is preceded by a growth arrest of the fruits destined to shed and ends with the activation of the abscission zones (AZs), special tissues devoted to undergo cell separation and leading to the physical separation of the organ from the plant (for a complete review see Roberts et al., 2002). Although the identity of the signal responsible for the developmental arrest of fruits destined to drop is still unknown, the correlative origin of fruitlet abscission is generally accepted. In apple the fruitlet physiological drop is due to the activation of the AZ located at the junction of the peduncle into the twig. It is generally accepted that abscission is a highly regulated development process that is activated in response to internal cues or environmental conditions (Bangerth, 2000). Ethylene and auxin are the major hormones involved, being proved their ability in enhancing or delaying the shedding process. Their role in abscission has been extensively reviewed by Taylor and Whitelaw (2001). As far as immature fruit drop is concerned, it has been demonstrated that the activation of peach AZ explants is preceded by a stimulation of ethylene biosynthesis occurring all over non zone and zone tissues (Ruperti et al., 1998). In apple abscission induction through chemical thinner allows a stimulation of ethylene biosynthesis (Dal Cin et al., 2005, 2007a, 2007b, 2009a), paralleled by the up-regulation of key regulatory genes for ethylene biosynthesis, such as *MdACO1* and *MdACS5B*. These results confirm the role of this hormone in coordinating abscission event in fruitlets prone to be shed.

From a horticultural point of view this self-regulatory mechanism is insufficient to guarantee fruit of acceptable commercial value. For this reason fruitlet thinning is a common practice in orchard management. The operation can be performed by hand, mechanically or chemically. Fruitlet chemical thinning has been regarded as the most interesting because it is a quick and low cost operation that can be performed at the proper time. In fact, in apple industry the timing of fruitlet thinning is extremely important not only to guarantee fruit of good marketable size at harvest but also to avoid biennial bearing. To

pursue both goals, apple fruitlet thinning should be performed within 5-6 weeks after full bloom (Wertheim, 2000). Bioregulators as NAA and its amide and citokinins (BA) are currently used as apple thinners. An important feature of fruit chemical thinners relies on the ability in magnifying fruitlet natural drop unexpressed at the moment of treatment (Wertheim, 2000). Given a natural fruit drop dynamics, the maximum thinning effect is exhibited when the chemical is sprayed at the beginning of the natural fruit drop (Ramina, 1981). Later on applications are less effective, and the chemical can be totally ineffective sprayed at the end of fruit drop (Bergh, 1990, 1992). It has to be underlined that the thinning action of bioregulators is quite variable and depends on environmental conditions and varieties. In apple there are varieties easy to thin and others difficult even though different chemicals or combination of them are used (Wertheim, 2000).

In recent years thinning methods have received considerable attention, confirmed by numerous published reviews (Williams, 1979; Miller, 1988; Dennis, 2000; Bangerth et al., 2000; Wertheim, 2000; Webster and Spencer, 1999) and are widely employed in response to the necessity for regulating fruit load, avoiding biennial bearing and improving fruit quality, in terms of both size and coloration for marketing, and firmness for good storability. Still, the degrees of adjustment to the crop load required depend on market requirements for the fruit: more severe reductions (thinning) are often needed where the fruits are sold for fresh consumption, as most of the multiple retailers pretend large fruits. Thinning can often be less rigorous where the fruits are grown for one of the many processing markets (e.g. for jams, canning, and alcoholic beverages).

Chemical thinners are separated in two categories, bloom (flower) and post-bloom (fruit) thinners. Blossom thinning is applied in order to prevent fruit set whereas fruitlet thinning to directly decrease crop load.

Blossom thinning

Fruit set can be prevented by reducing floral bud number by means of winter pruning, treatments with gibberellins (GAs), removing pollinators, performing hand thinning or applying blossom thinner at full bloom.

Winter pruning can be regarded as part of the routine winter pruning operation, and is considered an environmentally acceptable method. It gives the maximum benefit in terms of reducing competition, as early as possible, between flowers and fruits for assimilates and nutrients. However, this technique lacks precision in terms of the relative numbers of flowers removed and left on the tree, and is a high-risk strategy because it is carried out before flowering when the risk of frost damage may be elevated. Inhibition of flower bud formation can be achieved by treating fruit tree with GAs, responsible for the inhibition of floral bud initiation. Sprays of synthetic GAs in peach, applied when flower buds are initiating or in the early stages of development, can significantly reduce the number of flower buds (Webster, 2002). There is, however, almost no evidence that a similar strategy is effective in apple and pear, although it has been demonstrated that the GAs produced by the seeds of apple fruitlets may negatively affect the returning bloom (Chan and Cain, 1967). Besides being very difficult to control the degree of flower bud inhibition, the quality of flower buds (i.e. their ability to set fruits) produced in the subsequent season may be reduced. GAs may have also deleterious effects in the current season, since fruits may change their storage potential due to an increase of competition between fruits and shoots (Webster, 2002).

Prevention of fruit set can be obtained by reducing the number of potential pollinators of the orchard, thus negatively affecting pollination. Alternatively, the removal of flowers from trees can be performed manually, although the operation is feasible and economically viable only if mechanical devices are used. Mechanical flower thinning is an environmentally friendly technology and an alternative to the standard chemical thinning, which is limited by a decreasing number of registered (i.e. approved) chemical compounds whose success is very dependent on weather conditions, variety, flowering dynamics, and tree age (Wertheim, 2000). Mechanical thinning can be performed by means of a device newly developed at the University of Bonn (Germany) (Solomakhin and Blanke, 2009)(**Figure 1**), which consists in vertically rotating brushes attached on the front of a tractor able to remove

an adjustable amount of flowers by centrifugal force. Nowadays, apple tree is used as a model crop for studying the effect of this device on final fruit yield and return of flowering.

Figure 1 Blossom thinning throughout mechanical device.

All these methods for preventing fruit set are very high-risk strategies, and thus flower thinning at bloom by means of chemical sprays still remains the most popular practice. Many blossom thinning chemicals bring about their effect by desiccating the vital female organs (stigma/style or ovary) of the flower. Chemicals such as dinitro-ortho-cresol (DNOC), endothallic acid, pelargonic acid, sulfcarbamide, ammonium thiosulphate (ATS), urea, lime sulphur (calcium polysulphide), and ethephon (2-chlotoethyl-phosphonic acid) all function in this way.

DNOC, believed to prevent germination and pollen tube growth (Hildebrand, 1944), was one of the first chemicals to be used as an effective blossom thinner but its production was discontinued in 1989 because of environmental concerns and the high cost of re-registration (Greene, 2002). Endothall, perlagonic acid, and sulfurcarbamide can be phytotoxic and do not provide consistent thinning (Wertheim, 2000). ATS is widely employed in apple and peach because provide effective blossom thinning but it does not significantly increase fruit size (Fallahi and Willemsen, 2002; Greene et al., 2001). ATS efficiency depends upon the

flower developmental stage, since pistils of fully open flowers are significantly more susceptible to ATS damage than exposed pistils of closed flowers. Only in 18.8% of closed flowers pistils were damaged by ATS in apple (Janoudi and Flore, 2005). The reason for this difference in susceptibility has not been determined yet. Pistils of closed flowers may have a higher concentration of protective antioxidants than older fully developed flowers (Janoudi and Flore, 2005). Antioxidant compounds such as glutathione and enzymes such as catalases and superoxide dismutases have been reported to be present in flowers of various plant species (Acevedo and Scandalios, 1992; Mills et al., 1997). The thinning activity and phytotoxicity of ATS varies according to the cultivar and the dosage, respectively (Basak, 2000; Fallahi and Willemsen, 2002). It has negative effects on leaves causing damages when applied at concentration of 1.5% or more as observed in apple (Basak, 2000; Fallahi and Willemsen, 2002). On the other hand, this product may be safely used in peach because the trees have less foliage than apples at the time when blossom thinners are applied (Dennis, 2000). An additional blossom thinner in apple is urea, although it can damage spur leaves. Some loss of leaf area may occur without loss of fruit size but the supplement of calcium may be negatively affected (Volz et al., 1994). Further, urea may have other negative effects, such as diminished fruit color and flower-bud formation, and increased skin russet. Therefore, its thinning activity is not always satisfactory. Lime sulphur is a blossom thinner permitted also in organic apple production under EU legislation, and is considered an effective thinning agent on apple (Meland, 1998; Bertschinger et al., 2000, Stopar, 2004). However, its high phytotoxicity may be a limiting factor for its worldwide application on apple (Hobl et al., 2003). Another thinner is ethephon, a well-known chemical for blossom thinning (Irving et al., 1989; Williams, 1994) although it can give variable results. Ethephon effect is broad when applied at full bloom compared to its poor action near to petal fall. Ethephon, when applied to tree canopy, is hydrolyzed to release ethylene that gives rise to caustic effect on floral reproductive organ affecting final fruit set. Ethephon activity may depend on the flower developmental stage and weather conditions. The temperature during application is the most important parameter (Olien and Bukovac, 1978) since it affects both absorption and degradation of the chemical to ethylene (Biddle et al., 1976; Flore and Bukovac, 1982; Olien and Bukovac, 1978).

In the last decades, the relevant interest of the public opinion on healthy cultivated products have focused the attention of many farmers on chemical-free treatments. Therefore, natural products that can be employed in organic farming as substitutive of chemical products have begun to be used. Blossom and fruitlet chemical thinner will be succeeded to safer or lower phytotoxic products. As blossom thinners sodium chloride, acetic acid, rape oil, sunflower or soybean oil emulsions, and dextrins are used. These compounds have been tested on Golden Delicious apple trees (Stopar, 2004). Full bloom application of 3% oil emulsions (rape, sunflower, and soybean) achieved a significant fruitlet thinningh and enhanced the mean fruit weight. A decreased effect (not significant) was observed with full bloom applications of either 1 and 1.5% sodium chloride or 1 and 3% acetic acid. In spite of all, sodium chloride is a promising organic thinning compound because of its evidently positive effect on return bloom. Non-chemical techniques such as dragging knotted ropes through the trees and water sprayed at very high pressures have been proved to knock off blossom in peaches (Baugher et al., 1991; Byers, 1989). Water sprayed techniques resulted very successful in USA trials although water pressure needed is very high (i.e. >30 bars of atmosphere) and very careful design of equipment is required to satisfy the current legislation concerning operator's health and safety (Byers, 1989).

However, the efficacy of blossom thinners depend upon environmental conditions, post-flowering frost damages, and by the presence of flowers that are at different stages of development at the time of the application. These variables compromise the action of chemicals resulting in unpredictable cultivar responses in term of fruit set and percentage of small fruits at harvest. In order to avoid these unsuccessful findings a fruitlet thinner is often coupled to the flower ones.

Fruitlet thinning

Reducing the numbers of fruitlets on the tree after flowering is traditionally the most common strategy to adjust crop load, and is usually referred to as fruitlet thinning. The primary effect of fruit thinning on fruit size is more often a reduction in the number of smaller fruits than a dramatic increase in the size of the remaining fruits (Forshey and Elfving, 1977).

Fruitlet thinning can be achieved by removing fruitlets by hand, mechanically or throughout chemical sprays. Hand thinning has the advantage of being a low risk strategy, it can be

applied after spring frost period, and facilitates precise crop loading and fruitlet distribution within the tree. The right time for fruitlet removal can be controlled precisely with a significant effect on the final fruit size. Although this technique is environmentally acceptable, its high cost in terms of working power makes this practice not suitable. Mechanical thinning can be also applied during early fruitlet growth, although it is not selective and removes also the big fruitlets that would naturally persist on the three causing serious skin damages thus compromising their final marketing. For these reasons, the fruitlet chemical thinning appears the most promising technique, usually carried out by means of plant growth regulators and carbamate insecticides (Dennis, 2000), whose modes of action are still unknown.

The first chemical thinners to be applied were auxin-like compounds. Their promotion of post-bloom fruit abscission was first reported more than 60 years ago (Davidson et al., 1945) and since then, synthetic auxins, such as NAA (naphthaleneacetic acid) and its amide (NAD), have become the dominant fruit thinner, especially in apple (Dennis, 2000). NAA and NAD are thought to cause a temporary reduction in photosynthesis, movements of assimilates to the fruits, and reduction of the basipetal auxin transport from the fruitlet to the leaf (Patrick, 1987). In Elstar apples, after the application of 10 ppm of the NAA, a gradual decrease from 9 to 33.8% in tree canopy photosynthesis measured continuously over three days was reported. This result confirms unpublished results, obtained by Lakso in 2000, showing up to a 30% reduction of leaf photosynthesis in Empire and Delicious cultivars after the application of 10 ppm NAA, two weeks after full bloom when fruit size was between 10 and 14 mm. However, NAA drawback is attributed to its negative effect on fruit growth and size, even though crop load is substantially reduced (Stopar and Lokar, 2003). Some reports indicate that this undesired effect is even more evident with higher concentrations or late application of NAA (Bound, 2001). Therefore, the increase in size of the persisting fruit is not commensurate with the reduction in crop load (Greene and Autio, 1994; Luckwill, 1953; Marsh et al., 1960). Moreover, a wrong application in terms of time and concentration of NAA can block abscission of small and seedless fruitlets (< 65 mm diameter), known as pygmy fruitlets, thus consequently retained until harvest. This effect is usually more pronounced in late post-bloom applications and after NAD than NAA application (Hoffman et al., 1955; Wise et al., 2007).

The most successful chemical thinner is carbaryl (1-naphthyl-N-methylcarbamate), known also as Sevin (Knight, 1986). In Italy it was widely used in combination with several others chemicals such as NAD, NAA and benzyladenine. Carbaryl belongs to the group of carbamate insecticides and displays activity in most apple varieties (Bini and Raddi, 1967; Fukuda, 1991; Knight, 1978; Wertheim, 1973), in pears (Griggs et al., 1962), and also in stone fruits. Growers discovered the apple thinning activity of carbaryl in 1958 and it was researched aggressively (Batjer and Thompson, 1961). The negative side did appear neither for the tree nor for the fruits, apart from a possible increase in fruit-skin russeting. The latter may occur after early as well as repeated applications. Carbaryl was usually applied at 2 to 4 weeks after full bloom at 12 mm of diameter (Wertheim, 1973; Forshey, 1976). Three to four carbaryl sprays are recommended on apple cultivars with high fruit set (Thiéry, 1996), whereas two or three successive sprays of carbaryl did not thin more than one application in the cultivar Cox (Knight and Lovell, 1983). Carbaryl is toxic to bees when applied during bloom (Helson et al., 1994), although more bee-safe formulations (Sevin XLR) exist (Noga and Engel, 1986). The chemical was withdrawn from use in Austria, Denmark, Germany, Sweden and Switzerland several years ago and in almost all other European countries, including Italy, from 2008 (Dorigoni et al., 2003; Dorigoni and Lezzer, 2007; Costa et al., 2004). A potential new fruit thinner is metamitron, a commonly used herbicide that at low dosage reduces photosynthesis and consequently enhances fruit drop. It acts in a similar way to shading, by stressing competition between shoots and fruitlets, causing starvation and drop of the laterals. Dorigoni and Lezzer (2007) employed metamitron as thinner in Golden Delicious and Fuji. From a purely technical point of view, metamitron, although not currently registered in Europe as a thinner, showed great potential for crop load control. One of its key points is the dose-response, since double amounts of the active ingredient strongly increased its efficacy. Its simple and universally effective mode of action represents an important aspect since it thins several cultivars, from the relatively easy to thin Golden Delicious, to the most difficult ones such as Fuji, suggesting that unlike hormone-based thinners, whose activity is strongly related to specific cultivars, it can cover a wide spectrum of apple cultivars as well as other species.

In the last years benzyladenine (BA) was also extensively investigated as a fruitlet thinner. BA has been found to have chemical thinning activity (Greene and Miller, 1984; Greene

and Autio, 1989; Greene et al., 1990; Bound et al., 1991, 1993, 1997) in several apple cultivars (Dal Cin et al., 2007a; Elfving and Cline, 1993; Ferree, 1996; Greene, 1993; Greene et al., 1990; Stover et al., 2001). It reduced the crop load and increased the fruit size by increasing cell division in the fruit cortex in apple and pear fruit (Flaishman et al., 2005; Greenhalgh et al., 1977; Letham, 1968; Williams and Stahly, 1960; Wismer et al., 1995) and had a positive influence on the return bloom (McLaughlin and Greene, 1984; Elfving, 1989; Greene, 1989). In cultivar McIntosh, Yuan and Greene (2000) reported a dramatic increase in cytokinin zeatin riboside level after BA application, two weeks after full bloom in 10 mm fruitlets. It was suggested that BA promotes cell division in apple tissues possibly by increasing zeatin riboside levels in fruits. Additionally, BA has no drawback effects on mite predators (Thistlewood and Elfving, 1992; Elfving, 1994; Greene and Autio, 1994), it is not a persistent chemical, and is more likely to meet modern environmental and food quality guidelines. When applying BA for fruit thinning, the timing of spraying and its concentration depend first of all on the cultivar. To date, BA is applied when fruitlets have a diameter of 7 to 12 mm (most often 10 to 12 mm), i.e. 14 to 21 days after full bloom. Penetration of BA into the fruit is considerably higher than penetration into the leaves through either the abaxial or adaxial surface (Greene, 1993). Nevertheless, leaves may play a primary role because BA applied to the leaves alone, or both to the leaves and fruits resulted in comparable thinning (Greene, 1989, 1993). Results of experiments carried out over three years on the same apple cultivars, such as Golden Delicious, Idared and Wellspur Delicious, supported the temperature-dependent effect of BA on fruit thinning (Bubán and Lakatos, 1997, 2000). The optimum range of temperature is between 24-27 °C in the two to four days following application (Greene, 1997). The effect of BA on some quality traits, not related to abscission result, such as the increase in flesh firmness and soluble solid content, have also been observed in several experiments (Greene et al., 1990; Wertheim, 2000). The recorded increase in soluble solid content may be attributed to stimulation resulting from the import of sorbitol from the leaf, as demonstrated by Yuan and Greene (2000). In spite of all these additional positive effects of BA on fruit development, very little is known about its mode of action and, therefore, many different mechanisms are proposed to explain its activity. BA stimulates ethylene biosynthesis, since Greene et al. (1992) reported that, while ethylene production in both leaves and fruit increased linearly with increasing BA

concentration, the magnitude of this increase was not considered large enough to be the primary cause of thinning. Another suggested mode of action is the inhibition of photosynthesis. Yuan and Greene (2000) reported a reduction of 10–15% in photosynthesis and an increase in dark respiration at higher temperatures (30°C) but not lower (20°C) after application of BA. Another possible explanation is that thinning chemicals interfere with endogenous hormones controlling the supply of nutrients to the developing fruit. According to Ouma and Matta (2002), fruit abscission increased by chemical stress of any kind, and, consequently, embryo abortion may occur before or simultaneously with fruit abscission, although it is not considered as the main cause of abscission. It is probable that the thinning action of BA involves more than one of the mechanisms described, most likely including a combination between ethylene biosynthesis and a reduction and/or a block of transport of nutrients to the fruit.

By the time of carbaryl withdrawal from marketing, an alternative chemical thinning technique could be the combination of different thinners. Wertheim (1997) reported that combinations of thinning compounds might cause additional thinning compared to the thinning caused by a single one. This would agree with the report by Bukovac et al. (1994), in which the tank mix of NAA plus BA thinned Empire much stronger than application of NAA and BA alone. For example, in the hard to thin cultivar Fuji, BA was employed in combination with a double spraying of carbaryl to reach the best results (Dorigoni and Lezzer, 2007). The combination of NAA and BA showed a similar or lower thinning effect than NAA or BA applied alone, but they produced 2.5 to 5 fold more small fruits. The incidence of small fruits was also increased by a broad range of BA to NAA ratios (25:15 to 125:15 mg/L) in Redchief Delicious and Fuji, but not in Elstar, Gala and Jonagold cultivars (Bukovac et al., 2008) (**Figure 2**). The amount of small fruit formation was significantly reduced and mean fruit weight increased in the small-fruit cultivars Elstar and Gala. These data are consistent with other reports showing a positive effect of NAA and BA on fruit size in additional cultivars (Basak, 2006; Stopar and Lokar, 2003).

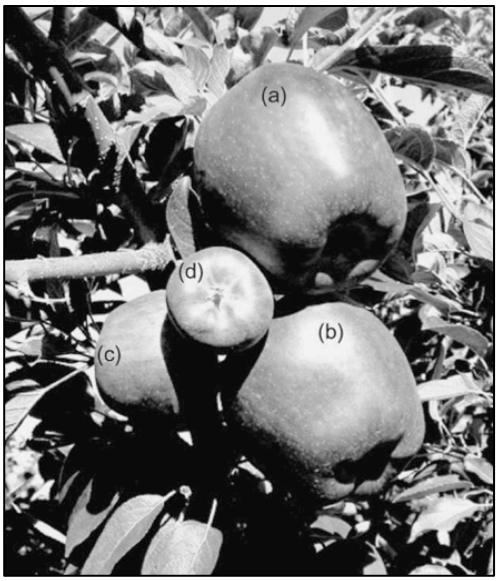

Figure 2 Redchief Delicious different fruit sizes resulting after NAA plus BA treatment. (a) King fruit per spur, (b) King fruit, (c) secondary and (d) tertiary lateral fruit per spur (from Bukovac et al., 2008).

Chemical thinners, such as NAA and BA, alone or in combination, can effectively thin apple when applied at fruit diameter of 5-16 mm (Byers, 2003; Marini, 1996). Furthermore, these chemical thinners have little or no effect on apple fruit thinning when fruit are larger than 16 mm in diameter. Ethephon, or ethrel (2-chloroethyl-phosphonic acid), is the only compound that can effectively thin apples when fruit are 18-30 mm in diameter (Byers, 2003; Jones et al., 1983; Marini, 1996; Veinbrants and Hutchinson, 1976). Therefore, ethephon has been suggested as a follow up thinner when previous chemical thinning sprays have failed to thin adequately (Byers, 2003). Ethephon hydrolysis releases ethylene that is the major hormone in stimulating fruitlet abscission by negatively affecting the auxin transport from fruitlets. Moreover, it positively affects return bloom (Jones et al., 1983; Stopar, 2000). Its effects depend on the temperature during and after application, being this the most important environmental factor affecting fruit abscission response to this chemical (Flore and Bukovac, 1982; Olien and Bukovac, 1978; Wittenbach and Bukovac, 1973; Jones and Koen, 1985; Yuan and Burns, 2004). Temperature affects both absorption and degradation of the chemical (Biddle et al., 1976; Flore and Bukovac, 1982; Olien and Bukovac, 1978). Low temperature after application results in a poor fruit abscission response, whereas high temperature may lead to over-thinning (Jones and Koen, 1985; Olien and Bukovac, 1978; Yuan and Burns, 2004).

Abscission

Abscission is a developmentally regulated and genetically programmed complex process, whereby multicellular organs such as leaves, flowers, or fruits become detached from the parent plant body (Osborne, 1989; Bleecker and Patterson, 1997; Taylor and Whitelaw, 2001; Roberts et al., 2002). In higher plants, abscission is achieved by dissolution of the middle lamellae of a specific group of positionally differentiated cells called 'abscission cells', and the region of layers of abscission cells at this point constitutes an abscission zone (AZ) (Osborne, 1989). The AZ is a predetermined site for specific inter- and intra-cellular signalling events, and the cells that constitute this zone respond in different ways with respect to their neighbours to the same hormonal cues (Brown, 1997; Gonzalez-Carranza et al., 1998; Roberts et al., 2000; Taylor and Whitelaw, 2001). For convenience, abscission can be divided into four steps (Patterson, 2001). Steps one and two involve the ontogeny of the abscission zone and the acquisition of competence to respond to abscission signals, respectively, while the third phase triggers the onset of the cell separation that leads to final organ shedding, the fourth stage (Patterson, 2001). One of the first model systems used for abscission zone characterization was bean (*Phaseolus vulgaris*). However, due to the limited number of layers comprised by its abscission zone, workers have focused their attention on other systems where the changes associated with cell separation may be co-ordinated over a greater area. The leaflet abscission zone of *Sambucus nigra* has proved a valuable system in this regard, being composed of 20-30 layers of cells (Osborne and Sargent, 1976).

AZs are generally formed during the development of the associated organ system and are characterized by a band of small densely cytoplasmic cells from a few to many cell layer thick (Addicott, 1982; Sexton and Roberts, 1982; Osborne, 1989). They are characterized by increased rough endoplasmic reticulum associated with the plasma-membrane and Golgi. This has been observed in many abscission systems (Sexton and Roberts, 1982) and is assumed to be involved in enzyme production and secretion (Ramina et al., 1989). Cells within AZ are characterized also by accumulation of microbodies and invaginations of the plasma membrane, associated with the swelling of cells on either the proximal or distal side of the abscission layer. These cells differentiate early within the time span of the organ to

which they are associated and remain in this state of arrested development (Addicott, 1982), while neighbouring cells enlarge, mature and become vacuolated.

However, it is clear that abscission cells, once differentiated early in development, are competent to respond to the appropriate stimulus. Osborne (1977) identified three types of cells with respect to their responses to hormonal stimuli. Type I cells elongate in response to auxin, while the addition of ethylene arrests this elongation growth and causes lateral expansion with the cell volume remaining essentially unchanged. Type II target cells are marked by expansive growth enhanced by ethylene, and not by auxin while the third type of target cortical cells expand and extend in response to both hormones (Osborne, 1977). The type II cells that comprise the abscission zones in higher plants have been well characterized in terms of their responses to both ethylene and auxin. Once abscission is commenced, a variety of many biochemical changes within the cells of the abscission zone are measured in association with the process of abscission. It has been hypothesized that abscission zone cells, especially those that line the separation point, may undergo programmed cell death (Roberts et al., 2000). Features of programmed cell death such as the breakdown of cellular compartmentalization have previously been observed in AZ cells during abscission of *Pelargonium* petals (Evensen et al., 1993), while abscission in *Delphinium belladonna* was shown to be preceded by DNA degradation and chromatin condensation in whole turgid petals, although abscission zones were not studied (Yamada et al., 2007). Tripathi et al. (2009) showed that petal abscission in *Rosa bourboniana* is associated with the expression of an ethylene-responsive cysteine protease gene, *RbCP1*, and the appearance of a 37 kDa cysteine protease leads to a decrease in protein content during abscission possibly associated with programmed cell death in the abscission zone. The role of *RbCP1* in petal abscission was confirmed by a reduction of its expression in 1-MCP-treated samples where a delay in petal abscission was also observed (Tripathi et al., 2009).

Modifications of the elemental composition of the cells, changes in hormones and increased expression of cell wall hydrolytic enzymes are some of the most frequent observations. To date, lower levels of calcium have been observed in active abscission zones in correlation with the conversion of insoluble pectins to soluble pectic acids. Changes in the levels of pectin methylesterases and pectate lyases are thought to be involved in demethylation of the pectins, and thus the breakdown of the middle lamella. Other cell wall hydrolytic enzymes

that have been demonstrated to be up-regulated in correlation with abscission include glucanases, xyloglucan hydrolyases, and polygalacturonases (PGs; Hadfield and Bennett, 1998; Roberts et al., 2000)(**Figure 3**).

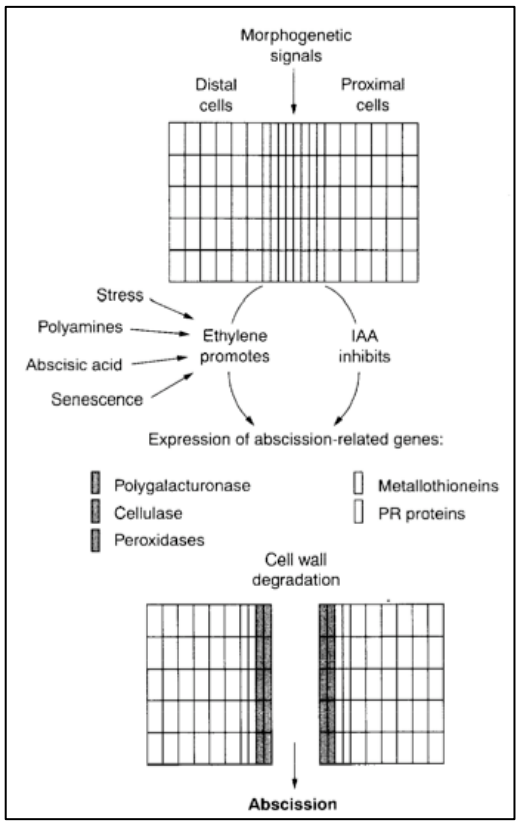

Figure 3 Schematic diagram of the abscission process (from Gonzalez-Carranza et al., 1998).

Herein below, the main results concerning AZ development and abscission related-genes will by briefly summarized as being already discussed in different reviews (Roberts et al., 2000, 2002). Different signalling pathways will be largely discussed as object of the present doctorate thesis.

Abscission zone development

A common strategy for examining AZ differentiation is to study mutants in which the normal development of AZ has in some way been impaired. Examples of phenotypes in which there is no abscission are rare. Two mutants of tomato, *jointless* (*j*) and *lateral suppressor* (*ls*), do not develop a pedicel AZ, and the mutated genes responsible for these phenotypes have been identified. *Jointless* is a simple recessive mutation and is genetically

mapped to chromosome 11 (Butler, 1936; Rick and Yoder, 1988). Analysis of *j* locus revealed a 939-basepair (bp) deletion including the 5' UTR and the first 33 bp of the gene (Mao et al., 2000, 2001). Protein sequence research revealed that *JOINTLESS* has profound homology with other MADS-box genes. Although there are several MADS-box genes in tomato, none of those resulted similar to *JOINTLESS* indicating that this gene represents a new kind of MADS-box gene (Mao et al., 2000). Tomato chimeras were generated from the *jointless* mutant (*j*) and wild-type plants (+) meristem layers (L1, L2 and L3) to gain insights into the process of pedicel abscission zone development. Chimeras *j*++ (L1 is *jointless*, *j*; L2 and L3 are wild type, +), *jj*+ and +*j*+ show a wild-type abscission zone phenotype and all the chimeras have wild-type L3-derived cells in the pedicel. Chimeras ++*j*, +*jj* and *j*+*j* all have *jointless* cells in L3 and the developed pedicels have neither abscission zone morphology nor the ability to undergo cell separation. This indicates a special role of the outermost L3-derived tissue in induction of abscission zone development (Szymkowiak and Irish, 1999). A second non-allelic mutation in tomato, *jointless-2* (*j-2*), prevents differentiation of the pedicel AZ. *j-2* mutation is located within the centromeric region of chromosome 12 (Budiman et al., 2004) and a candidate gene, *ToCPLI1*, have recently been identified closely related to Arabidopsis *C-terminal phosphatase-like gene 3* (*AtCPL3*). AtCPL3 has been shown to regulate gene expression as a response to plant stress and plays a role in growth and development (Koiwa et al., 2002). The *LATERAL SUPRESSOR* gene has also been characterized and was found to encode a member of a different group of putative transcriptional activators (Schumacher et al., 1999). The LS peptide exhibits homology to a group of VHIIID proteins that play a role in transducing signals associated with gibberellins (Peng et al., 1999). Two recently identified Arabidopsis genes encoding redundant regulators of leaf and flower patterning, *BLADE-ON-PETIOLE 1* (*BOP1*) and *2* (*BOP2*) (Ha et al., 2003, 2004, 2007; Hepworth et al., 2005; Norberg et al., 2005), were also shown to be required for floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). *Bop1 bop2* mutant floral organs fail to abscise. *BOP1* and *BOP2* genes are transcribed in early floral organ primordia and are localized to a region corresponding to the future AZ prior to other known abscission-related genes. The disappearance of AZ anatomy is the earliest identified event associated to the absence of both functional genes. *BOP1* and *BOP2* encode proteins with a BTB/POZ domain and four

ankyrin repeats (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). Of the 75 BTB/POZ domain-containing proteins in Arabidopsis, BOP1 and BOP2 are the most closely related to NON-EXPRESSOR OF PR GENES1 (NPR1)(Cao et al., 1997; Ha et al., 2004; Stogios et al., 2005). NPR1 mediates transcriptional changes during systemic acquired resistance through interaction with members of the TGA family of bZIP transcrition factors (Dong, 2004). It is likely that BOP1 and BOP2 interact with or activate unique transcription factors to promote floral organ patterning (Hepworth et al., 2005) and abscission.

Not all non-shedding mutants appear to be the consequence of a failure of the AZ to differentiate. In Australia, a spontaneous single gene mutant was discovered in the early 1990s among plants of *Lupinus angustifolius* cultivar Danja. In the mutant *abs1* any organ, despite an apparently normal pattern of growth and senescence, does not display abscission (Atkins and Pigeaire, 1993).

Abscission related-genes

Endo-β-1,4-glucanases (EGases)

One of the first abscission-specific enzymes isolated from the primary leaves of *Phaseolus vulgaris* was a bean abscission-specific cellulase (BAC), β-1,4-endo-glucanase (EGases)(Lewis and Varner, 1970, Koehler et al., 1980, Tucker et al., 1988, Koehler et al., 1996). Expression of the corresponding gene correlates precisely with the onset of abscission and is regulated by ethylene and auxin (Tucker et al., 1988). The BAC involved in shedding event belong to the α- and β- EGases subfamily (Libertini et al., 2004). Earlier studies on the BAC promoter in tomato (*Lycopersicon esculentum*) and bean explants demonstrated that 210 bp of the proximal 5-upstream BAC sequence was sufficient for ethylene- and auxin-regulated expression (Koehler et al., 1996). The position -103 upstream the starting codon achieves the positive regulation of the BAC promoter as it contains a TGA core motif recognized as a binding site for bZIP transcription factors (Tucker et al., 2002). Mutation of the TGA motif reduced ethylene-induced expression of BAC by 80%. A further regulatory motif involved in auxin responsiveness is located immediately upstream, at -113 bp. Mutation of this 10-bp element enhanced BAC expression and made it unresponsive to auxin. Therefore these site-directed mutagenesis gave insight into the regulation of this gene by both ethylene and auxin, and underlined the presence of core motifs directly responsible for different hormonal responsiveness.

In peach the expression of three different genes (*pCel10*, *pCel20* and *pCel30*) encoding EGases has been studied, indicating that only pCel10-related mRNA (PpEG1) highly accumulated in the abscission zone of leaves and fruits (Trainotti et al., 1997). The same results have been obtained in tomato in which the expression of seven cellulase genes (*Cel1-Cel7*) has been investigated in flower-pedicel abscission zones (Brummell et al., 1997a, 1997b; Calata et al., 1997; del Campillo and Bennet, 1996; Lashbrook et al., 1994), and the expression of *Cel1*, *Cel2*, and *Cel5* has been detected during the cell separation event (del Campillo and Bennet, 1996; Gonzalez-Bosch et al., 1997; Kalaitzis et al., 1999).

In the spontaneous mutant *abs* of *Lupinus angustifolius* cultivar Danja, the lack of abscission could be due to the absence of an inducible abscission-specific cellulase (Henderson et al., 2001; Clemens and Atkins, 2001).

A role in abscission signalling is attributed to *Phaseolus vulgaris* endo-β-1,4-glucanhydrolase. Thompson and Osborne (1994) have discovered that a signal coming from the stele tissues was necessary before the cells of the cortex can fully respond to defined abscission-promoting events or to hormonal treatments. A failure in endo-β-1,4-glucanhydrolase activity and cell separation was detected when abscission zone was separated from the stele tissue causing a block in shedding of the petiole (Thompson and Osborne, 1994). Sexton et al. (1981) have demonstrated that endo-β-1,4-glucanhydrolase, although being present in the abscission zone of pulvinus, was also detected in the stele tissue as closely involved in the development of tyloses (Scott et al., 1967; Poovaiah, 1974; del Campillo et al., 1990). It is also possible that very low levels of endo-β-1,4-glucanhydrolase activity induced in and around the vascular bundles are sufficient to release a signalling cytoplasmic or cell-wall fragment (Thompson and Osborne, 1994). Perhaps abscission is mediated by the interaction of endo-glucanhydrolases and glycosidases with abscission zone stele-specific polysaccharide breakdown products that either directly interact with the cell-wall enzymes or induce new gene expression in the target cells.

Polygalacturonases (PGs)

An increase in PG activity during abscission was first reported over 30 years ago (Taylor et al., 1991, 1993; Hadfield and Bennett, 1998). Not only the activity of PG has been shown to increase specifically in AZ tissue during the shedding of organs but also the expression of

PG genes has been found to increase prior to and during abscission (Bonghi et al., 1992). The identification of seven different PG genes in tomato has been reported and, among them, *TAPG1*, *TAPG2*, and *TAPG4* showed to be expressed during ethylene-induced leaf and flower abscission (Kalaitzis et al., 1997; Hong and Tucker, 2000). The time course of expression of *TAPG2* and *TAPG4*, accumulating in the pedicel zone prior to the onset of cell separation (at 6 h), differs from that of *TAPG1,* detected 6 h later at the time of shedding.
The expression of an abscission-related PG (*PGAZAT*) has recently been characterized in flower AZs of Arabidopsis (Gonzalez-Carranza et al., 2002). Recently, a null *PGAZAT* mutant has been identified in which the time course of ethylene-promoted flower abscission was slightly delayed compared to wild-type plants. This observation provides further evidence that the activity of PGAZAT contributes to the timing of organ abscission (Sander et al., 2001). In addition, in Arabidopsis, two other genes, *ADPG2* and *QRT2* belonging to the class of PG genes, have been discovered, and shown to be positively involved in controlling floral organs abscission. A delay in floral organ abscission was observed in the *adpg2* and *qrt2* single mutants and in the *adpg2 qrt2* double mutant (Ogawa et al., 2009). Besides the role of PGAZAT in the timing of abscission, ADPG2 and QRT2 could cover the same functions in determining the onset of the shedding process.

Expansins (EXP)

During abscission, the induction of cell wall degrading enzymes is paralleled by an up-regulation of expansin genes (Bonghi et al., 1993; Taylor et al., 1993, 1994; Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000). Evidences supporting a role for this class of cell wall proteins in abscission came from the report that expression of *AtEXP10* (a member of the expansin family in Arabidopsis) could be observed specifically at the base of the leaf petiole (Cho and Cosgrove, 2000). This hypothesis was strengthened by the demonstration that down-regulation of the gene resulted in an increased force necessary to separate the petiole from the body of the plant. Recent studies carried out conduced in *Sambucus nigra* have brought evidence on the role for expansins in organ shedding. An increase in activity of *SniEXP2* and *SniEXP4*, has been found in the leaflet AZ 12-24 hour after ethylene treatment (Belfield et al., 2005). RT-PCR amplification of two additional expansins, *SniEXP1* and *SniEXP3*, suggested that they are enriched in leaflet AZ cells as well (Belfield et al., 2005).

Pathogenesis-related (PR) genes

The shedding of plant organs provides an ideal site for invasion by pathogens, therefore before separation takes place, it is fundamental to induce genes involved in the protection against microbial infection. A range of PR proteins such as β-1,3-glucanases, multiple isoforms of chitinases, and thaumatin-like proteins were first shown to accumulate in response to ethylene treatments in bean abscission zones (del Campillo and Lewis, 1992). More recently, the accumulation of transcripts corresponding to genes encoding a PR4-like protein, a metallothionein-like protein, and an allergen-like protein, has been reported to occur during tomato and *Sambucus nigra* leaf abscission (Coupe et al., 1995; Harris et al., 1997; Ruperti et al., 1999).

Hormonal signalling

The differentiation of the AZ where cell separation will take place is just the first phase in the abscission process. The second step is to trigger the cells that make up the AZ so that cell wall hydrolysis occurs. The induction of abscission has been related to both a decrease in auxin at AZ level and an increase in ethylene (Sexton et al., 1985) that is the main hormone for promoting processes that lead to cell separation (Sexton et al., 1985; Ramina et al., 1986, 1989). The ability of exogenous ethylene to stimulate abscission and the possibility of preventing shedding by using inhibitors of ethylene biosynthesis or action or both confirm the crucial role played by the hormone also in peach (Ramina et al., 1989). It has been suggested that the endogenous concentrations of indole-3-acetic acid (IAA) must fall below a certain threshold in the abscission zone to promote abscission (Osborne, 1989). The IAA flux across the abscission zone of leaves, flowers, and fruit appears to determine the sensitivity of those organs to ethylene and subsequent abscission (Brown, 1997). In contrast to IAA, high concentrations of abscisic acid (ABA) seem to be related to promotion of fruit abscission.

Ethylene

The role of ethylene in accelerating abscission has been recognized for decades, although researchers have begun to address whether ethylene is absolutely essential for abscission (Addicott, 1982; Abeles et al., 1992). In order to underline the effective role of ethylene in abscission, mutants with deficiency in ethylene signalling were analyzed. A subset of genes

required for ethylene sensitivity, including *ETHYLENE RECEPTOR 1* (*ETR1*), *ETHYLENE-INSENSITIVITE 2* (*EIN2*) and members of the *EIN3* transcription factor family, are implicated in the proper timing of floral organ abscission in Arabidopsis (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004). Delayed floral abscission has been demonstrated in Arabidopsis plants expressing the mutant ethylene receptor allele *etr1-1* (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004) and also in leaves of tomato (*Lycopersicon esculentum*)(*LeETR1*; Barry et al., 2005). The ethylene receptor gene *ETR1* homolog (*tETR1*) was found to be specifically up-regulated in the flower AZ of tomato, as well as in other ethylene-mediated processes in other tissues (Payton et al., 1996). Considering the relationship between expression of ethylene receptor genes in the AZ and tissue sensitivity to ethylene, it is possible that IAA may control AZ sensitivity to ethylene by affecting components downstream to ETR1 or ERS1 receptors in the ethylene signal transduction pathway. *EIN2* has been reported to affect the timing of abscission in Arabidopsis indeed *ein2* mutants exhibit a strong ethylene-insensitive phenotype and floral shedding is not accelerated by treatment of ethylene (Patterson and Bleecker, 2004). On the contrary *ein3* mutants in Arabidopsis do not display, as expected, a delay in floral abscission whereas in tomato a nonspecific antisense RNA applied in order to knock down the expression of three EIN3-like gene, *LeEIL1, 2,* and *3* has confirmed a delay in flower shedding coupled to an ethylene-insensitive behaviour in plants (Tieman et al., 2001). Other mutants have been isolated and a delay in flower abscission has been observed. The semi-dominant *Never-ripe* (*Nr*) mutation affects the ethylene receptor LeETR3 (Wilkinson et al., 1995; Lashbrook et al., 1998), homolog to Arabidopsis ETR1 (Yen et al., 1995), and displays delayed floral abscission even if little acceleration in the process is observed after ethylene treatment. The same phenotype is displayed in two partially ethylene-insensitive, dominant mutations, *Never-ripe 2* (*Nr-2*) and *Green-ripe* (*Gr*) (Barry et al., 2005). *Nr* and *Gr* are predicted to have a disruption of a novel gene in the ethylene-signalling pathway, and support a model in which an ethylene-dependent pathway promotes abscission (Barry et al., 2005; Barry and Giovannoni, 2006).

Ethylene has been recently observed to promote abscission by interacting with other different signalling pathways. In fact, ethylene may induce leaf abscission by modulating interactions within the heterotrimeric G-protein complex (Gα, Gβ, and Gγ), thereby

affecting G-protein signalling. Heterotrimeric G-proteins play an important role in various signal transduction pathways (Assmann, 2002; Ashikari et al., 1999; Ueguchi-Tanaka et al., 2000; Coursol et al., 2003). The transduction of signals perceived by the G-protein pathway is achieved in part through interaction with phospholipases, enzyme that catalyses the hydrolysis of membrane phospholipids (Assman, 2002; Wang, 2005). D-type phospholipases (PLDs) can bind to and interact with Gα, a heterotrimeric G-protein subunit (Zhao and Wang, 2004), and the activation of heterotrimeric G-proteins through the release of the Gα subunit removes the inhibition of PLDs (Lein and Saalbach, 2001). Through direct or indirect enzymatic action of PLDs, a variety of lipid signal molecules are produced such as phosphatidic acid (PA). PLD activity and associated signalling molecules are regulated by various types of stress, wounding, ABA and ethylene and plant growth regulators often associated with senescence and/or abscission (Lee et al., 1998; Ritchie and Gilroy, 1998; Frank et al., 2000; Taylor and Whitelaw, 2001; Welti et al., 2002). In *Citrus*, Malladi and Burns (2008) demonstrated that, after ethephon treatment, CsPLDγ1 was rapidly induced in leaf blades during the early responses in leaf abscission. Modification of membrane phopsholipid composition by CsPLDγ1 activity and PA may further contribute to the acceleration of leaf and fruit abscission. PA has been shown to interact directly with of CTR1, a negative regulator of ethylene signalling, and inhibit its activity (Testerink et al., 2007). Hence, PA generated by CsPLDc1 activity may directly mediate ethylene-induced leaf abscission responses through the inhibition of CTR1 and the activation of ethylene signalling. In addition, PA may also modify auxin transport, another important regulator of ethylene-induced abscission (Patterson, 2001; Taylor and Whitelaw, 2001), through interaction with RCN1 (roots curl in NPA 1), a regulator of auxin transport (Testerink et al., 2004; Muday et al., 2006).

The findings above presented have pointed out the importance of ethylene in controlling the timing and speed of abscission, but no experiment has convincingly shown that ethylene perception and signalling are required for abscission (Patterson, 2001). Therefore other studies have been carried out in order to fully understand the role of ethylene in Arabidopsis floral abscission in mutants displaying floral organ separation defects. In these mutants the primary role of ethylene has been widely discussed, and the discovery of ethylene-dependent and independent pathway (Patterson and Bleckeer, 2004) leads to declare that

ethylene is not the only trigger in abscission. In particular, the assessment of an interaction between a ligand, IDA, and a receptor-like kinase, HAESA, (Cho et al., 2008; Stenvik et al., 2008) has led researchers to draw how the abscission signal is carried out and perceived in the AZ. A further implementation of this first model has required the characterization of other genes, *NER* (Liljegren et al., 2009) and *EVR* (Leslie et al., 2010), whose functions have been found to affect the IDA-HAESA interaction. Butenko et al. (2009) and Leslie et al. (2010) have been able to partially complete the ethylene-independent abscission signalling pathway also considering putative downstream elements of IDA-HAESA interaction.

Auxin (IAA)

The generally accepted model is that the basipetal polar IAA flux through the AZ prevents abscission by causing an ethylene insensitive. When the level of auxin decreases and the level of ethylene increases, the process of cell separation is initiated (Osborne, 1989). Prevention of organ abscission has been found to require a continuous and constant supply of auxin to the AZ (Morris, 1993; Roberts et al., 2002). Recently, in *Mirabilis jalapa*, Meir et al. (2006) investigated the role of auxin in regulating sensitivity of AZ tissues to ethylene and identified genes differentially expressed when zones became competent to abscise in response to exogenous ethylene. Gene expression was examined in leaf and stem AZs over the period they became ethylene competent followed by either indole-3-acetic acid (IAA) depletion with the IAA transport inhibitor naphthylphthalamic acid or leaf deblading treatment. *Mj-Aux/IAA1* and *Mj-Aux/IAA2* transcripts, encoding Aux/IAA proteins, and three other transcripts showing high identity to a polygalacturonase inhibitor, a β-expansin, and a β-tubulin, were down-regulated by auxin deblading. Auxin affects abscission not only by regulating sensitivity to ethylene, but also by affecting the intra-cellular transport of the hydrolytic enzyme polygalacturonase (PG) associated with cell wall degradation (Dal Degan et al., 2001). A similar correlation between the effectiveness of auxin in delaying abscission and induction of *Aux/IAA* gene expression was reported recently in *Cestrum elegans* (Abebie et al., 2005). Application of the synthetic auxin 2,4-diclorophenoxy acetic acid, which delayed floret abscission in this system (Abebie et al., 2005), induced a higher expression of *Aux/IAA* genes in the AZ. Therefore, *Aux/IAA* genes can be used as molecular markers to measure auxin activity and free auxin level in the AZ. These data indicate that a

certain critical threshold level of free auxin has to be supplied continuously to the AZ cells for effective and continuous expression of *Aux/IAA* genes. The regulation of the abscission process might require expression of specific *Aux/IAA* genes, localized in the AZ, rather than an expression of all *Aux/IAA* genes.

Functional analysis of *ARF1*, *ARF2*, *NONPHOTOTROPIC HYPOCOTYL4 (NPH4)/ARF7,* and *ARF19* genes in Arabidopsis has suggested that these transcription regulators act with partial redundancy to promote senescence and floral organ abscission (Ellis et al., 2005; Okushima et al., 2005a, 2005b). *arf2* single mutant flowers show a delay at the onset of both senescence and floral organ abscission. Although the single *arf1*, *nph4*, and *arf19* single mutants do not show any shedding defects, *arf1 arf2* double mutants and *arf2 nph4 arf19* triple mutants show an enhanced delay in abscission compared to arf2 single mutants (Ellis et al., 2005). Upon these findings ARF1 and ARF2 act like transcriptional repressor whereas NPH4 and ARF19 like stimulator (Ulmasov et al., 1997, 1999; Tiwari et al., 2003). Particularly interesting is the function of ARF2 in promoting ethylene biosynthesis prior to the shedding event. A decrease in the transcript levels of *1-aminocyclopropane-1-carboxylate* (ACC) *synthase* family (*ACS2*, *ACS6*, and *ACS8*) was observed in *arf2* flowers (Okushima et al., 2005a). But whether ACS family is regulated by ARF2 through AUX/IAA signalling (Yamagami et al., 2003; Ellis et al., 2005) it is still unknown. Changes in auxin gradients across the AZ may either delay or promote abscission, possibly by modulating ARF activity, as already observed in *Arabidopsis thaliana* floral organs (Taylor and Whitelaw, 2001; Ellis et al., 2005).

A recent work carried out in tomato (*Solanum lycopersicum* Mill, cv Shiran 1335), by means of the Affymetrix GeneChip® Tomato Genome Array release in 2005, has explored the role of auxin in regulating abscission at AZ level. Meir et al. (2010) have analysed the transcriptional profiles of flower AZ compared to the non-AZ (NAZ) tissue in response to auxin depletion, treated with or without 1-MCP. Tomato floral abscission process has been separated into two main phases: the early phase and late phase. After flower removal in the early phase, there is the acquisition of ethylene sensitivity and abscission competence whereas in the late phase active abscission processes start leading to the execution of pedicel abscission (Meir et al., 2010)(**Figure 4**). Consequently to the IAA depletion, genes directly regulated by IAA are down-regulated such as *IAA1*, *3*, *4*, *7* and *8*, *knotted*,

Homeobox-Leu zipper, and *bHLH*, whereas genes directly repressed by IAA are up-regulated (*PK7*, *ERF1c*, *WRKY lld-1*, *Protein phosphatase*). A third class of genes encodes other TF and/or post transcriptional regulators such as *LRR-RLK*, *PK7*, *TPRP-F1*, *Phantastica*, and *Ovate*, whose expression were modified later on by the variation of IAA within AZ tissues. Once IAA flow decreases, an increase in sensibility of AZ to ethylene induces its autocatalysis mediated by the early up-regulation of ethylene-related genes and the expression of genes belonging to ethylene signal transduction or abscission regulators such as *ETR4*, *CTR1*, *ERF1c*, *TAGL12*, *LRR-RLK*, and *PK7*. After 4 hours, an induction of genes encoding cell wall modifying enzymes and defence proteins such as *WRKY* TFs, *ERT10*, *Chitinase*, and *Peroxidases* involved in the last phase of abscission, was observed. This analysis establishes a clear sequence of events occurring during acquisition of tissue sensitivity to ethylene, and confirms the hypothesis that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes.

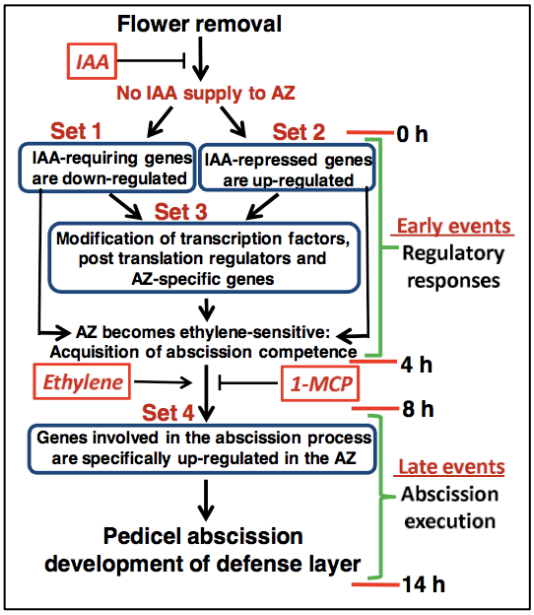

Figure 4 Events leading to tomato pedicel abscission in response to auxin depletion following flower removal, and the possible effects of exogenous application of IAA or 1-MCP (from Meir et al., 2010).

Abscisic acid (ABA)

It is well documented that endogenous ABA is positively associated with abscission in cotton (Guinn and Brummett, 1988), litchi (Yuan and Huang, 1988), and *citrus* (Gomez-Cadenas et al., 2000; Sagee and Erner, 1991; Talòn et al., 1990). Moreover, Talòn et al.

(1997) found a close relationship between ABA concentration and abscission suggesting a stimulating effect of ABA on *ACC* gene and ethylene biosynthesis, with the latter one being responsible for the increased fruit drop. Bangerth (2000) has pointed out that the increment of ABA content could be the effect of a disturbance at auxin transport level. Therefore, a hormonal cross-talk among IAA, ABA and ethylene could be responsible for the onset of shedding event. A recent study carried out by Hansen and Grossmann (2000) in cleavers (*Galium aparine*) treated with high concentrations of IAA determines a positive regulation of ethylene and ABA hormone. The increment of ABA levels is closely related to the up-regulation of ACC gene of ethylene biosynthetic pathway.

ABA signalling pathway is mediated by ROS production (Cho et al., 2009), whose level is closely related to NADPH oxidase activity, and by sugars (Arenas-Huertero et al., 2000), as consequence of an established stress status in plants. In fact, treating Arabidopsis with ABA or hydrogen peroxide results in the activation of two MAPKs, AtMPK3 and AtMPK6 (Kovtun et al., 2000), involved in the signalling cascade of ROS and ABA. As consequence of sugar treatment, Arabidopsis seedlings increases ABA levels whereas treatments with ABA increases sugar sensitivity, suggesting that regulation of ABA synthesis plays an important role in plant sugar responses (Arenas-Huertero et al., 2000). Therefore, stresses induced by sugars and ROS compounds have an important role in orchestrating the ABA transduction and biosynthetic pathway.

Other non-hormonal signalling pathways

Besides the role of hormonal signalling pathways regulating abscission, there are other two compounds (reactive oxygen species and sugars) showing putative role in this process as messengers.

Reactive oxygen species (ROS)

In plants, ROS (superoxide, hydrogen peroxide and hydroxyl radicals) function as second messenger for hormone signalling, development, programmed cell death, and defence mechanisms. Their production is linked to fundamental metabolic activities in different cell compartments, mainly mitochondria, peroxisomes and chloroplast. Due to their high toxicity, plants have developed a ROS oxidative stress scavenging machinery, including catalase, glutathione dehydrogenase and peroxidases enzymes, to remove ROS from cells

(Apel and Hirt, 2004). ROS level is positively regulated by the membrane-located NADPH oxidase (NOXs), one of the several possible sources of ROS, functioning in defence and hormone signalling in plants (Keller et al., 1998; Pei et al., 2000; Jiang and Zhang, 2002, 2003). The activation of NOXs is mediated by the small G-protein Rac in animal cells (Sumimoto, 2008), and also confirmed in rice (Ono et al., 2001; Wong et al., 2007). In Arabidopsis, genes coding for NOXs have been involved in plant development and defence (Foreman et al., 2003; Monshausen et al., 2007). ROS signalling mediated by mitogen activated protein kinase (MAPK) cascades is well documented by Pitzschke and Hirt (2009). ROS signalling through the MAPK cascades leads to the regulation of many different gene pools (Apel and Hirt, 2004; Miller et al., 2008; Desikan et al., 2001; Wang et al., 2006). TFs such as WRKY53 involved in the induction of stress- and defence-related genes as well as senescence-associated genes are positively induced (Miao et al., 2006). Promoter analysis of ROS regulated genes reveal the presence of phytohormone-, defence-, and stress-responsive elements suggesting the involvement of ROS in a variety of cellular signalling cascades (Mahalingam et al., 2006; Geisler et al., 2006). Among these pathways, the involvement of ROS in hormone signalling has been widely studied.

Concerning the role of ROS in abscission, ROS production was shown to mediate ethylene signalling (D'Haeze et al., 2003; Desikan et al., 2005). The observation of a ROS increasing production in the AZ during the onset of shedding is well documented (Hinman and Lang, 1965; Gahagan et al., 1968; Henry, 1975), although their role in the process of abscission is still unclear. It may be that peroxidases have more than one function during abscission, and changes in the activity of specific peroxidase isoenzymes have been detected in zone and non-zone tissues from the primary leaves of bean after exposure to ethylene (McManus, 1994). In ethylene-treated *citrus* leaf explants, a set of transcripts belonging to the oxidative stress scavenging machinery (a catalase, a glutathione dehydrogenase, an ascorbate peroxidase and two peroxidases) have previously been reported to be over-represented in petioles, whereas a peroxidase was transiently over-represented in manually-dissected LAZ-enriched tissues (Agustí et al., 2008) and in ethylene induced pedicel abscission in tobacco (Henry et al., 1974). Recently, hydrogen peroxide (H_2O_2) has been shown to be directly involved in ethylene-mediated abscission signalling *in vitro* in *Capsicum* leaves, where it appears to act as an intermediate molecule in the expression of ethylene-induced cell wall

hydrolases (Sakamoto et al., 2008). H_2O_2 accelerated abscission without modulating the expression of ethylene-responsive-genes, whereas IAA completely suppressed *in vitro* abscission accelerated by the addition of either H_2O_2 or ethylene. The relationship between auxin and ROS is also well documented (Joo et al., 2001; Kawano, 2003). Ethylene competence induced by the depletion of IAA is also indispensable for the H_2O_2-induced abscission signalling. It is possible that H_2O_2 activates the downstream abscission signalling cooperatively with unknown signal component(s), including those under the regulation of ethylene and/or IAA (**Figure 5**).

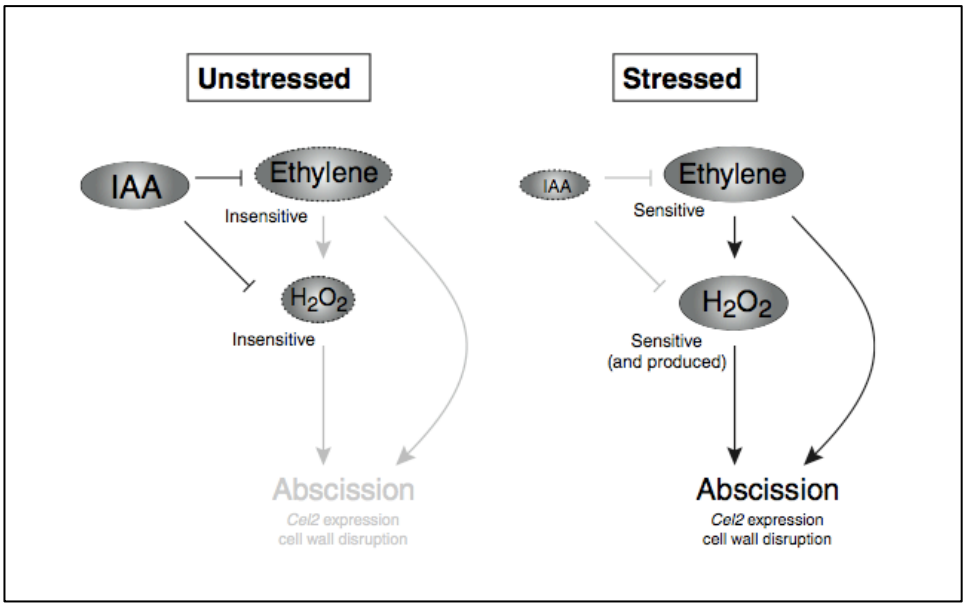

Figure 5 Proposed model of stress-induced leaf abscission signalling (from Sakamoto et al., 2008).

Sugars

Carbohydrate partitioning between different sinks have always had a putative role in abscission. In fact, a strong relationship between sugars available for fruitlets and their possibility of abscission has been suggested in apple (Berüter and Droz, 1991), *citrus* (Mehauachi et al., 1995; Iglesias et al., 2003, 2006), pistachio (Nzima et al., 1999), and cherry (Atkinson et al., 2002). Sugar competition may probably raises, concurrently with other factors (phosphate and nitrogen inorganic ions)(Sadka et al., 1994; Nielsen et al., 1998), signals that start the shedding event. In the last years, sugars are considered important signalling molecules involved in regulating cellular metabolism by inducing the transcription of a number of specific enzymes involved in sucrose breakdown and

metabolism and storage product synthesis in sink tissues (Koch, 1996; Godt and Roitsch, 1997).

Different sugar signalling transduction pathways have been identified in plants, among which the MAPK-mediated pathways are recognized to be important in regulating the response to a wide range of endogenous and exogenous stimuli (Ehness et al., 1997). Among MAPKs, the family of the SnRK is grouped into three subfamilies: SnRK1, SnRK2 and SnRK3. SnRK1 is closely related to yeast Snf1, whereas the other two subfamilies are probably unique to plant (Halford et al., 2003). Antisense knockdown of *SnRK1* causes a reduction in the expression of *SUS4* gene and loss of *SUS4* sucrose-inducibility in leaves of tomato cultivar Desirèe, and thus affecting sugar metabolism (Purcell et al., 1998). Further analyses revealed that SnRK1 activation is a consequence of a high cellular sucrose and/or low cellular glucose levels thus proving its involvement in sugar signalling (Halford et al., 2003).

Another important element for sugar signalling is the trehalose-6-phosphate (T6P) synthase (TPS) involved in trehalose metabolism, a sugar pathway that showed regulatory effect on plant growth, development and stress resistance. TPS synthesizes T6P from UDP-Glc and Glc-6-P, followed by dephosphorylation to thehalose by T6P phosphatase (TPP). T6P is essential for carbohydrate utilization for growth in Arabidopsis and the expression of trehalose metabolic genes is differentially regulated during embryo development and senescence (Schluepmann et al., 2003). In Arabidopsis a disruption of *AtTPS1* leads to an embryo lethal phenotype, whereas an over-expression of yeast *TPS1* cause drought tolerance and insensitivity to sugars and ABA, permitting the plants to normally growth and develop in high Glc and ABA concentration (Avonce et al., 2004). Concerning the role of trehalose metabolism in shedding events, Arabidopsis plants treated with CMNP, a growth regulator for inducing abscission in citrus mature fruits, show an early up-regulation of *AtTPS1*. Immature apple fruits, prone to drop as a consequence of abscission induction by BA, showed an up-regulation of TPS as well (Dal Cin et al., 2009a). The authors justified this finding as a consequence of the strong competition existing in the early fruit developmental stages for assimilates between different sink tissues. Therefore, in abscising fruitlets TPS could be involved in the stress signalling contributing to trigger abscission.

In addition, several studies have assessed a putative relationship between sugars and ROS in stress responses (Contento et al., 2004; Price et al., 2004; Loreti et al., 2005). The sugar-ROS cross-talk cannot be considered a one-way relationship in which high level of sugars correlate with a high amount of ROS. Studies have proved that in case of high level of sugars, in particular of glucose, ROS (Russel et al., 2002) and NADPH oxidase (Bonnefont-Rousselot, 2002) production increase. On the contrary, sucrose starvation activates catalase involved in ROS production (Contento et al., 2004). Hence high sugar level can induce the activation of some ROS production pathways whereas a condition of starvation can affect other ROS biosynthetic pathways. Glucose and sucrose, especially, parallel changes in sugar content within the cells, suggesting a relevant role in sugar sensing and signalling.

Massive gene approaches to investigate abscission at AZ level

Consequently to the onset of microarray technology, new researches have started to massively investigate biological processes such as abscission. Microarray coupled with laser capture micro-dissection (LCM) technology has been used to analyze stamen AZ of Arabidopsis (Cai and Lashbrook, 2008) and leaf laminar abscission zone (LAZ) of *citrus* (Agustí et al., 2009) where separation takes place. Investigating abscission at AZ level represents a challenge in terms of the experimental approach due to the restricted area. In fact, previous molecular approaches were generally hampered by the limited area and cell number constituting the AZ. Therefore, detailed studies at the single cell resolution by means of LCM are of great relevance in order to accurately describe the process and identify potential candidate genes for biotechnological applications. Cai and Lashbrook (2008) have identified 551 genes, differentially expressed during the time course of natural abscission, grouped into different eight classes according to their expression profile in the stamen AZ. Among all genes, those involved in cell wall modification such as expansins (*EXP*, three genes), extensin (*EXT4*), glycosyl hydrolase (three genes), pectin methylesterase (*PME*, five genes), peroxidase (*PER*, 11 genes), polygalacturonase (*PG*, three genes) and xyloglucan endotransglycosylase/hydrolase (*XTH*, four genes) were grouped into the largest class as mainly responsible for the disassembling of the cell wall for organ detachment. Differential expression of genes associated with cell wall metabolism during abscission was demonstrated also during ethylene-induced abscission of citrus leaves (Agustí et al., 2008, 2009). Agustí et al. (2009) have observed that LAZ layers activate both

catabolic and anabolic cell wall modification pathways during the abscission program to facilitate organ shedding and develop protective barriers. Their data also indicate that some transcripts preferentially expressed in LAZ associated with protein biosynthesis and modification (ubiquitination and phosphorylation) might play specific and relevant roles in ethylene-promoted citrus leaf abscission. On the other hand, the results highlight the activation of defensive programs in the contiguous petiole. In addition, new potential citrus abscission-regulatory genes were identified. The involvement of particular members of different transcription factor gene families (MADS-box, basic helix-loop-helix and homeodomain proteins) in the differentiation of the effective cell separation layer is also suggested. Moreover, Cai and Lashbrook (2008) have revealed differential gene expression for MYB factors, such as *Myb17* and *Myb 68* that are regulated by nitrogen and sugar status, hormone signalling, including *ERF1* induced by JA and ethylene in defence gene system, and a transcription factor, Zinc finger proteins (*AtZFP2*). A constitutive expression of *AtZFP2* has led to asynchronous and delayed abscission, supporting the role of this protein in partecipating to processes that initiate and/or coordinate cell separation in floral organ abscission zones. All these genes are putatively involved in different signalling pathways during the early shedding pathway suggesting the hypothesis that the primary abscission cue that initiates ethylene-independent and/or –dependent abscission may be derived from these or other events.

Nevertheless, there are several questions that need to be clarified. The place where the abscission signal is generated and the main signalling pathways involved in triggering this signal are the main questions that the scientific community must solve to further improving knowledge in abscission.

Apple fruitlet abscission

The control of fruit set is one of the major issues for apple producers to achieve a satisfactory yield and high quality fruits, and ensure a regular production in the following year. Foster et al. (2003) studied the early developmental phases of the meristem that will make up the new inflorescence in the following year. The transition from vegetative to floral meristem occurs within 100 days after full bloom (DAFB)(Foster et al., 2003), and the reduction of fruitlet number through different cultural practice, such as chemical thinners, growth regulators and defoliation, reduces the level of GA-hormone on the plant and

ensures a proper bloom the next year. A tree setting 10% of its blossom has a full crop load (Williams and Edgerton, 1981), but over-cropping results in a very poor fruit weight and size (Jones et al., 1992). Apple fruit size has been always a critical factor in determining the market value, and early removal of fruit results in larger fruit size at harvest (Preston and Quinlan, 1968; Quinlan and Preston, 1968; Jones et al., 1992). For these reasons, chemical thinning has been introduced as a common orchard practice, although the modes of action of the different chemicals is still not well understood and prevalently under control of environmental factors and genotypes. Thinning with chemical compounds is considered an amplification of the natural self-regulatory physiological drop. Fruitlet shedding occurs at a specific stage (called the June drop) within five to six weeks after full bloom. In a developing fruitlet population, there are differences due mainly to position of each fruitlet within cluster, time of blooming, pollination, number of seeds, spur vigour, and number of proximal developing fruits. Within a spur of apple tree there are typically four to five flowers surrounding a central one. The first flower to bloom is the central one, called king flower, developing the king fruit (KF). Being its development anticipated, it dominates over the lateral fruitlets (LFs) (**Figure 6**). Even among the lateral fruitlets a ranking of dominance exists, although not as great as between KF and LF. In particular, the length of the blooming period can influence uniformity of flower opening and as a consequence the fruitlet age.

Figure 6 Apple corymb at king flower (KF) blooming.

Analysis of the effect of fruit position (central versus lateral) and number of fruits per spur revealed that the KF, or a dominant lateral fruit (LF), in the absence of KF was consistently

the largest fruit with minimal variation (≈ 4%) in the presence of one or two LF. However, the size of a single LF in the presence of a KF was 20% less than the KF. If two LF were present, the secondary fruit was 10% and tertiary 28% smaller than the KF (Bukovac et al., 2008). Therefore, fruit size is well correlated with the presence of fruit on the same spur (intraspur competition) than the presence of fruits on different spur (interspur competition)(Bukovac et al., 2008).

In young apple fruitlets, auxin export increases shortly after fruit set (Gruber and Banghert, 1990) whereas ethylene production drops at low levels (Blanpied, 1972). Since the high IAA export from the fruit would not allow sensitization and activation of the AZ by ethylene, a down-regulation of the auxin export in some fruitlets would cause them to abscise. Suttle (1988) was able to demonstrate that the well-known IAA-transport inhibition is caused by ethylene and is a result of a reduction of the IAA export carriers, possibly caused by a decrease in their synthesis and/or stimulation of their degradation. The king fruit with high IAA diffusion over those fruitlets with lower IAA export may contribute to the abscission of the latters. It seems conceivable that where shoots, adiacent to fruitlets, are growing vigorously their high level of IAA export results in a inhibition of IAA export from young fruitlets. IAA export is increased by the application of gibberellins (GA4)(Callejas and Banghert, 1997), while application of growth retardants such as Alar or prohexadione-Ca, has the opposite effect (Callejas and Banghert, 1997). Lower concentration of free and conjugated IAA in smaller fruitlets may be due partially to a decreased IAA production. IAA may be destroyed by oxidation, as was found to happen in small peaches with high concentration of p-coumaric acid, a co-factor of IAA oxidation (Ramina and Masia, 1980). A close relationship had also been shown between the growth dynamics of various fruitlets and vascular development of their pedicels (Nii, 1980; Habdas et al., 1982; Bustan et al., 1995). Drazeta et al. (2004) assessed the role of IAA flow in affecting the vessel formation 2-4 days after petal fall in cv Granny Smith and used N-(1-Napthyl)phthalamic acid (NPA) because of its specific ability to inhibit auxin efflux carrier (Lomax et al., 1995). The accumulation of IAA measured upstream of the site of NPA application led to a feedback inhibition of IAA biosynthesis in the fruitlets and a reduction of auxin export from the fruitlets affecting also the vessel formation at the distal end of the pedicel. The characteristics of polar auxin transport (PAT) and the amount of endogenous IAA moving

through the PAT in pedicels of *Prunus avium* were determined in relation to abscission (Else et al., 2004). It was tested whether IAA exported via the PAT pathway was necessary for fruit retention and development using the PAT inhibitor 2,3,5- triiodobenzoic acid (TIBA), which blocks the membrane-trafficking of the putative efflux carrier PIN1 (Geldner et al., 2001; Muday et al., 2003). Application of TIBA to pedicels prompted fruitlet abscission. These data suggest that the ability of IAA to regulate the flow of assimilates to developing fruits may be an important factor in determining whether fruit of *Prunus avium* (Patrick, 1979, 1987) and *citrus* (Agustì et al., 2002) are retained. The capacity of the vascular system in the pedicel is one of the factor limiting the availability of essential photo-assimilates to the fruit. A strong correlation between fruit size and pedicel cross-section areas was also reported in *Citrus* fruitlets (Busta et al., 1995). Besides the direct influence on abscission, auxin has other roles in preventing abscission by attracting nutrients to its site (Luckwilll et al., 1969; Seth and Wareing, 1967). Disturbance in auxin flow is responsible of a reduction of assimilates from leaves to the dominated fruitlets bringing about their growth reduction and consequently activating their AZ. The arrest in growth rate of the developing fruitlets seems to be an outcome of disturbs happening at the seed level. Hormone balance in seed of shedding fruitlets of apple (*Malus domestica* L. Borkh) have been considered by Avanzi et al. (1988) and Vernieri et al. (1992), pointing out a role for hormones in the nutritional aspects of seed development influencing the synthesis localization, and sequestering of storage compounds. Studies carried out in seeds of abscised and non-abscised fruitlets pointed out an absence or a reduction of some storage compounds such as starch, lipids and proteins, and an altered pattern of their distribution in seeds of shed fruitlets (Forino et al., 2000). These changes could be a consequence of an altered supply of nutrients from maternal tissues and/or to an interruption in the nutrient routing and/or an inability of seed storage tissues to synthesize some storage compounds. Loss of carbohydrate production has been suggested as the causal agent in abscission of shaded organs such as leaves and flowers and fruits from field-grown plants or plants stored in the dark (Schou et al., 1978; Heindl and Brun, 1983; Force et al., 1988). The changes in accumulation and routing of storage compounds are important events that seem to be interrelated with complex hormonal controls. High levels of ABA present in seeds of shedding *Malus domestica* fruitlets (Vernieri et al., 1992) have been reported and a role of

this hormone was hypothesized in triggering the transition of tissue from a state of active growth to one prepared to survive a developmentally or environmentally induced stress (Hetherington and Quatrano, 1991). High levels of phenolic substances have been found in seeds of shed fruitlets. Phenols present in the integuments are likely to be secondary metabolites, such as tannins. Tannins and other substances that are assumed to have an adaptive value are accumulated in plants under nutritional stress (Gershenzon, 1984). Michaux-Ferriere et al. (1992) and Nour et al. (1993) reported that accumulation of phenol compounds, together with an early differentiation, is associated with process of tissue degeneration. In fact, abscised fruitlets may show degenerated ovule structure (Forino et al., 1987; Avanzi et al., 1988) or marked inhibition of seed growth (Marini and Byers, 1988) that increases if injury is imposed earlier (Proctor and Schechter, 1992). Abscising fruitlets with disturbance in embryogenesis show a reduction in IAA flow and a burst of ethylene able to activate AZ before shedding. Several studies on source-sink imbalances support the hypothesis that competition for limited photo-assimilates among developing fruitlets regulates fruit abscission (Polomski et al., 1988; Bangerth, 2000). Sucrose supplementation decreased fruit abscission rate in *citrus* (Iglesias et al., 2003), suggesting that fruit setting and development are highly dependent on carbohydrate supplied. Low levels of sucrose in the inflorescence after anthesis were associated with high abscission (Mehouachi et al., 1995; Gomez-Cadenas et al., 2000). During the post-bloom period, 10-20 days after bloom, demand for carbohydrates and other nutrients from various sinks exceeds what the sources can supply (Lakso et al., 1999). Carbon balance can be negatively affected by shading, photosynthetic inhibitors, elevate temperatures or combining effect of simultaneous shading and chemicals thinners (McArtney et al., 2004), thus accelerating the abscission event. Stopar et al. (2001) applied shadow in combination with two chemicals thinners, NAA and BA, to induce apple fruitlet abscission, and measured the level of carbohydrates between abscising and non-abscising fruitlets. The fruitlets prone to drop contained more starch, less glucose and slightly more fructose. The decrease of fructose can be considered as a parameter for determining fruitlets not prone to drop (Stopar et al., 2001). These findings are in agreement with the results obtained by Berüter and Droz (1991), investigating the effect of shading, girdling, seeds, and fruit removal on fruit abscission. In addition, it has

been discovered that abscission occurred only in those fruits in which the glucose content of the pedicel was below a critical level.

Dal Cin et al. (2005) showed that BA, besides inducing abscission, stimulates also ethylene biosynthesis. Abscising fruitlets (AFs) showed a high level of ethylene biosynthesis compared to non-abscising ones (NAFs). *MdACS5B* and *MdACO1* are two genes involved in the biosynthesis of ethylene during the abscission induction and are also considered as abscission molecular markers (Dal Cin et al., 2007a). Their transcript levels were found to be higher in AFs than in NAFs. Ethylene evolution shows a peak 13 days before abscission in fruitlets prone to drop suggesting a streaking correlated between ethylene and abscission (Dal Cin et al., 2005). This time lag between the ethylene peak and abscission may indicate that AZs need time to gain sensitivity to ethylene and cause the fruit to shed from the tree. These results reconfirm the involvement of this hormone in apple abscission as demonstrated in other fruit species such as peach (Ruperti et al., 1998). Ruperti et al. (1998) showed an increasing expression of *PpACO*, encoding the enzyme involved in the last step of ethylene biosynthesis, coupled with the onset of ethylene. When abscission is induced, *ACO* expression is stimulated in the distal and proximal tissue near the abscission zone but less in the latter, showing a formation of an ethylene biosynthesis gradient between these tissues (Ruperti et al., 1998)(**Figure 7**).

During June drop the fruitlets more prone to drop are the smallest ones and usually with a few number of seeds. In fact, fruit size is directly related to seed number (Heinicke, 1917; Denne, 1963; Dennis, 1986). Seed number or seed-derived hormones presumably affect fruit retention indirectly (Dennis, 1986). Fruits with more seeds would produce higher levels of hormones and thus they are strong sinks in competing for partitioning assimilates with other fruitlets and shoots (Addicott, 1982). Once ethylene biosynthesis is induced in pre-determined organs its signal has to be perceived. The ethylene sensitivity is acquired through the modulation of ethylene receptors. In apple six genes coding for ethylene receptors (*MdETR1*, *MdETR1b*, *MdETR2*, *MdETR5*, *MdERS1*, and *MdERS2*) have been identified (Dal Cin et al., 2005; Tatsuki and Endo 2006; Wiersma et al., 2007), and their expression patterns have been examined during different fruit stages, from development, through maturation and ripening (Dal Cin et al., 2007b; Tatsuki and Endo 2006; Tatsuki et al., 2009; Wiersma et al., 2007).

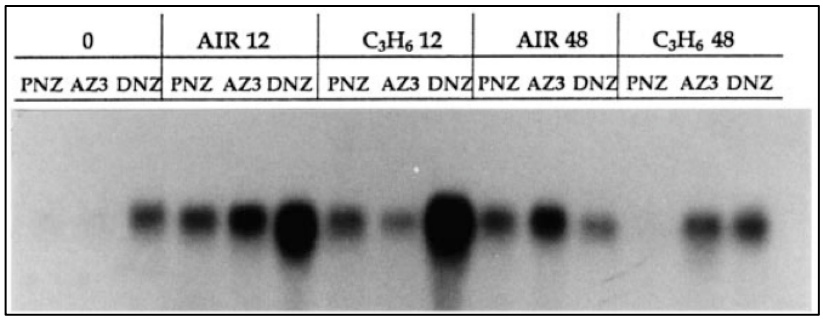

Figure 7 *ACO* transcript accumulation in tissues excided from explants treated with air or air + propylene at 0, 12 and 48h of flushing. PNZ: proximal non-zone, AZ3: abscission zone, DNZ: distal non-zone (from Ruperti et al., 1998).

These studies underlined the role of ethylene in inducing its receptors and the first element of its transduction pattern, MdCTR1. CTR1 is a negative regulator that, interacting with an empty ethylene receptor, blocks the ethylene response. However, in the presence of ethylene, CTR1 does not interact with the receptors and thus the hormone response occurs. As far as abscission is concerned, the sensing of ethylene is a necessary requirement for those organs over-producing ethylene, such as seed, cortex, peduncle, and AZ. The ethylene target genes may be different according to tissue. In AZ, targets encode specific cell wall hydrolases leading to cell separation (Bonghi et al., 1992, 2000). Dal Cin et al. (2005) measured the transcription level of *MdETR1*, *MdERS1*, and *MdCTR1* during abscission induced by BA. The transcription level of *MdETR1* was constant in AFs and NAFs throughout abscission whereas the transient increase of *MdERS1* expression was a 2-3 fold higher in AFs. *MdCTR1* was up-regulated in AZ and seed, and down-regulated in the peduncle and cortex of AFs with respect to the NAFs. High ethylene production may be coupled with an increasing concentration of ethylene receptors (O'Malley et al., 2005) or a modification of the ratio between ETR- and ERS-type receptors. Besides possible differences in terms of kinetic activity of the two receptors (Moussatche and Klee, 2004), ERSs, which miss the receiver domain, are thought to interact less efficiency with CTR1 (Clark et al., 1998). The variation in MdERS1/MdETR1 ratio increased in AFs and decreased in NAFs. An increase of this ratio, although transient in cortex, peduncle, and AZ of AFs, may determine a gain in sensitivity to ethylene, and thus playing a crucial role in regulating sensitivity to the hormone.

Massive gene approaches to apple fruitlet abscission

In the last years, massive gene approaches, based on microarray technologies, have been widely applied to study and isolate group of genes involved in abscission. Before the raising of microarray technology, cDNA-AFLP (Dal Cin et al., 2009a) and suppression-subtractive hybridization (Zhou et al., 2008) approaches were used in apple, in order to investigate gene expression during fruitlet abscission. Dal Cin et al. (2009a) identified 227 transcripts derived fragments (TDFs) differentially expressed in seed, cortex and peduncle tissues between two fruitlet population of cultivar Golden Delicious with different abscission potentials after BA treatment. Annotation analysis pointed out that the main changes in fruitlets with different destinies involved mainly membrane (20,1%), plastid (16,6%) and cytoplasm organelles (15,7%). TDF functions implicate mainly transport (15,3%), protein fate (12,7%), metabolism (9,6%) and transcription (8,1%). Among the genes associated with transport that are noteworthy a potassium transporter, ABC transporter family proteins and a sulfate transporter ATST1, in protein fate a carboxypeptidase and an aminopeptidase, in metabolism an alcohol dehydrogenase and a xilose epimerase and within transcription elements a basic helix–loop–helix and a histone acetyl transferase. Additionally an interesting finding supported the hypothetical role of sugar starvation or sugar sensing in abscission. A lack of sugars may occur as a consequence of the reallocation process, supported in this work by the isolation of an alkaline invertase (Rolland et al., 2002) and a putative trehalose-6-phosphate synthase (Gibson, 2005), respectively. Several TDFs were also involved at different steps of the auxin signal transduction: a BIG ortholog associated to polar auxin transport and to the positioning of the efflux carrier PIN1, an auxin hydrogen symporter, a PIN7, and auxin response factors (ARFs). Changes in the expression of auxin-related genes have also been observed by Dal Cin et al. (2009b), as a consequence of the ethylene burst preceding fruitlet abscission. Additionally, genes coding for an ABA responsive element binding factor 2 (*ABF2*), and a gibberellin response modulator scarecrow were also differentially expressed, supporting the hypothesis that abscission induction is the result of the antagonist role of auxin and ethylene supported by the action of other hormones such as ABA and GA. As a consequence of abscission induction through chemical treatment, a parallel pathway related to senescence process was also activated as

supported by the isolation of different TDFs encoding for senescence associated proteins, such as Staygreen protein, proteinases and asparagil synthetase (Dal Cin et al., 2009a).

Zhou et al. (2008) isolated and characterized genes associated with apple abscission after 24 and 48 hours of shading. Two cDNA libraries were constructed using the suppression-subtractive hybridization (SSH) method and 347 expressed sequence tags (ESTs) were obtained. 168 ESTs were preferentially expressed in fruitlets after 24h of shading, and the other 179 after 72h. Sequence analyses revealed that these clones represent 68 (24h) and 44 (72h) unique genes and belong to eight functional categories, mostly related to carbohydrate metabolism, and including the *sorbitol 6-phosphate dehydrogenase* (*S6PDH*) gene detected after 72 hour of shading. S6PDH is believed to play a key role in the biosynthesis of sorbitol in apple (Tao et al. 1995; Zhou et al. 2006). The *ACO* gene, one of the key genes in ethylene biosynthesis, is induced 72h after shading and maybe the final target of the pathway regulated by sugars to trigger abscission. Shading appeared to induce different response dependently to the length of time of its application. In apple, 24 hours of shadowing induced the expression of genes mainly involved in intracellular signalling, stress response and transcription but not those associated to abscission. Later on (72 h), an up-regulation of genes involved in the secondary metabolism, interaction with the environment, and cell rescue, defence and virulence was observed. Theses data pointed out that shade-induced fruit abscission in apple is very complex and could be regulated by a more elaborate mechanisms apart from carbohydrate and hormonal pathways.

Results obtained in these two researches have reinforced the importance of carbohydrates and hormone signalling in apple abscission induction. Their involvement could represent the consequence of primary signalling, yet unknown, or be the signalling request for the onset of events leading to the organ separation. Throughout massive gene approaches it could be possible to understand deeply the first step of abscission in order to answer to the question concerning the nature of the primary signalling.

As far as microarray analysis in apple is concerned, in the last years progress has been made with the release of more than 150,000 apple ESTs (Newcomb et al. 2006; Park et al. 2006), a useful tool that allowed the setting up of microarrays for the analysis, the isolation and identification of genes differentially expressed in apple (Lee et al. 2007) from floral bud

differentiation to fruit ripening (Janssen et al., 2008; Soglio et al., 2009). However, this technology has not yet been used to study apple fruitlet abscission induction.

Aims of the thesis

The primary aim of the present study was the development of molecular tools for elucidating abscission process in apple. Different approaches were followed in order to identify transcriptomic and metabolomic markers related to abscission:

1) A 30k CombiMatrix microarray was employed to study the transcriptomic changes occurring during abscission induction at both the cortex and seed level. BA was used as a tool for magnifying the physiological fruit drop. Differences in gene expression were examined within a 4-day timecourse, during which abscission is known to be induced by BA. According to the main results of the transcriptomic analyses, specific investigations were carried out by analyzing the carbohydrate status (sucrose, glucose, fructose, starch and sorbitol) and hydrogen peroxide concentration. Finally, a model was proposed summarizing the main findings.

2) Fruitlets with different abscission potentials were analyzed by means of PTR-MS to identify differential emission of volatile organic compounds (VOCs) between abscising and non-abscising fruitlet of two different cultivars, Golden Delicious and Redchief Delicious, in two subsequent year, treated with two different chemical thinners, BA and MET. Volatile markers were identified along with regulatory pathways controlling VOCs emission.

References

Abebie B, Goren R, Huberman M, Meir S, Philosoph-Hadas S, Riov J (2005) Prevention of bud and floret abscission in Cestrum cut flowers is related to the mode of transport and metabolism of synthetic auxins. Acta Hort **682**: 789-794

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology, Ed 2. Academic Press, San Diego

Acevedo A, Scandalios JG (1992) Differential expression of the catalase and superoxide dismutase genes in maize ear shoot tissues. Plant Cell Physiol **33**: 1079-1088

Addicott FT (1982) Abscission. Berkeley, Los Angeles, London: University of California Press

Agustí J, Merelo P, Cercos M, Tadeo FR, Talòn M (2008) Ethylene-induced differential gene expression during abscission of citrus leaves. J Exp Bot **59**: 2717-2733

Agustí J, Merelo P, Cercós M, Tadeo FR, Talón M (2009) Comparative transcriptional survey between laser-microdissected cells from laminar abscission zone and petiolar cortical tissue during ethylene-promoted abscission in citrus leaves. BMC Plant Biol **9**:127

Agustì M, Zaragoza S, Iglesias DJ, Almela V, Primo-Millo E, Talòn M (2002) The synthetic auxin 3, 5, 6-TPA stimulates carbohydrate accumulation and growth in citrus fruit. Plant Growth Regul **36**: 141-147

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55**: 373-399

Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, Leon P (2000) Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev **14**: 2085-2096

Ashikari M, Wu J, Yano M, Sasaki T, Yoshimura A (1999) Rice gibberellin-insensitive dwarf mutant gene *Dwarf 1* aspects encodes the α-subunit of GTP-binding protein. Proc Natl Acad Sci USA **96**: 10284-10289

Assmann SM (2002) Heterotrimeric and Unconventional GTP Binding Proteins in Plant Cell Signaling. The Plant Cell **14**: 355-373

Atkinson CJ, Else MA, Stankiewicz A, Webster AD (2002) The effects of phloem

girdling on the abscission of *Prunus avium* L. fruits. J Hortic Sci Biotech **77**: 22-27

Atkins CA, Pigeaire A (1993) Applications of cytokinins to flowers to increase pod set in *Lupinus angustifolius* L. A J Agri Res **44**: 1799-819

Avanzi S, Vernieri P, Ceccarelli M, Tagliasacchi AM, Forino LMC, Lorenzi R, Bonomo R (1988) Developmental stages and gibberellin activity in ovules of abscission affected fruits of *Malus domestica* Borkh. Plant Physiol **132**: 10-15

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The *Arabidopsis* trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signalling. Plant Physiology **136**: 3649-3659

Bangerth F (2000) Abscission and thinning of young fruit and their regulation by plant hormones and bioregulator. Plant Growth Regul **31**: 43-59

Bangerth F, Li CJ, Gruber J (2000) Mutual interaction of auxin and cytokinins in regulating correlative domaninca. Plant Growth Regul **32**: 205-217

Barry CS, Giovannoni JJ (2006) Ripening in the tomato *Green-ripe* mutant is inhibited by ectopic expression of a protein that disrupts ethylene signaling. Proc Natl Acad Sci USA **103**: 7923-7928

Barry CS, McQuinn RP, Thompson AJ, Seymour GB, Grierson D, Giovannoni JJ (2005) Ethylene insensitivity conferred by the *green-ripe* and *never-ripe 2* ripening mutants of tomato. Plant Physiol **138**: 267- 275

Basak A (2000) Use of benzyladenine, endothall and ammonium thiosulfate for fruitlet thinning in some apple cultivars. Acta Hort **517**: 217-225

Basak A (2006) The effect of ethephon, NAA and BA, used separately or jointly, on the efficiency of thinning in apple trees cultivars Jonagold, Golden Delicious and Elstar. Proc Plant Growth Reg Soc Amer **33**:144-150

Batjer LP, Thompson BJ (1961) Effect of 1-naphthyl-N-methylcarbamate (sevin) on thinning apples. Proc Amer Soc Hort Sci **77**: 1-8

Baugher, TA, Elliott KC, Leach DW, Horton BD, Miller S (1991) Improved methods of mechanically thinning peaches at full bloom. J Amer Soc Hort Sci **116**: 766-769

Belfield EJ, Ruperti B, Roberts JA, McQueen-Mason S (2005) Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in *Sambucus nigra*. J Exp Botany **56**: 817-823

Bergh O (1990) Effect of time of hand thinning on apple fruit size. S Afr J Plant and Soil Sci **7**: 1-10

Bergh O (1992) Cumulative effect of time of hand thinning on fruit size of 'Golden Delicious' and 'Granny Smith' apples. S Afr J Plant and Soil Sci **9**: 64-67

Bertschinger L, Weibel F, Stadtler W, Schmid A, Hohn H, Evequoz C, Pfammatter W (2000) Thinning procedures for regulating apple fruit quality and yield. Proc. 13th Int. IFIAM Sci Conf, Basel, Switzerland, 28-31: 248

Berüter J, Droz PH (1991) Studies on locating the signal for fruit abscission in the apple tree. Sci Hort **46**: 201-214

Biddle E, Kerfoot DGS, Kho YH, Russell KE (1976) Kinetic studies of the thermal decomposition of 2-chloroethylphosphonic acid in aqueous solution. Plant Physiol **58**: 700-702

Bini G, Raddi P (1967) Il diradamento dei frutti di melo con Sevin. Riv Ortoflorofruttic Ital **92**: 129-145

Blanpied GD (1972) A study of ethylene in apple, red raspberry and cherry. Plant Physiol **49**: 627-630

Bleecker AB, Patterson SE (1997) Last exit: senescence, abscission and meristem arrest in *Arabidopsis*. Plant Cell **9**: 1169-1179

Bonghi C, Casadoro G, Ramina A, Rascio N (1993) Abscission in leaf and fruit explants of *Prunus persica* (L) Batsch. New Phytol **123**: 555-565

Bonghi C, Rascio N, Ramina A, Casadoro G (1992) Cellulase and polygalacturonase involvement in the abscission of leaf and fruit explants of peach. Plant Mol Biol **20**: 839-848

Bonghi C, Tonutti P, Ramina A (2000) Biochemical and molecular aspects of fruitlet abscission. Plant Growth Regul **31**: 35-42

Bonnefont-Rousselot D (2002) Glucose and reactive oxygen species. Curr Opin in Clinical Nutrition and Metabolic Care **5**: 561-568

Bound SA (2001) Managing crop load. In: Dris R, Niskanen R, Jain SM. (Eds.), Crop management and postharvest handling of horticultural products, Volume I, Inc. Plymouth, UK, Science Publisher: 89-109

Bound SA, Jones KM, Graham B, Oakford MJ, Tichon M (1993) Modelling the effects

of timing and rates of application of benzyladenine as a secondary thinner on Fuji apple after ethephon. J Hort Sci **68**: 967-973

Bound SA, Jones KM, Koen TB, Oakford MJ (1991) The thinning effect of benzyladenine on red 'Fuji' apple trees. J Hortic Sci **66**: 789-794

Bound SA, Jones KM, Oakford MJ (1997) Post-bloom thinning with 6-benzyladenine. Acta Hort **463**: 493-499

Bray EA, Beachy RN (1985) Regulation by ABA of β-conglicinin expression in cultured developing soybean cotyledons. Plant Physiol **79**: 746-750

Brown KM (1997) Ethylene and abscission. Physiol Plant **100**: 567-76

Brummell DA, Bird CR, Schuch W, Bennett AB (1997a) An endo-1,4-β-glucanase expressed at high levels in rapidly expanding tissues. Plant Mol Biol **33**: 87-95

Brummell DA, Catala C, Lashbrook CC, Bennett AB (1997b) A membrane-anchored E-type endo-β-1,4-glucanase is localized on Golgi and plasma membranes of higher plants. Proc Natl Acad Sci USA **94**: 4794-4799

Bubán T, Lakatos T (1997) Fruit thinning with benzyladenine in trees of hard-to-thin apple cultivars. Hortic Sci **29**: 31-35

Bubán T, Lakatos T (2000) Contributions to the efficacy of benzyladenine as a fruit thinning agent for apple cultivars. Acta Hort **514**: 59-67

Budiman MA, Chang SB, Lee S, Yang TJ, Zhang HB, de Jong JH, Wing RA (2004) Localization of *jointless-2* gene in the centromeric region of tomato chromosome 12 based on high resolution genetic and physical mapping. Theor Appl Genet **108**: 190-196

Bukovac MJ, Black BL, Hull J, Stopar M (1994) Interaction between NAA and BA on cropping and fruit size in 'Delicious' and 'Empire' apples. HortScience **29**: 472

Bukovac MJ, Sabbatini P, Schwallier PG, Schroeder M (2008) Characterizing the interaction between NAA and BA on apple fruit abscission and development. HortScience **43**: 1794-1801

Bustan A, Erner Y, Goldschmidt EE (1995) Interactions between developing *Citrus* fruit and their supportive vascular system. Annals of Botany **76**: 657-666

Butenko MA, Vie AK, Brembu T, Aalen RB, Bones AM (2009) Plant peptides in signalling: looking for new partners. Trends Plant Sci **14**: 255-63

Butler L (1936) Inherited characters in the tomato. II. Jointless pedicel. J Hered **37**: 25-26

Byers RE (1989) High pressure water stream bloom thinning and GA3 sprays on flower bud numbers and distribution in peach trees. Annual Report of the State Horticultural Society, Michigan, USA, **119**

Byers RE (2003) Flower and fruit thinning and vegetative: fruiting balance. In: Ferree, D.C., Warrington, IJ. (Eds.), Apples: Botany, Production and Uses. CABI Publ, Cambridge, MA, USA: 409-436

Cai S, Lashbrook CC (2008) Stamen abscission zone transcriptome profiling reveals new candidates for abscission control: enhanced retention of floral organs in transgenic plants overexpressing *Arabidopsis* ZINC FINGER PROTEIN2. Plant Physiol **146**: 1305-1321

Callejas R, Bangerth F (1997) Is auxin export of apple fruit an alternative signal for inhibition of flower bud induction? Acta Hort **463**: 271-277

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88**: 57-63

Catala C, Rose JKC, Bennett AB (1997) Auxin regulation and spatial localization of an endo-1,4-β-D-glucanase and a xyloglucan endotransglycosylase in expanding tomato hypocotyls. Plant J **12**: 417-26

Chan BG, Cain JC (1967) The effect of seed formation on subsequent flowering in apple. Proceeding of the American Society for Horticultural Science **91**: 63-67

Cho D, Shin D, Jeon BW, Kwak JM (2009) ROS-mediated ABA signaling. J Plant Biol **52**:102-113

Cho HT, Cosgrove DJ (2000) Altered expression of expansin modulates leaf growth and pedicel abscission in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **97**: 9783-9788

Cho SK, Larue CT, Chevalier D, Wang H, Jinn T-L, Zhang S, Walker JC (2008) Regulation of floral organ abscission in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **105**: 15629-15634

Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS1 ethylene receptors. Proc Natl Acad Sci USA **95**: 5401-5406

Clements J, Atkins C (2001) Characterization of a non-abscission mutant in *Lupinus angustifolius* L. I. Genetic and structural aspects. Am J Bot **88**: 31-42

Contento AL, Kim S-J, Bassham DC (2004) Transcriptome profiling of the response of Arabidopsis suspension culture cells to Suc starvation. Plant Physiol **135**: 2330-2347

Costa G, Bonany J, Carbo J, Casala M, Stopar M, Lafer G, Dorigoni A, Vizzotto G, Bomben C (2004) Multilocation analysis of ATS and BA thinning efficacy on 'Golden delicious'. Acta Hort **636**: 303-313

Coupe SA, Taylor JE, Roberts JA (1995) Characterization of an mRNA encoding a metallothionein-like protein that accumulates during ethylene-promoted abscission of *Sambucus nigra* L. leaflets. Planta **197**: 442-447

Coursol S, Fan LM, Le Stunff H, Spiegel S, Gilroy S, Assmann SM (2003) Sphingolipid signalling in *Arabidopsis* guard cells involves heterotrimeric G proteins. Nature **423**: 651-654

D'Haeze W, Rycke RD, Mathis R, Goormachtig S, Pagnotta S, Verplancke C, Capoen W, Holsters M (2003) Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. Proc Natl Acad USA **100**: 11789-11794

Dal Cin V, Barbaro E, Danesin M, Murayama H, Velasco R, Ramina A (2009a) Fruitlet abscission: A cDNA-AFLP approach to study genes differentially expressed during shedding of immature fruits reveals the involvement of a putative auxin hydrogen symporter in apple (Malus domestica L. Borkh). Gene **442**: 26–36

Dal Cin V, Boschetti A, Dorigoni A, Ramina A (2007a) Benzylaminopurine application on two different apple cultivars (*Malus domestica*) displays new and unexpected fruitlet abscission features. Annals of Botany **99**: 1195–1202

Dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A (2005) Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borck). J Exp Bot **56**: 2995–3005

Dal Cin V, Danesin M, Botton A, Boschetti A, Dorigoni A, Ramina A (2007b) Fruit load and elevation affect ethylene biosynthesis and action in apple fruit (*Malus domestica* L. Borkh) during development, maturation and ripening. Plant Cell Environ **30**: 1480-5

Dal Cin V, Velasco R, Ramina A (2009b) Dominance induction of fruitlet shedding in *Malus X domestica* (L. Borkh): molecular changes associated with polar auxin transport. BMC Plant Biol **9**: 139

Dal Degan F, Child R, Svendsen I, Ulvskov P (2001) The cleavable N-terminal domain of plant endopolygalacturonases from clade B may be involved in a regulated secretion mechanism. J Biol Chem **276**: 35297-35304

Davidson JH, Hammer OH, Reimer CA, Dutton WC (1945) Thinning apples with the sodium salt of naphthylacetic acid. Mich Agr Expt Quart Bul **27**: 352-356

del Campillo E, Bennett AB (1996) Pedicel breakstrength and cellulase gene expression during tomato flower abscission. Plant Physiol **111**: 813-20

del Campillo E, Lewis LN (1992) Identification and kinetics of accumulation of proteins induced by ethylene in bean abscission zones. Plant Physiol **98**: 955-961

del Campillo E, Reid PD, Sexton R, Lewis LN (1990) Occurrence and localization of 9.5 cellulase in abscising and nonabscising tissues. Plant Cell **2**: 245-254

Denne MP (1963) Fruit development and some tree factors affecting it. NZ J Bot **1**: 265-294

Dennis FG (1986) Apple. In: MonseliseSP, eds. CRC Handbook of fruit-set and development. Bocaraton, FL, USA: CRC Press: 1-44

Dennis FG (2000) The history of fruit thinning. Plant Growth Reg **31**: 1-16

Desikan R, Hancock JT, Bright J, Harrison J, Weir I, Hooley R, Neill SJ (2005) A role for ETR1 in hydrogen peroxide signaling in stomatal guard cells. Plant Physiol **137**: 831-834.

Desikan R, Mackerness SAH, Hancock JT, Neill SJ (2001) Regulation of the Arabidopsis transcriptonprme by oxidative stress. Plant Physiol **127**: 159-172

Dong X (2004) NPR1, all things considered. Curr Opin Plant Biol **7**: 547- 552

Dorigoni A, Comai M, Dallabetta N, Piffer I, Angeli D (2003) Bezyladenine: a new thinner for apple to supplement those already in use. Rivista di Frutticoltura e di Ortofloricoltura **65**: 69-75

Dorigoni A, Lezzer P (2007) Chemical thinning of apple with new compounds. Erwerbs-Obstbau **49**: 93-96

Drazeta L, Lang A, Cappellini C, Hall AJ, Volz RK, Jameson PE (2004) Vessel

differentiation in the pedicel of apple and the effects of auxin transport inhibition. Physiol Plant **120**: 162-170

Ehness R, Ecker M, Godt DE, Roitsch TH (1997) Glucose and stress independently regulate source and sink metabolism and defence mechanisms via signal transduction pathways involving protein phosphorylation. Plant Cell **9**: 1825-1841

Elfving DC (1989) N-(phenylmethyl)-1H-purine-6-amine (BA) as a chemical thinner for 'Idared' apple. Acta Hort **239**: 357-362

Elfving DC (1994) Benzyladenine: chemical thinner for the Northwest. Good Fruit Grower **45**: 47–48

Elfving DC, Cline RA (1993) Benzyladenine and other chemicals for thinning 'Empire' apple trees. J Am Soc Hortic Sci **118**: 593-598

Ellis CM, Nagpal P, Young JC, Hagen G, Guilfoyle TJ, Reed JW (2005) *AUXIN RESPONSE FACTOR*1 and *AUXIN RESPONSE FACTOR*2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*. Development **132**: 4563-4574

Else MA, Stankiewicz-Davies AP, Crisp CM, Atkinson CJ (2004) The role of polar auxin transport through pedicels of *Prunus avium* L. in relation to fruit development and retention. J Exp Bot **55**: 2099-2109

Evensen KB, Page AM, Stead AD (1993) Anatomy of ethylene-induced petal abscission in *Pelargonium x hortorum*. Annals of Botany **71**: 559-566

Fallahi E, Willemsen KM (2002) Blossom thinning of pome and stone fruit. HortScience **37**: 474-477

Ferree D (1996) Performance of benzyladenine as a chemical thinner on eight apple cultivars. J Tree Fruit Prod **1**: 33-50

Flaishman M, Shargal A, Shilzerman L, Lev-Yadun S, Stern RA, Grafi G (2005) The synthetic cytokinins CPPU and TDZ prolong the phase of cell division in developing pear (*Pyrus communis* L.) fruit. Acta Hort **671**: 151-157

Flore JA, Bukovac MJ (1982) Factors influencing absorption of ^{14}C(2- chloroethyl) phosphonic acid by leaves of cherry. J Am Soc Hort Sci **107**: 965-968

Force AR, Lawton KA, Woodson WR (1988) Dark-induced abscission of hibiscus flower buds. Hortscience **23**: 592-593

Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead

P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422**: 442-446

Forino LMC, Andreucci AC, ìGiraldi E, Tagliasacchi AM (2000) Cytohistochemical Analysis of Malus domestica Borkh. Seeds from Shedding and Nonshedding Fruits. I lnt J Plant Sci **161**: 463-472

Forino LMC, Tagliasacchi AM, Avanzi S (1987) Embryo-sacs frequency in ovules of abscission affected fruits in *Malus domestica* Borkh. Adv Hort Sci **1**: 65-67

Forshey CG (1976) Factors affecting chemical thinning of apples. NY Food Life Sci Bull **64**: 7

Forshey CG, Elfving DC (1977) Fruit numbers, fruit size, and yield relationships in'McIntosh' apples. J Amer Soc Hort Sci **102**: 399-402

Foster T, Jonhston R, Seleznyove A (2003) A morphological and quantitative characterization of early floral development in apple (*Malus x domestica* Borkh.). Annals of Botany **92**: 199-206

Frank W, Munnik T, Kerkmann K, Salamini F, Bartels D (2000) Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. The Plant Cell **12**: 111-123

Fukuda H (1991) Difference in the efficacy of NAC as thinning agent of apple fruit among several cultivars. Bull Fruit Tree Res Stat Min Agric Forest Fish Japan **19**: 39-48

Gahagan HE, Holm RE, Abeles FB (1968) Effect of ethylene on peroxidase activity. Physiologia Plantarum **21**: 1270-1279

Geisler M, Kleczkowski LA, Karpinski S (2006) A universal algorithm for genome-wide in silicio identification of biologically significant gene promoter putative cis-regulatory-elements; Identification of new elements for reactive oxygen species and sucrose signaling in Arabidopsis. Plant J **45**: 384-398

Geldner M, Friml J, Stierhof Y-D, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature **413**: 425-428

Gershenzon G (1984) Changes in the levels of plant secondary metabolites production under water and nutrient stress. Recent Adv Phytochem **18**: 273-320

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. Curr Opin Plant Biol **8**: 93-102

Godt DE, Roitsch T (1997) Regulation and tissue-specific ditribution of mRNA for three extracellular invertase isoenzymes of tomato suggests an important function in establishing and maintaining sink metabolism. Plant Physiol **115**: 273-282

Gomez-Cadenas A, Mehouachi J, Tadeo FR, Primo-Millo E, Talon M (2000) Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. Planta **210**: 636-643

Gonzalez-Bosch C, del Campillo E, Bennett AB (1997) Immunodetection and characterization of tomato endo-1,4-β-glucanase cel1 protein in flower abscission zones. Plant Physiol **114**:1541-46

Gonzalez-Carranza ZH, Lozoya-Gloria E, Roberts JA (1998) Recent developments in abscission: shedding light on the shedding process. Trends Plant Sci **3**: 10-14

Gonzalez-Carranza ZH, Whitelaw CA, Swarup R, Roberts JA (2002) Temporal and spatial expression of a polygalacturonase during leaf and flower abscission in oilseed rape and Arabidopsis. Plant Physiol **128**: 534-543

Goren R (1993) Anatomical, physiological, and hormonal aspects of abscission in citrus. Hort Rev **15**: 33-46

Greene DW (1989) Regulation of fruit set in tree fruits with plant growth regulators. Acta Hort **239**: 323-334

Greene DW (1993) A review of the use of benzyladenine (BA) as a chemical thinner for apples. Acta Hort **329**: 231-236

Greene DW (1997) Chemical thinning strategies for the future. In: Proc. New England Fruit Meetings: 22-26

Greene DW (2002) Chemicals, timing, and environmental factors involved in thinner efficacy on apple. HortScience **37**: 477-481

Greene DW, Autio WR (1989) Evaluation of benzyladenine as a chemical thinner on 'McIntosh' apples. J Amer Soc Hort Sci **114**: 68-73

Greene DW, Autio WR (1994) Suggestions for use of the new postbloom thinner Accel. Fruit Notes Spring: 18-20

Greene DW, Autio WR, Erf JA, Mao ZY (1992) Mode of action of benzyladenine when used as a chemical thinner on apples. J Am Soc Hortic Sci **117**: 775-779

Greene DW, Autio WR, Miller P (1990) Thinning activity of benzyladenine on several

apple cultivars. J Amer Soc Hort Sci **115**: 394-400

Greene DW, Hauschild KI, Krupa J (2001) Effect of blossom thinners on fruit set and fruit size of peaches. HortTechnology **11**: 179-183

Greene DW, Miller P (1984) Uses of 6-benzyladenine as a chemical thinner for apples. HortScience **19**: 528

Greenhalgh WJ, Godley GL, Menzies R (1977) Studies of fruit shape in apples: Response to gibberellin and cytokinin sprays. Aust J Expt Agr Anim Husb **17**: 505-509

Griggs WH, Iwakiri BT, Madsen HF (1962) Effect of dilute and concentrated sprays of 1-naphthyl-N-methylcarbamate (sevin) on fruit set and seed content of Bartlett pears. Proc Amer Soc Hort Sci **81**: 93-97

Gruber JD, Bangerth F (1990) Diffusible IAA and dominance phenomena in fruits of apple and tomato. Physiol Plant **79**: 354-358

Guinn G, Brummett DL (1988) Changes in free and conjugated indole 3-acetic acid and abscisic acid in young cotton fruits and their abscission zones in relation to fruit retention during and after moisture Stress. Plant Physiol **86**: 28-31

Ha CM, Jun JH, Nam HG, Fletcher JC (2004) *BLADE-ON-PETIOLE1* encodes a BTB/POZ domain protein required for leaf morphogenesis in *Arabidopsis thaliana*. Plant Cell Physiol **45**: 1361-1370

Ha CM, Jun JH, Nam HG, Fletcher JC (2007) *BLADE-ON-PETIOLE1* and *2* control *Arabidopsis* lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell **19**: 1809-1825

Ha CM, Kim GT, Kim BC, Jun JH, Soh MS, Ueno Y, Machida Y, Tsukaya H, Nam HG (2003) The *BLADE-ON-PETIOLE1* gene controls leaf pattern formation through the modulation of meristematic activity in *Arabidopsis*. Development **130**: 161-172

Habdas H, Jankiewicz LS, Borkowska B (1982) Changes in anatomical structure of apple fruitlet pedicels preceding June drop. Acta Agrobot **35**: 11-23

Hadfield KA, Bennett AB (1998) Polygalacturonases: many genes in search of a function. Plant Physiol **117**: 337-343

Halford NG, Hey S, Jhurreea D, Laurie S, McKibbin RS, Paul M, Zhang Y (2003) Metabolic signalling and carbon partitioning: role of Snf1-related (SnRK1) protein kinase. J Exp Bot **54**: 467-75

Hansen H, Grossmann K (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. Plant Physiol **124**: 1437-1448

Harris N, Taylor JE, Roberts JA (1997) Characterization and expression of an mRNA encoding a wound-induced (win) protein from ethylene-treated tomato leaf abscission zone tissue. J Exp Bot **48**: 1223-1227

Heindl JC, Brun WA (1983) Light and shade effects on abscission and C-photoassimilate partitioning among reproductive structures in soybean. Plant Physiol **73**: 434-439

Heinicke AJ (1917) Factors influencing the abscission of flowers and partially developed fruits of the apple *(Pyrus mains* L.). Cornell agric Exji Sta Bull **393**: 43-114

Helson BV, Barber KN, Kingsbury PD (1994) Laboratory toxicology of six forestry insecticides to four species of bee (Hymenoptera: Apoidea). Arch Environ Toxicol **27**: 107-114

Henderson J, Lyne L, Osborne DJ (2001) Failed expression of an endo-β-1,4-glucanhydrolase (cellulase) in a non-abscinding mutant of *lupinus angustifolius* cv Danja. Phytochemistry **58**: 1025-1034

Henry EW, Valdovinos JG, Jensen TE (1974) Peroxidases in tobacco abscission zone tissue: II. Time course studies of peroxidase activity during ethylene-induced abscission. Plant Physiol **54**: 192-196.

Hepworth SR, Zhang Y, McKim S, Li X, Haughn G (2005) BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in Arabidopsis. Development **17**: 1434-1448

Hetherington AM, Quatrano RS (1991) Mechanisms of action of abscisic acid at the cellular level. New Phytol **119**: 9-12

Hildebrand EM (1944) The mode of action of the pollenicide Elgetol. Proceedings of the American Soc Hort Sci **45**: 53-58

Hinman RL, Lang J (1965) Peroxidase-catalyzed oxidation of indole-3-acetic acid. Biochemistry **4**: 144-158

Hobl I, Jong J, Heijne PF (2003) Efficacy and phytotoxicity of lime sulphur in organic apple production. Ann Applied Biol **142**: 225-233

Hoffman MB, Edgerton LJ, Fisher EG (1955) Comparison of naphthaleneacetic acid and naphthaleneacetamide for thinning apples. Proc Amer Soc Hort Sci **65**: 63-70

Hong SB, Tucker ML (2000) Molecular characterization of a tomato ploygalacturonase gene abundantly expressed in the upper third of pistils from opened and unopened flowers. Plant Cell Rep **19**: 680-683

Iglesias DJ, Tadeo FR, Primo-Millo E, Talòn M (2003) Fruit set dependence on carbohydrate availability in citrus trees. Tree Physiol **23**: 199-204

Iglesias DJ, Tadeo FR, Primo-Millo E, Talòn M (2006) Carbohydrate and ethylene levels related to fruitlet drop through abscission zone A in citrus. Trees **20**: 348-355

Irving DE, Pallesen JC, Drost JH (1989) Preliminary results on chemical thinning of apple blossom with ammonium thiosulphate, NAA, and ethephon. NZ J Crop Hort Sci **17**: 363-365

Janoudi A, Flore JA (2005) Application of ammonium thiosulfate for blossom thinning in apples. Sci Hort **104**: 161-168

Janssen BJ, Thodey K, Schaffer RJ, Alba R, Balakrishnan L, Bishop R, Bowen JH, Crowhurst RS, Gleave AP, Ledger S, McArtney S, Pichler FB, Snowden KC, Ward S (2008) Global gene expression analysis of apple fruit development from the floral bud to ripe fruit. BMC Plant Biol **8**: 16

Jiang M, Zhang J (2002) Involvement of plasma-membrane NADPH oxidase in abscisic acid- and water stress-induced antioxidant defense in leaves of maize seedlings. Planta **215**:1022-1030

Jiang M, Zhang J (2003) Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defence in leaves of maize seedlings. Plant Cell Environ **26**: 929-939

Jones KM, Bound SA, Oakford MJ (1992) Identifying the optimum thinning time for red 'Fuji' apples. J Hort Sci **67**: 685-694

Jones KM, Koen TB (1985) Temperature effects on ethephon thinning of apples. J Hort Sci **60**: 13-19

Jones KM, Koen TB, Meridith RJ (1983) Thinning Golden Delicious apples using ethephon sprays. J Hort Sci **58**: 381–388

Joo JH, Bae YS, Lee JS (2001) Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiol. **126**: 1055-1060

Kalaitzis P, Hong S-B, Solomos T, Tucker ML (1999) Molecular characterization of a

tomato endo-β-1,4-glucanase gene expressed in mature pistils, abscission zones and fruit. Plant Cell Physiol **40**: 905-8

Kalaitzis P, Solomos T, Tucker ML (1997) Three different polygalacturonases are expressed in tomato leaf and flower abscission, each with a different temporal expression pattern. Plant Physiol **113**: 1303-1308

Kawano T (2003) Role of reactive oxygen species-generating peroxidase reactions in plant defense and growth induction. Plant Cell Rep **21**: 829-837

Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C (1998) A plant homolog of the neutrophil NADPH oxidase gp91phox subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. Plant Cell **10**: 255-266

Knight JN (1978) Chemical thinning of the apple cultivar, Laxton's Superb. J Hort Sci **53**: 63-66

Knight JN (1986) Fruit thinning with carbaryl. Acta Hort **179**: 707-708

Knight JN, Lovell JD (1983) Chemical thinning with carbaryl. A R East Malling Res Stat 1982: 29-30

Koch KE (1996) Carbohydrate-modulated gene expression in plants. Annu Rev Plant Physiol Plant Mol Biol **47**: 509-540

Koehler DE, Lewis LN, Shannon LM, Durbin ML (1980) Purification of abscission zone cellulase. Phytochemistry **20**: 409-412

Koehler SM, Matters GM, Nath P, Kemmerer EC, Tucker ML (1996) The gene promoter for a bean abscission cellulase is ethylene-induced in transgenic tomato and shows high sequence conservation with a soybean abscission cellulase. Plant Mol Biol **31**: 595-606

Koiwa H, Barb AW, Xiong L, Li F, McCully MG, Lee BH, Sokolchik I, Zhu J, Gong Z, Reddy M, Sharkhuu A, Manabe Y, Yokoi S, Zhu JK, Bressan RA, Hasegawa PM (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate Arabidopsis thaliana abiotic stress signaling, growth, and development. Proc Natl Acad Sci USA **99**: 10893-10898

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci USA **97**: 2940–294

Lakso AN, Wünsche JN, Palmer JW, Grappadelli LC (1999) Measurement and modeling of carbon balance of the apple tree. HortScience **34**: 1040-1047

Lashbrook CC, Gonzalez-Boch C, Bennett AB (1994) Two divergent endo-β-1,4-glucanase genes exhibit overlapping expression in ripening fruit and abscising flowers. Plant Cell **6**: 1485-93

Lashbrook CC, Tieman DM, Klee HJ (1998) Differential regulation of the tomato *ETR* gene family throughout plant development. Plant J **15**: 243-252

Lee SH, Chae HS, Lee TK, Kim SH, Shin SH, Cho BH, Cho SH, Kang BG, Lee WS (1998) Ethylene-mediated phospholipid catabolic pathway in glucose-starved carrot suspension cells. Plant Physiol **116**: 223-229

Lee YP, Yu GH, Seo YS, Han SE, Choi YO, Kim D, Mok IG, Kim WT, Sung SK (2007) Microarray analysis of apple gene expression engaged in early fruit development. Plant Cell Rep **26**: 917-926

Lein W, Saalbach G (2001) Cloning and direct G protein regulation of phospholipase D from tobacco. Biochim Biophys Acta **1530**: 172-183

Leslie ME, Lewis MW, Youn JY, Daniels MJ, Liljegren SJ (2010) The EVERSHED receptor-like kinase modulates floral organ shedding in *Arabidopsis*. Development **137**: 467-476

Letham DS (1968) Regulation of cell division in plant tissue. I. Inhibitors and stimulants of cell division in developing fruits: Their properties and activity in relation to the cell division period. NZ J Bot **1**: 336-350

Lewis LN, Varner JE (1970) Synthesis of cellulase during abscission of *Phaseolus vulgaris* leaf explants. Plant Physiol **46**: 194-199

Libertini E, Li Y, McQueen-Mason SJ (2004) Phylogenetic analysis of the plant endo-beta-1,4-glucanase gene family. J Mol Evol **58**: 506-515

Liljegren SJ, Leslie ME, Darnielle L, Lewis MW, Taylor SM, Luo R, Geldner N, Chory J, Randazzo PA, Yanofsky MF, Ecker JR (2009) Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. Development **136**: 1909-1918

Lomax TL, Muday GK, Rubery PH (1995) Auxin transport. In: Davies PJ ed. Plant Hormones. Physiology, Biochemistry and Molecular Biology, 2nd edn. Kluwer

Academic Publishers, Dordrecht: 509–530

Loreti E, Poggi A, Novi G, Alpi A, Perata P (2005) A genome-wide analysis of the effects of sucrose on gene expression in Arabidopsis seedlings under anoxia. Plant Physiol **137**: 1130-1138

Luckwill LC (1953) Studies on fruit development in relation to plant hormones. I. Hormone production by the developing apple seed in relation to fruit drop. J Hort Sci **28**: 14-24

Luckwill LC, Weaver P, MacMillan J (1969) Gibberellins and other growth hormones in apple seeds. J Hort Sci **44**: 413-424

Mahalingam R, Jambunathan N, Gunjan SK, Faustin E, Weng H, Ayoubi P (2006) Analysis of oxidative signalling induced by ozone in Arabidopsis thaliana. Plant Cell Environ **29**: 1357-1371

Malladi A, Burns JK (2008) *CsPLDα1* and *CsPLDγ1* are differentially induced during leaf and fruit abscission and diurnally regulated in *Citrus sinensi.* J Exp Bot **59**: 3729-3739

Mao L, Begum D, Chuang H, Budiman MA, Szymkowiak EJ, Irish EE, Wing RA (2000) *JOINTLESS* is a MADS-box gene controlling tomato flower abscission zone development. Nature **406**: 910-3

Mao L, Begum D, Goff SA, Wing RA (2001) Sequence and analysis of the tomato *JOINTLESS* locus. Plant Physiol **126**: 1331-40

Marini R, Byers R (1988) Methods for evaluating chemical inhibitors of apple abscission. Hortscience **23**: 849-851

Marini RP (1996) Chemically thinning spur 'Delicious' apples with carbaryl, NAA, and ethephon at various stages of fruit development. HortTechnology **6**: 241-246

Marsh HV, Southwick FW, Weeks WD (1960) The influence of chemical thinners on fruit set and size, seed development and preharvest drop of apples. Proc Amer Soc Hort Sci **75**: 5-21

McArtney S, Latter MWI, Campbell J (2004) Individual and combined effects of shading and thinning chemicals on abscission and dry-matter accumulation of "Royal Gala" apple fruit. J Hortic Sci Biotechnol **79**: 441-448

McLaughlin JM, Greene DW (1984) Effects of BA, GA 4+7, daminozide on fruit set, fruit quality, vegetative growth, flower initiation, and flower quality of 'Golden Delicious'

apples. J Amer Soc Hort Sci **109**: 34-39

McManus MT (1994) Peroxidases in the separation zone during ethylene-induced bean leaf abscission. Phytochemistry **35**: 567-572

Mehouachi J, Serna D, Zaragoza S, Agusti M, Talon M, Primo-Millo E (1995) Defoliation increases fruit abscission and reduces carbohydrate levels in developing fruits and woody tissues of Citrus unshiu. Plant Science **107**: 189-197

Meir S, Hunter DA, Chen JC, Halaly V, Reid MS (2006) Molecular changes occurring during acquisition of abscission competence following auxin depletion in *Mirabilis jalapa*. Plant Physiol **141**: 1604-1616

Meir S, Philosoph-Hadas S, Srivignesh Sundaresan KS, Selvaraj V, Burd S, Ophir R, Kochanek B, Reid MS, Jiang C-Z, Lers A (2010) Microarray analysis of the abscission-related transcriptome in the tomato flower abscission zone in response to auxin depletion. Plant Physiol **154**: 1929-1956.

Meland M (1998) Thinning apples and pears in a Nordic climate. III. The effect of NAA, ethephon and lime sulphur on fruit set, yiapple cultivars. Acta Hort **463**: 517-525

Miao Y, Lv D, Wang P, Wang XC, Chen J, Miao C, Song CP (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18**: 2749-2766

Michaux-Ferriere N, Grout H, Carron MP (1992) Origin and ontogenesis of somatic embryos in *Hevea brasiliensis* (Euphorbiaceae). Am J Bot **79**: 174-180

Miller G, Shulaev V, Mittler R (2008) Reactive oxygen signaling and abiotic stress. Physiol Plant **133**:481–489

Miller SS (1988) Plant niotegulators in appe and pear culture. Horicultural Review **10**: 309-401

Mills BJ, Stinson CT, Liu MC, Lang CA (1997) Glutathione and cyst(e)ine profiles of vegetables using high performance liquid chromatography with dual electrochemical detection. J Food Compos Anal **10**: 90-91

Monshausen GB, Bibikova TN, Messerli MA, Shi C, Gilroy S (2007) Oscillations in extracellular pH and reactive oxygen species modulate tip growth of Arabidopsis root hairs. Proc Natl Acad Sci USA **104**: 20996-21001

Morris DA (1993) The role of auxin in the apical regulation of leaf abscission in cotton

(*Gossypium hirsutum* L.). J Exp Bot **44**: 807-814

Moussatche P, Klee HJ (2004) Autophosphorylation activity of the Arabidopsis ethylene receptor multigene family. J Biol Chem **279**: 48734-48741

Muday GK, Brady SR, Argueso C, Deruère J, Kieber JJ, DeLong A (2006) RCN1-regulated phosphatase activity and EIN2 modulate hypocotyl gravitropism by a mechanism that does not require ethylene signaling. Plant Physiol **141**: 617-1629

Muday GK, Peer WA, Murphy AS (2003) Vesicular cycling mechanisms that control auxin transport polarity. Trends in Plant Science **8**: 301-304

Newcomb RD, Crowhurst RN, Gleave AP, Rikkerink EHA, Allan AC, Beuning LL, Bowen JH, Gera E, Jamieson KR, Janssen BJ, Laing WA, McArtney S, Nain B, Ross GS, Snowden KC, Souleyre EJF, Walton EF, Yauk Y-K (2006) Analyses of expressed sequence tags from apple. Plant Physiol **141**: 147-166

Nielsen TH, Krapp A, RBper-Schwarz U, Stitt M (1998) The sugar-mediated regulation of genes encoding the small subunit of Rubisco and the regulatory subunit of ADP glucose pyrophosphorylase is modified by phosphate and nitrogen. Plant Cell Env **21**: 443-454

Nii N (1980) Seasonal changes in growth and enlargement of the Japanese pear fruit, *Pyrus serotina* cv Shinsheiki, in relation to vascular bundle development in the pedicel and flesh. J Hort Sci **55**: 385-396

Noga G, Engel G (1986) Chemische Ausdünnung von Äpfeln mit Hilfe von Netzmitteln – neue Ansätze für die Praxis. Erwerbsobst **28**: 141-143

Norberg M, Holmlund M, Nilsson O (2005) The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs. Development **132**: 2203-2213

Nour KA, EC Yeung, TA Thorpe (1993) Shoot bud histogenesis from mature embryos and shoots of eastern white cedar (*Thuja occidentalis* L.) cultured in vitro. Int J Plant Sci **154**: 378-385

Nzima MDS, Martin GC, Nishijima C (1999) Effect of fall defoliation and spring shading on shoot carbohydrate and growth parameters among individual branches of alternate bearing `Kerman' pistachio trees. J Am Soc Hort Sci **124**: 52-60

O'Malley RC, Rodriguez FI, Esch JJ, Binder BM, O'Donnel P, Klee HJ, Bleecker AB

(2005) Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J **41**: 651-659

Ogawa M, Kay P, Wilson S, Swain SM (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 are polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. Plant Cell **21**: 216-233

Okushima Y, Mitina I, Quach HL, Theologis A (2005a) *Auxin response factor 2* (*ARF2*): A pleiotropic development regulator. Plant J **43**: 29-46

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005b) Functional genomic analysis of the *AUXIN RESPONSE FACTOR* gene family members in Arabidopsis thaliana: Unique and overlapping functions of *ARF7* and *ARF19*. Plant Cell **17**: 444-463

Olien WC, Bukovac MJ (1978) The effect of temperature on rate of ethylene evolution from ethephon and from ethephon-treated leaves of sour cherry. J Am Soc Hort Sci **103**: 199-202

Ono E, Wong HL, Kawasaki T, Hasegawa M, Kodama O, Shimamoto K (2001) Essential role of the small GTPase Rac in disease resistance of rice. Proc Natl Acad Sci USA **98**: 759-764

Osborne DJ (1977) Ethylene and target cells in the growth of plants. Science progress **64**: 51-63

Osborne DJ (1989) Abscission. Crit Rev Plant Sci **8**: 103-29

Osborne DJ, Sargent J (1976) The positional di erentiation of ethylene-responsive cells in rachis abscission zones in leaves of *Sambucus nigra* and their growth and ultrastructural changes at senescence and separation. Planta **130**: 203-210

Ouma G, Matta F (2002) Responses of several apple cultivars to chemical thinning sprays. J Food Technol Afr **7**: 16-20

Park S, Sugimoto N, Larson MD, Beaudry R, van Nocker S (2006) Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. Plant Physiol **141**: 811-824

Patrick JW (1979) Auxin-promoted transport of metabolites in stems of *Phaseolus vulgaris* L. J Exp Bot **114**: 1-13

Patrick JW (1987) Are hormones involved in assimilate transport? In: Hoad GV, Lenton JR, Jackson MB, Atkin RK, eds. Hormone action in plant development-a critical appraisal. London: Butterworths: 175-188

Patterson SE (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. Plant Physiol **126**: 494-500

Patterson SE, Bleecker AB (2004) Ethylene-dependent and independent processes associated with floral organ abscission in Arabidopsis. Plant Physiol **134**: 194-203

Payton S, Fray RG, Brown S, Grierson D (1996) Ethylene receptor expression is regulated during fruit ripening, flower senescence and abscission. Plant Mol Biol **31**: 1227-1231

Pei ZM, Murata Y, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406**: 731-734

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, Sudhakar D, Christou P, Snape JW, Gale MD, Harberd NP (1999) "*Green revolution*" genes encode mutant gibberellin response modulators. Nature **400**: 256-61

Pitzschke A, Hirt H (2009) Disentangling the complexity of mitogen activate protein kinases and reactive oxygen species signaling. Plant Physiol **149**: 606-615

Polomski RF, Barden JA, Byers RE, Wolf DD (1988) Apple fruit non-structural carbohydrates and abscission as influenced by shade and terbacil. J Am Soc Hortic Sci **113**: 506-511

Poovaiah BW (1974) Formation of callose and lignin during leaf abscission. Am J Bot **61**: 829-834

Preston AP, Quinlan JD (1968) A fruit thinning experiment with 'Cox's Orange Pippin' apple. Rep E Malling Res Stn: 75-78

Price J, Laxmi A, Martin SKS, Jang JC (2004) Global transcription profiling reveals multiple sugar signal transduction mechanisms in Arabidopsis. The Plant Cell **16**: 2128-2150

Proctor JTA, Schechter I (1992) Effect of ovule damage on fruit development in three apple cultivars. Hortscience **27**: 18-19

Purcell PC, Smith AM, Halford NG (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. Plant J **14**: 195-202

Quinlan, JD, Preston AP (1968) Effects of thinning blossom and fruitlets on growth and cropping of sunset apple. J Hort Sci **43**: 373-381

Ramina A (1981) La dinamica della cascola ed alcuni aspetti fisiologici della abscission nel diradamento chimico dei frutti di pesco (*Prunus Persica*, L. Batsch). I fitoregolatori nel controllo della produzione degli alberi da frutto. Ferrara, 26 Marzo: 9-32.

Ramina A, Masia A (1980) Levels of extractable abscisic acid in the mesocarp and seed of persisting and abscising peach fruit. J Amer Soc Hortic Sci **105**: 465-468

Ramina A, Masia A, Vizzotto G (1986) Ethylene and auxin transport and metabolism in peach fruit abscission. J Am Soc Hort Sci **111**: 760-764

Ramina A, Rascio N, Masia A (1989) The abscission processes in peach: structural, biochemical and hormonal aspects. In: Osborne DJ, Jackson MB, eds. Cell separation in plants: physiology, biochemistry and molecular biologv Berlin Heidelberg: NATO ASI, Springer Verlag: 233-238

Rick CM, Yoder JI (1988) Classical and molecular genetics of tomato: highlights and perspectives. Annu Rev Genet **22**: 281-300

Ritchie S, Gilroy S (1998) Abscisic acid stimulation of phospholipase D in the barley aleurone is G-protein-mediated and localized to the plasma membrane. Plant Physiol**124**: 693-702

Roberts JA, Elliott KA, Gonzalez-Carranza ZH (2002) Abscission, dehiscence, and other cell separation processes. Annu Rev Plant Biol **53**: 131-58

Roberts JA, Whitelaw CA, Gonzalez-Carranza ZH, McManus MT (2000) Cell separation processes in plants: models, mechanisms and manipulation. Annals of Botany **86:** 223-235

Rolland F, Moore B, Sheen J (2002) Sugar sensing and signaling in plants. Plant Cell **14**: 185-205

Ruperti B, Bonghi C, Tonutti P, Ramina A (1998) Ethylene biosynthesis in peach fruitlet abscission. Plant Cell Environ **21**: 731-737

Ruperti B, Whitelaw CA, Roberts JA (1999) Isolation and expression of an allergen-like mRNA from ethylene treated *Sambucus nigra* leaflet abscission zones. J Exp Bot **50**: 733-734

Russell JW, Golovoy D, Vincent AM, Mahendru P, Olzmann JA, Mentzer A, Feldmann EL (2002) High glucose-induced oxidative stress and mitochondrial dysfunction in neurons. FASEB Journal **16**: 1738-1748

Sadka A, DeWald DB, May GD, Park WD, Mullet JE (1994) Phosphate modulates transcription of soybean *VspS* and other sugar-inducible genes. Plant Cell **6**: 737-749

Sagee O, Erner Y (1991) Gibberellin and abscisic acid contents during flowering and fruit set of Shamouti orange. Scientia Hort **53**: 99-107

Sakamoto M, Munemura I, Tomita R, Kobayashi K (2008) Involvement of hydrogen peroxide in leaf abscission signaling, revealed by analysis with an in vitro abscission system in *Capsicum* plants. Plant J **56**: 13-27

Sander L, Child R, Ulvskov P, Albrechtsen M, Borkhardt B (2001) Analysis of a dehiscence zone endo-polygalacturonase in oilseed rape (*Brassica napus*) and *Arabidopsis thaliana*: evidence for roles in cell separation in dehiscence and abscission zones, and in stylar tissues during pollen tube growth. Plant Mol Biol **46**: 469-479

Schluepmann H, Pellny T, van Dijken A, Smeekens S, Paul M (2003) Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth in *Arabidopsis thaliana*. Proc Natl Acad Sci USA **100**: 6849-6854

Schou JB, Jeffers DL, Streeter JG (1978) Effects of reflectors, black boards or shades applied at different stages of plant development on yield of soybeans. Crop Sci **18**: 29-34

Schumacher K, Schmitt T, Rossberg M, Schmitz G, Theres K (1999) The *Lateral suppressor* (*Ls*) gene of tomato encodes a new member of the VHIID protein family. Proc Natl Acad Sci USA **96**: 290-95

Scott PC, Miller LW, Webster BD, Leopold AC (1967) Structural changes during leaf abscission. Am J Bot **54**: 730-743

Seth AK, Wareing PF (1967) Hormone directed transport of metabolites and its possible role in plant senescence. J Exp Bot **18**: 65-77

Sexton R, Durbin ML, Lewis LN, Thomson WW (1981) The immunocytochemical localization of 9.5 cellulase in abscission zones of bean (*Phaseolus vulgaris* cv. Red Kidney). Protoplasma **109**: 335-347

Sexton R, Lewis LN, Trewavas AJ, Kelly P (1985) Ethylene and abscission. In: Roberts JA, Tucker G, eds. Ethylene and plant development. Butterworths, London: 173-196

Sexton R, Roberts JA (1982) Cell biology of abscission. Annual Review of Plant Physiology **33**: 133-162

Soglio V, Costa F, Molthoff JW, Weemen-Hendriks WMJ, Schouten HJ, Gianfranceschi L (2009) Transcription analysis of apple fruit development using cDNA microarrays. Tree Genet and Gen **5**: 685-698

Solano R, Stepanova A, Chao Q, Ecker JR (1998) Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev **12**: 3703-3714

Solomakhin AA, Blanke MM (2009) Mechanical flower thinning improves the fruit quality of apples. J Sci Food Agric **90**: 735-741

Stenvik GE, Tandstad NM, Guo Y, Shi CL, Kristiansen W, Holmgren A, Clark SE, Aalen RB, Butenko MA (2008) The EPIP peptide of INFLORESCENCE DEFICIENT IN ABSCISSION is sufficient to induce abscission in *Arabidopsis* through the receptor-like kinases HAESA and HAESA-LIKE2. Plant Cell **20**: 1805-17

Stogios PJ, Downs GS, Jauhal JJ, Nandra SK, Privé GG (2005) Sequence and structural analysis of BTB domain proteins. Genome Biol **6**: 82

Stopar M (2000) Comparison of the most frequently used thinning compounds for the thinning of 'Jonagold', 'Elstar' and 'Golden Delicious' apples. Res Agriculture **75**: 89-94

Stopar M (2004) Thinning of flowers/fruitlets in organic apple production. Journal of Fruit and Ornamental Plant Research **12**

Stopar M, Lokar V (2003) The effect of ethephon, NAA, BA, and their combination on thinning intensity of ‘Summered’ apples. J Central European Agr **4**: 399-403

Stopar M, Resnik M, Pongrac VD (2001) Non-structural carbohydrate status and CO2

exchange rate of apple fruitlets at the time of abscission influenced by shade, NAA or BA. Sci Hort **87**: 65-76

Stover E, Fargione M, Risio R, Yang X, Robinson T (2001) Fruit weight, crop load, and return bloom of'Empire' apple following thinning with 6-benzyladenine and NAA at several physiological stages. HortScience **36**: 1077-1081

Sumimoto H (2008) Structure, regulation and evolution of Nox-family NADPH oxidases that produce reactive oxygen species. FEBS J **275**: 3249-3277

Suttle JC (1988) Effect of ethylene treatment on polar IAA transport, net IAA uptake and specific binding of N-l-naphtylphthalamic acid in tissues and microsomes isolated from etiolated pea epicotyls. Plant Physiol **88**: 795-799

Szymkowiak EJ, Irish EE (1999) Interactions between *jointless* and wild-type tomato tissues during development of the pedicel abscission zone and the inflorescence meristem. Plant Cell **11**: 159-175

Talòn M, Hedden P, Primo-Millo E (1990) Hormonal changes associated with fruit set and development in mandarins differing in their parthenocarpic ability. Physiol Plant **79**: 400-406

Talòn M, Tadeo FR, Ben-Cheikh W, Gomez-Cadenas A, Mehouachi J, Perez-Botella J, Primo-Millo E (1997) Hormonal regulation of fruit set and abscission in Citrus: Classical concepts and new evidence. Acta Hort **463**: 209–217

Tao R, Uratsu SL, Dandekar AM (1995) Sorbitol synthesis in transgenic tobacco with apple cDNA-encoding NADP-dependent sorbitol-6-phosphate dehydrogenase. Plant Cell Physiol **36**: 525-532

Tatsuki M, Endo A (2006) Analyses of expression patterns of ethylene receptor genes in apple (*Malus domestica* Borkh.) fruit treated with or without 1-methylcyclopropene (1-MCP). J Jpn Soc Hort Sci **75**: 481-487

Tatsuki M, Hayama H, Nakamura Y (2009) Apple ethylene receptor protein concentrations are affected by ethylene, and differ in cultivars that have different storage life. Planta **230**: 407-417

Taylor JE, Coupe SA, Picton SJ, Roberts JA (1994) Isolation and expression of a mRNA encoding an abscission-related β1,4 glucanase from *Sambucus nigra*. Plant Mol Biol **24**: 961-64

Taylor JE, Tucker GA, Lasslett Y, Smith C, Arnold C, Watson C, Schuch W, Grierson D, Roberts JA (1991) Polygalacturonase expression during leaf abscission of normal and transgenic tomato plants. Planta **183**: 133-138

Taylor JE, Webb TS, Coupe SA, Tucker GA, Roberts JA (1993) Changes in polygalacturonase activity and solubility of polyuronides during ethylene-stimulated leaf abscission in *Sambucus nigra*. J Exp Bot **44**: 93-98

Taylor JE, Whitelaw CA (2001) Signals in abscission. New Phytol **151**: 323-339

Testerink C, Dekker HL, Lim ZY, Johns MK, Holmes AB, de Koster CG, Ktistakis NT, Munnik T (2004) Isolation and identification of phosphatidic acid targets from plants. The Plant Journal **39**: 527- 536

Testerink C, Larsen PB, Van der Does D, van Himbergen JAJ, Munnik T (2007) Phosphatidic acid binds to and inhibits the activity of Aradidopsis CTR1. J Exp Bot **58**: 3905-3914

Thiéry D (1996) L'éclaircissage chimique du pommier. Arboric fruit **493**: 25-29

Thistlewood HMA, Elfving DC (1992) Laboratory and field effects of chemical fruit thinners on Tetranychid and predatory mites (Acari) of apple. J Econ Entomol **85**: 477-485

Thompson DS, Osborne DJ (1994) A role for the stele in intertissue signaling in the initiation of abscission in bean leaves (*Phaseolus vulgaris* L.). Plant Physiol **105:** 341-347

Tieman DM, Ciardi JA, Taylor MG, Klee HJ (2001) Members of the tomato *LeEIL* gene family are functionally redundant and regulate ethylene responses throughout plant development. The Plant Journal **26**: 47-58

Trainotti L, Spolaore S, Ferrarese L, Casadoro G (1997) Characterization of *ppEGl*, a member of a multi-gene family which encodes endo-P-l,4-glucanase in peach. Plant Mol Biol **34**: 791-802

Tripathi SK, Singh AP, Sane AP, Nath P (2009) Transcriptional activation of a 37 kDa ethylene responsive cysteine protease gene, *RbCP1*, is associated with protein degradation during petal abscission in rose. J Exp Bot **60**: 2035-2044

Tucker ML, Sexton R, del Campillo E, Lewis LN (1988) Bean abscission cellulase: characterization of a cDNA clone and regulation of gene expression by ethylene and

auxin. Plant Physiol **88**: 1257-1262

Tucker ML, Whitelaw CA, Lyssenko NN, Nath P (2002) Functional analysis of regulatory elements in the gene promoter for an abscission-specific cellulase from bean and isolation, expression, and binding affinity of three TGA-type basic leucine zipper transcription factors. Plant Physiol **130**: 1487-1496

Ueguchi-Tanaka M, Fujisawa Y, Kobayashi M, Ashikari M, Iwasaki Y, Kitano H, Matsuoka M (2000) Rice dwarf mutant *d1*, which is defective in the α subunit of the heterotrimeric G protein, affects gibberellin signal transduction. Proc Natl Acad Sci USA **97**: 11638-11643

Ulmasov T, Hagen G, Guilfoyle TJ (1997) ARF1, a transcription factor that binds auxin response elements. Science **276**: 1865-1868

Ulmasov T, Hagen G, Guilfoyle TJ (1999) Activation and repression of transcription by auxin-response factors. Proc Natl Acad Sci **96**: 5844-5849

Veinbrants N, Hutchinson JF (1976) Studies on the use of 2-chloroethylphosphonic acid (ethephon) as a thinning agent for Jonathan apples. Aust J Exp Agric Anim Husbandry **16**: 937-942

Vernieri P, Tagliasacchi AM, Forino L, Lanfranchi A, Lorenzi R, Avanzi S (1992) Abscisic acid levels and cell structure in single seed tissues of shedding affected fruits of *Malus domestica* Borkh. J Plant Physiol **140**: 699-706

Volz RK, Ferguson IB, Hewett EW, Woolley DJ (1994) Wood age and leaf area influence fruit size and mineral composition of apple fruit. J Hort Sci **69**: 385-395

Wang PC, Du YY, An GY, Zhou Y, Miao C, Song CP (2006) Analysis of global expression profiles of Arabidopsis genes under abscisic acid and H_2O_2 applications. J Integr Plant Biol **48**: 62-74

Wang X (2005) Regulatory functions of phospholipase D and phosphatidic acid in plant growth, development, and stress responses. Plant Physiol **139**: 566-573

Webster AD, Spencer JE (1999) New strategies for the chemical thinning of apple (*Malus domestica* Borkh.) cultivars Queen Cox and Royal Gala. J Hort Sci & Biotechnology **74**: 337-34

Webster T (2002) Current approved thinning strategies for apples and pears and recent thinning research trials in Europe. The compact fruit tree **35**

Welti R, Li WQ, Li MY, Sang YM, Biesiada H, Zhou HE, Rajashekar CB, Williams TD, Wang XM (2002) Profiling membrane lipids in plant stress responses: role of phospholipase Dα in freezing-induced lipid changes in *Arabidopsis*. J Biol Chem **277**: 31994-32002

Wertheim SJ (1973) Chemical control of flower and fruit abscission in apple and pear. Acta Hort **34**: 321-331

Wertheim SJ (1997) Chemical thinning of deciduous fruit trees. Acta Hort **463**: 445-462

Wertheim SJ (2000) Developments in the chemical thinning of apples and pears. Plant Growth Regul **31**: 85-100

Wiersma PA, Zhang H, Lu C, Quail A, Toivonen PMA (2007) Survey of the expression of genes for ethylene synthesis and perception during maturation and ripening of 'Sunrise' and 'Golden Delicious' apple fruit. Postharvest Biol Technol **44**: 204-211

Wilkinson JQ, Lanahan MB, Yen H-C, Giovannoni JJ, Klee HJ (1995) An ethylene-inducible component of signal transduction encoded by Never-ripe. Science **270**: 1807-1809

Williams MW (1979) Chemical thinning of apples. Hort Reviews **1**: 270-300

Williams MW (1994) Factors influencing chemical thinning and update on new chemical thinning agents. Comp Fruit Tree **27**: 115-122

Williams MW, Edgerton LJ (1981) Fruit thinning of apples and pears with chemicals. US Dept Agr Info Bul 289

Williams MW, Stahly EA (1960) Effect of cytokinins and gibberellins on shape of Delicious apple fruits. Proc Amer Soc Hort Sci **94**: 17-18

Wise JC, Gut LJ, Isaacs R, Schilder AMC, Sundin GW, Zandstra B, Beaudry R, Lang G (2007) Michigan fruit management guide. Mich State Univ Ext Bul E-154: 162-165

Wismer PT, Proctor JTA, Elfving DC (1995) Benzyladenine affects cell division and cell size during apple fruit thinning. J Am Soc Hort Sci **120**: 802-807

Wittenbach VA, Bukovac MJ (1973) Cherry fruit abscission: effect of growth substances, metabolic inhibitors and environmental factors. J Am Soc Hort Sci **98**: 348-351

Wong HL, Pinontoan R, Hayashi K, Tabata R, Yaeno T, Hasegawa K, Kojima C, Yoshioka H, Iba K, Kawasaki T, Shimamoto K (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell **19**: 4022-

4034

Yamada T, Ichimura K, van Doorn WG (2007) Relationship between petal abscission and programmed cell death in *Prunus yedoensis* and *Delphinium belladonna*. Planta **226**: 1195-1205

Yamagami T, Tsuchisaka A, Yamada K, Haddon WF, Harden LA, Theologis A (2003) Biochemical diversity among the 1-amino-cyclopropane-1-carboxylate synthase isozymes encoded by the Arabidopsis gene family. J Biol Chem **278**: 49102-49112

Yen H-C, Lee S, Tanksley SD, Lanahan MB, Klee HJ, Giovannoni JJ (1995) The tomato *never-ripe* locus regulates ethylene-inducible gene expression and is linked to a homolog of the *Arabidopsis ETR1* gene. Plant Physiol **107**: 1343-1353

Yuan R, Burns JK (2004) Temperature factors affecting the abscission response of mature fruit and leaves to CMN-pyrazole and ethephon in 'Hamlin' oranges. J Am Soc Hort Sci **129**: 287-293

Yuan R, Greene DW (2000) `McIntosh' apple fruit thinning by benzyladenine in relation to seed number and endogenous cytokinin levels in fruit and leaves. Sci Hort **86**: 127-134

Yuan R, Huang H (1988) Litchi fruit abscission: its patterns, effect of shading and relation to endogenous abscisic acid. Sci Hort **36**: 281-292

Zhao J, Wang XM (2004) Arabidopsis phospholipase Dα1 interacts with the heterotrimeric G-protein α-subunit through a motif analogous to the DRY motif in G-protein-coupled receptors. J Biol Chem **279**: 1794-1800

Zhou C, Lakso AN, Robinson TL, Gan S (2008) Isolation and characterization of genes associated with shade-induced apple abscission. Mol Gen Genomics **280**: 83-92

Zhou R, Cheng LL, Dandekar AM (2006) Down-regulation of sorbitol dehydrogenase and up-regulation of sucrose synthase in shoot tips of the transgenic apple trees with decreased sorbitol synthesis. J Exp Bot **57**: 3647-3657

Chapter 2 – A transcriptomic approach to apple fruitlet abscission

Signalling pathways mediating the induction of apple fruitlet abscission

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Abstract

Apple (*Malus* x *domestica* L. Borkh) represents an interesting model tree crop for studying fruit abscission. The physiological fruitlet drop occurring in this species can be easily magnified by using thinning chemicals, such as benzyladenine (BA), to obtain fruits with improved quality and marketability. Despite the economical importance of this process, the molecular determinants of apple fruitlet abscission are still unknown. In the present research, BA was used to obtain fruitlet populations with different abscission potentials, to be analysed by means of a newly released 30K oligonucleotide microarray. RNAs were extracted from cortex and seed of apple fruitlets sampled over a 4-day timecourse, during which BA triggers fruit drop, and used for microarray hybridisation. Transcriptomic profiles of persisting and abscising fruitlets were tested for statistical association with abscission potential, allowing to identify molecular signatures strictly related to fruit destiny. A hypothetical model for apple fruitlet abscission was obtained by putting together available transcriptomic and metabolomic data. According to this model, BA treatment would establish a nutritional stress within the tree that is primarily perceived by the fruitlet cortex whose growth is blocked by resembling the ovary growth inhibition found in other species. In weaker fruits, this stress is soon visible also at the seed level, likely transduced via ROS/sugar and hormones signalling crosstalks, and followed by a block of embryogenesis and the consequent activation of the abscission zone.

Introduction

Fruit development is an exquisitely plant specific process under the control of a complex interplay of endogenous and environmental factors. Many molecular studies have focused on aspects of the last phases of fruit development and, mostly, for its important economical impacts, on the ripening process (Giovannoni, 2004). A large body of experimental data, obtained in tomato as a model system, supports the master role played by the hormone ethylene for the control of ripening in climacteric fruits (reviewed by Giovannoni 2004). A significantly minor body of research has been so far devoted to the molecular factors involved in fruit set and early fruit development. Despite of the detailed characterization of growth dynamics and hormonal balance during the early steps of fruit development (Ozga and Reinecke, 2003; Nitsch, 2009), the molecular aspects underpinning these events have only recently been started to be unravelled.

The process of fruit set, defined as the commitment of the ovary tissues to undergo transformation into a fruit (Gillaspy, 1993), is gaining increasing interest also for its potential exploitation to control parthenocarpic fruit development, in the absence of pollination/fertilization. Auxins and gibberellins play a pivotal role in the inductive phase of fruit set and parthenocarpic development of fruits (De Jong et al, 2009; Pandolfini et al., 2007; Gillaspy et al, 1993). Several evidences support the view that auxins may represent the master signal triggering cell divisions, and their interplay with gibberellins may be required for sustaining cell expansion (reviewed by de Jong et al., 2009). In detail, data obtained from both tomato and Arabidopsis have suggested that the transformation of the ovary into fruit is prevented by a negative control exerted by AUX/IAA and ARF proteins. The removal of this negative regulation, following pollination/fertilization or treatment with auxins, leads to cell proliferation and to fruit set. Consistently, the de-repression of auxin responses through antisense inhibition of AUX/IAA9 (Wang et al., 2005) and ARF7 (de Jong et al., 2009) in tomato, and loss of function of ARF8 in Arabidopsis (Goetz et al., 2006) leads to parthenocarpy.

Transcriptomic profiling studies carried out in tomato (Vriezen et al., 2008) have reinforced the view that fruit development appears to rely on the removal of a negative feedback regulation of ovary growth. This inhibition is established by a negative control exerted mainly by ABA- and ethylene-dependent pathways. In fact, as soon as fruit set is triggered,

the molecular machineries of both ethylene and ABA biosynthesis and action appear to be significantly and promptly down regulated and, concomitantly, those of auxin and gibberellins biosynthesis and action are activated (Vriezen et al., 2008; Nitsch et al., 2009). These data are progressively giving a hint on the spatio-temporal regulation of the molecular factors involved in early steps of fruit set and development. However, very little or no information is available on how these factors could be modulated by the plant to restrain the development of a fraction of fruits in response to endogenous/environmental perturbations.

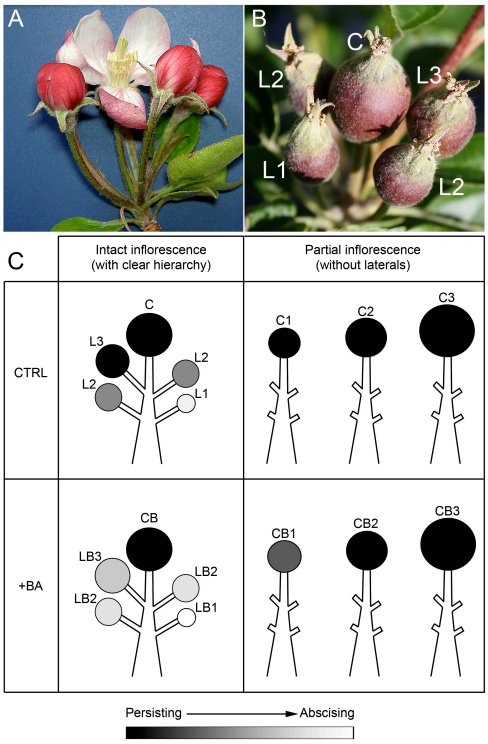

Figure 1. A. The apple corymb with the central flower at bloom, whose anticipation with respect to the lateral flowers is clearly visible. **B.** Apple cluster with a clear hierarchy, as indicated by progressive numbers (C = central fruitlet; L1 = small lateral fruitlets; L2 = medium lateral fruitlets; L3 = big lateral fruitlets). **C.** Schematic representation of the different abscission potentials ascribed to fruitlets within either intact or partial inflorescence (black: strongly persisting; white: strongly abscising) of control (CTRL, top panels) or BA-treated (+BA, bottom panels) trees. Sample fruitlets were labelled according to their size, position within the cluster, and eventual treatment (L: lateral; C: central; B: treated with BA; 1:small size; 2: medium size; 3:big size).

Fruit trees have evolved a system to control and adapt the size of the fruit population they bring to final maturity in relation to their nutritional status, thus allowing the plant to make an efficient use of resources. This is accomplished by a process called ‘physiological drop’, consisting on the abscission of young fruitlets during the early phases of development. In

apple, the physiological drop is eminently a correlative phenomenon and has to be distinguished from the senescence driven abscission of ripe fruits (Bangerth, 2000). Therefore, drop of young fruits can be interpreted as a developmental arrest that the plant exerts selectively on fruitlets representing weaker sinks, during early phases of development, in response to nutritional shortage. In this scenario, studying the fruit physiological drop can provide important additional insights into the molecular mechanisms regulating early fruit development and the fruit developmental plasticity in response to endogenous and environmental changes. Apple trees are an interesting model system for such a study since they develop flower/fruit clusters in which a clear gradient of correlative dominance exists in relation to the position of the fruit within the cluster (Figure 1). This dominance can be further exacerbated by means of shading or treatments with chemicals that can induce fruit drop (Bangerth, 2000; Greene, 1992), a practice called 'fruit thinning'. Fruit thinning is adopted by horticulturists to reduce the number of fruits on the tree therefore improving their final size and quality. Benzyladenine (BA) is a widely known chemical thinner exerting its action by stimulating shoot growth and, as a consequence, effecting fruit drop by exacerbating competitions between shoots and fruit clusters, between the different clusters (inter-cluster competition) and, prominently, between fruits of the same cluster (intra-cluster competition)(Bangerth, 2000; Buban, 2000). A number of studies have elucidated this mechanism of action showing that BA treatment has in fact no effect on fruit drop when applied directly only to fruits (Greene et al., 1992). Therefore, BA represents an interesting tool to evoke a fruit developmental arrest and abscission in a controlled, inducible and selective way through the enhancement of correlative inhibitions and for the identification of the molecular factors underpinning this developmental arrest.

In the present study, BA treatments on apple trees were exploited to selectively induce drop of lateral fruits that usually persist and develop. Their global transcriptional profiles were assessed by means of a new 30K microarray in both cortex and seeds tissues and compared with those of persisting fruits. Candidate genes with a potential involvement in driving fruit developmental arrest finally leading to fruit abscission were identified. Although apple fruit is a pseudocarpic fruit, in which cells of the cortex arise mainly from the receptacle, our data suggest that the developmental arrest leading to fruit drop relies on the re-establishment of negative constraints based on activation of ABA and ethylene signalling and inhibition of

gibberellins biosynthesis, similarly to those that are removed from the ovary to initiate fruit development. The involvement of sugars and ROS signalling may also be hypothesized.

Results

Establishing classes of fruitlets with different abscission potentials

In order to identify the molecular events responsible for apple fruitlet abscission, fruitlet sub-populations characterised by different abscission potentials and fruit drop dynamics were first obtained and sampled. Apple fruitlets develop in clusters, each including a central fruit (also called the 'king fruit') and four lateral fruits (Figure 1). The position within the cluster is an important determinant of the hierarchy between competing fruits and, consequently, of their tendency to abscise, defined as abscission potential: the central fruit develops earlier, since it originates from an earlier flowering event (Figure 1A), and exerts a correlative dominance over the lateral fruits making the latter ones weaker sinks and significantly more prone to abscise (Bangerth, 2000) (Figure 1B). A hierarchy also exists between lateral fruitlets. In fact, those deriving from earlier blooming flowers (L3 fruitlets in Figure 1) reach a bigger size, a stronger sink activity and a lower abscission potential, and exert a correlative dominance over the smaller ones, inserted below, that finally display the highest abscission potential (Figure 1B-C, L2 and L1). In the absence of external perturbations, the central (C) and biggest lateral fruits (L3) are less prone to abscise and virtually bound to stay on the plant, while L2 and L1 undergo shedding. Therefore, in apple clusters a correlative reproductive dominance exists, starting from the central fruit towards the basal lateral ones, that, in turn, is reflected by an opposed increasing gradient of abscission potentials as represented by grey to black scales in Figure 1C. As a consequence, fruit size and position within the cluster, being strongly correlated with the capacity of attracting assimilates (Bangerth, 2000), may be considered reliable parameters for predicting the fruitlet abscission potential. Taking these aspects into account, the experimental plan was aimed at triggering the induction of abscission mainly on L3 fruits by treating trees with BA (Figure 1C, LB3), a well known chemical thinner. BA exerts its action mainly by enhancing shoot growth and branching (Dal Cin et al., 2007) and therefore exacerbating the correlative competition between fruits, resulting on the abscission of an increased number of lateral fruits of the L3 class, while leaving unaffected the L2 and L1

fruit abscission potential (Angeli et al., 2002). In parallel, a subpopulation of non-abscising persisting fruitlets (NAF) 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 (Figure 1C, right top panel). However, since the smallest sized central fruits did show a minimal tendency to drop after BA treatment (less than 10%), these were considered 'borderline' samples (Figure 1C, right bottom panel). By means of this approach, eight populations were sampled and classified for transcript profiling, and assigned to the following four predicted different abscission potentials (APs) based upon previous experiments (unpublished data): naturally abscising fruitlets (NA: L1 fruitlets; AP ≈ 90% < AP < 100%), strongly abscising fruitlets (SA: LB1 and LB3 fruitlets; 90% < AP < 100%), probably persisting fruitlets (PP: CB1 fruitlets; AP < 10%), strongly persisting fruitlets (SP: L3, C1, C3 and CB3; AP ≈ 0%).

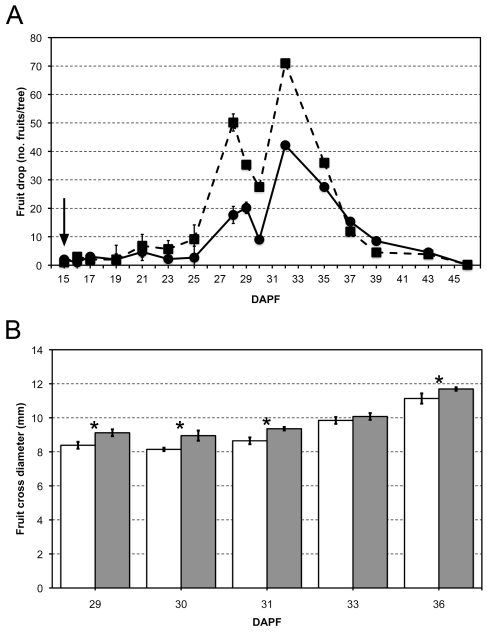

Figure 2. A. Fruit drop dynamics in control (circles and continuous line) and BA-treated (squares and dot-line) trees, expressed as number of drop fruitlets per tree. The arrow represent the time of BA treatment. **B.** Mean cross diameter of dropped fruits in control (white) and BA-treated (dark grey) trees. Only the five most divergent dates are reported. Bars represent standard deviation, whereas asterisks show the statistically significant differences ($P \leq 0.05$).

In order to test the reliability of the predicted abscission potentials of fruitlets and their actual representativeness (i.e. sample fruits with different destinies) for global transcriptomic analyses, the fruit drop dynamics was followed, in relation to the position and size of dropped fruits, and their ethylene biosynthesis and expression of ethylene

biosynthetic genes previously proved to be reliable diagnostic markers of the apple fruitlets destiny (Dal Cin et al., 2005, 2009a, 2009b) measured.

The fruit drop dynamics appeared biphasic, with a first peak occurring at 29 and 28 DAPF for control and BA treated trees, respectively, and a second one at 32 DAPF in both theses (Figure 2A). BA treatment did not result in changes of fruit shedding dynamics, yet on a magnification of the process. In fact, at the end of the fruit shedding period (around 46 DAPF), BA treated trees showed overall about a two-fold increase of fruit drop, measured throughout the entire experimental period, in comparison to that observed in the control ones (Figure 2A). The average cross diameter of abscised fruitlets resulted to be significantly higher ($P < 0.01$) in treated trees than in the untreated ones. In particular, the most significant differences were assessed at 29, 30, 31, and 36 DAPF, as shown in Figure 2B. This was also confirmed by the number of clusters with only one fruit left on the tree after BA treatment (data not shown). These data provide further evidence that BA dependent magnification of abscission affected mainly the bigger lateral fruitlets (L3) that would normally persist on the tree. A sporadic non-significant fruit drop was also observed for the smallest central fruitlets of the BA treated trees (CB1)(data not shown).

The drop potential of the fruit categories was verified by evaluating their different behaviour in terms of ethylene biosynthesis and *MdACO1* gene expression, widely accepted indicators of an actual abscission induction and the earliest markers of fruitlet abscission in apple (Dal Cin et al., 2005, 2009a, 2009b). In previous experiments, ethylene biosynthesis was shown to peak in abscising fruits, regardless of their size, around three days after BA treatment in AFs and correlated well with the increase of *MdACO1* transcripts in the fruit cortex (Dal Cin et al., 2005).

Concerning ethylene biosynthesis, the small lateral fruitlets of control trees (L1) showed a minor peak of ethylene production at 21 DAPF that remained at basal levels thereafter. BA treated fruits of the same size class (LB1) showed an increased ethylene biosynthesis already two days after the treatment peaking at 23 DAPF, eight days from the beginning of the experiments (Figure 3A). Concerning the bigger laterals (L3), ethylene peaked at 19 DAPF in control samples (L3) and remained at basal levels thereafter, whereas the treated fruitlets (LB3) showed the highest ethylene biosynthesis at 21 DAPF with a fast decreasing trend thereafter (Figure 3B). In central fruits, a different situation was pointed out in terms

of ethylene production, which was lower than that found in the laterals, both in control and treated samples.

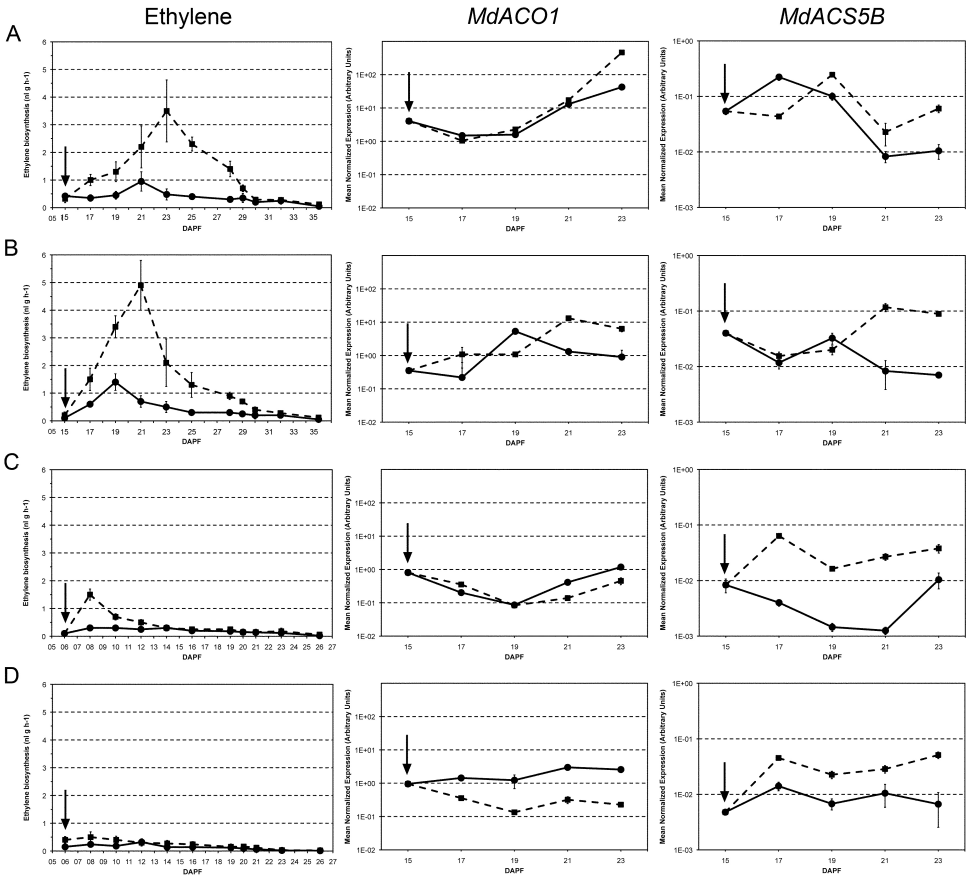

Figure 3. Ethylene biosynthesis (left), expression of *MdACO1* (centre) and *MdACS5B* (right) in small (**A**) and big (**B**) lateral fruitlets, and in small (**C**) and big (**D**) central fruitlets, either untreated (circles and continuous line) or treated with BA (squares and dot-line). Bars represent standard deviation.

As far as *MdACO1* expression in LB1 fruitlets is concerned, a significant divergence from the control was observed at 23 DAPF (Figure 3A), correlated with ethylene levels. *MdACO1* transcripts peaked earlier at 21 DAPF in LB3, paralleling ethylene production (Figure 3B). In small central fruitlets no significant difference was observed (Figures 3C), whereas, concerning the big central ones, lower *MdACO1* levels were measured in the treated samples (CB3) starting from 17 DAPF, throughout the experiment (Figure 3D). *MdACS5B* gene expression levels were also assessed and shown to correlate with those of *MdACO1,* although only in lateral fruitlets. In both C1 and C3, divergent expression trends were observed, most likely responsible for the slightly enhanced ethylene biosynthesis found upon treatments with BA at least in C1 (Figure 3).

The 30K apple microarray

The apple oligonucleotide microarray herein set up by means of the CombiMatrix platform represents one of the most complete transcriptomic tools available for this species, allowing to analyze more than 30,000 transcripts, with three technical replicates in a single experiment. Similar molecular tools were previously set up based upon apple sequence sets obtained almost exclusively by means of the publicly available TGICL tool (Pertea et al., 2003), and further empirical fine-tuning procedures such as elimination of short sequences and duplicates. Schaffer et al. (2007) set up a microarray with 15,720 sequences chosen among a total number of 42,938 nonredundant records, comprising 17,460 temptative contigs and 25,478 singletons, obtained from 151,687 expressed sequence tags (ESTs) from different tissues and cultivars (Newcomb et al., 2006). More recently, an apple oligonucleotide microarray with 55,230 sequences was built starting from 184,132 publicly available records (Jensen et al., 2009). In the present research, a total number of 255,950 ESTs and mRNAs were retrieved from public databases, clustered and assembled by means of a dedicated pipeline (Figure S1), allowing to obtain 41,927 final non-redundant sequences, including tentative contigs and singletons, among which 30,419 with transcription orientation were chosen to be spotted on the slide. Differently from previous researches, additional steps were introduced both before and after the TGICL elaboration phase, in order to improve contig reliability and to further decrease redundancy (see material and methods section). In particular, before carrying out the additional steps introduced at the end of the clustering/assembly pipeline, 26,658 tentative contigs and 50,382 singletons were obtained, for a total of 77,040 nonredundant sequences. At this stage, the proportion between the starting number of ESTs/mRNAs and final non-redundant sequences (3.2 : 1) was closer to those previously achieved by Newcomb et al. (3.6 : 1) and Jensen et al. (3.3 : 1), than that (4.5 : 1) by Park et al. (2006). After the additional processing, the proportion was 6.2 : 1, indicating that a strong reduction occurred most likely due to redundancy elimination. On one hand, this approach was effective in decreasing redundancy based upon a 'functional model', since sequences contained in the same Unigene (*i.e.* with putative identical functions) were considered once by retaining just the longest record. By this way, different alleles and eventual duplicated genes were most

likely clustered together. On the other hand, paralogs were not clustered together because of the high stringency parameters adopted.

Annotation of apple sequences spotted on the microarray was based on similarity to Uniprot hits and transfer of their Gene Ontology (GO) annotation terms and descriptions to apple sequences. Among all spotted sequences, 39.1% were not annotated for the molecular function (MF) category, 45.4% for the biological process (BP), and 54% for the cellular compartment (CC) subvocabulary. The relative proportions of each GO category on the total within each subvocabulary (i.e. MF, BP, and CC) were well correlated with the annotation distribution found for other species, such as *Arabidopsis thaliana*, *Vitis vinifera*, *Prunus persica*, and *Populus* (Figure S3). The worst correlation was found for the BP subvocabulary, probably due to the known higher fragmentation existing in this GO section (Figure S3) generated by the higher total number of terms (18,189) than in the other two subvocabularies (8,671 in MF and 2,672 in CC; see www.geneontology.org for more information).

The apple sequence set used by Jensen et al. (2010) had a relatively low coverage with respect to *Arabidopsis thaliana* proteome, assessed as equal to 52.1% with homology to 14,266 unique proteins on a total of 27,379 records (TAIR9 database), as well as a high redundancy level. Twenty five thousand five hundred eighty (84.1%) apple contigs and singletons herein used for the microarray construction were shown to match with 13,706 unique Arabidopsis proteins (blastx algorithm with 1E-3 cutoff), giving a 50.1% coverage. Considering the total number of genes (57,386) recently predicted on the genome of domesticated apple (Velasco et al., 2010), a 53% coverage is achieved. Based upon these data and taking into account that the majority of ESTs used to set up the microarray derives from fruit tissues (data not shown), it is likely that almost the whole fruit transcriptome is represented along with a relevant part of genes expressed in the seed.

Global test analysis of apple fruitlet transcriptomes

The Globaltest package (Goeman et al., 2004) of Bioconductor was used to assess whether significant associations exist between global gene expression profiles, in cortex and seed, and 'phenotypes' or physiological responses in terms of abscission potential (fruitlet destiny), fruit weight and fruit position within the cluster (herein called the 'response variables'). Globaltest analysis was performed either on the whole gene set (30,419 genes)

or on the subset of significantly variable genes identified, separately in cortex and seed, as described in the materials and methods section. It has to be highlighted that this approach allows to identify static associations between the transcriptome and a given variable/phenotype, regardless of the time course of expression profiles of the genes considered in the analysis. Therefore, a second complementary and confirmatory approach was also performed to identify genes displaying divergent kinetics (see following paragraph), related to different abscission potentials.

As far as the overall gene expression data in the cortex are concerned, no significant association with fruitlet weight was identified. Significant associations were found with the position within the cluster ($P < 0.05$) and the treatment ($P < 0.07$), whereas a highly significant association was pointed out with fruitlet destiny ($P < 0.004$). When only the significantly differentially expressed (DE) genes were considered, the significance level increased for all the considered response variables, except for 'treatment', reaching a P value equal to 0.001 for the association with abscission potential (see Table S1 for the overall statistics for the cortex). Considering the seed transcriptome, highly significant and significant associations were detected only with fruitlet weight ($P < 0.008$) and abscission probability ($P < 0.01$). The significance level improved in all cases when the subset of DE genes was considered, indicating the reliability of the statistical analysis. In particular it has to be pointed out that the association between gene expression data and weight reached a P value equal to 0.0008 (extremely significant), whereas the statistical test on fruitlet destiny was highly significant ($P < 0.004$). In order to test the time course association between transcriptomic data and response variables, gene expression data at each sampling date (beginning of the experiment, T0; after 2 days, T2; after 4 days, T3) were processed separately, taking into account that the lower number of samples analysed in each test may have partially biased the statistical calculation resulting in lower levels of significance. As far as the cortex is concerned, no significant association was reported at T0, whereas significant levels at T2 ($P < 0.03$) and T3 ($P < 0.01$) were pointed out for fruit destiny. At T3, also the response variable 'treatment' showed a significant level ($P < 0.05$). A statistically relevant association was reported between the seed transcriptome and fruitlet weight ($P < 0.08$) already at T0 that became non-significant at T2, and again significant at T3 ($P < 0.06$). Also the abscission potential was significantly associated with the seed

transcriptome, but only later at T3 ($P < 0.02$). All the statistics for the seed are reported in Table S2.

The association of the response variables with expression data of gene subsets encoding elements involved in hormone biosynthesis, metabolism, perception, signal transduction, and cross-talk was also investigated. The statistical analyses were performed separately for the five major plant hormones (abscisic acid, auxin, cytokinin, ethylene, and gibberellin), and as a whole for minor plant growth regulators (jasmonates, salicylic acid, polyamines, brassinosteroids).

As far as the overall gene expression data are concerned, the highest levels of significance were found again in the cortex. In detail, extremely significant associations with fruit destiny were found for genes related to abscisic acid, cytokinin, and gibberellins (GAs), with $P < 0.0005$ in all cases, whereas for auxin and ethylene the statistics were highly significant in both cases ($P < 0.002$ and $P < 0.005$, respectively). In the same tissue, GA-related genes were highly associated also with the position of the fruit within the cluster, with $P < 0.005$. Concerning the time course, the ABA-related genes were significantly correlated ($P < 0.01$) with the abscission potential already at T2 (2 days after the BA treatment). All the other major plant hormones-related genes showed a significant P level of association with the same response variable. Concerning the position within the cluster, GA-related genes were already correlated at T2, with $P < 0.03$. The group of genes related to the minor hormones showed highly significant and significant statistics at T2, concerning the association with the abscission potential ($P < 0.007$) and BA treatment ($P < 0.09$), respectively. At T0, only non-significant P levels were found. Statistics for all samples and gene subsets are shown in Table S1.

Concerning the seed, a slightly different situation with respect to the cortex was pointed out, both in terms of significance levels in overall samples and the time course of the associations. The global test evidenced a highly significant association with fruit weight and destiny for the gene subsets related to ABA, auxin, and GAs. For ethylene and minor hormones the statistic was significant only with respect to the former response variable, whereas for cytokinin lower levels of significance were assessed (see Table S2 for P values). Concerning the time course statistics, a significant P level was calculated already at T0, with the exception of for genes related to GAs. For the major hormones, this level of

significance was kept up to T3, when a highly significant test was reported for the association between auxin-related genes and abscission potential ($P < 0.01$), being the earliest in the seed considering all the plant hormones with respect to fruit destiny. In Table S2, all the statistics are reported for the seed.

Hierarchical clustering of abscission-related genes

According to the Globaltests, a highly significant association exists between the expression of DE gene subsets in cortex and seed, and the fruits' probability of abscising. When the same genes were clustered according to their expression levels in all samples, these genes were not able to finely discriminate samples according to the corresponding abscission potential. This analysis instead pointed out that the BA treatment had a relevant weight on the overall gene expression profiles and, in turn, on the hierarchical clustering process, as evidenced in Figure S6, thus masking the clustering of genes associated with fruitlet destiny. Therefore, further clustering analyses were carried out only with genes highly correlated with the fruit destiny, choosing a highly stringent cut-off Z score (> 7) and selecting among the whole set regardless of significant differential expression. In detail, samples with a high probability of persisting clustered together, whereas those with higher abscission potentials grouped in a distinct cluster, confirming Globaltest analyses (Figures 4 and 5). Moreover, it is worth noting that BA treated central fruitlets of small size (CB1) were split into two different clusters, at T2 (CB12) in the 'persisting' group, and at T3 (CB13) in the 'abscising' one. This is consistent with the 'borderline' condition of 'probably persisting' fruitlets ascribed to this sample class. A temporal shift was shown for LB3 samples, in that LB32 clustered closer to naturally abscising fruits whereas LB33 grouped together with treated samples with equally high abscission potential.

According to the Globaltest analyses, the seed transcriptomes appeared to be associated to fruitlet destiny to a lesser extent than those of the cortex. Consistently, only 24 genes in the seed compared to 83 in the cortex had a Z score higher than 7. Also in this case, a clear distinction was reported, when the genes were hierarchically clustered, in terms of expression levels in association with the abscission potential (Figure 5). Indeed, the same 'borderline' samples that were split into two distinct clusters in the cortex were separated also in the seed, although inversely (*i.e.* CB12 with abscising samples and CB13 with the persisting ones), strengthening the hypothesis that the small central fruitlets may have a

higher probability to abscise than the bigger ones. Remarkably, transcriptomes of L3 lateral fruitlets (untreated) clearly clustered together within the persisting ones, while BA treatment reverted this and forced their clustering together with abscising samples at all time points in both seed and cortex. This finding confirmed that BA treatment had a significant effect in inducing abscission of L3 fruits thus changing their developmental destiny, and this effect could be linked to transcriptional signatures in cortex and seed that are specifically associated with the induction of the abscission response. For microarray data validation, quantitative (q)PCR experiments were performed on a subset of selected genes and revealed similar expression patterns and strong correlations (Figure S7).

Signatures of fruitlet abscission in cortex

Genes representing the abscission-specific transcriptional signatures in cortex are clustered in Figure 4 and listed in Table 1. Concerning those involved in metabolism, a marker of high abscission potential encodes a trehalose-6-phosphate synthase (Md_15339:1:a; EC 2.4.1.15), discriminating also the 'borderline' CB13 sample. Blast analysis allowed to point out a 62% identity with Arabidopsis *AtTPS10* (At1g60140), a class II TPS gene induced by sugar starvation (Osuna et al., 2007), cytokinins (Brenner et al., 2005), and ABA (Paul, 2007). A transcript for a sorbitol transporter (Md_97721:s) co-regulated with the previous one showed a high level of identity (77%) with *MdSOT5* (Accession no. BAD42345), functioning either in import or export of sorbitol in/from leaves (Watari et al., 2004). Five genes (Md_ 6319:1:a, Md_373:3:a, Md_6808:1:a, Md_8451:1:a, and Md_6021:1:a) encoding UDP-glucosyltransferases (EC 2.4.1.115) were highly expressed in abscising samples. In Arabidopsis and in *Beta vulgaris* the transcription of genes belonging to this family was induced during superoxide-dependent cell death (Mazel and Levine, 2002) and oxidative stress (Sepulveda-Jimenez et al., 2005), respectively. A UDP-glucose-4-epimerase (EC 5.1.3.2) gene (Md_1619:3:a), coregulated with the previous ones, displayed a high degree of similarity (79% identity) with *UGE5* of Arabidopsis (At4g10960), induced by abscisic acid and coregulated with carbohydrate biosynthetic enzymes (Rösti et al., 2007). Md_400:5:a, encoding an asparagine synthetase (EC 6.3.5.4) similar to *AtASN1* of Arabidopsis (At3g47340), was up-regulated in abscising samples, although at low levels in LB32. In other species, genes encoding this class of enzymes are controlled by sugar

starvation and involved in resource mobilization (Herrera-Rodríguez et al., 2004; Rose et al., 2006; Rook et al., 2006).

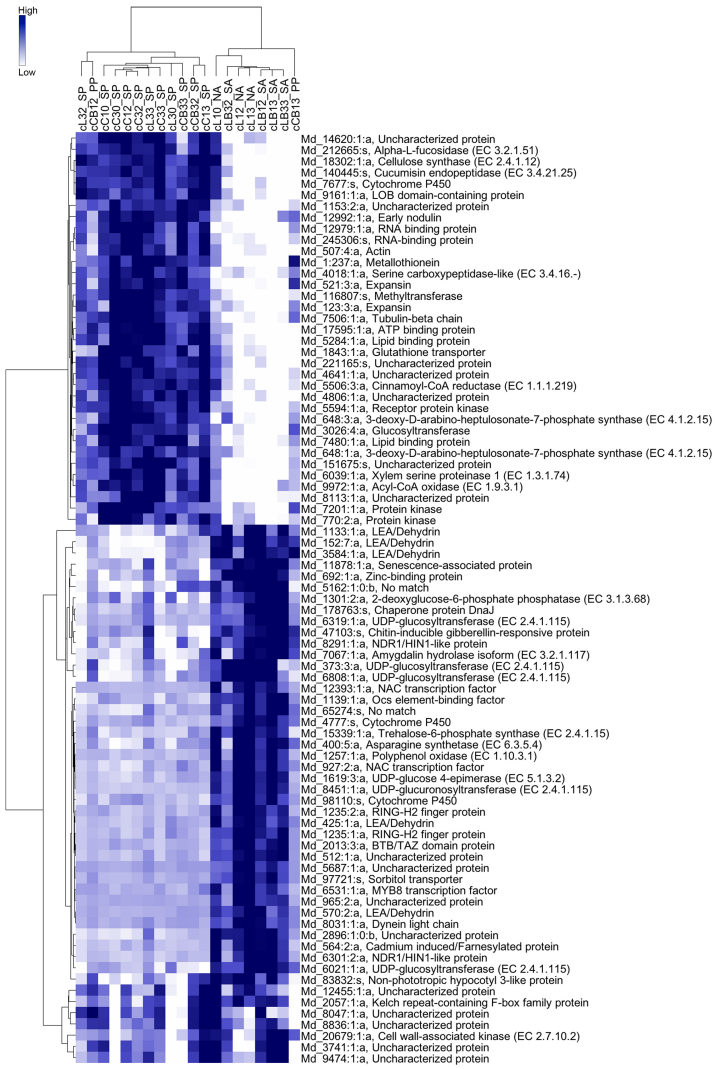

Figure 4. Hierarchical clustering of genes with expression levels in the cortex that are highly associated with the fruitlet abscission potentials. Only genes with a Z score ≥ 7 are reported, according to the Globaltest analysis. Samples are reported on the top side of the heatmap with the following codes: c: cortex; L: lateral, C: central, B: treated with BA; first number indicates fruit size category: 1: small fruitlets, 3: big fruitlets; second number indicates sampling time: 0: T0, 2:T2. 3:T3. SP: strongly persisting, PP: probably persisting, NA: naturally abscising, SA: strongly abscising.

A different gene set showed a high discriminating power in terms of higher expression in persisting fruitlets (Figure 4). This set comprised genes coding for cellulose synthase (Md_18302:1:a; EC 2.4.1.12), cinnamoyl-CoA reductase (Md_5506:3:a; EC 1.1.1.219), acyl-CoA oxidase (Md_9972:1:a; EC 1.9.3.1), and 3-deoxy-d-arabino-heptulosonate-7-phosphate synthase (two genes: Md_648:3:a and Md_648:1:a; EC 4.1.2.15), that are typically expressed in developing organs (Lauvergeat et al., 2001; Pedersen and Henriksen,

2005; Sato et al., 2006) such as the persisting apple fruitlets. High transcription rates were reported in the persisting fruitlets also for a gene encoding a cucumisin endopeptidase (Md_140445:s; EC 3.4.21.25), which is expressed at high levels also during the early developmental stages in melon (Choi et al., 2004), and an α–L-fucosidase (Md_212665:s; EC 3.2.1.51), probably involved in fruit growth (Desveaux et al., 1998).

Table I Genes with expression data in the cortex associated with abscission potential with a Z score ≥ 7. A tentative annotation, the influence on the whole association, the statistical score (Z), and the expression pattern (NA=high expression in naturally abscising fruitlets; SA=high expression in strongly abscising fruitlets; SP=high expression in strongly persisting fruitlets) are reported for each gene.

Gene ID	Tentative annotation	Influence	Z	Exp.
Md_8451:1:a	UDP-glucosyltransferase (EC=2.4.1.115)	1987.67	8.80	NA
Md_521:3:a	Expansin	209.73	8.74	SP
Md_245306:s	RNA-binding region-containing protein	569.08	8.67	SP
Md_9972:1:a	Acyl-CoA oxidase (EC=1.9.3.1)	123.73	8.40	SP
Md_4641:1:a	Uncharacterized protein	167.99	8.35	SP
Md_12979:1:a	RNA binding protein	323.19	8.34	SP
Md_7480:1:a	Lipid binding protein	210.18	8.29	SP
Md_570:2:a	LEA/Dehydrin	2686.19	8.22	NA
Md_8836:1:a	Uncharacterized protein	26.56	8.08	SA
Md_151675:s	Uncharacterized protein	121.54	8.06	SP
Md_4018:1:a	Serine carboxypeptidase-like (EC=3.4.16.-)	112.73	8.05	SP
Md_2896:1:0:b	Uncharacterized protein	562.33	7.94	NA
Md_152:7:a	LEA/Dehydrin	964.27	7.88	SA
Md_15339:1:a	Trehalose-6-phosphate synthase (EC=2.4.1.15)	375.72	7.87	NA
Md_97721:s	Sorbitol transporter	1185.32	7.87	NA
Md_1257:1:a	Polyphenol oxidase (EC=1.10.3.1)	1532.64	7.85	NA
Md_116807:s	Generic methyltransferase	164.21	7.79	SP
Md_507:4:a	Actin	231.25	7.78	SP
Md_2057:1:a	Kelch repeat-containing F-box family protein	55.08	7.73	NA
Md_7506:1:a	Tubulin-beta chain	122.74	7.73	SP
Md_1619:3:a	UDP-glucose 4-epimerase (EC=5.1.3.2)	680.49	7.67	NA
Md_12393:1:a	NAC transcription factor	2144.47	7.64	NA
Md_8047:1:a	Uncharacterized protein	23.37	7.64	SA
Md_1133:1:a	LEA/Dehydrin	546.19	7.59	SA
Md_648:3:a	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 4.1.2.15)	294.22	7.57	SP
Md_6039:1:a	Xylem serine proteinase 1 (EC=1.3.1.74)	111.24	7.56	SP
Md_3741:1:a	Uncharacterized protein	35.15	7.55	SA
Md_3026:4:a	Glucosyltransferase	160.01	7.55	SP
Md_4806:1:a	Uncharacterized protein	242.23	7.54	SP
Md_1235:2:a	RING-H2 finger protein	806.57	7.52	NA
Md_8291:1:a	NDR1/HIN1-like protein	322.13	7.52	SA
Md_12992:1:a	Early nodulin	172.86	7.50	SP
Md_7677:s	Cytochrome P450	86.16	7.50	SP
Md_512:1:a	Uncharacterized protein	962.45	7.49	NA
Md_8113:1:a	Uncharacterized protein	161.13	7.46	SP
Md_47103:s	Chitin-inducible gibberellin-responsive protein	134.13	7.44	SA
Md_3584:1:a	LEA/Dehydrin	1491.95	7.43	SA
Md_1153:2:a	Uncharacterized protein	280.40	7.38	SP
Md_770:2:a	Protein kinase family protein	139.58	7.38	SP
Md_20679:1:a	Cell wall-associated kinase (EC=2.7.10.2)	101.45	7.35	SA
Md_18302:1:a	Cellulose synthase (EC=2.4.1.12)	104.04	7.34	SP
Md_221165:s	Uncharacterized protein	91.34	7.34	SP
Md_11878:1:a	Senescence-associated protein	114.63	7.33	SA
Md_373:3:a	UDP-glucosyltransferase (EC=2.4.1.115)	372.45	7.32	SA
Md_1:237:a	Metallothionein	348.11	7.32	SP
Md_123:3:a	Expansin	701.08	7.32	SP
Md_425:1:a	LEA/Dehydrin	767.30	7.31	NA
Md_140445:s	Cucumisin endopeptidase (EC=3.4.21.25)	84.62	7.31	SP
Md_5594:1:a	Receptor protein kinase	154.01	7.28	SP
Md_2013:3:a	BTB/TAZ domain protein	711.00	7.27	NA

Md_6319:1:a	UDP-glucosyltransferase (EC=2.4.1.115)	255.14	7.24	SA
Md_400:5:a	Asparagine synthetase (EC=6.3.5.4)	561.06	7.23	NA
Md_5284:1:a	Lipid binding protein	142.63	7.23	SP
Md_6021:1:a	UDP-glucosyltransferase (EC=2.4.1.115)	257.50	7.22	SA
Md_1235:1:a	RING-H2 finger protein	849.17	7.21	NA
Md_17595:1:a	ATP binding protein	89.54	7.21	SP
Md_6808:1:a	UDP-glucosyltransferase (EC=2.4.1.115)	256.94	7.20	SA
Md_6531:1:a	MYB8 transcription factor	1273.41	7.19	NA
Md_7201:1:a	Protein kinase	214.48	7.19	SP
Md_927:2:a	NAC transcription factor	693.00	7.16	NA
Md_9474:1:a	Uncharacterized protein	30.35	7.15	SA
Md_1301:2:a	2-deoxyglucose-6-phosphate phosphatase (EC 3.1.3.68)	83.26	7.14	SA
Md_5162:1:0:b	No match	141.10	7.13	NA
Md_6301:2:a	NDR1/HIN1-like protein	907.91	7.13	NA
Md_9161:1:a	LOB domain-containing protein	77.16	7.11	SP
Md_7067:1:a	Amygdalin hydrolase isoform (EC=3.2.1.117)	264.52	7.10	NA
Md_65274:s	No match	312.20	7.09	NA
Md_178763:s	Chaperone protein DnaJ	338.06	7.09	SA
Md_14620:1:a	Uncharacterized protein	41.32	7.09	SP
Md_12455:1:a	Uncharacterized protein	97.40	7.07	NA
Md_4777:s	Cytochrome P450	764.14	7.07	NA
Md_8031:1:a	Dynein light chain	1044.35	7.07	NA
Md_98110:s	Cytochrome P450	977.28	7.07	NA
Md_564:2:a	Cadmium induced/Farnesylated protein-like	542.16	7.06	NA
Md_5506:3:a	Cinnamoyl-CoA reductase (EC=1.1.1.219)	133.48	7.06	SP
Md_5687:1:a	Uncharacterized protein	1511.38	7.05	NA
Md_1843:1:a	Glutathione transporter	56.80	7.05	SP
Md_83832:s	Non-phototropic hypocotyl 3-like protein	144.01	7.03	NA
Md_692:1:a	Zinc-binding protein	207.75	7.03	SA
Md_648:1:a	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (EC 4.1.2.15)	179.34	7.03	SP
Md_1139:1:a	Ocs element-binding factor	466.11	7.02	NA
Md_965:2:a	Uncharacterized protein	1487.27	7.00	NA
Md_212665:s	Alpha-L-fucosidase	118.65	7.00	SP

As far as the genes encoding structural elements are concerned, it is worthy to note that in persisting fruitlets an up-regulation of genes encoding actin (Md_507:4:a), tubulin (Md_7506:1:a), and expansins (Md_521:3:a and Md_123:3:a) was detected, consistently with the active growth characterizing this fruitlet class. Among the transcripts up-regulated in the abscising samples, no structural element was reported, except for a dynein gene (Md_8031:1:a) probably involved in the organization and control of vesicle trafficking (Lawrence et al., 2001). In the same samples, dehydrin/LEA protein genes represent a clear genetic signature. In fact, five transcripts related to this class of proteins (Md_1133:1:a, Md_152:7:a, Md_3584:1:a, Md_425:1:a, and Md_570:2:a), which are known to be expressed in senescing organs strictly upon ABA control (Hong-Bo et al., 2005; Rorat, 2006), are strongly up-regulated in abscising fruitlets also during the early stages of shedding induction, consistently with the destiny of these samples.

Genes encoding elements of signalling pathways were found among the most discriminating transcriptional signatures. A LOB (Lateral Organ Boundaries) gene (Md_9161:1:a) was expressed at very lower levels in the abscising fruitlets than in the persisting ones. A 75% identity was assessed between this gene and *ASL12/LOB21* of *Arabidopsis thaliana*

(At3g11090), which is expressed at high levels in the silique and belongs to a gene family whose members promote lateral organ fate and polarity, thereby restricting the developmental potential of the organ-forming cells (Ha et al., 2007; Matsumura et al., 2009). Similar expression patterns were observed for metallothionein-like protein transcripts (Md_1:237:a), closely similar to senescence-induced Arabidopsis *MT3* (At3g15353). Interestingly, two genes encoding protein kinases (Md_7201:1:a and Md_770:2:a) were very powerful in discriminating strongly abscising fruitlets showing very low expression levels. Abscising fruitlets showed higher expression levels for genes encoding a zinc-binding protein (Md_692:1:a), a chaperone protein DnaJ (Md_178763:s), and a GA-responsive protein (Md_47103:s), putatively involved in cell cycle regulation, senescence, and GA signalling, respectively (TAIR data). A similar transcriptional profile was reported for an ocs element-binding factor gene (Md_1139:1:a), involved in ethylene signalling (Büttner and Singh, 1997; Singh et al., 2002), and for two coregulated NAC genes (Md_12393:1:a and Md_927:2:a), which were shown to be involved also in senescence-associated mobilization of resources (Uauy et al., 2006). Specifically, the former was closely similar to cotton *NAC5*, promptly induced by exogenous ABA (Meng et al., 2009) whereas the latter showed a significant degree of identity with senescence associated *ANAC083* (At5g13180) of Arabidopsis (Ay et al., 2009). Among the other signalling elements, transcripts for a MYB transcription factor (Md_6531:1:a), and two RING-H2 finger proteins (Md_1235:2:a and Md_1235:1:a) were overexpressed in abscising fruits. The former is apple *MdMYB8* (DQ267899) whereas the latters were both similar to *XERICO* gene of *A. thaliana* (At2g04240), a positive regulator of ABA signalling (Ko et al., 2006). Finally, a BTB/TAZ-domain protein was most likely encoded by the gene Md_2013:3:a, highly expressed in fruitlets with high abscission potential as well. This gene showed a 63% identity with *BT1* of Arabidopsis, promptly induced by treatments with hydrogen peroxide (Du and Poovaiah, 2004). In Figure 4, the transcriptional profiles of all the most discriminating genes are shown.

Signatures of fruitlet abscission in seed

As far as the seed transcriptome is concerned, 24 highly discriminating genes were clustered, among which nine were up-regulated in the abscising samples and fifteen in the persisting ones (Figure 5 and Table 2). Among the former, *MdERS2* (Md_247145:s) and a

diacylglicerol kinase gene (Md_69518:s; EC 2.7.1.107) were found. The second gene showed 62% identity with Arabidopsis *ATDGK5* (At2g20900), induced by ozone, ethylene and jasmonic acid (Tamaoki et al., 2003), and also *MdERS2* was shown to be ethylene-inducible (Tatsuki et al., 2009). A zinc finger protein (Md_2869:1:a), similar to *LZF1* of Arabidopsis (At1g78600), and a cytochrome P450 gene (Md_2596:1:a), similar to *CYP714A1* (At5g24910), grouped in the same cluster. These genes were shown to be involved in seedling photomorphogenesis and seed development, respectively, of *A. thaliana* (Chang et al., 2008; Datta et al., 2008; Kushiro et al., 2004). Persisting fruitlets expressed at higher levels some genes encoding proteins involved in metabolism, such as a NADP-dependent malic enzyme (Md_132905:s) similar to ATNADP-ME2 (At5g11670), a mannosyltransferase (Md_140389:s) similar to PEANUT1 (At5g22130), a UDP-glucose-4-epimerase (Md_3500:1:a) similar to At4g20460. Their putative Arabidopsis orthologs were shown to have fundamental roles in embryo development (Wheeler et al., 2005; Gillmor et al., 2005; Gómez et al., 2006). Other co-regulated genes involved in metabolism are reported in Figure 5. Two genes encoding structural elements (actin, Md_507:4:a, and a proline-rich cell wall protein, Md_97336:s) were also expressed at high levels in the seed of persisting fruitlets, consistently with an actively developing status. Also the overexpression of a DEAD-box gene (Md_4964:1:a) similar to mitochondrial RNA helicase may be representative of a very active metabolism.

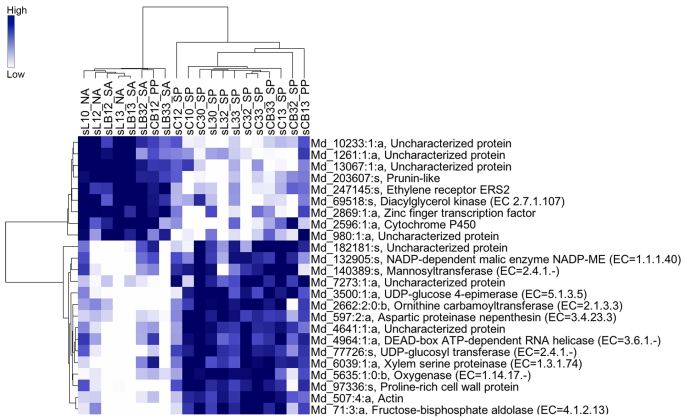

Figure 5. Hierarchical clustering of genes with expression levels in the seed that are highly associated with the fruitlet abscission potentials. Only genes with a Z score ≥ 7 are reported, according to the Globaltest analysis. Samples are reported on the top side of the heatmap with the following codes: s: seed; L: lateral, C: central, B: treated with BA; first number indicates fruit size category: 1: small fruitlets, 3: big fruitlets; second number indicates sampling time: 0: T0, 2:T2. 3:T3. SP: strongly persisting, PP: probably persisting, NA: naturally abscising, SA: strongly abscising.

It has to be noted that the transcriptional profiles of the above genes in the seed are clearly less discriminative than those found in the cortex. In fact some samples (i.e. L10, L12, LB32), despite the hierarchical clustering, showed expression levels somehow different from those of other members of the same cluster (Figure 5). Whereas, considering only fruitlets at T3, the same genes were very reliable in discriminating fruit's destiny, therefore confirming the Globaltest results indicating an earlier association of the cortex transcriptome than the seed's with fruit abscission potential.

Table II Genes with expression data in the seed associated with abscission potential with a Z score ≥ 7. A tentative annotation, the influence on the whole association, the statistical score (Z), and the expression pattern (NA=high expression in naturally abscising fruitlets; SA=high expression in strongly abscising fruitlets; SP=high expression in strongly persisting fruitlets) are reported for each gene.

Gene ID	Tentative annotation	Influence	Z	Exp.
Md_140389:s	Mannosyltransferase (EC=2.4.1.-)	39.91	9.37	SP
Md_4641:1:a	Uncharacterized protein	135.78	8.74	SP
Md_6039:1:a	Xylem serine proteinase (EC=1.3.1.74)	95.01	8.25	SP
Md_247145:s	Ethylene receptor ERS2	173.68	8.15	SA
Md_77726:s	UDP-glucosyl transferase (EC=2.4.1.-)	104.49	8.04	SP
Md_507:4:a	Actin	286.42	7.86	SP
Md_203607:s	Prunin-like	316.63	7.79	SA
Md_10233:1:a	Uncharacterized protein	224.81	7.64	NA
Md_4964:1:a	DEAD-box ATP-dependent RNA helicase (EC=3.6.1.-)	75.52	7.48	SP
Md_2869:1:a	Zinc finger transcription factor	49.21	7.34	NA
Md_182181:s	Uncharacterized protein	67.95	7.32	SP
Md_2662:2:0:b	Ornithine carbamoyltransferase (EC=2.1.3.3)	68.18	7.31	SP
Md_97336:s	Proline-rich cell wall protein	317.78	7.28	SP
Md_3500:1:a	UDP-glucose 4-epimerase (EC=5.1.3.5)	176.60	7.27	SP
Md_132905:s	NADP-dependent malic enzyme NADP-ME (EC=1.1.1.40)	155.09	7.21	SP
Md_980:1:a	Uncharacterized protein	681.02	7.18	SA
Md_1261:1:a	Uncharacterized protein	800.80	7.13	NA
Md_5635:1:0:b	Oxygenase (EC=1.14.17.-)	146.93	7.13	SP
Md_7273:1:a	Uncharacterized protein	48.74	7.11	SP
Md_597:2:a	Aspartic proteinase nepenthesin (EC=3.4.23.3)	201.44	7.08	SP
Md_69518:s	Diacylglycerol kinase (EC 2.7.1.107)	38.81	7.07	NA
Md_71:3:a	Fructose-bisphosphate aldolase (EC=4.1.2.13)	188.95	7.06	SP
Md_13067:1:a	Uncharacterized protein	58.76	7.03	NA
Md_2596:1:a	Cytochrome P450 (EC=1.3.3.9)	77.41	7.01	NA

Genes involved in BA-induced abscission

A parallel approach was adopted along with the Globaltest by subtracting gene pools either developmentally or pharmacologically regulated by BA to the overall DE genes in the LB3 fruitlets. This 'subtractive' approach is detailed in materials and methods section, and represents a validation of the Globaltest analysis. A detailed description of DE genes is reported below only for the most interesting categories. The number of DE genes for each comparison and an overall list along with a tentative annotation, molecular function classification, and expression pattern, are reported in Tables S3, S4, and S5, whereas in

Tables III and IV only the most interesting genes are listed. For microarray data validation, quantitative (q)PCR experiments were performed on a subset of selected genes and showed similar expression patterns and reliable correlations (Figure S7).

It is worthy to note that also in this case the cortex showed the most relevant transcriptional response, at least in terms of number of genes up- or down-regulated during abscission induction because of BA-specific action and in a development-independent manner (J_{ABS} and K_{ABS} sets). Specifically, from T0 to T2, 218 DE genes included 26 and 182 down- and up-regulated transcripts, respectively. From T2 to T3, only 10 DE genes were detected, six and four of which down- and up-regulated, respectively.

Twenty genes putatively encoding transcription factors (TFs) of diverse families were differentially expressed in the cortex, most of which with an up-regulation pattern from T0 to T2. From T2 to T3, no TF-encoding gene was either up- or down-regulated with statistical significance (Table III). Besides some of the TFs, additional hormone-related genes were differentially expressed during abscission induction, among which some indicating an ongoing recovery of auxin homeostasis, and an extensive inactivation of GAs and cytokinins. ABA signalling seemed to be strongly affected by abscission induction as well, particularly concerning the cross-talk with ethylene, ROS, and sugars. The expression of some jasmonate (JA)-inducible genes along with an indicator of active brassinosteroid (BR)-JA cross-talk may point towards the involvement of these two hormones. Ethylene (ET) signalling was clearly affected during abscission induction, as shown for some transcription factors. Two additional key elements of ET signal transduction pathway were also found, coding for a MAP kinase (MPK) and a MAP kinase kinase (MAKK), the former probably involved in ET-ABA cross talk (Xin et al., 2005). Key elements were found among the DE genes, that are likely involved in ROS-sugars-hormones cross talk, and three up-regulated genes showed close similarity with ROS-induced, -detoxifying, or –producing elements. Another interesting category concerns the vesicular trafficking, since at least six DE genes may encode elements involved either in endo- or exo-cytosis. Finally, as far as protein degradation is concerned, four genes were up-regulated during abscission induction, indicating a likely remobilization of resources. According to this analysis, the Globaltest results were largely confirmed not only from a quantitative point of view but also qualitatively, especially concerning the involvement of ABA, sugars and ROS (Table III).

Table III A selection of DE genes in the cortex. The Gene ID is reported along with a short annotation, the pattern of expression from T0 to T2, and from T2 to T3, likely correlations with hormones, metabolites or physiological events, and references reporting specific information about the genes. In the first column categories are indicated as follows: H = hormone biosynthesis, metabolism and action; H/S = hormone-sugars cross talk; P = protein synthesis and metabolism; R = reactive oxygen species synthesis, metabolism and signalling; TF = transcription factors; VT = vesicle trafficking. The complete list along with further details are available in Table S4.

	Gene ID	Tentative annotation	T0-T2	T2-T3	Notes[a]	References
H	Md_1133:1:a	LEA/dehydrin	up	-	+ABA	-
	Md_131178:s	14-3-3-like protein	down	-	ABA/ET	Lancien and Roberts, 2006
	Md_14070:1:a	Gibberellin 2-oxidase	up	-	-GA	-
	Md_140962:s	BRI1-associated receptor kinase 1 (BAK1)	up	-	+ROS, BR/JA	Xia et al., 2009
	Md_214104:s	Gibberellin 2-oxidase	up	-	-GA	-
	Md_246936:s	Mitogen-activated protein kinase (MAPK)	up	-	+ABA, ABA/ET	Xin et al., 2005
	Md_25179:s	Gibberellin 2-oxidase	up	-	-GA	-
	Md_2556:1:a	GASA4-like protein	down	-	+GA	Chen et al., 2007
	Md_2750:1:a	LEA/dehydrin	up	-	+ABA	
	Md_4451:1:a	Jasmonate induced protein	-	down	+JA	-
	Md_4451:1:a	Jasmonate induced protein	up	-	+JA	-
	Md_5550:1:a	IAA-amino acid hydrolase, ILR1	up	-	+IAA	Seidel et al., 2006
	Md_5793:1:0:b	Mitogen-activated protein kinase kinase (MAPKK)	up	-	ET	-
	Md_7045:1:a	Gibberellin 2-oxidase	up	-	-GA	-
	Md_74377:s	Cytokinin dehydrogenase	up	-	-CK	Frebortova et al., 2004
	Md_93:4:a	14-3-3-like protein	down	-	ABA/ET	Lancien and Roberts, 2006
H/S	Md_12387:1:a	SNF1-related kinase 3.10	up	-	+ST	Chikano et al., 2001; Purcell et al., 1998
	Md_253006:s	AMP-activated protein kinase, gamma regulatory subunit	up	-	+ABA	Genevestigator
	Md_9662:1:a	Sucrose synthase	up	-	+SUC	Chikano et al., 2001

P	Md_20453:1:a	Aspartic proteinase nepenthesin	up	-	-	-
	Md_240669:s	Ubiquitin-protein ligase	up	-	-	-
	Md_6142:1:a	Subtilisin-like protease	up	-	-	-
	Md_66411:s	Ubiquitin-protein ligase	up	-	-	-
R	Md_288:2:a	Ferritin	up	-	+ROS	Ravet et al., 2009
	Md_5375:1:a	Respiratory burst NADPH-oxidase	up	-	+ROS	Torres et al., 2002; Kwak et al., 2003
	Md_67394:s	Peroxidase	up	-	+ROS	Almagro et al., 2008
TF	Md_1109:1:a	WRKY53 transcription factor	up	-	+JA, +ROS, +SEN	Miao et al., 2004; Miao and Zentgraf, 2007; Zentgraf et al., 2010; Pitschke and Hirt, 2009
	Md_1122:1:a	NAC/NAM transcription factor	up	-	+ABA	Tran et al., 2004; Fujita et al., 2004
	Md_117252:s	WRKY53 transcription factor	up	-	+JA, +ROS, +SEN	Miao et al., 2004; Miao and Zentgraf, 2007; Zentgraf et al., 2010; Pitschke and Hirt, 2009
	Md_119754:s	MYC1 transcription factor	up	-	+STR	Smolen et al., 2002
	Md_121294:s	Ethylene-responsive AP2/ERF transcription factor	up	-	ET/JA	Lorenzo et al., 2003
	Md_12393:1:a	NAC/NAM transcription factor	up	-	+ABA	Meng et al., 2009; Uauy et al., 2006
	Md_1709:2:a	EIL2 (EIN3-like)	up	-	+ET, +ROS	Huang et al., 2010; Zhong et al., 2009
	Md_19496:1:a	Zinc finger DHHC domain-containing protein	up	-	-	-
	Md_200958:s	Zinc-finger C2H2 protein SERRATE	up	-	-	-
	Md_249698:s	Ethylene-responsive AP2/ERF transcription factor	up	-	ET/JA	Lorenzo et al., 2003
	Md_2575:1:a	MdMYB6 transcription factor	up	-	+SUC	Genevestigator
	Md_3896:1:0:b	WRKY4 transcription factor	up	-	+JA	Fonseca et al., 2009
	Md_40605:s	Ethylene-responsive AP2/ERF transcription factor	up	-	+JA	Onate-Sànchez and Singh, 2002
	Md_5724:1:a	Zinc finger protein CONSTANS-LIKE 5	down	-	-	-
	Md_6240:1:a	Zinc finger homeodomain protein SZF-HD1	down	-	-	-
	Md_6531:1:a	MdMYB8 transcription factor	up	-	+SA	Yanhui et al., 2006
	Md_7112:2:a	WRKY19 transcription factor	up	-	-	-
	Md_9390:1:a	Auxin response factor 3 (ARF3)	up	-	+ROS, +ST, +SUC, +ABA	Genevestigator
VT	Md_135689:s	ADP-ribosylation factor, ARF	down	-	-	-
	Md_16163:1:a	Protein transport protein Sec23	up	-	-	-

	Md_179063:s	Dynamin	up	-	-	Jin et al., 2001; Zhang and Hu, 2010; Buban, 2000
	Md_6659:1:a	Synaptotagmin	up	-	-	Schapire et al., 2008
	Md_7571:1:a	Coatomer beta subunit	up	-	-	Sanderfoot and Raikhel, 2003
	Md_8558:1:a	Clathrin assembly protein	up	-	-	Legendre-Guillemin et al., 2004

a) '+' = positive correlation; '-' = negative correlation; '/' = cross-talk; ABA = abscisic acid; BR = brassinosteroids; CK = cytokinin; GA = gibberellin; ET = ethylene; IAA = indole acetic acid; JA = jasmonic acid; ROS = reactive oxygen species; SA = salicylic acid; SEN = senescence; ST = starvation; STR = stress; SUC = sucrose.

Table IV A selection of DE genes in the seed. The Gene ID is reported along with a short annotation, the pattern of expression from T0 to T2, and from T2 to T3, likely correlations with hormones, metabolites or physiological events, and references reporting specific information about the genes. In the first column categories are indicated as follows: H = hormone biosynthesis, metabolism and action; P = protein synthesis and metabolism; R = reactive oxygen species synthesis, metabolism and signalling; TF = transcription factors; TR = transport. The complete list along with further details are available in Table S5.

	Gene ID	Tentative annotation	T0-T2	T2-T3	Notes[a]	References
H	Md_570:2:a	Dehydrin, ABA responsive	-	up	+ABA	-
	Md_74377:s	Cytokinin dehydrogenase	-	up	-CK	-
P	Md_11646:1:a	Protein synthesis inhibitor, DPH2	up	-	-	-
	Md_13925:1:a	Subtilisin	-	up	-	-
	Md_20453:1:a	Aspartic proteinase, nepenthesin-1	-	up	-	-
R	Md_288:2:a	Ferritin	-	up	+ROS	Ravet et al., 2009
TF	Md_114537:s	Homeobox-leucine zipper protein ATHB-40	-	up	+ABA	Henriksson et al., 2005
	Md_19486:1:a	Bel1 homeotic protein	-	up	+T6P	Ray et al., 1994; Skinner et al., 2004; Schluepmann, 2004; Dong et al., 2000
	Md_3290:2:a	MADS-box transcription factor, STK/AGL11	down	-	-	Yao et al., 1999; Tani et al., 2009
	Md_3329:1:a	Ethylene responsive AP2/ERF transcription factor	-	up	+ET	Genevestigator
TR	Md_4628:1:a	High affinity nitrate transporter	-	up	+ST	Chopin et al., 2007; Remans et al., 2006

a) '+' = positive correlation; '-' = negative correlation; '/' = cross-talk; ABA = abscisic acid; BR = brassinosteroids; CK = cytokinin; GA = gibberellin; ET = ethylene; IAA = indole acetic acid; JA = jasmonic acid; ROS = reactive oxygen species; SA = salicylic acid; SEN = senescence; ST = starvation; STR = stress; SUC = sucrose; T6P = treahalose-6-phosphate.

Concerning the seed, a situation very close to that assessed by the Globaltest analysis was again reported, in that a consistent transcriptional response was detected later, but at a less overall extent, than in the cortex. From T0 to T2, only 31 genes were differentially expressed (13 down- and 18 up-regulated), whereas 45 transcripts differentially accumulated from T2 to T3, most of which up-regulated.

Among the four DE seed genes that were shown to putatively encode TFs, Md_3290:2:a was the only one early down-regulated from T0 to T2. It showed a high identity (90%) with *AGL11*-like *MdMADS10*, expressed in apple after pollination (Yao et al., 1999), and with a MADS-box transcription factor STK of *Prunus persica* thought to be important for embryo development (Tani et al., 2009). Although less relevant than in the cortex, the hormonal response of the seed herein pointed out seemed to resemble the Globaltest results, in that active ABA and ET signallings were found, along with a likely degradation of cytokinins, an ongoing oxidative stress, and a probable inhibition of protein synthesis concurrent with a later increase of protein degradation (Table IV).

Carbohydrates and peroxides

Since transcriptomic analyses pointed out components of sugar metabolism and genes related to responses to H_2O_2, the main sugars along with H_2O_2 content were measured in persisting and abscising fruitlets. Sucrose, glucose, fructose, sorbitol, and starch levels were assessed in all samples up to 23 DAPF. Significant variations throughout the experiment were found only for sucrose and starch, the former being correlated with abscission induction (Figure 6) whereas the latter most likely depending on the BA treatments (Figure S4). Concerning sucrose, a significant increase of its levels was observed immediately after the treatment only in LB3 fruitlets, remaining higher than in untreated samples throughout the experiment. In all the other samples, no significant variations were observed in this phase. Another relevant aspect of sucrose behaviour was pointed out in central fruitlets, both CB1 and CB3, in which a significant decrease was assessed later on after abscission induction. The other carbohydrates did not show significant variations associated with abscission potential (Figure S4).

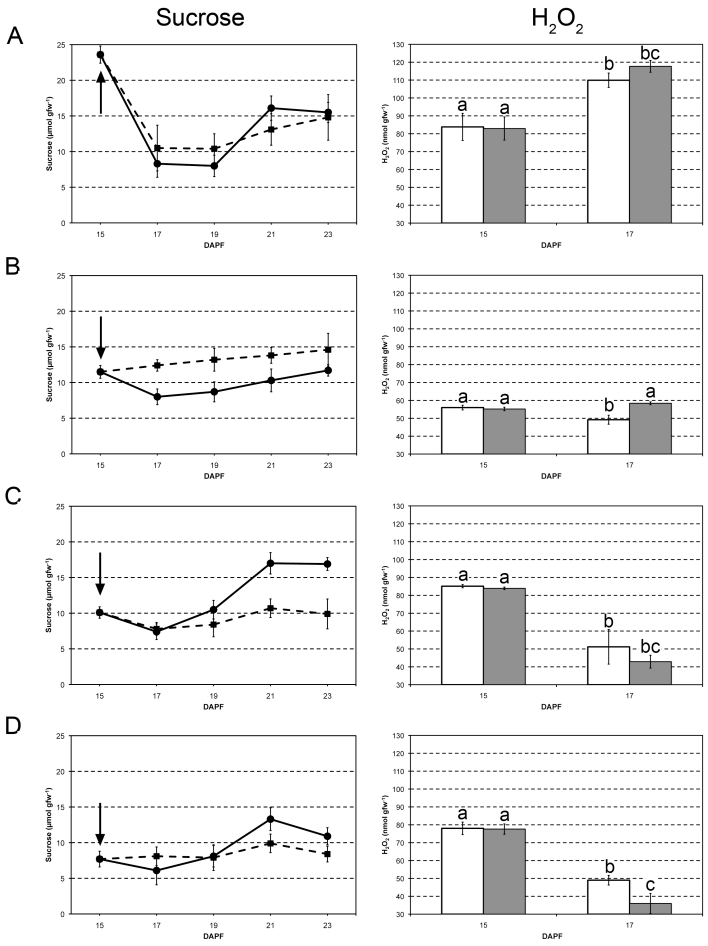

Figure 6. Sucrose concentration (left) and hydrogen peroxide amount (right) in small (**A**) and big (**B**) lateral fruitlets, and in small (**C**) and big (**D**) central fruitlets, either untreated (circles and continuous line for sucrose, white bars for H_2O_2) or treated with BA (squares and dot-line for sucrose, grey bars for H_2O_2). Letters indicate significant differences as pointed out by LSD test ($P < 0.05$). Bars represent standard deviation.

Since the majority of ROS-related genes were differentially expressed in the cortex from T0 to T2, hydrogen peroxide was measured only in this time lapse, which is crucial for abscission induction. Also in this case, divergent trends were observed upon BA treatment in LB3 fruits with respect to the centrals. The latter showed a decreasing trend in untreated samples with a magnifying effect of the treatment resulting into lower levels of peroxides. On the other hand, control L3 fruitlets displayed a decreasing trend in peroxide levels as in the previous ones, although at a lower extent, but had an opposite reaction when treated with BA. In fact, the significant increase observed in treated LB3 fruitlets at T2 was well correlated with expression data of ROS-related genes and, therefore, with the abscission potential. It is noteworthy that L1 fruitlets showed increasing levels of peroxide, being

highest at T2, along with a positive effect of BA treatment, although less significant than in LB3 (Figure 6).

Discussion

The apple inflorescence is almost a unique model system for studying correlatively-driven abscission, and the availability of chemical thinning tools able to selectively induce fruit drop allows the setting up of controlled experimental plans in the field aimed at magnifying the natural abscission potential. Indeed, the inter-fruitlets dominance relationships existing within the apple cluster can be assessed with good approximation in order to predict the destiny of each fruitlet, in terms of probability of abscise. However, a deep knowledge of the molecular events occurring during the early phases of apple fruitlet abscission induction is still lacking. Previous studies carried out by Dal Cin et al. (2009a) addressed this issue with a preliminary approach, by using a cDNA-AFLP-based differential display, but without prompting out an overall model of the early inductive events. A different study of the same authors (Dal Cin et al., 2009b) pointed out interesting evidences in terms of PAT (polar auxin transport) elements transcription in relation to the ethylene burst occurring in the post-induction phase. Therefore, the present research was focused on the earlier events occurring at the fruit level (the cortex and the seed), which is the place where the abscission signal is thought to be generated.

A model was prompted out for apple fruitlet abscission taking into account the overall transcriptomic data herein obtained and some key metabolic analyses aimed at strengthening and confirming the crucial steps pointed out by microarray experiments (Figure 7). According to this model, apple fruitlet abscission takes place in four main steps, corresponding to the four structural levels where the key events may occur (*i.e.* the tree, the fruit cortex, the seed, and the abscission zone). The initial steps arise at the tree level, where a nutritional stress is established either naturally or upon a thinning treatment with BA. Such a condition is plausible since at the beginning of the vegetative and reproductive season the growth of shoots and fruitlets is supported at a large extent by stored assimilates. As a consequence, a strong competition for storage assimilate reallocation exists among shoots, between shoots and fruits, among fruits of different clusters, and fruits of the same cluster. Since young growing shoots act as stronger sinks than fruits, the tree is unable to support all the growing fruitlets, causing the ‘weaker’ ones to naturally abscise thus

generating the fruitlet physiological drop. When BA is used as a thinner, a magnification of the already existing nutritional stress occurs. This cytokinin is known to induce lateral bud outgrowth, thus enhancing the shoot sink activity and, consequently, the competition for assimilates (Bubàn, 2000; Bangerth, 2000; Bangerth et al., 2000). At this point, how does this nutritional stress trigger fruitlet abscission? And how is this nutritional signal transduced into the abscission signal?

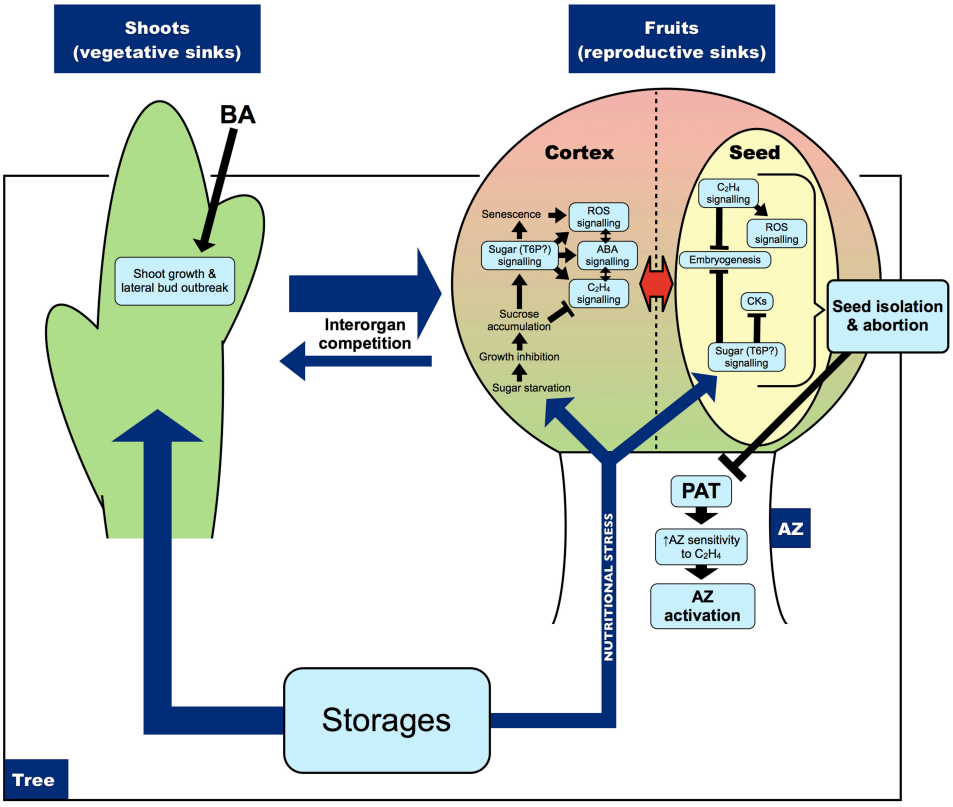

Figure 7. Hypothetical model for immature fruit abscission in apple. The interorgan competition for stored assimilates existing within the tree is magnified by BA which stimulates shoot growth and bud outbreak. This condition is perceived by weaker fruitlets as a nutritional stress, which is translated at both the cortex and seed level throughout cross-talking signalling pathways, mainly involving sugars, ROS, abscisic acid, and ethylene. When the seed perceives the situation as unrecoverable, a block of embryo development occurs leading to seed isolation and abortion. This crucial step would determine the depolarization of auxin transport, the enhancement of AZ sensitivity to ethylene, and its activation (Abbreviations: BA = benzyladenine; AZ = abscission zone; T6P = trehalose-6-phosphate; ROS = reactive oxygen species; CKs = cytokinins; PAT = polar auxin transport). The thickness of arrows related to interorgan competition and storage partitioning is proportional to the strength of the organ as a sink.

Our transcriptomic data suggest that the cortex is the primary response tissue perceiving this nutritional stress, at least in quantitative terms. Nutrient and sugar starvation affect its transcriptomic profiles already at 2 days after treatment, whereas significant changes related to abscission in the seed transcriptome appear later, at 4 days after BA spray (Tables S1 and

S2). During the early steps of abscission induction, a sugar signal, most likely involving trehalose-6-phosphate (T6P), induces a prompt reaction to nutritional stress. The involvement of T6P is suggested by the high expression levels of a class II TPS gene in abscising fruitlets (AFs), as found also during induced abscission of citrus fruits (Alferez et al., 2007). A significant increase of sucrose concentration in AFs with respect to the non-abscising ones (NAFs) was also found (Figure 6) promptly after the treatment, as previously demonstrated by Stopar et al. (2001). Accumulation of sucrose is often reported as a reaction to sugar starvation and has generally been considered to be an adaptive response to the stress condition (Roitsch, 1999). It is also associated to senescence, whose regulation in plants is known to be triggered by sugars (Wingler et al., 2009). Also an increased ROS production may be linked to sugar starvation (Hooks et al., 1995; Contento et al., 2004), and sucrose accumulation may also represent an oxidative stress balance mechanism (Couée et al., 2006). Moreover, the idea that high sugar (carbon)/low nitrogen conditions and not starvation would trigger changes in gene expression that are characteristic of developmental senescence is supported by experimental evidences, at least in leaf (Wingler et al., 2009). According to this view, sugar starvation would not directly trigger senescence-associated gene expression in the cortex of AFs, but rather contribute to the installation of the sugar signalling causing in turn the transcriptomic reaction associated to abscission induction. This reaction would be most likely mediated by ROS accumulation, since a higher concentration of hydrogen peroxide in the AFs than the NAFs was herein assessed during early abscission induction (Figure 6). These evidences are further supported by ROS-related gene expression, as described in the previous section, especially concerning a NADPH oxidase gene highly similar to *AtRBOHD* involved in ROS production and a gene coding for a class III peroxidase with diverse possible roles (Cosio and Dunand, 2009), both up-regulated in the cortex of fruitlets induced to abscise. In this context, ABA signalling concurrently orchestrates sugars-ROS cross-talk, as pointed out by transcriptomic data indicating typical signatures of ABA action. The TPS gene found over-expressed in AFs may also regulate ABA signalling as found in Arabidopsis (Avonce et al., 2004). During the early phases of abscission induction, an active resource mobilization is already established in the cortex according to gene expression data. Moreover, persisting fruitlets show transcriptomic profiles typical of actively growing organs, in contrast with the abscising

ones displaying expression levels for the same genes compatible with a block of their growth, especially in terms of transcripts encoding structural and metabolic elements.

As far as the involvement of transcription factors (TFs) is concerned, some key elements were shown to be active during abscission induction in the cortex. However, most of these may possibly regulate downstream processes mostly related to ongoing senescence, rather than to the early inductive events. In fact, the two *NAC* genes *MdMYB8* and the two RING-H2 finger genes over-expressed in the AFs are closely similar to senescence-associated or ABA-induced TFs found in other species and putatively involved in ABA signalling downstream the abscission induction signal (Ko et al., 2006; Uauy et al., 2006; Meng et al., 2009; Ay et al., 2009). On the other hand, the subtractive approach focused on the fruitlets induced to abscise allowed to point out a likely involvement of some TFs during the earlier events, mostly with an up-regulation profile at T2. Specifically, two *WRKY* genes possibly involved in ROS signalling (Pitzschke and Hirt, 2009), a *MYB* gene similar to sucrose-induced TFs, a *MYC* putatively triggering stress-responsive genes (Smolen et al., 2002), and, interestingly, an *ARF* (auxin response factor) similar to Arabidopsis *AtARF3* whose transcription is induced by hydrogen peroxide, nitrogen starvation, sucrose and ABA. All these genes may act as early regulators of the abscission induction, probably involved in the translation of the initial stress condition into abscission signal at the cortex level. A functional validation is in progress to elucidate their relative importance and roles in the generation of the signal cascade triggering fruit shedding.

Part of this signalling may also include specific kinase cascades, such as those found to be up-regulated in AFs from T0 to T2. However, also in this case the majority of these genes are most likely involved downstream the abscission signal generation, except for an SNF1-related kinase gene closely similar to Arabidopsis *SnRK3*. The latter was shown to be induced by exogenous cytokinin (Chikano et al., 2001), and involved in sucrose-dependent transcription stimulation of sucrose synthase (SuSy) genes (Purcell et al., 1998) and in SuSy phosphorylation. Interestingly, a gene coding for a SuSy closely similar to Arabidopsis SUS3, induced by sucrose and regulated by SnRK3 itself (Chicano et al., 2001), was coregulated in the same samples. These two elements, the *SnRK3*-like gene and the *SUS3*-like sucrose synthase gene, may determine the early sugar sensing/signalling generating the

abscission signal as a response to nutrient starvation, thus representing a key regulation point leading to sucrose accumulation in the shedding fruitlets.

Gene expression data indicate not only an active resource mobilization, but also active protein degradation and vesicular trafficking, all of which are most likely triggered later on when the abscission signal is fully installed.

Hormones seems to play a relatively important role during the early phases of abscission in the cortex, since the majority of the transcriptionally activated elements involved in hormone signalling seem to be downstream the abscission induction. The earliest association with the abscission potential was found at T2 for ABA-related transcriptome (highly significant, $P < 0.001$). Behind the genes discussed in the above paragraphs, early ABA signalling involves also a down-regulation of 14-3-3 genes in the AFs. The related proteins may trigger ABA-ethylene cross talk and responses to sugar starvation (Lancien and Roberts, 2006). ABA-sugar cross talk may also involve a gene encoding an AMP-activated protein kinase similar to an Arabidopsis ABA-induced SNF1-related kinase, which was found to be up-regulated in the AFs. Interesting data concern the hormone metabolic pathways, especially regarding auxin, GAs, and cytokinins. In fact, a gene for an IAA-amidohydrolase thought to disjoin IAA from specific amino acids was up-regulated at T2 in AFs, probably as a homeostatic response. Concerning GAs, four over-expressed genes encoding deactivating enzymes (GA2-oxidases) and a down-regulated *GASA4*-like transcript would indicate a decrease of active GA levels in the cortex. As far as cytokinins are concerned, the up-regulation of a deactivating gene coding for a cytokinin dehydrogenase was found during abscission induction, pointing also in this case towards a decrease of the active hormone amount. Finally, ethylene signalling was also found to be triggered, but only downstream the abscission signal generation and as a consequence of the cross-talk with ABA and ROS. Two elements are to be considered pivotal in this context, both up-regulated and putatively involved in ethylene signal transduction and cross-talk with other transductive pathways. The first is an *AtMPK11*-like gene, induced by ABA, putatively involved in ethylene-ABA cross-talk and in modulation of ABA signalling (Xin et al., 2005), whereas the second is an *AtMKK9*-like gene, probably downstream *AtMPK11*, and involved in the up-regulation of *ACS* and *ERF* genes. Both elements are positioned in the same transductive pathway and most likely upstream the regulation of ethylene

biosynthesis induction occurring later on in AFs. Therefore, the ethylene burst usually found in abscising fruitlets may result from the cross-talk between ABA and ROS, generated during the early inductive phases, immediately after the perception and signalling of the sugar starvation status. In this context, it is worthy to note that hormone-related transcriptomic signatures assessed 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 (Vriezen et al., 2008). In fact, our data show that ABA and ET signalling are strongly up-regulated concurrently with a down-regulation of GA signalling specifically in fruits induced to abscise.

After the early reaction of the cortex, it may be hypothesized that a link is established with the seed when the abscission inductive process reaches an irreversible status. The seed appeared indeed affected at the transcriptional level at a later stage (Tables S1 and S2), at least from a quantitative point of view. Furthermore, the seed is a structure with a stronger homeostasis than the cortex, since it represents the reproductive endeavour carried out by the tree, and thus protected until the abscission process may become unrecoverable. Ethylene may function as the signal generated within the cortex and, through diffusion, carrying to the seed the abscission signal, as suggested by the transcription rates of several elements of its transductive pathway. In the seed, the signalling cascade activated by the abscission induction causes a block of the embryogenesis, as suggested by the significant down-regulation of *MdMADS10*, an *AGL11*-like gene differentially expressed only in the lateral fruitlets induced to abscise by BA, whose role is strictly linked either to ovule or embryo development (Yao et al., 1999; Tani et al., 2009). Several other genes linked to embryo and seed development were differentially expressed in AFs, among which are some ethylene-responsive genes, such as *MdERS2*, and an *AP2/ERF*, up-regulated in AFs, and a series of genes involved in metabolism that are expressed at higher levels in the NAFs. Again, like in the cortex, persisting fruitlets show a more dynamic metabolism and the active transcription of genes coding for structural proteins. Interestingly, a nitrate transporter gene closely similar to *AtNRT2.7* was over-expressed in LB3 fruits. In Arabidopsis, this gene controls nitrate content in the seed (Chopin et al., 2007), and is induced by nitrogen starvation (Remans et al., 2006). Moreover, actively growing organs, such as the young apple fruitlets, are a relevant source of auxin, whose main biosynthesis

site is the seed. The hormone is actively transported from the fruit through the pedicel, and its continuous flow would keep the abscission zone (AZ) insensitive to ethylene (Sexton and Roberts, 1982; Taylor and Whitelaw, 2001). Reactive oxygen species may play an important role also in this case, as demonstrated for leaf abscission by Sakamoto et al. (2008). In fact, some typical transcriptional signatures of high ROS levels were found also in the seed, although later than in cortex, such as a ferritin gene whose putative orthologs in Arabidopsis and rice are induced by hydrogen peroxide as a protective mechanism (Ravet et al., 2009). Therefore, the oxidative atmosphere where the seed is constricted at this stage along with the nutritional stress and the signals coming from the cortex may contribute to the increase of ROS production, which in turn would disrupt metabolism and suppress the synthesis of IAA as previously described (Sakamoto et al., 2008). The reduced supply of auxin to the AZ concurrently 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).

Conclusions

To the best of our knowledge, this study provides the first global monitoring of gene expression changes occurring during the early phases of apple fruitlet abscission induction. The model herein proposed takes into account both the temporal evolution of differential gene expression and its static association with abscission potential in both cortex and seed. According to this approach, the cortex would be the place where the primary abscission signal is generated whereas the seed would function as a modulator of the physiological response translating this signal to the abscission zone. However, as the time course of the inductive events in the two organs was based mainly upon massive transcriptomic data, a more targeted approach is now necessary to identify the actual key elements in charge for generating the abscission signal, and the temporal sequence of these molecular events. It cannot be ruled out that the cortex reaction may be due to an amplification of biologically relevant transcriptomic changes occurring at the seed level, herein not detected because of the high stringency adopted in the statistical analyses. Future studies will be focused on the transductive pathways pointed out in the present research as to be responsible for early abscission induction, such as the ROS-sugar-ABA cross talk. Besides these aspects, the downstream effectors, especially at the seed level, will be investigated with particular

attention devoted to MADS box and homeotic genes such as those evidenced in the transcriptomic analyses.

Materials and methods

Plant material and treatments

Experiments were carried out in 2008 on 8-year-old apple trees (cv. Golden Delicious/M9) trained with standard horticultural practices at the experimental farm of the IASMA (Trento, Italy). Populations of fruits with different abscission potentials (abscising fruitlets versus persisting fruitlets), were established as described by Dal Cin et al. (2005, 2007, 2009a), Angeli et al. (2002), and other preliminary experiments (unpublished data). Briefly, the abscising population was made up of lateral fruitlets treated with benzylaminopurine (BA) at 200 ppm (commercial name 'Brancher-Dirado'), when fruits had an average size of 13 mm (about 15 days after petal fall, DAPF). The population of central persisting fruitlets was generated by removing all the laterals from each cluster at petal fall, and leaving exclusively the central flower that had been hand-pollinated at full bloom with compatible pollen (cv. Stark Red). Samples of the two populations were collected at defined time points from groups of twenty homogeneous trees randomly distributed in the orchard in four blocks. Fruits were collected and categorised into three classes of size (class 1, smaller fruits; class 2, medium fruits; class 3, bigger fruits), two classes related to the position within the clusters (lateral versus central fruits), and two classes on the base of the treatment (BA-treated versus untreated fruits). Fruits of the intermediate size (labelled with number 2 in Figure 1) were not considered for sampling and subsequent molecular analyses, and only fruits of the two more divergent size classes 1 and 3 were kept. This resulted in a combined categorisation of fruits into 4 classes: 1) untreated lateral fruitlets, 2) lateral fruitlets treated with BA, 3) untreated central fruitlets, and 4) central fruitlets treated with BA (Figure 1). Each class was further distinguished into the two size categories 1 (small fruits) and 3 (bigger fruits), for a total of eight experimental theses. Fruitlet shedding and ethylene evolution were monitored throughout the physiological drop from the beginning of the experiments up to 46 DAPF in all fruitlet classes, separately. Seed and cortex (including epidermis) samples were collected from all classes of fruitlets at 0 (T0), 1 (T1), 2 (T2), 4 (T3), 6 (T4), and 8 (T5) days after the BA treatment from control and treated trees, and

according to their position within the clusters (central vs lateral) and size (small vs big), as described. The latter parameter was decided at each sampling date based upon the mean cross diameter of the whole population of lateral fruits, calculated over a sample of 100 fruits measured randomly on twenty trees. The small ones had a cross diameter below the mean - s.d. (standard deviation), whereas the big ones had a cross diameter above the mean + s.d. Lateral fruitlets were collected from intact clusters showing a clear hierarchy in terms of fruit size (*i.e.* with a clearly distinguishable central fruit, bigger than any lateral). Acronyms were ascribed to samples according to the following code: the first letter(s) describes fruitlet position within the cluster and the presence of BA treatment (L=untreated lateral, C=untreated central, LB=treated lateral, CB=treated central), then a digit to describe the size (1=small, 3=big), and finally a digit to described the time point (0=time of the treatment, 1=1 day after treatment, 2=2 days after treatment, 3=4 days after treatment, etc.). For example, LB32 is a lateral fruit, treated with BA, big sized, 2 days after treatment. All samples were frozen in liquid nitrogen, and stored at -80°C for later molecular analyses.

RNA isolation

Total RNA was extracted from cortex and seed following the method of Ruperti et al. (2001), with few adaptations due to the differences between tissues. The extraction buffer volume was set at 10 mL for the cortex and 1 mL for the seed, and the starting amount of tissue was 0.60 g and 0.02 g, respectively. In order to achieve the final quality of the extract, 30 (cortex) and 3 μL (seed) of a calcium hydroxide suspension at 60 g/L were added just before the first centrifugation step (Botton et al., 2008; Botton et al., 2009a, 2009b). Total RNA was quantified spectrophotometrically and its integrity checked by running 1 μg in a 1% agarose gel stained with SYBR® Safe (Invitrogen, Carlsbad, CA).

Microarray analysis

The 30k custom microarray was set up by means of the Combimatrix technique starting from publicly available apple sequences (see supplemental data for a detailed description of the whole pipeline). For hybridizations, 1 μg of total RNA was amplified and 6 μg of antisense RNA labelled using the RNA Ampulse amplification and labelling kit with Cy5 for Combimatrix arrays (Kreatech Diagnostics, The Netherlands) according to manufacturer instructions, and were hybridized to arrays according to CombiMatrix protocols. Scanning

was performed on a GenePix 4000B scanner. Data extraction was done using CombiMatrix Microarray Imager software.

Global test analysis of transcriptional profiles and hierarchical clustering

Global transcriptional profile testing was carried out with the Globaltest package v 4.14.4 (Goeman et al., 2004) of R software v 2.9.1 (http://www.r-project.org/). This package tests the overall gene expression for significant association with a given variable. The test gives a unique *P*-value for the whole group, therefore avoiding a multiple testing adjustment (Goeman et al., 2004). If the statistic is significant, the genes in the group are, on average, more associated with the response variable than would be expected. The strength of this association is given by a Z score, calculated for each gene. In this way, at least part of the variance of the response variable can be predicted from the gene expression measurements of the gene set, or vice versa. Raw intensity data were used as input for the package and normalized using the *vsn2* function within R. Association was considered significant with $P < 0.1$, highly significant with $P < 0.01$, and extremely significant with $P < 0.001$. The response variables considered were: 'position' (the position of the fruit within the cluster: central versus lateral), 'weight' (fruit weight, a likely indicator of the fruit developmental stage), 'treatment' (untreated versus treated with BA), and 'destiny' (five classes of abscission potential, as described above). The analysis was performed considering as biological replicates the samples with the same predicted abscission potential (AP). The choice of replicates was done in order to reduce the total number of samples and hybridizations, concurrently taking into account a large part of the variation seen at the biological level (*i.e.* treated/untreated fruits with different dimensions but with the same AP). Four classes were established that are: naturally abscising fruitlets (NA: L1 fruitlets; $AP \approx 90\% < AP < 100\%$), strongly abscising fruitlets (SA: LB1 and LB3 fruitlets; $90\% < AP < 100\%$), probably persisting fruitlets (PP: CB1 fruitlets; $AP < 10\%$), strongly persisting fruitlets (SP: L3, C1, C3 and CB3; $AP \approx 0\%$). Summarizing, 3 biological replicates for NA fruits, 4 for SA fruits, 2 for PP fruits, and 11 for the SP fruits were used (Figure S5).

For hierarchical clustering, raw intensity data were mean-centred, normalized, and clustered by means of Cluster 3.0 software (de Hoon et al., 2004), using the uncentered correlation similarity matrix and the centroid linkage clustering method.

All the experimental procedures comply with MIAME standards for array data (Brazma et al., 2001). Gene expression data have been submitted to ArrayExpress (accession no. A-MEXP-1852).

Subtractive analysis

In order to validate the results of the Globaltest analysis, a subtractive approach was carried out on single-slide data by means of the Nudge package of R (Dean and Raftery, 2005). This method can be used also for non-repeated experiments and was herein applied to estimate genes with significant differential expression according to an all-against-all comparison, using *nudge1* function and posterior probability of at least 0.5. Raw intensity data were used as input for the program, since it already implements a normalization step (*loess* mean normalization, with default parameters). Negative controls spotted on the microarray were used for the false positive discovery. Accordingly, such control genes were correctly identified as non-differentially expressed at the end of the analysis. By means of this approach (Figure S5), it was assessed if the differential expression specifically induced by the BA treatment in L3 fruitlets concerned the same functional networks pointed out by the Globaltest analysis carried out above.

The gene sets differentially expressed during the 4 days time course, when abscission is thought to be induced, were considered (each group was labelled as described in Figure S2), and a series of operations performed, separately for up- and down-regulated genes, as follows:

Genes regulated from T0 to T2: $\{(J - G) - [(J - G) \cap H]\} - \{(V - S) - [(V - S) \cap T]\} = J_{ABS}$

Genes regulated from T2 to T3: $\{(K - H) - [(K - H) \cap G]\} - \{(W - T) - [(W - T) \cap S]\} = K_{ABS}$

In detail, the gene sets G and H were subtracted from J and K, respectively, to remove genes that are naturally regulated during fruitlet development from T0 to T2 (15 to 17 DAPF), and from T2 to T3 (17 to 19 DAPF), respectively. Genes with a delayed regulation, given by $(J - G) \cap H$, and those regulated in advance, given by $(K - H) \cap G$, were further subtracted. At this step, development-independent genes whose regulation was affected by BA from T0 to T2, and from T2 to T3 were obtained. However, among these genes, also those pharmacologically regulated by BA, thus with no specific correlation with abscission, were

included. Therefore, development-independent genes regulated by BA in strongly persisting fruitlets (*i.e.* not directly correlated with abscission) were further subtracted, giving the final sets of development-independent/ BA-regulated/ abscission-related genes differentially expressed from T0 to T2 (J_{ABS}), and T2 to T3 (K_{ABS}).

Quantitative (q)PCR expression analyses

cDNA for expression analyses was synthesized from 2 µg of DNA-free total RNA in a final volume of 25 µL containing 200 Units of MMLV Reverse Transcriptase (Promega, Madison, WI), 1X MMLV Buffer, 25 Units of RNasin (RNase inhibitor, Amersham Biosciences, Piscataway, NJ), 1 µg of Random Hexamers (Invitrogen, Carlsbad, CA) and 2mM dNTPs. The reaction was carried out for 1 h at 37°C in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

Real-time PCR relative quantification was performed in triplicate on two biological replicates, in a total volume of 10 µL using the Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) with 3 pmol of every primer and 2 µL of a 1:10 dilution of cDNA. Primers (Table S6) were designed with Primer3 software version 0.4.0 (http://frodo.wi.mit.edu/primer3/) according to the instructions given by Applied Biosystems. The specificity of amplification was assessed by subsequent subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The reaction mixture was amplified in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions: initial activation step at 95°C for 10 min, 50 cycles including 3 s of denaturation at 95°C, 15 s of annealing/extension at 60°C. After each PCR cycle, a data acquisition step was introduced to record the fluorescent signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product. Data were acquired, elaborated and exported with the StepOne Software v2.1 (Applied Biosystems, Foster City, CA), whereas all the final calculations were carried out with the automated Excel spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001). Besides those found in literature, additional reference genes were selected among those spotted on the microarray according to the criteria of Vandesompele et al. (2002). The genes were *MdUBI*, *Md18S* (Dal Cin et al., 2005), *MdACT* (Li and Yuan, 2008), Md_8283:1:a, and Md_4592:1:a for the cortex, and *Md18S* for the

seed, the latter being sufficiently stable to be used alone. Gene expression values were normalized to the housekeeping genes identified above and reported as arbitrary units (A.U.) of Mean Normalized Expression, using the equation 2 of Q-Gene. The correct size of the amplification products was checked by running each reaction in a 1.5% agarose gel stained with SYBR® Safe (Invitrogen, Carlsbad, CA) and viewed under UV light.

Quantification of carbohydrates and hydrogen peroxide

For carbohydrate measurements, carried out in three biological replicates, 50 mg of frozen flesh powder were extracted in 1.5 mL of 80% ethanol and 20% water, containing 100 mM Hepes-KOH (pH 7.1) and 10 mM MgCl2, for 45 min at 80°C. After cooling at room temperature, the extract was centrifuged at 15,800 g for 5 min. The supernatant, containing soluble sugars (glucose, fructose, sucrose and sorbitol), was either analysed immediately for the sugar content or stored at -20°C until analysis. The pellet, containing starch, was re-suspended and washed, at least four times, with 40 mM acetate buffer (pH 4.5). After washing, the pellet was autoclaved in 1mL of the washing buffer for 45 min at 120 °C to solubilise the starch. After autoclaving, 4 units of α-amylase and 40 units of amyloglucosidase were added to the pellet and the mixture was incubated for 1 h at 50°C to allow complete starch hydrolysis. After starch hydrolysis, the samples were centrifuged and the supernatant was analysed immediately for glucose or stored at -20°C until analysis. Soluble sugars, as well as glucose originated from the starch hydrolysis, were analysed enzymatically as described by Jones et al. (1977) with minor modifications as described by Antognozzi et al. (1996). Sucrose was analysed in sequence after glucose and fructose following the addition of 100 units of invertase to the assay mixture. Sorbitol was measured enzimatically following the reduction of NAD+ coupled to sorbitol oxidation to fructose mediated by sorbitol dehydrogenase. The assay was set up to allow the use of a plate reader. The assay contained 100 mM Bicine pH 9.2, 5 mM MgCl2, 0.01 % (w/v) BSA, 1 mM NAD+, 2U SDH, and the appropriate amount of sample. The carbohydrate extracts were used directly up to 40 µL in the assay without problems. All carbohydrate measurements were performed in dual-wavelength mode (340 - 405 nm) in an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzburg, Austria).

Hydrogen peroxide was quantified by means of the PeroXOquant™ Quantitative Peroxide Assay kit (Pierce, Rockford, IL, USA), following the instructions provided by the

manufacturer. Briefly, 130 mg of fruit cortex were ground to a fine powder in liquid nitrogen, and processed as indicated by the manufacturer in three biological replicates.

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Literature cited

Alferez F, Zhong GY, Burns JK (2007) A citrus abscission agent induces anoxia- and senescence-related gene expression in Arabidopsis. J Exp Bot **58:** 2451-2462

Almagro L, Gómez Ros LV, Belchi-Navarro S, Bru R, Ros Barceló A, Pedreño MA (2008) Class III peroxidases in plant defence reactions. J Exp Bot **60:** 377-390

Angeli D, Comai M, Danesin M, Dorigoni A, Ruperti B, Ramina A (2002) Interazione tra citochinine ed etilene nel controllo del diradamento del melo. Rivista di Frutticoltura e di ortofloricoltura **5:** 56–59

Antognozzi E, Battistelli A, Famiani F, Moscatello S, Stanica F, Tombesi A (1996) Influence of CPPU on carbohydrate accumulation and metabolism in fruits of *Actinidia deliciosa* (A Chev). Sci Hortic **65:** 37–47

Avonce N, Leyman B, Mascorro-Gallardo JO, Van Dijck P, Thevelein JM, Iturriaga G (2004) The Arabidopsis trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signalling. Plant Physiol **136:** 3649–3659

Ay N, Irmler K, Fischer AM, Uhlemann R, Reuter G, Humbeck K (2009) Epigenetic programming via histone methylation at *WRKY53* controls leaf senescence in *Arabidopsis thaliana*. Plant J **58:** 333–346

Bangerth F (2000) Abscission and thinning of young fruit and thier regulation by plant hormones and bioregulators. Plant Growth Regul **31:** 43–59

Bangerth F, Li CJ, Gruber J (2000) Mutual interaction of auxin and cytokinins in regulating correlative dominance. Plant Growth Regul **32**: 205–217

Botton A, Lezzer P, Dorigoni A, Barcaccia G, Ruperti B, Ramina A (2008) Genetic and environmental factors affecting allergen-related gene expression in apple fruit (*Malus domestica* L. Borkh). J Agric Food Chem **56:** 6707–6716

Botton A, Andreotti C, Costa G, Ramina A (2009a) Peach (*Prunus persica* L. Batsch)

allergen-encoding genes are developmentally regulated and affected by fruit load and light radiation. J Agric Food Chem **57:** 724–734

Botton A, Lezzer P, Dorigoni A, Ruperti B, Ramina A (2009b) Environmental factors affecting the expression of apple (*Malus domestica* L. Borkh) allergen-encoding genes. J Hortic Sci Biotech **ISAFRUIT Special Issue:** 182–187

Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FC, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U, Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAME) - toward standards for microarray data. Nat Genet **29:** 365–371

Brenner WG, Romanov GA, Köllmer I, Bürkle L, Schmülling T (2005) Immediate-early and delayed cytokinin response genes of *Arabidopsis thaliana* identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J **44:** 314–333

Buban T (2000) The use of benzyladenine in orchard fruit growing: a mini review. Plant Growth Regul **32:** 381–390

Büttner M, Singh KB (1997) *Arabidopsis thaliana* ethylene-responsive element binding protein (AtEBP), an ethylene-inducible, GCC box DNA-binding protein interacts with an ocs element binding protein. Proc Natl Acad Sci USA **94:** 5961–5966

Chang CS, Li YH, Chen LT, Chen WC, Hsieh WP, Shin J, Jane WN, Chou SJ, Choi G, Hu JM, Somerville S, Wu S (2008) LZF1, a HY5-regulated transcriptional factor, functions in Arabidopsis de-etiolation. Plant J **54:** 205–219

Chen I, Lee S, Pan S, Hsieh H (2007) *GASA4*, a GA-stimulated gene, participates in light signalling in Arabidopsis. Plant Sci **172:** 1062–1071

Chikano H, Ogawa M, Ikeda Y, Koizumi N, Kusano T, Sano H (2001) Two novel genes encoding SNF1-related protein kinases from *Arabidopsis thaliana*: differential accumulation of *AtSR1* and *AtSR2* transcripts in response to cytokinins and sugars, and phosphorylation of sucrose synthase by AtSR2. Mol Gen Genet **264:** 674–681

Choi JW, Kimi GB, Huh YC, Kwon MR, Mok IG, Kim JW, Lee TS, Kim S, Im KH (2004) Cloning of genes differentially expressed during the initial stage of fruit

development in melon (*Cucumis melo* cv. Reticulatus). Mol Cells **17:** 237–241

Chopin F, Orsel M, Dorbe M, Chardon F, Truong H, Miller AJ, Krapp A, Daniel-Vedele F (2007) The Arabidopsis ATNRT2.7 nitrate transporter controls nitrate content in seeds. Plant Cell **19:** 1590–1602

Contento AL, Kim SJ, Bassham DC (2004) Transcriptome profiling of the response of Arabidopsis suspension culture cells to Suc starvation. Plant physiol **135:** 2330–2347

Cosio C, Dunand C (2009) Specific functions of individual class III peroxidase genes. J Exp Bot **60:** 391-408

Couée I, Sulmon C, Gouesbet G, El Amrani A (2006) Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. J Exp Bot **57:** 449–459

Dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A (2005) Ethylene biosynthesis and perception in apple fruitlet abscission (Malus domestica L. Borck). J Exp Bot **56:** 2995–3005

Dal Cin V, Boschetti A, Dorigoni A, Ramina A (2007) Benzylaminopurine application on two different apple cultivars (Malus domestica) displays new and unexpected fruitlet abscission features. Ann Bot (Lond) **99:** 1195–1202

Dal Cin V, Barbaro E, Danesin M, Murayama H, Velasco R, Ramina A (2009a) Fruitlet abscission: A cDNA-AFLP approach to study genes differentially expressed during shedding of immature fruits reveals the involvement of a putative auxin hydrogen symporter in apple (*Malus domestica* L. Borkh). Gene **442:** 26–36

Dal Cin V, Velasco R, Ramina A (2009b) Dominance induction of fruitlet shedding in *Malus* X *domestica* (L. Borkh): molecular changes associated with polar auxin transport. BMC Plant Biol **9:** 139

Datta S, Johansson H, Hettiarachchi C, Irigoyen ML, Desai M, Rubio V, Holm M (2008) LZF1/SALT TOLERANCE HOMOLOG3, an Arabidopsis B-Box protein involved in light-dependent development and gene expression, undergoes COP1-mediated ubiquitination. Plant Cell **20:** 2324

de Hoon MJ, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. Bioinformatics **20:** 1453–1454

de Jong M, Mariani C, Vriezen WH (2009) The role of auxin and gibberellin in tomato

fruit set. J Exp Bot **60:** 1523-1532

Dean N, Raftery AE (2005) Normal uniform mixture differential gene expression detection for cDNA microarrays. BMC Bioinformatics **6:** 173

Desveaux D, Faik A, Maclachlan G (1998) Fucosyltransferase and the biosynthesis of storage and structural xyloglucan in developing nasturtium fruits. Plant Physiol **118:** 885–894

Dong YH, Yao JL, Atkinson RG, Putterill JJ, Morris BA, Gardner RC (2000) *MDH1*: an apple homeobox gene belonging to the BEL1 family. Plant Mol Biol **42:** 623–633

Du L, Poovaiah BW (2004) A novel family of Ca2+/calmodulin-binding proteins involved in transcriptional regulation: interaction with fsh/Ring3 class transcription activators. Plant Mol Biol **54:** 549–569

Espley RV, Hellens RP, Putterill J, Stevenson DE, Kutty-Amma S, Allan AC (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J **49:** 414–427

Fonseca S, Chico JM, Solano R (2009) The jasmonate pathway: the ligand, the receptor and the core signalling module. Curr Opin Plant Biol **12:** 539–547

Frebortova J, Fraaije MW, Galuszka P, Sebela M, Pec P, Hrbac J, Novak O, Bilyeu KD, English JT, Frebort I (2004) Catalytic reaction of cytokinin dehydrogenase: preference for quinones as electron acceptors. Biochem J **380:** 121–130

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LSP, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signalling pathway. Plant J **39:** 863–876

Gillaspy G, Ben-David H, Gruissem W (1993) Fruits: A developmental perspective. Plant Cell **5:** 1439–1451

Gillmor CS, Lukowitz W, Brininstool G, Sedbrook JC, Hamann T, Poindexter P, Somerville C (2005) Glycosylphosphatidylinositol-anchored proteins are required for cell wall synthesis and morphogenesis in Arabidopsis. Plant Cell **17:** 1128-1140

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. Plant Cell **16:** S170–180

Goeman JJ, van de Geer SA, De Kort F, van Houwelingen HC (2004) A global test for

groups of genes: testing association with a clinical outcome. Bioinformatics **20:** 93–99

Goetz M, Vivian-Smith A, Johnson SD, Koltunow AM (2006) AUXIN RESPONSE FACTOR8 is a negative regulator of fruit initiation in Arabidopsis. Plant Cell **18:** 1873–1886

Gomez LD, Baud S, Gilday A, Li Y, Graham IA (2006) Delayed embryo development in the ARABIDOPSIS TREHALOSE-6-PHOSPHATE SYNTHASE 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. Plant J **46:** 69–84

Greene DW, Autio WR, Erf JA, Mao ZY (1992) Mode of action of benzyladenine when used as a chemical thinner on apples. J Am Soc Hortic Sci **117:** 775–779

Ha CM, Jun JH, Nam HG, Fletcher JC (2007) BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell **19:** 1809

Henriksson E, Olsson ASB, Johannesson H, Johansson H, Hanson J, Engstrom P, Soderman E (2005) Homeodomain leucine zipper Class I genes in Arabidopsis. Expression patterns and phylogenetic relationships. Plant Physiol **139:** 509

Herrera-Rodríguez MB, Maldonado JM, Pérez-Vicente R (2004) Light and metabolic regulation of HAS1, HAS1.1 and HAS2, three asparagine synthetase genes in *Helianthus annuus*. Plant Physiol Biochem **42:** 511–518

Hong-Bo S, Zong-Suo L, Ming-An S (2005) LEA proteins in higher plants: structure, function, gene expression and regulation. Colloids Surf B Biointerfaces **45:** 131–135

Hooks MA, Bode K, Couee I (1995) Regulation of Acyl-CoA oxidases in maize seedlings. Phytochemistry **40:** 657–660

Huang S, Sawaki T, Takahashi A, Mizuno S, Takezawa K, Matsumura A, Yokotsuka M, Hirasawa Y, Sonoda M, Nakagawa H, Sato T (2010) Melon EIN3-like transcription factors (CmEIL1 and CmEIL2) are positive regulators of an ethylene- and ripening-induced 1-aminocyclopropane-1-carboxylic acid oxidase gene (*CM-ACO1*). Plant Sci **178:** 251–257

Jensen P, Makalowska I, Altman N, Fazio G, Praul C, Maximova S, Crassweller R, Travis J, Mcnellis T (2009) Rootstock-regulated gene expression patterns in apple tree scions. Tree Genet Genomes **6:** 57–72

Jin JB, Kim YA, Kim SJ, Lee SH, Kim DH, Cheong G, Hwang I (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the trans-golgi network to the central vacuole in Arabidopsis. Plant Cell **13:** 1511

Jones MG, Outlaw WH, Lowry OH (1977) Enzymic assay of 10^{-7} to 10^{-14} moles of sucrose in plant tissues. Plant Physiol **60:** 379–383

Ko J, Yang SH, Han K (2006) Upregulation of an Arabidopsis RING-H2 gene, *XERICO*, confers drought tolerance through increased abscisic acid biosynthesis. Plant J **47:** 343–355

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The Arabidopsis cytochrome P450 *CYP707A* encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. EMBO J **23:** 1647–1656

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI (2003) NADPH oxidase *AtRBOHD* and *AtRBOHF* genes function in ROS-dependent ABA signalling in Arabidopsis. EMBO J **22:** 2623–2633

Lancien M, Roberts MR (2006) Regulation of *Arabidopsis thaliana* 14-3-3 gene expression by gamma-aminobutyric acid. Plant Cell Environ **29:** 1430–1436

Lauvergeat V, Lacomme C, Lacombe E, Lasserre E, Roby D, Grima-Pettenati J (2001) Two cinnamoyl-CoA reductase (*CCR*) genes from *Arabidopsis thaliana* are differentially expressed during development and in response to infection with pathogenic bacteria. Phytochemistry **57:** 1187–1195

Lawrence CJ, Morris NR, Meagher RB, Dawe RK (2001) Dyneins have run their course in plant lineage. Traffic **2:** 362–363

Legendre-Guillemin V, Wasiak S, Hussain NK, Angers A, McPherson PS (2004) ENTH/ANTH proteins and clathrin-mediated membrane budding. J Cell Sci **117:** 9–18

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defence. Plant Cell **15:** 165–178

Matsumura Y, Iwakawa H, Machida Y, Machida C (2009) Characterization of genes in the *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES* (*AS2/LOB*) family in

Arabidopsis thaliana, and functional and molecular comparisons between *AS2* and other family members. Plant J **58:** 525–537

Mazel A, Levine A (2002) Induction of glucosyltransferase transcription and activity during superoxide-dependent cell death in Arabidopsis plants. Plant Physiol Biochem **40:** 133–140

Meng C, Cai C, Zhang T, Guo W (2009) Characterization of six novel NAC genes and their responses to abiotic stresses in *Gossypium hirsutum* L. Plant Science **176:** 352–359

Miao Y, Laun T, Zimmermann P, Zentgraf U (2004) Targets of the WRKY53 transcription factor and its role during leaf senescence in Arabidopsis. Plant Mol Biol **55:** 853–867

Miao Y, Zentgraf U (2007) The antagonist function of Arabidopsis WRKY53 and ESR/ESP in leaf senescence is modulated by the jasmonic and salicylic acid equilibrium. Plant Cell **19:** 819-830

Newcomb RD, Crowhurst RN, Gleave AP, Rikkerink EHA, Allan AC, Beuning LL, Bowen JH, Gera E, Jamieson KR, Janssen BJ, Laing WA, McArtney S, Nain B, Ross GS, Snowden KC, Souleyre EJF, Walton EF, Yauk YK (2006) Analyses of expressed sequence tags from apple. Plant Physiol **141:** 147–166

Nitsch LMC, Oplaat C, Feron R, Ma Q, Wolters-Arts M, Hedden P, Mariani C, Vriezen WH (2009) Abscisic acid levels in tomato ovaries are regulated by *LeNCED1* and *SlCYP707A1*. Planta **229:** 1335–1346

Oñate-Sanchez L, Singh K (2002) Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. Plant Physiol **128:** 1313-1322

Osuna D, Usadel B, Morcuende R, Gibon Y, Bläsing OE, Höhne M, Günter M, Kamlage B, Trethewey R, Scheible WR, Stitt M (2007) Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived Arabidopsis seedlings. Plant J **49:** 463–491

Ozga JA, Reinecke DM (2003) Hormonal interactions in fruit development. J Plant Growth Regul **22:** 73–81

Pandolfini T, Molesini B, Spena A (2007) Molecular dissection of the role of auxin in fruit

initiation. Trends Plant Sci **12:** 327–329

Park S, Sugimoto N, Larson MD, Beaudry R, van Nocker S (2006) Identification of genes with potential roles in apple fruit development and biochemistry through large-scale statistical analysis of expressed sequence tags. Plant Physiol **141**: 811–824

Paul M (2007) Trehalose 6-phosphate. Curr Opin Plant Biol **10:** 303–309

Pedersen L, Henriksen A (2005) Acyl-CoA oxidase 1 from *Arabidopsis thaliana*. Structure of a key enzyme in plant lipid metabolism. J Mol Biol **345:** 487–500

Pertea G, Huang XQ, Liang F, Antonescu V, Sultana R, Karamycheva S, Lee Y, White J, Cheung F, Parvizi B, Tsai J, Quackenbush J (2003) TIGR Gene Indices clustering tools (TGICL): a software system for fast clustering of large EST datasets. Bioinformatics **19:** 651–652

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:** e45

Pitzschke A, Hirt H (2009) Disentangling the complexity of mitogen-activated protein kinases and reactive oxygen species signalling. Plant Physiol **149:** 606–615

Purcell PC, Smith AM, Halford NG (1998) Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. Plant J **14:** 195–202

Ravet K, Touraine B, Boucherez J, Briat JF, Gaymard F, Cellier F (2009) Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. Plant J **57:** 400–412

Ray A, Robinson-Beers K, Ray S, Baker SC, Lang JD, Preuss D, Milligan SB, Gasser CS (1994) Arabidopsis floral homeotic gene *BELL* (*BEL1*) controls ovule development through negative regulation of *AGAMOUS* gene (*AG*). Proc Natl Acad Sci USA **91:** 5761-5765

Remans T, Nacry P, Pervent M, Girin T, Tillard P, Lepetit M, Gojon A (2006) A central role for the nitrate transporter NRT2.1 in the integrated morphological and physiological responses of the root system to nitrogen limitation in Arabidopsis. Plant Physiol **140:** 909

Roitsch T (1999) Source-sink regulation by sugar and stress. Curr Opin Plant Biol **2:** 198–

206

Rook F, Corke F, Baier M, Holman R, May AG, Bevan MW (2006) *Impaired sucrose induction1* encodes a conserved plant-specific protein that couples carbohydrate availability to gene expression and plant growth. Plant J **46**: 1045–1058

Rorat T (2006) Plant dehydrins--tissue location, structure and function. Cell Mol Biol Lett **11:** 536–556

Rose TL, Bonneau L, Der C, Marty-Mazars D, Marty F (2006) Starvation-induced expression of autophagy-related genes in Arabidopsis. Biol Cell **98:** 53–67

Rösti J, Barton CJ, Albrecht S, Dupree P, Pauly M, Findlay K, Roberts K, Seifert GJ (2007) UDP-Glucose 4-Epimerase isoforms UGE2 and UGE4 cooperate in providing UDP-galactose for cell wall biosynthesis and growth of *Arabidopsis thaliana*. Plant Cell **19:** 1565

Ruperti B, Bonghi C, Rasori A, Ramina A, Tonutti P (2001) Characterization and expression of two members of the peach *1-aminocyclopropane-1-carboxylate oxidase* gene family. Physiol Plantarum **111:** 336–344

Sakamoto M, Munemura I, Tomita R, Kobayashi K (2008) Reactive oxygen species in leaf abscission signalling. Plant Signal Behav **3:** 1014-1015

Sanderfoot A, Raikhel N (2003) The secretory system of Arabidopsis. The Arabidopsis Book

Sato K, Mase K, Nakano Y, Nishikubo N, Sugita R, Tsuboi Y, Kajita S, Zhou J, Kitano H, Katayama Y (2006) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase is regulated for the accumulation of polysaccharide-linked hydroxycinnamoyl esters in rice (*Oryza sativa* L.) internode cell walls. Plant Cell Rep **25:** 676–688

Schaffer RJ, Friel EN, Souleyre EJF, Bolitho K, Thodey K, Ledger S, Bowen JH, Ma J, Nain B, Cohen D, Gleave AP, Crowhurst RN, Janssen BJ, Yao J, Newcomb RD (2007) A genomics approach reveals that aroma production in apple is controlled by ethylene predominantly at the final step in each biosynthetic pathway. Plant Physiol **144:** 1899–1912

Schapire AL, Voigt B, Jasik J, Rosado A, Lopez-Cobollo R, Menzel D, Salinas J, Mancuso S, Valpuesta V, Baluska F, Botella MA (2008) Arabidopsis synaptotagmin 1 is required for the maintenance of plasma membrane integrity and

cell viability. Plant Cell **20:** 3374

Schluepmann H, Van Dijken A, Aghdasi M, Wobbes B, Paul M, Smeekens S (2004) Trehalose mediated growth inhibition of Arabidopsis seedlings is due to trehalose-6-phosphate accumulation. Plant Physiol **135:** 879–890

Seidel C, Walz A, Park S, Cohen JD, Ludwig-Müller J (2006) Indole-3-acetic acid protein conjugates: novel players in auxin homeostasis. Plant Biol **8:** 340–345

Sepulveda-Jimenez G, Rueda-Benitez P, Porta H, Rocha-Sosa M (2005) A red beet (*Beta vulgaris*) UDP-glucosyltransferase gene induced by wounding, bacterial infiltration and oxidative stress. J Exp Bot **56:** 605

Sexton R, Roberts J (1982) Cell biology of abscission. Ann Revi Plant Physiol **33:** 133–162

Simon P (2003) Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics **19:** 1439–1440

Singh K, Foley RC, Oñate-Sánchez L (2002) Transcription factors in plant defence and stress responses. Curr Opin Plant Biol **5:** 430–436

Skinner DJ, Hill TA, Gasser CS (2004) Regulation of ovule development. Plant Cell **16:** S32

Smolen GA, Pawlowski L, Wilensky SE, Bender J (2002) Dominant alleles of the basic helix-loop-helix transcription factor ATR2 activate stress-responsive genes in Arabidopsis. Genetics **161:** 1235

Stopar M, Resnik M, Pongrac VZ (2001) Non-structural carbohydrate status and CO2 exchange rate of apple fruitlets at the time of abscission influenced by shade, NAA or BA. Sci Hortic **87:** 65–76

Tamaoki M, Nakajima N, Kubo A, Aono M, Matsuyama T, Saji H (2003) Transcriptome analysis of O3-exposed Arabidopsis reveals that multiple signal pathways act mutually antagonistically to induce gene expression. Plant Mol Biol **53:** 443–456

Tani E, Polidoros AN, Flemetakis E, Stedel C, Kalloniati C, Demetriou K, Katinakis P, Tsaftaris AS (2009) Characterization and expression analysis of *AGAMOUS*-like, *SEEDSTICK*-like, and *SEPALLATA*-like MADS-box genes in peach (*Prunus persica*) fruit. Plant Physiol Biochem **47:** 690–700

Tatsuki M, Hayama H, Nakamura Y (2009) Apple ethylene receptor protein concentrations are affected by ethylene, and differ in cultivars that have different storage life. Planta **230:** 407–417

Taylor JE, Whitelaw CA (2001) Signals in abscission. New Phytol **151:** 323–339

Torres MA, Dangl JL, Jones JD (2002) Arabidopsis gp91phox homologues AtRBOHD and AtRBOHF are required for accumulation of reactive oxygen intermediates in the plant defence response. Proc Natl Acad Sci USA **99:** 517–522

Tran LP, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell **16:** 2481

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A NAC Gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science **314:** 1298–1301

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3:** research0034

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D, Salvi S, Pindo M, Baldi P, Castelletti S, Cavaiuolo M, Coppola G, Costa F, Cova V, Ri AD, Goremykin V, Komjanc M, Longhi S, Magnago P, Malacarne G, Malnoy M, Micheletti D, Moretto M, Perazzolli M, Si-Ammour A, Vezzulli S, Zini E, Eldredge G, Fitzgerald LM, Gutin N, Lanchbury J, Macalma T, Mitchell JT, Reid J, Wardell B, Kodira C, Chen Z, Desany B, Niazi F, Palmer M, Koepke T, Jiwan D, Schaeffer S, Krishnan V, Wu C, Chu VT, King ST, Vick J, Tao Q, Mraz A, Stormo A, Stormo K, Bogden R, Ederle D, Stella A, Vecchietti A, Kater MM, Masiero S, Lasserre P, Lespinasse Y, Allan AC, Bus V, Chagné D, Crowhurst RN, Gleave AP, Lavezzo E, Fawcett JA, Proost S, Rouzé P, Sterck L, Toppo S, Lazzari B, Hellens RP, Durel C, Gutin A, Bumgarner RE, Gardiner SE, Skolnick M, Egholm M, Peer YVd, Salamini F, Viola R (2010) The genome of the domesticated apple (*Malus* x

domestica Borkh.). Nat Genet (published online) **doi:10.1038/ng.654**

Vriezen WH, Feron R, Maretto F, Keijman J, Mariani C (2008) Changes in tomato ovary transcriptome demonstrate complex hormonal regulation of fruit set. New Phytol **177:** 60–76

Wang H, Jones B, Li Z, Frasse P, Delalande C, Regad F, Chaabouni S, Latché A, Pech J, Bouzayen M (2005) The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. Plant Cell **17:** 2676–2692

Watari J, Kobae Y, Yamaki S, Yamada K, Toyofuku K, Tabuchi T, Shiratake K (2004) Identification of sorbitol transporters expressed in the phloem of apple source leaves. Plant Cell Physiol **45:** 1032–1041

Wheeler MCG, Tronconi MA, Drincovich MF, Andreo CS, Flügge U, Maurino VG (2005) A comprehensive analysis of the NADP-malic enzyme gene family of Arabidopsis. Plant Physiol **139:** 39-51

Wingler A, Masclaux-Daubresse C, Fischer AM (2009) Sugars, senescence, and ageing in plants and heterotrophic organisms. J Exp Bot **60:** 1063-1066

Xia X, Wang Y, Zhou Y, Tao Y, Mao W, Shi K, Asami T, Chen Z, Yu J (2009) Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol **150:** 801

Xin Z, Zhao Y, Zheng Z (2005) Transcriptome analysis reveals specific modulation of abscisic acid signalling by ROP10 Small GTPase in Arabidopsis. Plant Physiol **139:** 1350-1365

Yanhui C, Xiaoyuan Y, Kun H, Meihua L, Jigang L, Zhaofeng G, Zhiqiang L, Yunfei Z, Xiaoxiao W, Xiaoming Q, Yunping S, Li Z, Xiaohui D, Jingchu L, Xing-Wang D, Zhangliang C, Hongya G, Li-Jia Q (2006) The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol **60:** 107–124

Yao JL, Dong YH, Kvarnheden A, Morris B (1999) Seven MADS-box genes in apple are expressed in different parts of the fruit. J Am Soc Hortic Sci **124:** 8–13

Zentgraf U, Laun T, Miao Y (2010) The complex regulation of *WRKY53* during leaf senescence of *Arabidopsis thaliana*. Eu J Cell Biol **89:** 133–137

Zhang X, Hu J (2010) The Arabidopsis chloroplast division protein DYNAMIN-

RELATED PROTEIN5B also mediates peroxisome division. Plant Cell **22:** 431

Zhong S, Zhao M, Shi T, Shi H, An F, Zhao Q, Guo H (2009) EIN3/EIL1 cooperate with PIF1 to prevent photo-oxidation and to promote greening of Arabidopsis seedlings. Proc Natl Acad Sci USA **106:** 21431-21436

Supplemental materials and methods - The construction of the microarray

Clustering and assembly pipeline

A dedicated pipeline was set up for clustering and assembling apple sequences for the construction of a 30k microarray (Figure S1). Different parameters were preliminary tested at each step of the clustering process to assess the specificity and reliability of the whole procedure. Only the best final setups are reported below along with a step-by-step description of the bioinformatic tools used to obtain the final temptative contigs.

Malus x *domestica* sequences were downloaded from NCBI Taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi), in the nucleotide EST section, and included 539 mRNAs (complete or partial CDS), and 255,411 ESTs, for a total of 255,950 sequences (sequences were retrieved in January 2009). Database records were filtered for uninformative and/or spurious nucleotides (not ACGTN). In the following step sequences were further cleaned and validated. This process was performed with SeqClean (http://compbio.dfci.harvard.edu/tgi/software/) tool and consisted of i) low-quality and low-complexity sequences trimming, ii) polyA/T tails removal, and iii) vectors/adaptors contamination removal. UniVec_core (http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) was used to recover vectors, adapters, linkers, and primers commonly used to isolate cDNAs. This lead to the selection of a dataset consisting of 252,300 sequences. RepeatMasker (http://www.repeatmasker.org/) tool was used to screen for interspersed repeats and low-complexity sequences. The screening was made using *Malus* x *domestica* libraries and the option '-small' returning repetitive regions in lowercase. A total of 1,445,369 bp were masked, including retroelements, small RNAs and simple repeats. Sequences with stretches of masked nucleotides near the ends (within 35 bp from the end) were trimmed. Only sequences longer than 100 bp were considered for the next steps.

The clustering and assembly phase was accomplished by TGIcl tool (http://compbio.dfci.harvard.edu/tgi/software/), a software using Megablast (Zhang et al., 2000) for the clustering step (not considering lowercase masked nucleotides) and Cap3 (http://dendrome.ucdavis.edu/resources/tooldocs/Cap3/readme.html) for the assembly step

(Huang and Madan, 1999). To this end, the following three parameters were chosen: i) 95% of sequence identity between the overlapping region; ii) 60 bp minimum overlap between 2 sequences; iii) 30 bp maximum number of overhanging, unaligned bases. The assembly step produced 77,040 sequences (26,658 contigs and 50,382 singletons). An additional filtering phase was carried out by developing *ad hoc* Perl scripts to eliminate putative chimera or incorrect clusters, based upon ACE file and quality values produced by CAP3, leading to an increase of the dataset to a total 77,074 sequences, due to the breaking of some clusters. In order to reduce the number of unigenes and remove the redundancy caused by the high-level of stringency, CDhit (Li et al., 2001; Li et al., 2002; Li and Godzik, 2006; http://bioinformatics.ljcrf.edu/cd-hi/) tool was launched to group together different unigenes with a strong overlap (90% identity and 70% coverage of shorter sequence). The longest sequence for each group was considered. This process removed similar sequences, but did not affect the contig reliability, because discarded sequences did not contribute to the contig formation. The resulting dataset (48,045 seqs) was blasted against NCBI UniGene db for *Malus* x *domestica* and the best-hits were collected with high-stringency (90% identity and 80% coverage of UniGene ESTs). The sequences belonging to the same UniGene were grouped, and the longest representative considered, allowing to obtain 41,927 sequences. The transcription orientation was determined by using the relative orientation of the protein best-hit (UniProt). The best-hits were selected according to a minimum 30% identity, 60% similarity and 50% coverage.

Annotation of sequences

Tentative contigs and singletons obtained above were initially annotated with Gene Ontology (GO) terms recovered from the five Uniprot best-hits. Annotations were imported in Blast2GO v2.4.0 (Conesa et al., 2005; Conesa and Goetz, 2008) and further enriched using the built-in tools as described by Botton et al. (2008). In particular, the Annex function (Myhre et al., 2006) was used to increase the total number of annotations. Direct acyclic graphs were traced to recover father GO terms allowing to select the most suitable annotation level for comparisons with other species. Level 2 annotations were finally adopted and compared with those of *Arabidopsis thaliana*, *Vitis vinifera*, *Prunus persica*, and *Populus* publicly available in The Gene Index website (http://compbio.dfci.harvard.edu/tgi/plant.html). Pearson correlation coefficient was

calculated separately for each GO subvocabulary (molecular function, biological process, and cellular component), in order to assess if the relative proportion of GO categories of apple sequences may resemble those of other dicot species.

Microarray design and preparation

A total of 30,419 oligos were designed with OligoArray 2.1 (Rouillard et al., 2002) by adopting parameters compatible with CombiMatrix protocols (http://www.combimatrix.com/support_docs.htm). Sequences for oligo design were chosen among the 41,927 obtained as described above, with priority given to those with annotation and orientation. Chip layout was planned using Layout Designer 4.2.1 CombiMatrix software (CombiMatrix, Mulkiteo, USA) with each probe randomly spotted in triplicate. In addition to standard CombiMatrix negative and quality controls included by default in factory layouts, 21 negative controls were designed on bacterial and viral sequences (*Bacillus anthracis* phage Gamma genome, *Haemophilus ducreyi* genome, *Alteromonas* phage PM2 genome) using the same parameters adopted for apple oligos, and checked for cross-hybridization by BLAST searches against all available apple sequences. Microarrays were prepared on Blank 90K CustomArray chips using a 90K/12K CombiMatrix Synthesizer (CombiMatrix, Mulkiteo, USA) with the standard manufacturing and quality control protocols provided by CombiMatrix.

Supplemental references

Botton A, Galla G, Conesa A, Bachem C, Ramina A, Barcaccia G (2008) Large-scale Gene Ontology analysis of plant transcriptome-derived sequences retrieved by AFLP technology. BMC Genomics **9:** 347

Conesa A, Götz S, García-Gómez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics **21:** 3674–3676

Conesa A, Götz S (2008) Blast2GO: A comprehensive suite for functional analysis in plant genomics. Int J Plant Genomics **2008:** 1-12**Huang X, Madan A** (1999) CAP3: A DNA sequence assembly program. Genome Res **9:** 868–877

Li W, Godzik A (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics **22:** 1658

Li W, Jaroszewski L, Godzik A (2001) Clustering of highly homologous sequences to reduce the size of large protein databases. Bioinformatics **17:** 282

Li W, Jaroszewski L, Godzik A (2002) Tolerating some redundancy significantly speeds up clustering of large protein databases. Bioinformatics **18:** 77

Myhre S, Tveit H, Mollestad T, Laegreid A (2006) Additional gene ontology structure for improved biological reasoning. Bioinformatics **22:** 2020–2027

Rouillard JM, Herbert CJ, Zuker M (2002) OligoArray: genome-scale oligonucleotide design for microarrays. Bioinformatics **18:** 486–487

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol **7:** 203–214

Supplemental tables

Table S1 – Globaltest statistics summarizing the associations between expression data in the cortex and fruitlet destiny.

Gene set	No. Genes	Response variable	T0		T2		T3		Overall	
			P-value	Sign.	P-value	Sign.	P-value	Sign.	P-value	Sign.
Spotted genes	30,419	Position[a]	0.66667	ns	0.17143	ns	0.17143	ns	0.05040	*
		Weight	0.12500	ns	0.36493	ns	0.49528	ns	0.21072	ns
		Treatment[b]	0.75000	ns	0.91429	ns	0.05714	*	0.06500	*
		Destiny[c]	0.25000	ns	0.02857	*	0.01190	*	0.00359	**
Variable	4,361	Position[a]	0.66667	ns	0.14286	ns	0.17143	ns	0.02371	*
		Weight	0.16667	ns	0.29722	ns	0.43178	ns	0.14749	ns
		Treatment[b]	0.75000	ns	0.62857	ns	0.05714	*	0.07296	*
		Destiny[c]	0.25000	ns	0.02143	*	0.00714	**	0.00117	**
ABA	190	Position[a]	0.66667	ns	0.11429	ns	0.08571	*	0.01061	*
		Weight	0.25000	ns	0.26541	ns	0.32293	ns	0.06722	*
		Treatment[b]	0.50000	ns	0.45714	ns	0.14286	ns	0.10849	ns
		Destiny[c]	0.25000	ns	0.00952	**	0.00476	**	0.00045	***
Auxin	313	Position[a]	0.66667	ns	0.11429	ns	0.28571	ns	0.03930	*
		Weight	0.12500	ns	0.39521	ns	0.54183	ns	0.18662	ns
		Treatment[b]	0.50000	ns	0.94286	ns	0.14286	ns	0.12741	ns
		Destiny[c]	0.25000	ns	0.06429	*	0.04048	*	0.00228	**
Cytokinin	70	Position[a]	0.66667	ns	0.14286	ns	0.11429	ns	0.01285	*
		Weight	0.29167	ns	0.19734	ns	0.26819	ns	0.13789	ns
		Treatment[b]	0.50000	ns	0.25714	ns	0.08571	*	0.06448	*
		Destiny[c]	0.25000	ns	0.01429	*	0.00238	**	0.00051	***
Ethylene	165	Position[a]	0.66667	ns	0.20000	ns	0.37143	ns	0.07157	*
		Weight	0.12500	ns	0.37615	ns	0.48997	ns	0.24528	ns
		Treatment[b]	0.75000	ns	0.82857	ns	0.14286	ns	0.14176	ns
		Destiny[c]	0.25000	ns	0.03333	*	0.07381	*	0.00524	**
Gibberellin	72	Position[a]	0.66667	ns	0.02857	*	0.08571	*	0.00504	**
		Weight	0.16667	ns	0.22502	ns	0.29211	ns	0.04685	*
		Treatment[b]	0.75000	ns	0.97143	ns	0.14286	ns	0.23505	ns
		Destiny[c]	0.25000	ns	0.05000	*	0.00238	**	0.00048	***
Minor hormones	152	Position[a]	0.66667	ns	0.34286	ns	0.14286	ns	0.07432	*
		Weight	0.12500	ns	0.56089	ns	0.45846	ns	0.19304	ns
		Treatment[b]	1.00000	ns	0.71429	ns	0.08571	*	0.17825	ns
		Destiny[c]	0.25000	ns	0.40000	ns	0.00714	**	0.01085	*
Transcription factors	977	Position[a]	1.00000	ns	0.17143	ns	0.11429	ns	0.04591	*
		Weight	0.25000	ns	0.34399	ns	0.35985	ns	0.19634	ns
		Treatment[b]	0.75000	ns	0.91429	ns	0.08571	*	0.12823	ns
		Destiny[c]	0.25000	ns	0.03095	*	0.00476	**	0.00191	**

a) Lateral vs central
b) Untreated vs treated with BA
c) Abscising vs persisting
ns=non-significant (P>=0.1)
*=significant (P<0.1)
**=highly significant (P<0.01)
***=extremely significant (P<0.001)

Table S2 – Globaltest statistics summarizing the associations between expression data in the seed and fruitlet destiny.

Gene set	No. Genes	Response variable	T0		T2		T3		Overall	
			P-value	Sign.	P-value	Sign.	P-value	Sign.	P-value	Sign.
Spotted genes	30,419	Position[a]	0.66667	ns	0.45714	ns	0.17143	ns	0.18350	ns
		Weight	0.08333	*	0.12233	ns	0.06858	*	0.00827	**
		Treatment[b]	1.00000	ns	0.11429	ns	0.37143	ns	0.20484	ns
		Destiny[c]	0.50000	ns	0.43571	ns	0.01667	*	0.01170	*
Variable	4,276	Position[a]	0.66667	ns	0.45714	ns	0.20000	ns	0.09562	*
		Weight	0.08333	*	0.08679	*	0.04510	*	0.00084	***
		Treatment[b]	1.00000	ns	0.05714	*	0.40000	ns	0.16630	ns
		Destiny[c]	0.25000	ns	0.36190	ns	0.03333	*	0.00478	**
ABA	190	Position[a]	0.66667	ns	0.42857	ns	0.22857	ns	0.03741	*
		Weight	0.08333	*	0.05896	*	0.07442	*	0.00424	**
		Treatment[b]	1.00000	ns	0.17143	ns	0.31429	ns	0.50632	ns
		Destiny[c]	0.25000	ns	0.69286	ns	0.01905	*	0.00355	**
Auxin	313	Position[a]	1.00000	ns	0.57143	ns	0.14286	ns	0.06846	*
		Weight	0.04167	*	0.07862	*	0.05381	*	0.00148	**
		Treatment[b]	1.00000	ns	0.05714	*	0.31429	ns	0.24623	ns
		Destiny[c]	0.50000	ns	0.47381	ns	0.00952	**	0.00971	**
Cytokinin	70	Position[a]	1.00000	ns	0.82857	ns	0.20000	ns	0.23853	ns
		Weight	0.04167	*	0.08257	*	0.08890	*	0.01813	*
		Treatment[b]	1.00000	ns	0.17143	ns	0.22857	ns	0.34261	ns
		Destiny[c]	0.50000	ns	0.74286	ns	0.01667	*	0.01162	*
Ethylene	165	Position[a]	0.66667	ns	0.51429	ns	0.20000	ns	0.10813	ns
		Weight	0.08333	*	0.07559	*	0.04037	*	0.00173	**
		Treatment[b]	1.00000	ns	0.11429	ns	0.42857	ns	0.43325	ns
		Destiny[c]	0.50000	ns	0.84048	ns	0.03095	*	0.01865	*
Gibberellin	72	Position[a]	1.00000	ns	0.74286	ns	0.20000	ns	0.05382	*
		Weight	0.37500	ns	0.06059	*	0.05542	*	0.00423	**
		Treatment[b]	1.00000	ns	0.11429	ns	0.48571	ns	0.40545	ns
		Destiny[c]	0.25000	ns	0.48095	ns	0.02143	*	0.00260	**
Minor hormones	152	Position[a]	0.66667	ns	0.48571	ns	0.14286	ns	0.05859	*
		Weight	0.08333	*	0.12111	ns	0.04041	*	0.00130	**
		Treatment[b]	1.00000	ns	0.08571	*	0.54286	ns	0.28445	ns
		Destiny[c]	0.25000	ns	0.60952	ns	0.04286	*	0.01077	*
Trans. factors	977	Position[a]	0.66667	ns	0.60000	ns	0.20000	ns	0.11529	ns
		Weight	0.16667	ns	0.12182	ns	0.05369	*	0.00457	**
		Treatment[b]	1.00000	ns	0.05714	*	0.34286	ns	0.27578	ns
		Destiny[c]	0.25000	ns	0.60238	ns	0.01905	*	0.00977	**

a) Lateral vs central
b) Untreated vs treated with BA
c) Abscising vs persisting
ns=non-significant (P>=0.1)
*=significant (P<0.1)
**=highly significant (P<0.01)
***=extremely significant (P<0.001)

Table S3 – Number of DE genes in all the sample combinations with a score ≥0.5 (see materials and methods section for a detailed description of the statistical analyses).

				Lateral fruits										Central fruits									
				CTRL						+BA				CTRL						+BA			
				L1			L3			LB1		LB3		C1			C3			CB1		CB3	
				T0	T2	T3	T0	T2	T3	T2	T3	T2	T3	T0	T2	T3	T0	T2	T3	T2	T3	T2	T3
Lateral fruits	CTRL	L1	T0		188	198	203	456	222	51	32	219	31	492	373	225	242	255	238	225	146	340	192
			T2	97		225	458	336	291	46	10	270	20	385	368	107	479	260	219	436	152	299	229
			T3	181	78		334	282	360	527	13	458	12	267	321	139	383	359	179	242	450	337	326
		L3	T0	116	141	264		65	352	130	40	303	19	62	258	78	77	165	120	111	169	132	115
			T2	178	224	271	56		430	191	41	220	33	205	261	193	56	118	96	110	137	198	167
			T3	514	359	480	38	104		215	35	598	54	193	274	97	305	226	315	293	207	267	379
	+BA	LB1	T2	183	273	151	163	382	338		15	102	27	193	126	122	96	126	119	83	124	90	124
			T3	269	185	299	283	378	547	319		17	783	50	30	59	21	48	93	39	60	29	18
		LB3	T2	362	157	347	52	53	68	29	358		16	253	351	117	295	251	150	138	87	127	235
			T3	278	302	536	31	56	83	428	532	79		30	24	41	8	32	76	38	13	26	1
Central fruits	CTRL	C1	T0	312	579	474	59	136	200	185	432	118	132		170	78	50	105	74	183	98	211	197
			T2	168	141	182	195	217	448	318	324	165	404	207		108	282	228	167	207	123	267	243
			T3	563	322	525	31	62	125	249	643	116	146	151	412		53	94	84	173	55	297	57
		C3	T0	203	204	320	40	123	48	283	493	54	100	84	368	47		107	94	62	44	138	93
			T2	195	435	532	171	107	23	227	554	62	77	255	430	55	56		69	100	62	114	97
			T3	445	485	516	57	184	171	642	605	96	212	259	559	91	145	63		183	80	114	67
	+BA	CB1	T2	360	68	125	47	57	119	43	131	41	81	79	93	123	45	65	169		68	114	105
			T3	188	80	112	400	232	366	293	73	114	306	218	178	299	248	381	466	87		47	16
		CB3	T2	319	138	186	84	88	77	89	316	17	102	123	182	116	61	62	123	40	174		128
			T3	367	345	469	238	155	171	344	641	86	238	238	672	165	142	46	248	152	845	102	

Cortex

Seed

Table S4 – Genes differentially expressed in the cortex of fruitlets that were induced to abscise upon BA treatment.

A temptative annotation (with EC number when available), and the expression pattern is reported for each gene (up_J = up-regulated from T0 to T2; down_J = down-regulated from T0 to T2; up_K = up-regulated from T2 to T3; down_K = down-regulated from T2 to T3).

Gene ID	Temptative annotation (EC)	Molecular function	Exp.
Md_1:85:a	Extensin	Structural constituent of cell wall	up_J
Md_10757:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_10798:1:a	Amino acid permease	Substrate-specific transporter activity	up_J
Md_10973:1:a	Heat shock protein binding protein	Protein binding	down_J
Md_1109:1:a	WRKY53 transcription factor	Nucleic acid binding	up_J
Md_11157:1:a	3'-N-debenzoyl-2'-deoxytaxol N-benzoyltransferase (EC=2.3.1.15)	Transferase activity	up_J
Md_1122:1:a	NAC/NAM transcription factor	Nucleic acid binding	up_J
Md_1133:1:a	LEA/dehydrin	Unknown molecular function	up_J
Md_116911:s	No match found	Unknown molecular function	down_J
Md_117252:s	WRKY53 transcription factor	Nucleic acid binding	up_J
Md_118786:s	Cytochrome P450 (EC=1.14.13.88)	Ion binding, oxidoreductase activity	up_J
Md_11946:1:a	No match found	Unknown molecular function	down_J
Md_119754:s	MYC1 transcription factor	Nucleic acid binding	up_J
Md_12040:1:a	F-box family protein	Protein binding	up_J

Md_121294:s	Ethylene-responsive AP2/ERF transcription factor	Nucleic acid binding	up_J
Md_12188:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_12387:1:a	SNF1-related kinase 3.10 (EC=2.7.11.1)	Ion binding, transferase activity, nucleotide binding, protein binding	up_J
Md_12393:1:a	NAC/NAM transcription factor	Nucleic acid binding	up_J
Md_124:5:a	Repetitive proline-rich cell wall protein	Structural constituent of cell wall	down_K
Md_125:1:a	No match found	Unknown molecular function	up_J
Md_12505:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_1257:1:a	Polyphenol oxidase, chloroplastic (EC=1.10.3.1)	Ion binding, oxidoreductase activity	up_J
Md_126596:s	Ribulose bisphosphate carboxylase large chain (EC=4.1.1.39)	Oxidoreductase activity, lyase activity, transmembrane transporter activity	up_J
Md_128142:s	Aldehyde dehydrogenase family 2 member B7, mitochondrial (EC=1.2.1.3)	Oxidoreductase activity	up_J
Md_128177:s	Extensin	Structural constituent of cell wall	up_J
Md_131178:s	14-3-3-like protein	Protein binding	down_J
Md_132098:s	Pectate lyase (EC=4.2.2.2)	Ion binding, lyase activity, transmembrane transporter activity	up_J
Md_13311:1:a	Uncharacterized protein	Transferase activity	up_K
Md_135689:s	ADP-ribosylation factor, ARF	Nucleotide binding, signal transducer activity	down_J
Md_136185:s	Major allergen Mal d 1.03G	Unknown molecular function	up_J
Md_136858:s	No match found	Unknown molecular function	up_J
Md_137415:s	Amino acid permease	Substrate-specific transporter activity	up_J

Md_13801:s	No match found	Unknown molecular function	down_J
Md_13809:1:a	Uncharacterized protein	Unknown molecular function	down_J
Md_13835:1:a	Uncharacterized protein	Unknown molecular function	down_J
Md_14070:1:a	Gibberellin 2-oxidase (EC=1.14.11.-)	Ion binding, oxidoreductase activity	up_J
Md_140962:s	BRI1-associated receptor kinase 1 (BAK1) (EC=2.7.11.23)	Transferase activity, nucleotide binding	up_J
Md_1412:1:a	FERONIA receptor-like kinase	Transferase activity, nucleotide binding, signal transducer activity	up_J
Md_14276:1:a	No match found	Unknown molecular function	up_J
Md_14497:1:a	Xyloglucan-specific endoglucanase inhibitor protein	Hydrolase activity	up_J
Md_1457:2:a	Sieve element-occluding protein	Unknown molecular function	up_J
Md_153461:s	Uncharacterized protein	Unknown molecular function	up_J
Md_154937:s	Calmodulin binding protein	Protein binding	down_J
Md_15501:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_157343:s	Uncharacterized protein	Nucleotide binding, ligase activity	up_J
Md_160:4:a	Quinone oxidoreductase (EC=1.6.5.5)	Ion binding, oxidoreductase activity	down_K
Md_16050:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_16163:1:a	Protein transport protein Sec23	Ion binding, protein binding	up_J
Md_16256:1:a	Multidrug resistance protein 1, 2 (EC=3.6.3.27, EC=3.6.3.44)	Hydrolase activity, nucleotide binding	up_J
Md_16774:1:a	23S rRNA methyltransferase (EC=2.1.1.-)	Transferase activity, nucleic acid binding	up_J

Md_16774:1:a	23S rRNA methyltransferase (EC=2.1.1.48)	Transferase activity, nucleic acid binding	down_K
Md_167974:s	DNA-directed RNA polymerase subunit beta (EC=2.7.7.6)	Transferase activity, nucleic acid binding	up_J
Md_169133:s	ATPase subunit 8 (EC=3.6.3.14)	Hydrolase activity, substrate-specific transporter activity	up_J
Md_1709:2:a	EIL2 (EIN3-like)	Nucleotide binding, signal transducer activity	up_J
Md_171:2:a	Chloroplast envelope membrane protein	Substrate-specific transporter activity	up_J
Md_17245:1:a	Serine-threonine protein kinase (EC=1.3.1.74)	Transferase activity, nucleotide binding, protein binding	up_J
Md_1741:2:a	Senescence-associated protein	Unknown molecular function	up_J
Md_174442:s	Uncharacterized protein	Structural constituent of ribosome	up_J
Md_17792:1:a	Cytochrome C oxidase subunit (EC=1.9.3.1)	Oxidoreductase activity, substrate-specific transporter activity	up_J
Md_179063:s	Dynamin	Hydrolase activity, nucleotide binding, lipid binding	up_J
Md_1791:1:a	DNA-directed RNA polymerase subunit beta (EC=2.7.7.6)	Transferase activity, nucleic acid binding	up_J
Md_179488:s	Transcription factor	Hydrolase activity, protein binding, nucleic acid binding	up_J
Md_18018:1:a	No match found	Unknown molecular function	up_J
Md_182062:s	No match found	Unknown molecular function	up_J
Md_18337:1:a	NADH-ubiquinone oxidoreductase chain 1 (EC=1.6.5.3)	Oxidoreductase activity	up_J
Md_18421:1:a	Uncharacterized protein	Ion binding, hydrolase activity, nucleotide binding, substrate-specific transporter activity	down_J
Md_185136:s	Uracil phosphoribosyltransferase (EC=2.4.2.9)	Transferase activity, nucleotide binding	up_J
Md_18993:1:a	Disease resistance response protein	Unknown molecular function	down_J

Md_19496:1:a	Zinc finger DHHC domain-containing protein At3g22180	Ion binding, transferase activity	up_J
Md_19664:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_19747:1:a	Laccase (EC=1.10.3.3)	Ion binding, oxidoreductase activity	up_J
Md_19796:1:a	Uncharacterized protein	Signal transducer activity	up_J
Md_2007:2:a	Cytochrome P450 (EC=1.14.13.-)	Ion binding, oxidoreductase activity	up_J
Md_2007:4:a	Cytochrome P450 (EC=1.14.13.21)	Ion binding, oxidoreductase activity	up_J
Md_200958:s	Zinc-finger C2H2 protein SERRATE	Ion binding	up_J
Md_201734:s	Nucleic acid binding protein	Nucleotide binding, nucleic acid binding	up_J
Md_20223:1:a	Xylan endohydrolase (EC=3.2.1.8)	Ion binding, hydrolase activity	up_J
Md_203119:s	Ceramidase (EC=3.5.1.23)	Hydrolase activity	up_J
Md_20392:1:a	Anthocyanin 5-aromatic acyltransferase (EC=2.3.1.153)	Transferase activity	down_J
Md_20453:1:a	Aspartic proteinase nepenthesin (EC=3.4.23.12)	Hydrolase activity, nucleic acid binding	up_J
Md_20585:1:a	Cleavage and polyadenylation specificity factor, 73 kDa subunit	Hydrolase activity, protein binding	up_J
Md_207445:s	Peroxisomal membrane ABC transporter family	Hydrolase activity, nucleotide binding	up_J
Md_210798:s	No match found	Unknown molecular function	down_J
Md_2112:1:a	Nucleic acid binding protein	Nucleotide binding, nucleic acid binding	up_J
Md_212068:s	Uncharacterized protein	Unknown molecular function	down_J
Md_214104:s	Gibberellin 2-oxidase (EC=1.14.11.-)	Ion binding, oxidoreductase activity	up_J

Md_214706:s	Kinase (EC=2.7.10.2)	Ion binding, transferase activity, oxidoreductase activity, nucleotide binding	up_J
Md_219419:s	No match found	Unknown molecular function	up_J
Md_219650:s	No match found	Unknown molecular function	down_J
Md_22:8:a	Hydrolase, hydrolyzing O-glycosyl compounds (EC=2.4.1.67)	Hydrolase activity	up_J
Md_225721:s	ATP binding protein	Hydrolase activity, nucleotide binding	up_J
Md_2258:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_226587:s	TIP GROWTH DEFECTIVE 1	Ion binding, transferase activity, acyl binding	up_J
Md_2280:1:0:b	Leucoanthocyanidin dioxygenase (EC=1.14.11.19)	Ion binding, oxidoreductase activity	up_J
Md_232357:s	Uncharacterized protein	Ion binding, transferase activity, nucleotide binding, nucleic acid binding	up_K
Md_2328:2:a	ATP sulfurylase (EC=2.7.7.4)	Transferase activity	up_J
Md_2343:1:a	Carboxylesterase (EC=3.1.1.1)	Hydrolase activity	up_J
Md_235374:s	No match found	Unknown molecular function	up_J
Md_240669:s	Ubiquitin-protein ligase (EC=6.3.2.19)	Ligase activity	up_J
Md_241340:s	No match found	Unknown molecular function	down_J
Md_241578:s	Fructose-1,6-bisphosphatase, chloroplastic (EC=3.1.3.11)	Ion binding, hydrolase activity	down_J
Md_242520:s	Fimbrin	Protein binding	up_J
Md_246922:s	Terpene synthase	Ion binding, lyase activity, transmembrane transporter activity	up_J
Md_246936:s	Mitogen-activated protein kinase (MAPK) (EC=2.7.11.24)	Transferase activity, nucleotide binding, signal transducer activity	up_J

Md_249698:s	Ethylene-responsive AP2/ERF transcription factor	Nucleic acid binding	up_J
Md_25179:s	Gibberellin 2-oxidase (EC=1.14.11.-)	Ion binding, oxidoreductase activity	up_J
Md_253006:s	AMP-activated protein kinase, gamma regulatory subunit (EC=2.7.11.3)	Transferase activity	up_J
Md_255371:s	26S protease regulatory subunit 7	Hydrolase activity, nucleotide binding	down_J
Md_2556:1:a	GASA4-like protein	Unknown molecular function	down_J
Md_2568:2:a	Leucoanthocyanidin dioxygenase (EC=1.14.11.19)	Ion binding, oxidoreductase activity	up_J
Md_2575:1:a	MdMYB6 transcription factor	Nucleic acid binding	up_J
Md_2582:1:a	Lipoxygenase (EC=1.13.11.12)	Ion binding, oxidoreductase activity	up_J
Md_259:7:a	Uncharacterized protein	Hydrolase activity	up_J
Md_2594:1:0:b	Vacuolar acid invertase (EC=3.2.1.26)	Hydrolase activity	down_J
Md_2750:1:a	LEA/dehydrin	Unknown molecular function	up_J
Md_27611:s	Initiation factor eIF-4 gamma, middle	Protein binding, nucleic acid binding	up_J
Md_2788:1:a	No match found	Unknown molecular function	down_J
Md_2827:2:a	Cryptochrome 1	Lyase activity, transmembrane transporter activity	up_J
Md_288:2:a	Ferritin	Ion binding, oxidoreductase activity	up_J
Md_2896:1:0:b	Uncharacterized protein	Unknown molecular function	up_J
Md_294:2:a	NADH dehydrogenase ND 4 subunit (EC=1.6.5.3)	Oxidoreductase activity, cofactor binding, tetrapyrrole binding	up_J
Md_2976:1:a	E6-1 protein kinase (EC=2.7.1.-)	Transferase activity	down_J

Md_3129:1:a	No match found	Unknown molecular function	up_J
Md_3190:1:a	3-ketoacyl-CoA thiolase B (EC=2.3.1.16)	Transferase activity	up_J
Md_3203:1:a	ATP binding protein	Transferase activity, nucleotide binding, protein binding	up_J
Md_323:1:a	Ripening-induced protein 1	Structural constituent of cell wall	up_J
Md_323:2:a	Proline-rich cell wall protein	Structural constituent of cell wall	up_J
Md_33654:s	Endoglucanase (EC=3.2.1.4)	Hydrolase activity, carbohydrate binding	up_J
Md_34857:s	Uncharacterized protein	Nucleic acid binding	up_J
Md_3600:3:a	PsbP domain-containing protein 1, chloroplastic	Ion binding	down_J
Md_3669:1:a	Hydrolase, hydrolyzing O-glycosyl compounds (EC=2.4.1.67)	Hydrolase activity	up_J
Md_370:s	Glycosyl hydrolase (EC=3.2.1.-)	Hydrolase activity	up_J
Md_373:3:a	UDP-glucose glucosyltransferase (EC=2.4.1.11)	Transferase activity	up_J
Md_3747:1:a	Protein SSM1	Hydrolase activity	up_J
Md_382:1:a	Cysteine synthase (EC=2.5.1.47)	Transferase activity, lyase activity, transmembrane transporter activity, cofactor binding, tetrapyrrole binding, lipid binding, vitamin binding	up_K
Md_3836:1:a	Polyneuridine-aldehyde esterase (EC=3.1.1.78)	Hydrolase activity	up_J
Md_3896:1:0:b	WRKY4 transcription factor	Nucleic acid binding	up_J
Md_403:5:a	Glycine-rich protein	Structural constituent of cell wall	down_J
Md_4050:1:a	No match found	Unknown molecular function	up_J
Md_40605:s	Ethylene-responsive AP2/ERF transcription factor	Nucleic acid binding	up_J

Md_40927:s	NB-ARC domain-containing protein	Hydrolase activity, nucleotide binding, protein binding	up_J
Md_41108:s	bHLH transcription factor	Nucleotide binding, nucleic acid binding	up_J
Md_419:3:a	Cell wall-plasma membrane linker protein	Structural constituent of cell wall	up_J
Md_4328:1:a	Alpha-glucosidase (EC=3.2.1.20)	Hydrolase activity	up_J
Md_4363:1:a	Uncharacterized protein	Transferase activity, isomerase activity	up_J
Md_4451:1:a	Jasmonate induced protein	Unknown molecular function	down_K
Md_4451:1:a	Jasmonate induced protein	Unknown molecular function	up_J
Md_48:1:a	Aldehyde dehydrogenase, RF2 (EC=1.2.1.3)	Hydrolase activity, nucleotide binding	up_J
Md_4837:1:a	No match found	Unknown molecular function	down_J
Md_4900:1:a	Receptor protein kinase, CLAVATA1 (EC=1.3.1.74, EC=2.7.10.1)	Hydrolase activity, transferase activity, nucleotide binding, protein binding, signal transducer activity	up_J
Md_4972:1:a	Fructose-bisphosphate aldolase (EC=4.1.2.13)	Lyase activity, transmembrane transporter activity	up_J
Md_50213:s	Chromatin remodeling complex subunit	Hydrolase activity, nucleotide binding, nucleic acid binding	up_J
Md_5069:1:a	Uncharacterized protein	Ion binding, lyase activity, transmembrane transporter activity	down_K
Md_50991:s	Anthranilate N-hydroxycinnamoyl/benzoyltransferase (EC=2.3.1.144)	Transferase activity	up_J
Md_51:1:a	No match found	Unknown molecular function	up_J
Md_5321:1:a	Uncharacterized protein At5g11550	Protein binding	down_J
Md_5375:1:a	Respiratory burst NADPH-oxidase (EC=1.6.99.-)	Ion binding, oxidoreductase activity, nucleotide binding, cofactor binding, tetrapyrrole binding	up_J
Md_5413:2:a	Ankyrin repeat-containing protein	Unknown molecular function	up_J

Md_5550:1:a	IAA-amino acid hydrolase, ILR1 (EC=3.5.1.32)	Hydrolase activity, protein binding	up_J
Md_564:1:a	Cadmium induced protein CdI19	Ion binding	up_J
Md_5687:1:a	Uncharacterized protein	Hydrolase activity, protein binding	up_J
Md_5724:1:a	Zinc finger protein CONSTANS-LIKE 5	Ion binding	down_J
Md_57702:s	Uncharacterized protein At5g05840	Unknown molecular function	down_J
Md_5793:1:0:b	Mitogen-activated protein kinase kinase (MAPKK) (EC=2.7.11.25)	Transferase activity, nucleotide binding, protein binding, kinase regulator activity, enzyme activator activity	up_J
Md_6:53:a	Endonuclease/exonuclease/phosphatase	Hydrolase activity	up_J
Md_60011:s	No match found	Unknown molecular function	down_J
Md_60263:s	Metal ion binding protein	Ion binding	up_J
Md_6068:1:a	No match found	Unknown molecular function	up_J
Md_6098:1:a	No match found	Unknown molecular function	up_J
Md_612:1:a	Beta-galactosidase (EC=3.2.1.23)	Ion binding, hydrolase activity, carbohydrate binding	up_J
Md_6142:1:a	Subtilisin-like protease (EC=3.4.21)	Ion binding, hydrolase activity, protein binding	up_J
Md_6192:1:a	Omega-3 fatty acid desaturase (EC=1.14.99.-)	Oxidoreductase activity	up_J
Md_6231:1:a	Microtubule-associated protein, TORTIFOLIA1	Protein binding	up_J
Md_6240:1:a	Zinc finger homeodomain protein SZF-HD1	Nucleic acid binding	down_J
Md_6435:1:a	Uncharacterized protein	Signal transducer activity	up_J
Md_6531:1:a	MdMYB8 transcription factor	Nucleic acid binding	up_J

Md_66:2:a	Carbonic anhydrase, chloroplastic (EC=4.2.1.1)	Ion binding, lyase activity, transmembrane transporter activity	up_J
Md_66411:s	Ubiquitin-protein ligase (EC=6.3.2.19)	Protein binding, ligase activity	up_J
Md_6659:1:a	Synaptotagmin	Lipid binding	up_J
Md_6703:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_67394:s	Peroxidase (EC=1.11.1.7)	Ion binding, oxidoreductase activity	up_J
Md_674:s	Plasma membrane H+ ATPase (EC=3.6.1.3)	Ion binding, hydrolase activity, nucleotide binding, substrate-specific transporter activity	up_J
Md_6808:1:a	UDP-glucose glucosyltransferase (EC=2.4.1.11)	Transferase activity	up_J
Md_69440:s	SGT1-like protein	Protein binding	down_J
Md_6974:1:0:b	Uncharacterized protein	Unknown molecular function	up_J
Md_6997:1:0:b	Uncharacterized protein	Unknown molecular function	down_J
Md_7045:1:a	Gibberellin 2-oxidase (EC=1.14.11.-)	Ion binding, oxidoreductase activity	up_J
Md_7112:2:a	WRKY19 transcription factor	Hydrolase activity, protein binding, nucleic acid binding	up_J
Md_71285:s	Myo-inositol oxygenase (EC=1.13.99.1)	Ion binding, oxidoreductase activity	up_J
Md_7162:1:a	WD-repeat protein (EC=2.7.11.7)	Unknown molecular function	up_J
Md_7242:1:a	No match found	Unknown molecular function	up_J
Md_7330:1:a	Oligopeptide transporter	Substrate-specific transporter activity	up_J
Md_7412:1:a	Chloroplast-targeted copper chaperone	Ion binding	up_J
Md_74377:s	Cytokinin dehydrogenase (EC=1.5.99.12)	Oxidoreductase activity, nucleotide binding, cofactor binding, tetrapyrrole binding	up_J

Md_74434:s	Myo-inositol oxygenase (EC=1.13.99.1)	Ion binding, oxidoreductase activity	up_J
Md_75:5:a	Class III chitinase (EC=3.2.1.14)	Ion binding, hydrolase activity	up_J
Md_7571:1:a	Coatomer beta subunit (EC=2.7.11.7)	Protein binding	up_J
Md_7638:1:a	Uncharacterized protein At1g21390	Unknown molecular function	up_J
Md_770:2:a	Uncharacterized protein	Transferase activity, nucleotide binding, structural constituent of cell wall	down_J
Md_77189:s	Uncharacterized protein	Oxidoreductase activity	up_J
Md_77513:s	Uncharacterized protein	Unknown molecular function	up_J
Md_78298:s	L-aspartate oxidase (EC=1.4.3.16)	Oxidoreductase activity	up_J
Md_79388:s	Ribulose bisphosphate carboxylase small chain (EC=4.1.1.39)	Oxidoreductase activity, lyase activity, transmembrane transporter activity	up_J
Md_8:16:a	Polyphenol oxidase, chloroplastic (EC=1.10.3.1)	Ion binding, oxidoreductase activity	up_J
Md_81431:s	Uncharacterized protein At5g23890	Unknown molecular function	up_J
Md_8451:1:a	UDP-glucuronosyltransferase (EC=2.4.1.115)	Transferase activity	up_J
Md_8531:1:a	30S ribosomal protein S15, chloroplastic	Structural constituent of ribosome	up_J
Md_8558:1:a	Clathrin assembly protein At2g25430	Protein binding, lipid binding	up_J
Md_869:1:a	Malate dehydrogenase, malic enzyme (EC=1.1.1.40)	Ion binding, oxidoreductase activity, cofactor binding, tetrapyrrole binding	up_J
Md_8693:1:a	Chitin-inducible protein	Unknown molecular function	up_J
Md_8742:1:a	No match found	Unknown molecular function	up_J
Md_8837:1:a	Uncharacterized protein	Unknown molecular function	up_J

Md_8959:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_9063:1:a	Phosphoenolpyruvate carboxylase (EC=1.3.1.74)	Lyase activity, transmembrane transporter activity	up_J
Md_9130:1:a	Leucine-rich repeat receptor-like kinase	Transferase activity, nucleotide binding, protein binding, signal transducer activity	up_J
Md_9183:1:a	Uncharacterized protein	Unknown molecular function	up_J
Md_92075:s	Uncharacterized protein At1g09290	Unknown molecular function	up_J
Md_92669:s	Ribulose bisphosphate carboxylase small chain (EC=4.1.1.39)	Oxidoreductase activity, lyase activity, transmembrane transporter activity	down_J
Md_93:4:a	14-3-3-like protein	Protein binding	down_J
Md_93451:s	ATP-binding cassette transporter (EC=3.6.3.28)	Hydrolase activity, nucleotide binding	down_J
Md_9390:1:a	Auxin response factor 3 (ARF3)	Nucleic acid binding	up_J
Md_93992:s	Uncharacterized protein	Unknown molecular function	up_K
Md_94413:s	Arabidopsis Mei2-like 1, AML1	Nucleotide binding, nucleic acid binding	up_J
Md_9554:1:a	(-)-germacrene D synthase (EC=4.2.3.22)	Ion binding, lyase activity, transmembrane transporter activity	down_K
Md_9554:1:a	Sesquiterpene synthase (EC=4.2.3.22)	Ion binding, lyase activity, transmembrane transporter activity	up_J
Md_9662:1:a	Sucrose synthase (EC= 2.4.1.13)	Transferase activity	up_J
Md_9772:1:a	ATP binding protein	Transferase activity, nucleotide binding, protein binding	up_J
Md_9928:1:a	ANP32/acidic nuclear phosphoprotein	Protein binding	up_J

Table S5 – Genes differentially expressed in the seed of fruitlets that were induced to abscise upon BA treatment.

A temptative annotation (with EC number when available), and the expression pattern is reported for each gene (up_J = up-regulated from T0 to T2; down_J = down-regulated from T0 to T2; up_K = up-regulated from T2 to T3; down_K = down-regulated from T2 to T3).

Gene_ID	Temptative annotation (EC)	Molecular function	Exp.
Md_10154:1:a	1-acyl-sn-glycerol-3-phosphate acyltransferase (EC=2.3.1.51)	Transferase activity	down_K
Md_1024:1:a	Polyphenol oxidase, chloroplast (EC=1.10.3.1)	Ion binding, oxidoreductase activity	down_J
Md_102479:s	No match found	Unknown molecular function	up_K
Md_103049:s	Uncharacterized protein	Protein binding	up_K
Md_105836:s	Amino acid transporter	Transmembrane transporter activity, substrate-specific transporter activity	up_J
Md_10661:1:a	No match found	Unknown molecular function	up_J
Md_114537:s	Homeobox-leucine zipper protein ATHB-40	Nucleic acid binding	up_K
Md_11646:1:a	Protein synthesis inhibitor, DPH2	Unknown molecular function	up_J
Md_12112:1:a	No match found	Unknown molecular function	up_J
Md_1225:1:a	Glycerol-3-phosphate acyltransferase (EC=2.3.1.15)	Transferase activity	up_K
Md_124360:s	H+-translocating pyrophosphatase (EC=3.6.1.1)	Hydrolase activity, ion binding, transmembrane transporter activity, substrate-specific transporter activity	up_J
Md_125286:s	Heat shock protein 70-2	Nucleotide binding	down_J

Md_1263:1:a	Metal ion binding protein	Ion binding	down_J
Md_132121:s	Probable inositol transporter	Transmembrane transporter activity, substrate-specific transporter activity	down_K
Md_134601:s	Hydrolase, hydrolyzing O-glycosyl compounds (EC=3.1.1.-)	Hydrolase activity, ion binding	down_J
Md_13925:1:a	Subtilisin	Hydrolase activity, protein binding	up_K
Md_14561:s	Uncharacterized protein	Unknown molecular function	up_J
Md_150042:s	Septum site-determining protein minD	Hydrolase activity, protein binding	down_J
Md_150042:s	Septum site-determining protein minD	Hydrolase activity, protein binding	up_K
Md_15425:1:a	2-oxoglutarate/malate translocator	Transmembrane transporter activity, substrate-specific transporter activity	up_J
Md_15698:1:a	Uncharacterized protein	Ion binding, nucleic acid binding	up_J
Md_160835:s	Uncharacterized protein	Unknown molecular function	up_K
Md_176079:s	Nucleoid chloroplast DNA-binding protein	Hydrolase activity, nucleic acid binding	up_J
Md_176335:s	Uncharacterized protein	Ion binding	up_K
Md_177051:s	Uncharacterized protein	Transferase activity	up_K
Md_17879:1:a	Uncharacterized protein	Unknown molecular function	down_J
Md_17879:1:a	Uncharacterized protein	Unknown molecular function	up_K
Md_1844:2:a	ALG2-interacting protein	Protein binding	up_K

Md_1867:1:a	No match found	Unknown molecular function	up_K
Md_19486:1:a	Bel1 homeotic protein	Nucleic acid binding	up_K
Md_20016:1:a	Uncharacterized protein	Structural constituent of cell wall	up_J
Md_20453:1:a	Aspartic proteinase, nepenthesin-1 (EC=3.4.23.6)	Hydrolase activity, nucleic acid binding	up_K
Md_20513:1:a	DEAD-box ATP-dependent RNA helicase (EC=3.6.1.-)	Hydrolase activity, nucleotide binding, nucleic acid binding	up_K
Md_20585:1:a	Cleavage and polyadenylation specificity factor, 73 kDa subunit	Hydrolase activity, protein binding	up_J
Md_219638:s	Uncharacterized protein	Transferase activity, nucleotide binding	down_J
Md_219638:s	Uncharacterized protein	Transferase activity, nucleotide binding	up_K
Md_219708:s	No match found	Unknown molecular function	up_J
Md_220544:s	No match found	Unknown molecular function	up_K
Md_237503:s	One helix protein	Unknown molecular function	up_J
Md_238804:s	Uncharacterized protein	Hydrolase activity, transferase activity, nucleotide binding, structural constituent of cell wall, signal transducer activity, enzyme inhibitor activity	up_K
Md_241578:s	Fructose-1,6-bisphosphatase, chloroplastic (EC=3.1.3.11)	Hydrolase activity, ion binding	up_J
Md_248247:s	Serine/threonine-protein kinase (EC=2.7.11.17)	Hydrolase activity, transferase activity, nucleotide binding, protein binding	up_J
Md_288:2:a	Ferritin (EC=1.16.3.1)	Ion binding, oxidoreductase activity	up_K
Md_2943:1:a	Uncharacterized protein	Unknown molecular function	up_K

Md_3290:2:a	MADS-box transcription factor, STK/AGL11	Nucleic acid binding	down_J
Md_3329:1:a	Ethylene responsive AP2/ERF transcription factor	Nucleic acid binding	up_K
Md_4091:1:a	Uncharacterized protein	Transferase activity, ion binding	up_K
Md_4146:1:a	Dynein	Hydrolase activity	up_K
Md_4628:1:a	High affinity nitrate transporter	Transmembrane transporter activity, substrate-specific transporter activity	up_K
Md_4950:1:a	F-box family protein	Protein binding	up_K
Md_4981:1:a	No match found	Unknown molecular function	up_K
Md_5177:1:a	Purple acid phosphatase (EC=3.1.3.2)	Hydrolase activity	up_K
Md_5177:1:a	Purple acid phosphatase (EC=3.1.4.46)	Hydrolase activity	down_J
Md_5416:1:a	Probable glycerophosphoryl diester phosphodiesterase	Hydrolase activity, transferase activity, nucleotide binding	up_J
Md_556:2:a	Uncharacterized protein	Unknown molecular function	up_K
Md_570:2:a	Dehydrin, ABA responsive	Unknown molecular function	up_K
Md_59405:s	NADH-Monodehydroascorbate reductase (EC=1.6.5.4)	Nucleotide binding, oxidoreductase activity, cofactor binding	up_K
Md_60462:s	Inositol polyphosphate multikinase alpha (EC=2.7.1.140)	Transferase activity	up_J
Md_618:1:a	Hydrolase, hydrolyzing O-glycosyl compounds (EC=3.1.1.-)	Hydrolase activity, ion binding	down_J
Md_74377:s	Cytokinin dehydrogenase (EC=1.5.92.12)	Nucleotide binding, oxidoreductase activity, cofactor binding	up_K

Md_770:1:a	Proline-rich cell wall protein	Transferase activity, nucleotide binding, structural constituent of cell wall	up_K
Md_78200:s	CC-NBS-LRR resistance protein	Nucleotide binding, protein binding	up_K
Md_7948:1:a	Uncharacterized protein	Unknown molecular function	down_J
Md_7974:1:a	Uncharacterized protein	Transferase activity, nucleic acid binding	up_K
Md_82931:s	Laccase (EC=1.10.3.2)	Ion binding, oxidoreductase activity	up_K
Md_843:4:a	Ubiquitin carboxyl-terminal hydrolase (EC=3.1.2.15)	Hydrolase activity	down_J
Md_843:4:a	Ubiquitin carboxyl-terminal hydrolase (EC=3.1.2.15)	Hydrolase activity	up_K
Md_89293:s	Uncharacterized protein	Unknown molecular function	up_K
Md_9315:1:a	Galactinol synthase (EC=2.4.1.123)	Transferase activity	up_K
Md_9430:1:a	Uncharacterized protein	Unknown molecular function	up_K
Md_9597:1:a	ATP synthase subunit beta (EC=3.6.3.14)	Hydrolase activity, nucleotide binding, transmembrane transporter activity, substrate-specific transporter activity	down_J
Md_9597:1:a	ATP synthase subunit beta (EC=3.6.3.14)	Hydrolase activity, nucleotide binding, transmembrane transporter activity, substrate-specific transporter activity	up_K
Md_965:2:a	Uncharacterized protein	Hydrolase activity	up_K
Md_9712:1:a	No match found	Unknown molecular function	up_K
Md_97343:s	Ribosomal protein L19	Structural constituent of ribosome	up_K
Md_98050:s	Alpha/beta fold family protein hydrolase	Hydrolase activity	up_J

Table S6 – Primers used in qPCR experiments given in 5'-3' orientation.

The last five genes were used as reference for qPCR normalization in the cortex (indicated by asterisks). As far as the seed, only *Md18S* was used as a reference (indicated by two asterisks).

Gene (Array ID)	Forward primer	Reverse primer	Reference
MdACO1	CAGTCGGATGGGACCAGAA	GCTTGGAATTTCAGGCCAGA	Dal Cin et al., 2005
MdACS5B	CACATGCGCTTCACAATG	CTTGAGCAGCCTACGCAA	Dal Cin et al., 2005
ERS2 (Md_247145:s)	ATTGCCTGTTCATCCAACTT	TTGTCGATACCCTCGCTTTC	-
HB40 (Md_114537:s)	AAGGGCTCGTTGGAAGAACA	GCCTCCGACAGTTGATCCTT	-
MADS10 (Md_3290:2:a)	TGGGTCAGAGATGAATGCAA	TGGCTAAAGAAATGGCGAGAA	-
AP2/ERF (Md_3329:1:a)	CAAAGTGTGGAGGTTTCGGTA	CCACCCTTTGGTCAACACG	-
ACTIN3 (Md_507:4:a)	ATCCCTCAGCACCTTCCAAC	AGAAGCATTTCCTGTGAACAA	-
TPS (Md_15339:1:a)	GCGAATGAGCCCGTAGTTGT	CGCATTCCCATTACCAATCA	-
MYB8 (Md_6531:1:a)	GGCATCTCCGAATCGACAC	GGCTGCGAAATGGTATGGTT	-
JAI (Md_4451:1:a)	CCACTTCAAACCGCAGCAC	CTGTTATTCCCGCCATGAGT	-
LEA/DEH2 (Md_570:2:a)	TGGCGGTGCTACTACAGGTG	CTGCCGTAATCGTGACCAGT	-
LEA/DEH (Md_425:1:a)	GACCCACACGTATTCCACGA	TGCGGACATCTGGTGAGTTC	-
*MdUBI**	CATCCCCCCAGACCAGCAGA	ACCACGGAGACGCAACACCAA	Dal Cin et al., 2005
*Md18S***	GTTACTTTTAGGACTCCGCC	TTCCTTTAAGTTTCAGCCTTG	Dal Cin et al., 2005

*MdACT**	TGACCGAATGAGCAAGGAAATTACT	TACTCAGCTTTGGCAATCCACATC	Li and Yuan, 2008
Md_8283:1:a*	CTCGTCGTCTTGTTCCCTGA	GCCTAAGGACAGGTGGTCTATG	-
Md_4592:1:a*	GTCGAAATGGTCAGCGGTAG	GCAATGGCAAACTCCACCTT	-

Supplemental figures

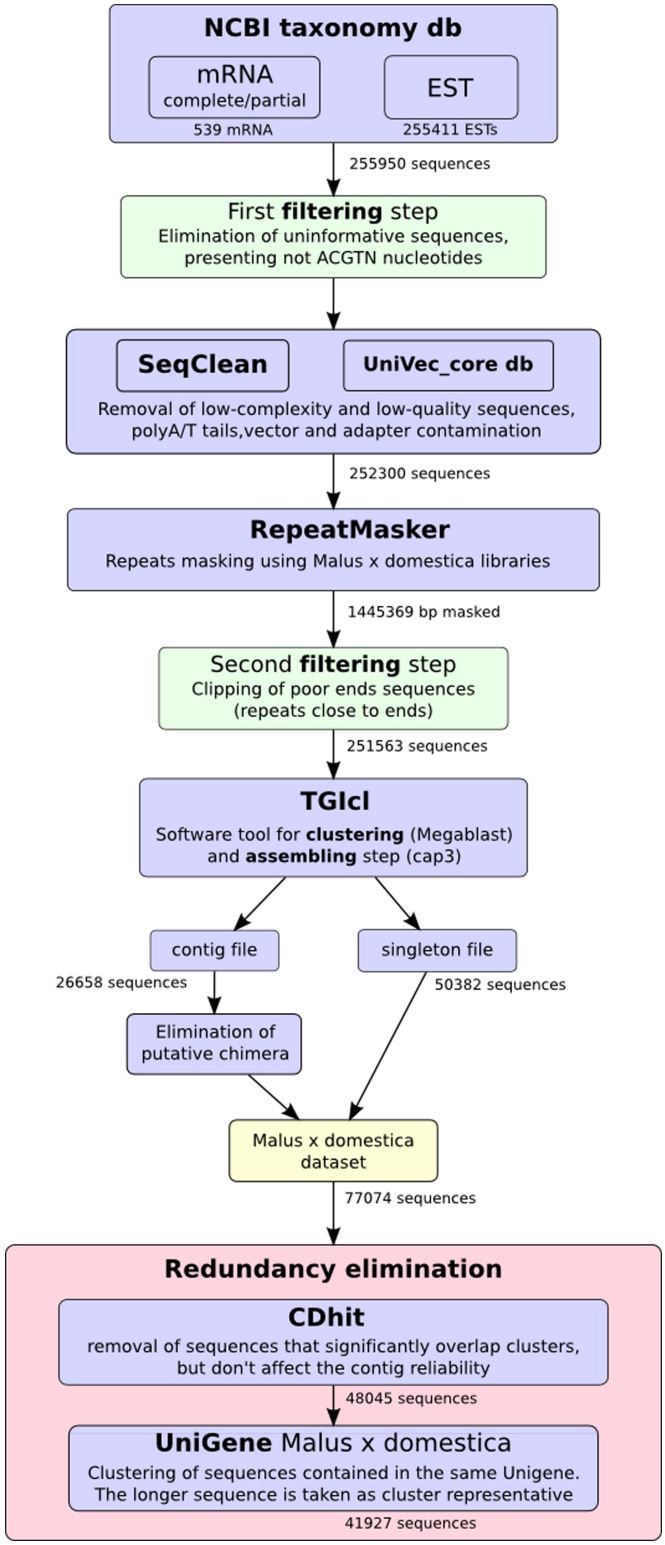

Figure S1 – The sequence clustering/assembling pipeline specifically set up for apple sequences. See materials and methods section for a detailed description.

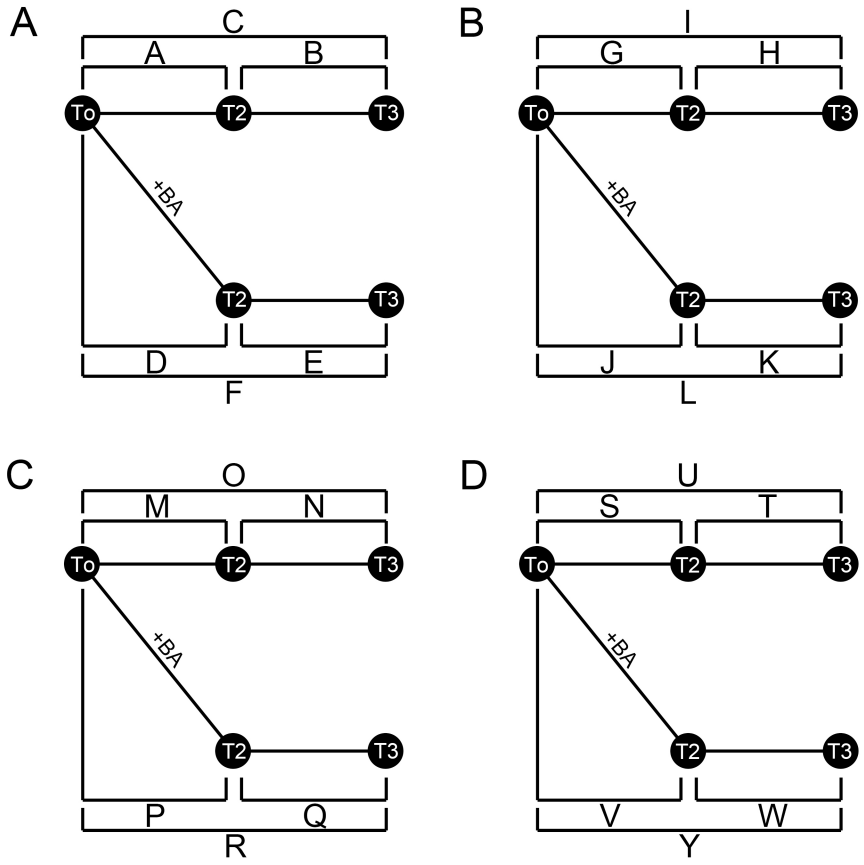

Figure S2 – Schematic representation of the experimental plan for microarray experiments (**A** = small lateral fruitlets; **B** = big lateral fruitlets; **C** = small central fruitlets; **D** = big central fruitlets; +BA = treatment with BA; To= 15 days after petal fall; T2 = 17 days after petal fall; T3 = 19 days after petal fall). Each letter on the square brackets refers to a comparison between two samples.

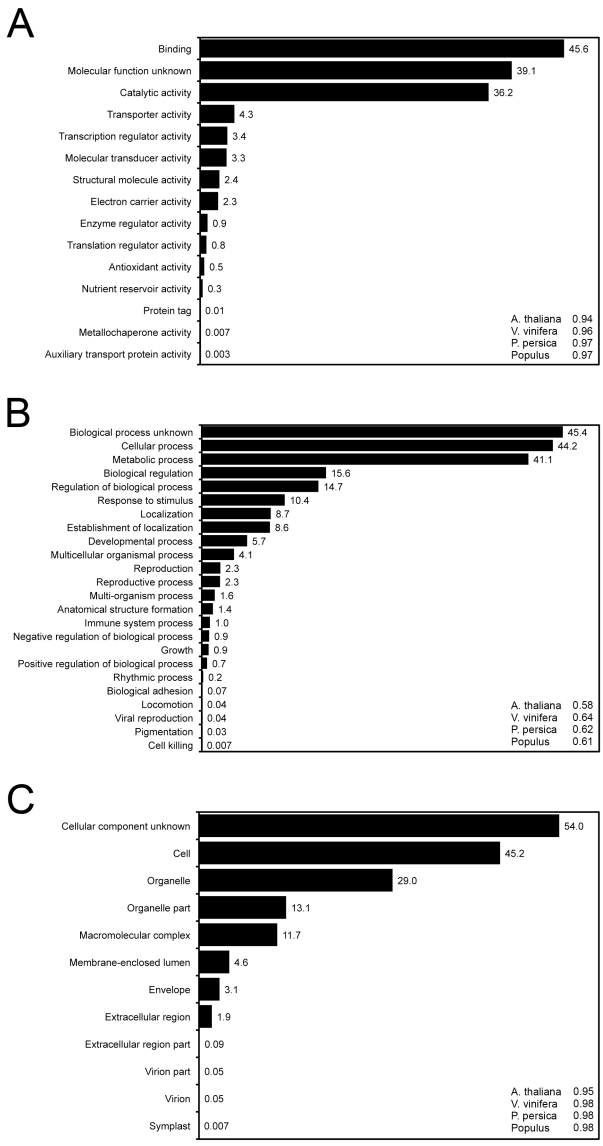

Figure S3 – Gene Ontology (GO) annotation of the 30,419 probes spotted on the apple microarray according to the molecular function (**A**), biological process (**B**), and cellular component (**C**) subvocabularies reported as percentage of the total sequences. In each chart, the Pearson correlation coefficients are reported with respect to the GO annotation pools of other important dicots for which a relevant number of genes have been annotated (*Arabidopsis thaliana*, *Vitis vinifera, Prunus persica*, and *Populus*).

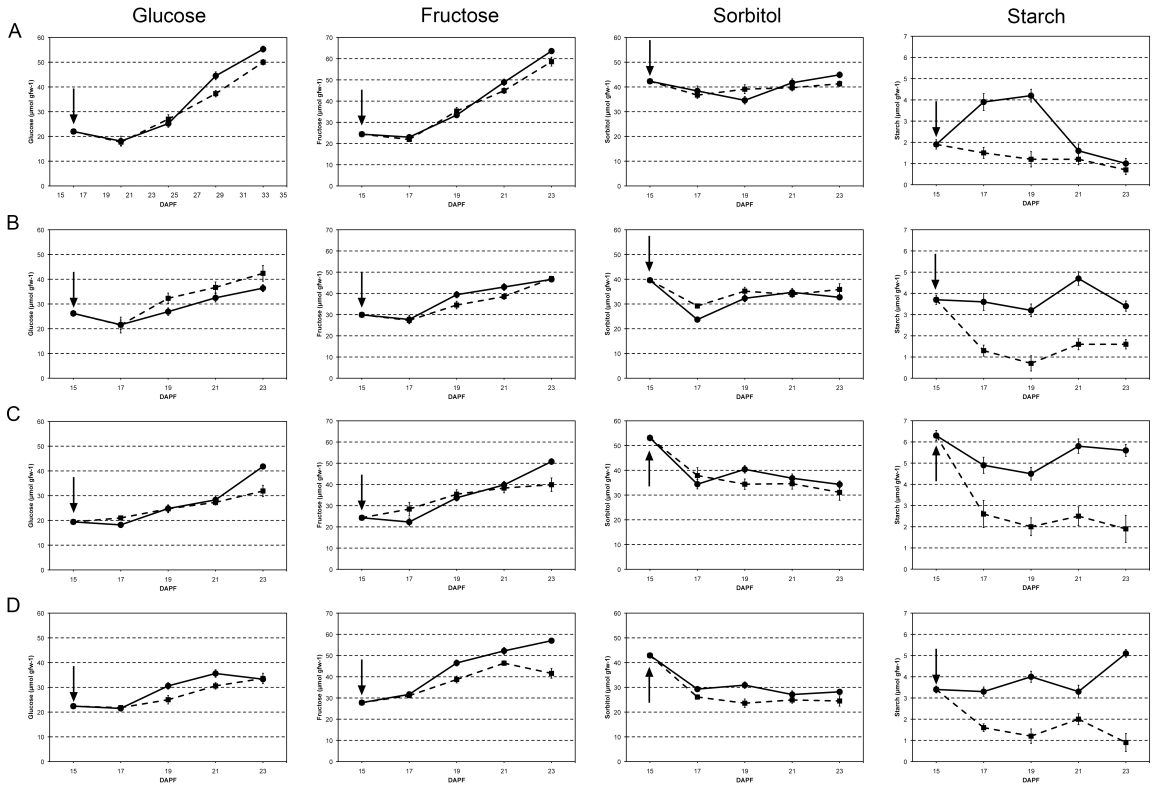

Figure S4 – Concentration of glucose, fructose, sorbitol, and starch in small (**A**) and big (**B**) lateral fruitlets, and in small (**C**) and big (**D**) central fruitlets, either untreated (circles and continuous line) or treated with BA (squares and dot-line). Bars represent standard deviation. DAPF: days after petal fall.

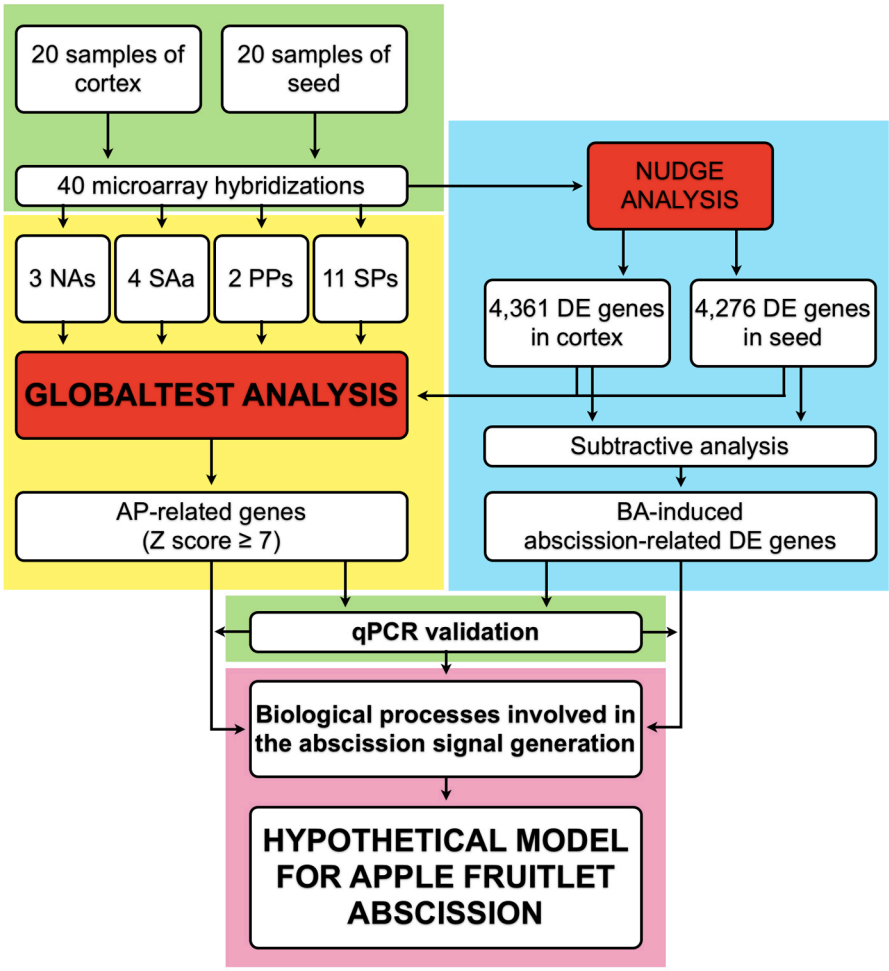

Figure S5 – Flow-chart of the data processing. Laboratory work is evidenced by a green box, the Globaltest analysis by a yellow box, the differentially expressed (DE) genes discovery by a cyan box, the setting-up of the model by a pink box. The red boxes represent the two main statistical analyses carried out to obtain the candidate abscission-related genes. The number of biological replicates is indicated for the samples undergone Globaltest analysis (AP = abscission potential; NA = naturally abscising fruitlets; SA = strongly abscising fruitlets; PP = probably persisting fruitlets; SP = strongly persisting fruitlets).

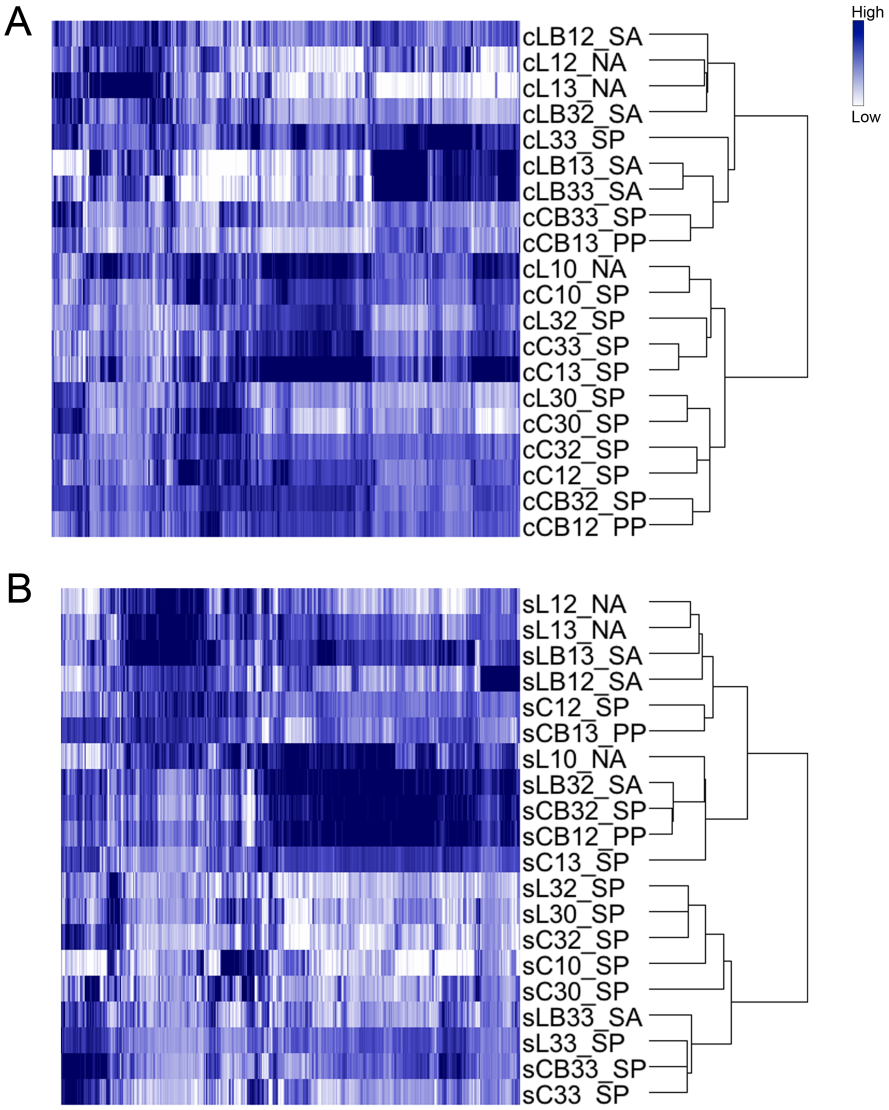

Figure S6 – Hierarchical clustering of the significantly variable transcriptome (see materials and methods section) in all samples of cortex (**A**) and seed (**B**). Samples are reported on the right side of the heatmap with the following codes: c: cortex, s: seed; L: lateral, C: central, B: treated with BA; first number indicates fruit size category: 1: small fruitlets, 3: big fruitlets; second number indicates sampling time: 0: T0, 2:T2. 3:T3. SP: strongly persisting, PP: probably persisting, NA: naturally abscising, SA: strongly abscising.

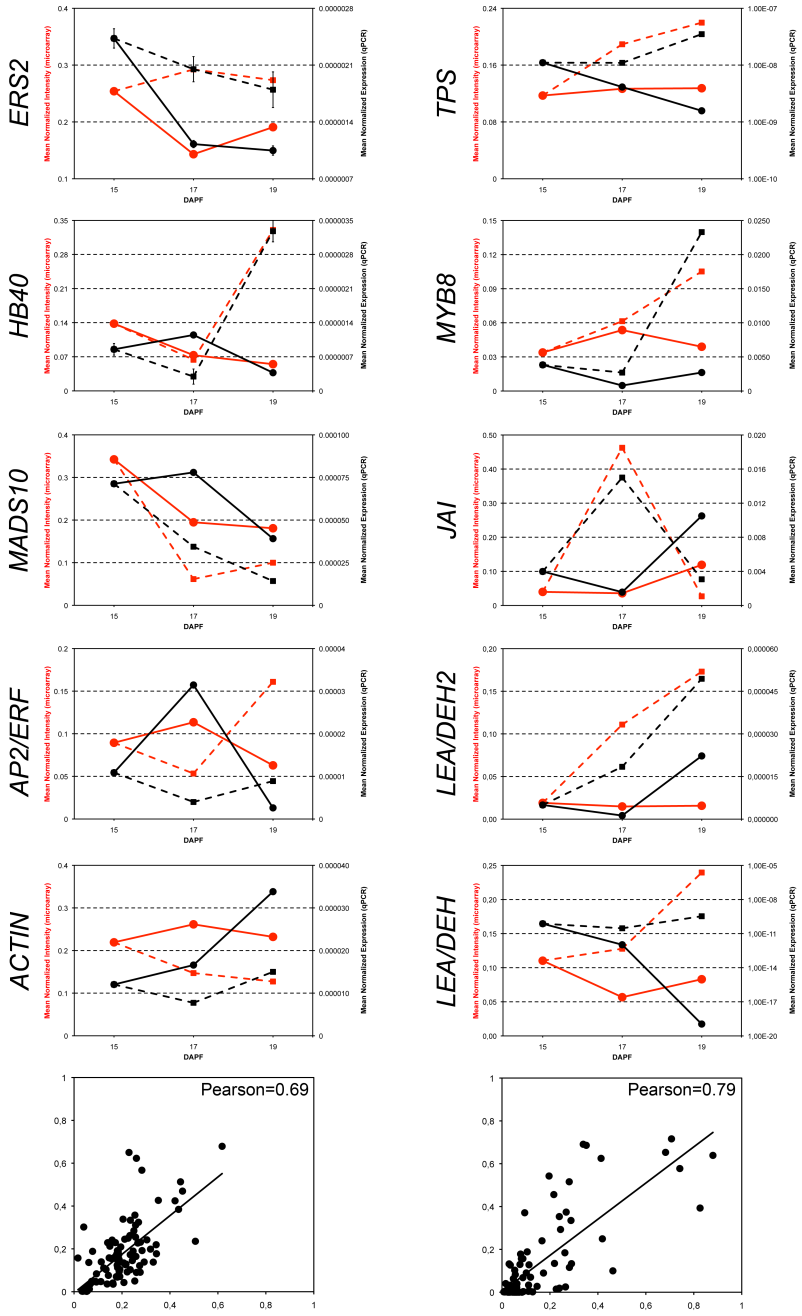

Figure S7 – Quantitative real-time PCR (qPCR) validation of selected genes in seed (left) and cortex (right) of L3 and LB3 samples. Transcript levels are reported for five genes for each tissue as normalized intensities for microarray analyses (in red coloration), and as Mean Normalized Expression for qPCR (in black coloration), for both untreated (circles and continuous line) and treated samples (squares and dot-line). Selected genes are: *ERS2* (Md_247145:s), *HB40* (Md_114537:s), *MADS10* (Md_3290:2:a), *AP2/ERF* (Md_3329:1:a), *ACTIN* (Md_507:4:a), *TPS* (Md_15339:1:a), *MYB8* (Md_6531:1:a), *JAI* (Md_4451:1:a), *LEA/DEH2* (Md_570:2:a), *LEA/DEH* (Md_425:1:a). In the bottom charts, correlations are shown for the same genes along with Pearson coefficient, considering normalized expression values of microarrays and qPCRs in all samples analyzed. DAPF: days after petal fall.

Chapter 3 – A PTR-MS approach for the characterization of abscission-related volatile organic compounds

Early induction of apple fruitlet abscission is characterized by isoprene emission and increased abscisic acid content

(article being submitted)

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Abstract

Apple fruitlet abscission represents an interesting model system to study the early phases of the shedding process, during which deep transcriptomic changes and metabolic rearrangements occur at the fruit level. Furthermore, fruit drop can be selectively magnified by means of thinning chemicals, such as benzyladenine and metamitron, which act by mimicking tha natural causes of fruitlet abscission. In the present research, a metabolomic study carried out on the volatile organic compounds emitted by fruitlets allowed to identify the isoprene as a reliable marker of the early phases of abscission induction. Its emission was strongly correlated with abscisic acid content (ABA) of the fruit cortex. A major regulatory role was hypothesised for ABA in controlling the transcription of both its biosynthetic genes and those involved in isoprene biosynthesis, as a ROS (reactive oxygen species)-detoxifying mechanism. The main findings were placed in the existing models and their potential practical implications were critically discussed.

Introduction

Fruit trees have evolved a system to control fruit load in relation to their nutritional status, thus allowing the plant to make an efficient use of resources. This is achieved by a process called 'physiological drop', consisting in the abscission of young developing fruitlets mainly due to a correlative phenomenon, differently from the senescence-driven abscission of ripe fruits (Bangerth, 2000). Apple represent an interesting model system to study the molecular mechanisms regulating early fruit development and the fruit developmental plasticity in response to endogenous and environmental changes, since it develop flower/fruit clusters in which a clear gradient of correlative dominance exists in relation to the position of the fruit within the cluster. This dominance can be controlled by means of chemical treatments that can induce fruit drop (Bangerth, 2000; Greene et al., 1992), a practice called 'fruit thinning', adopted by horticulturists to reduce fruit load therefore improving the final size and quality. Benzyladenine (BA) is a widely known chemical thinner exerting its action by stimulating shoot growth and, as a consequence, indirectly achieving fruit drop by enhancing inter-organ (between shoots and fruits), inter-cluster, and intra-cluster competitions (Bangerth, 2000; Buban, 2000). Therefore, BA represents an interesting tool to evoke a fruit developmental arrest and abscission in a controlled, inducible and selective way through the enhancement of correlative inhibitions. Because of its features, this chemical was recently used by Botton et al. (2011) to shed light on the signalling pathways mediating the induction of apple fruitlet abscission. A hypothetical model was obtained based upon massive transcriptomic data, indicating that the nutritional stress established within the tree and magnified by the BA treatment is primarily perceived by the fruitlet cortex, whose growth is blocked by resembling the ovary growth inhibition found in other species. A pivotal role in this developmental block seems to be played by abscisic acid (ABA), whose signalling and cross-talk with other hormones were shown to be very active in the cortex during the early abscission induction phases, along with an increased reactive oxygen species (ROS) production already at two days after the thinning treatment. The primary upstream signalling is most likely mediated by sugars, which transduce the nutritional stress downstream to the secondary signalling.

The same fruitlet samples assessed by Botton et al. (2011) from a global transcriptional point of view concurrently underwent a proton transfer reaction mass spectrometer (PTR-

MS) analysis (Lindinger et al., 1998), in order to identify possible volatile organic compounds (VOCs) to be used as markers of the abscission potential and shed light on the physiological processes which characterized the early phases of abscission induction. In the present study, VOCs emissions characterizing apple fruitlet abscission are shown, and a potentially reliable volatile marker of abscission induction (*i.e.* the isoprene) identified. A relationship between isoprene emission and ABA content in the fruit cortex was also pointed out, along with a specific activation of the corresponding biosynthetic genes. A delayed transcriptional activation of a biosynthetic gene involved in a key step of methyl-erythritol phosphate (MEP) pathway, supplying the precursors for both isoprene and ABA biosynthesis, was also shown. According to the main findings, a role for isoprene as a ROS-detoxifying mechanism mediated and transcriptionally controlled by ABA may be hypothesized. Finally, potential applications of these results are outlined and discussed.

Results

Fruit drop dynamics

Natural fruit drop followed the well-known biphasic dynamics (Botton et al., 2011) in both the genotypes used in the experiments, and the thinning treatments were shown to amplify the phenomenon, although with quantitative differences depending both on the cultivar and the chemical. In Golden Delicious (Figure 1A), both the thinning agents were effective in magnifying fruit drop. BA increased natural fruitlet abscission up to about 66%, whereas MET enhanced it of about 91%. The application of the first chemical, which is known to be very selective in certain conditions (Botton et al., 2011), resulted into an amplification of both the drop peaks, occurring at 29 and 33 DAPF, respectively, with a greater effect pointed out in correspondence of the latter one. On the other hand, the second chemical exerted its overall effect almost exclusively on the first abscission peak. Concerning the cv Red Chief (Figure 1B), the two peaks occurred at 28 and 35 DAPF, but in this case only MET was effective in magnifying abscission (up to about 12%), following the same dynamics reported above.

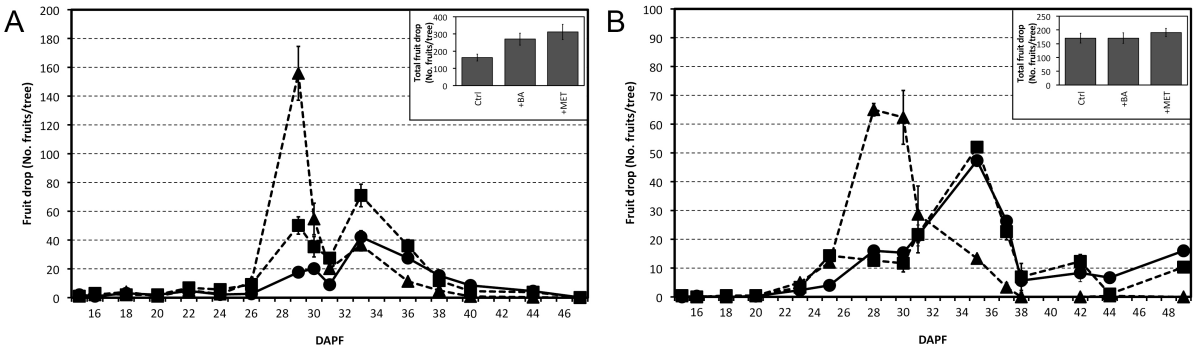

Figure 1 Fruit drop dynamics in control (circles and continuous line), BA-treated (squares and dot-line), and MET-treated (triangles and dot-line) trees of cvs Golden Delicious (**A**) and Red Chief (**B**), expressed as number of drop fruitlets per tree. In each chart the total fruit drop is also reported at the top-right side, expressed as total number of drop fruitlets per tree (Ctrl: untreated control trees; +BA: BA-treated trees; +MET: MET-treated trees). Bars represent standard deviation.

According to these results, an effective magnification of the natural abscission potential was achieved in both cultivars, although at different extents and depending upon the chemical used. Therefore, fruit samples collected from both genotypes and treatments may give an

overall representation of the biological variation due to both the genotype and the different mode of action of the thinning agents.

VOCs emissions

VOCs emissions were recorded in all fruitlet samples during abscission induction, which is thought to occur within four days after the thinning treatment, and in the following days (up to 25 and 22 DAPF, in Golden Delicious and Red Chief, respectively). In order to point out possible associations between the abscission potential and the emission of VOCs, the Globaltest package was used resulting into a statistical score (Z) for each mass measured by the PTR-MS. Concerning the 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, with scores higher than 10, and all the others showing lower statistical scores (Table I).

Table I Statistical scores of globaltest analyses in cv Golden Delicious. All the samples (control, treated with BA, and treated with MET) of cv Golden Delicious (year 2008) were considered for the analysis. Z-scores describe the association between VOCs emission and fruitlet abscission potentials (f = fragments).

m/z	Candidate/s	Z-score	Association
69	Pentanal, octanal, nonanal, 1- octen-3-ol, isoprene, methylbutanal	26.03	High in AFs
75	Methyl acetate, ethyl propionate	19.75	High in AFs
71	Alcohol fragment (2-pentanol, octanol), 3-methyl-1-butanol, ethylacetate	18.80	High in AFs
43	Acetic acid, acetate esters, propanol, hexanol	15.19	High in AFs
61	Acetic acid, ethyl acetate (f)	10.24	High in AFs
57	(E)-2-hexenal (f), butanol, propanol, ethyl propionate	9.07	High in AFs
63	Dimethyl sulphide	7.75	High in AFs
51	Cluster methanol+water	7.38	High in AFs
33	Methanol	3.98	High in AFs
65	Cluster ethanol+water	3.55	High in AFs
45	Acetaldehyde	2.10	High in AFs
47	Ethanol	0.85	High in AFs
85	Hexanol	-0.63	High in NAFs
73	Methyl ethylketone, 2-butanone	-0.65	High in AFs

In the case of cv Red Chief, the highest score was found for m/z 57 (15.35), followed by masses 69, 51, and 71, with Z scores higher than 10, and other masses with less significant statistics (Table II). When average statistical scores were calculated for all the volatiles in both cultivars, m/z 69 resulted the mass with the most significant association with abscission potential, with a mean Z score as high as 19.96, followed by masses 71, 57, and 51, with mean scores higher than 10, and all the others (Table III). Considering that in

plants the protonated mass 69 is univocally identified as the isoprene (Barta and Loreto, 2006; Cinege et al., 2009; Behnke et al., 2010; Rasulov et al., 2010), whereas the other masses herein shown are not univocally identified, the attention was focused on the former.

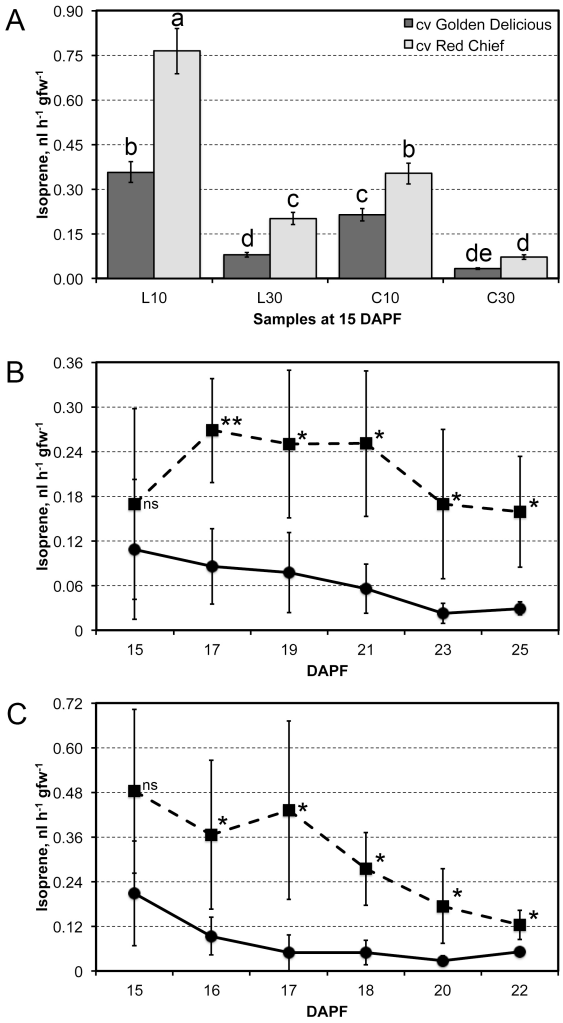

Figure 2. A. Isoprene emission in fruitlet samples of cvs Golden Delicious (dark grey) and Red Chief (light grey) at the beginning of the experiments (15 DAPF). Samples are named as follows: L10 = small lateral fruitlets; L30 = big lateral fruitlets; C10 = small central fruitlets; C30 = big central fruitlets. Bars represent standard deviation, whereas letters indicate significant differences as pointed out by LSD test ($P < 0.05$). Isoprene emission of abscising (squares and dot-line) and non-abscising (circles and continuous line) fruitlets of cvs Golden Delicious (**B**) and Red Chief (**C**). Statistically significant differences are also indicated (two asterisks at $P \leq 0.001$; one asterisk at $P \leq 0.01$; ns = non-significant). Bars represent standard deviation.

At the time when experiments began (15 DAPF for both cultivars), the destiny of some fruitlet classes was already defined: the L1 would have abscised, whereas the C3 would have persisted, both regardless of the thinning treatments. Isoprene emissions of these samples well resembled their abscission potential in both cultivars, being the highest for the

former class and the lowest for the latter one, whereas L3 and C1 classes showed intermediate emissions, higher in C1 than in L3.

Table II Statistical scores of globaltest analyses in cv Red Chief. All the samples (control, treated with BA, and treated with MET) of cv Red chief (year 2009) were considered for the analysis. Z-scores describe the association between VOCs emission and fruitlet abscission potentials (f = fragments).

m/z	Candidate/s	Z-score	Association
57	(E)-2-hexenal (f), butanol, propanol, ethyl propionate	15.35	High in AFs
69	Pentanal, octanal, nonanal, 1- octen-3-ol, isoprene, methylbutanal	13.89	High in AFs
51	Cluster methanol+water	10.46	High in AFs
71	Alcohol fragment (2-pentanol, octanol), 3-methyl-1-butanol, ethylacetate	10.21	High in AFs
33	Methanol	9.70	High in AFs
85	Hexanol	6.90	High in AFs
63	Dimethyl sulphide	3.72	High in AFs
73	Methyl ethylketone, 2-butanone	2.66	High in AFs
43	Acetic acid, acetate esters, propanol, hexanol	0.45	High in AFs
75	Methyl acetate, ethyl propionate	0.34	High in AFs
61	Acetic acid, ethyl acetate (f)	-0.48	High in AFs
47	Ethanol	-0.70	High in NAFs
45	Acetaldehyde	-0.70	High in AFs
65	Cluster ethanol+water	-0.71	High in AFs

Red Chief emitted significantly more isoprene than Golden Delicious in L1, L3, C1, and less significantly also in C3 (Figure 2A). Considering these data as a whole, isoprene emissions were inversely correlated with fruit size (data not shown). Taking into account the evolution of isoprene emissions separately in abscising (AFs) and non-abscising (NAFs) fruitlets, higher levels of the volatile can be observed in AFs of both Golden Delicious (Figure 2B) and Red Chief (Figure 2C), although more statistically significant in the former genotype and in samples at 17 DAPF.

Table III Average statistical scores of globaltest analyses. Z scores of Tables I and II were averaged. Only associations with a mean score higher than 2 are reported (f = fragments).

m/z	Candidate/s	Mean Z-score
69	Pentanal, octanal, nonanal, 1- octen-3-ol, isoprene, methylbutanal	19.96
71	Alcohol fragment (2-pentanol, octanol), 3-methyl-1-butanol, ethylacetate	14.51
57	(E)-2-hexenal (f), butanol, propanol, ethyl propionate	12.21
51	Cluster methanol+water	10.04
75	Methyl acetate, ethyl propionate	8.92
33	Methanol	7.82
43	Acetic acid, acetate esters, propanol, hexanol	6.84
85	Hexanol	5.73
63	Dimethyl sulphide	4.88
61	Acetic acid, ethyl acetate (f)	3.14

Since the behaviour of cv Golden Delicious with respect to isoprene emission, abscission potential of the different fruit classes, and drop dynamics was better defined and already characterized in previous studies (Botton et al., 2011), further investigations were carried out only in this genotype. Considering the single fruit classes separately (Figure 3), the peak in isoprene emission pointed out by AFs at 17 DAPF was shown to be due mainly to the contribution of L3 fruitlets treated with both the chemicals. Indeed, L1 fruitlets emitted more isoprene only when treated with MET, whereas BA had the opposite effect. Nevertheless, central fruitlets (C1 and C3) did not show any significant difference at 17 DAPF, regardless of both the thinning treatments.

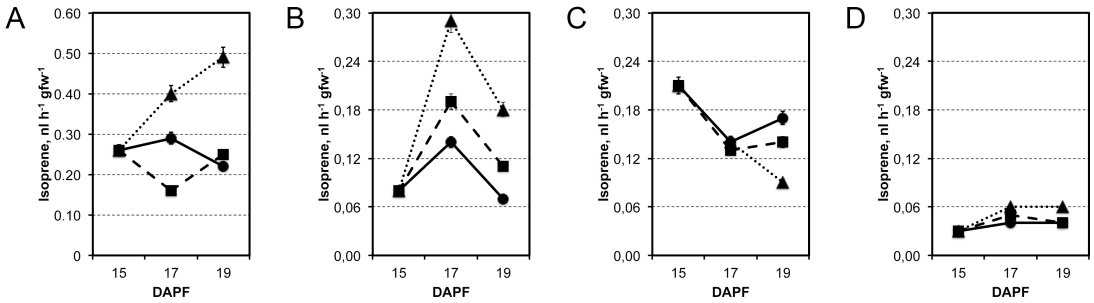

Figure 3. Isoprene emission in single fruit classes of cv Golden Delicious during early abscission induction. Small lateral (**A**), big lateral (**B**), small central (**C**), and big central (**D**) fruitlets of control (circles and continuous line), BA-treated (squares and dot-line), and MET-treated (triangles and dot-line) trees are shown. Bars represent standard deviation.

Abscisic acid content

Previous studies carried out in isoprene-emitting species by Barta and Loreto (2006) pointed out a correlation between isoprene emission and abscisic acid (ABA) content in leaves. Moreover, the key role played by ABA signalling and its cross-talk with other hormones in the early phases of apple fruitlet abscission induction was recently demonstrated (Botton et al., 2011). Taking into account this information, ABA content was measured in the cortex of the same Golden Delicious fruitlet samples used for global transcriptomic analyses by Botton et al. (2011), which did not include the samples treated with MET but only those treated with BA. At the beginning of experiments, ABA levels well correlated with abscission potential, being significantly higher in L1 than in the other classes (Figure 4A). AFs always showed higher ABA levels than NAFs, with a weak but significant peak at 17 DAPF, slightly decreasing at 19 DAPF (Figure 4B).

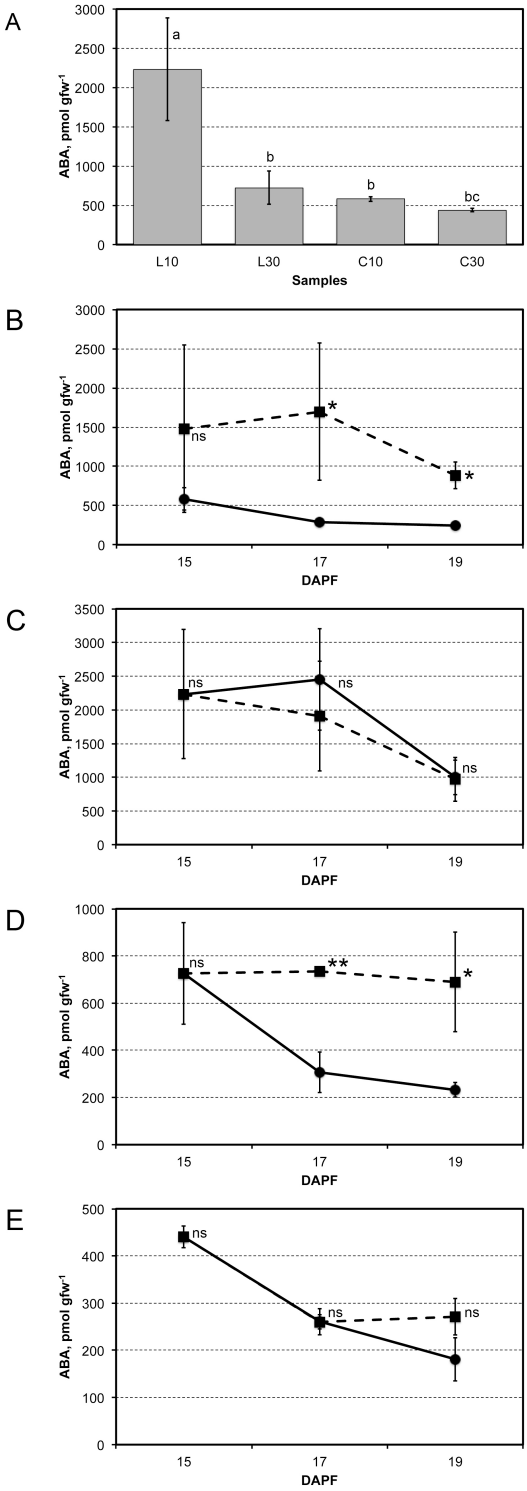

Figure 4. A. Abscisic acid (ABA) content measured in single fruit classes of cv Golden Delicious at the beginning of the experiments (15 DAPF). Samples are named as follows: L10 = small lateral fruitlets; L30 = big lateral fruitlets; C10 = small central fruitlets; C30 = big central fruitlets. Bars represent standard deviation, whereas letters indicate significant differences as pointed out by LSD test ($P < 0.05$). **B.** ABA content measured in abscising (squares and dot-line) and non-abscising (circles and continuous line) fruitlets of cv Golden Delicious during early abscission induction. Measurements are also reported separately for small lateral (**C**), big lateral (**D**), and big central (**E**) fruitlets, either BA-treated (squares and dot-line) or untreated

(next page)

(circles and continuous line). Statistically significant differences are indicated (two asterisks at P ≤ 0.001; one asterisk at P ≤ 0.01; ns = non-significant). Bars represent standard deviation.

A detailed analysis of the single fruit classes pointed out a trend resembling that of isoprene emission, with L1 and C3 being unaffected by the thinning treatment (Figure 4C and 4E), and L3 showing significantly higher levels when induced to abscise by BA (Figure 4D). Data concerning the C1 class are not shown. A statistically significant correlation was calculated between isoprene emission and ABA content, as high as 0.78 (Pearson coefficient) with $P < 0.0001$ (Figure 5).

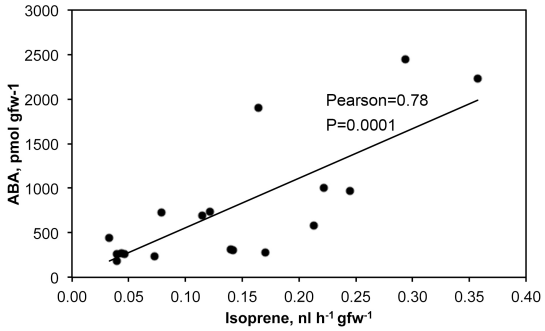

Figure 5. Relationship between isoprene emission and ABA content of Golden Delicious fruitlets. Also shown are the Pearson correlation coefficient and the *P* level.

Expression of putative ISPS-encoding genes

The enzyme responsible for isoprene biosynthesis from dimethylallyl pyrophosphate (DMAPP) is the isoprene synthase (ISPS), present in different isoforms and localized in the chloroplast (Sharkey and Yeh, 2001). A database search was performed in order to identify candidate *ISPS* genes, and qPCR expression studies carried out on them to assess possible correlations with isoprene emissions as measured by PTR-MS. The database set up as described by Botton et al. (2011), based upon publicly available EST sequences, was used for this search.

Thirteen candidates were identified and tested for their expression patterns in the cortex of apple fruitlet samples of cv Golden Delicious (untreated and BA-treated). Among them, only seven showed reliably detectable expression levels (data not shown). According to the subcellular localization prediction carried out with AtSubP, six of them are most likely chloroplast-localized, and may thus be considered putative ISPSs (Table IV).

Table IV Subcellular localization of putative ISPSs, NCEDs, and DXSs**.** Predictions were carried out with AtSubP (http://bioinfo3.noble.org/AtSubP/submit.html). Only positive scores are to be considered statistically reliable.

Apple_ID[a]	EST	Genome ID[b]	Annotation (acronym)[c]	Length[d]	CHL[e]	CYT[e]	GOL[e]	MIT[e]	EX[e]	NUC[e]	CM[e]	Prediction
Md_11874:1:a	EH034629	MDP0000398063	Isoprene synthase (ISPS)	553	1.17	-1.25	-1.29	-1.12	-1.12	-1.21	-1.23	Chloroplast
Md_1281:1:a	EG631361	MDP0000199152		475	1.15	-0.86	-1.29	-1.32	-1.06	-1.09	-1.08	Chloroplast
Md_246922:s	CN491852	MDP0000205617		394	1.30	-1.17	-1.29	-1.78	-1.04	-1.06	-1.23	Chloroplast
Md_29044:s	EB133707	MDP0000322688		494	1.30	-1.01	-1.29	-1.60	-1.07	-1.20	-1.13	Chloroplast
Md_5069:1:a	EG631334	MDP0000245233		762	1.00	-0.92	-1.29	-1.11	-1.06	-1.10	-1.27	Chloroplast
Md_67394:s	DR994337	MDP0000233961		362	-1.33	-1.36	-0.89	-1.16	1.27	-1.92	-0.90	Extracellular
Md_9554:1:a	DR992924	MDP0000203143		515	0.50	-0.56	-1.29	-1.00	-1.00	-0.87	-1.26	Chloroplast
Md_372:s	AY347797	MDP0000555220	9-cis-epoxycarotenoid dioxygenase (NCED)	571	1.82	-1.40	-1.29	-1.55	-1.10	-1.34	-1.11	Chloroplast
Md_48052:s	EB114699	MDP0000813805		615	1.47	-1.47	-1.29	-1.37	-1.07	-1.23	-1.13	Chloroplast
Md_48078:s	EB114673	MDP0000228070		614	1.23	-1.30	-1.29	-1.50	-1.01	-1.06	-1.10	Chloroplast
Md_52168:s	EB110583	MDP0000246006		235	0.55	-1.10	-1.29	-0.53	-1.02	-0.96	-1.14	Chloroplast
Md_629:3:a	EB121925	MDP0000164529		560	-1.93	1.90	-1.29	-2.06	-1.19	-1.06	-1.20	Cytoplasm
Md_711:3:a	CV986431	MDP0000547655		621	1.79	-1.55	-1.29	-1.64	-1.09	-1.26	-1.14	Chloroplast
Md_9575:1:a	CN995338	MDP0000929213		607	1.80	-1.32	-1.29	-1.95	-1.11	-1.18	-1.10	Chloroplast
Md_17066:1:a	CN851542	MDP0000253952	1-deoxy-D-xylulose 5-phosphate synthase (DXS)	714	1.77	-1.10	-1.29	-1.66	-1.11	-1.30	-1.28	Chloroplast
Md_191957:s	CN900023	MDP0000793656		713	1.45	-1.08	-1.29	-1.39	-1.11	-1.28	-1.29	Chloroplast
Md_787:4:a	CN883461	MDP0000798878		714	1.60	-1.23	-1.29	-1.64	-1.09	-1.23	-1.25	Chloroplast

[a] Identifier of the cluster/singleton as assembled by Botton et al. (2011).

[b] Identifier of the predicted coding sequence recovered at http://genomics.research.iasma.it/gb2/gbrowse/apple/.

[c] Tentative annotation.

[d] Length of the deduced amino acid sequence.

[e] CHL: chloroplast; CYT: cytoplasm; GOL: golgi; MIT: mitochondrion; EX: extracellular; NUC: nucleus; CM: cell membrane.

Expression analyses showed that the majority of the corresponding genes are expressed at higher levels in AFs than NAFs, and a significant correlation was pointed out for one of them (Md_9554:1:a) with isoprene production, with a peak of expression in AFs at 17 DAPF (Figures 6A).

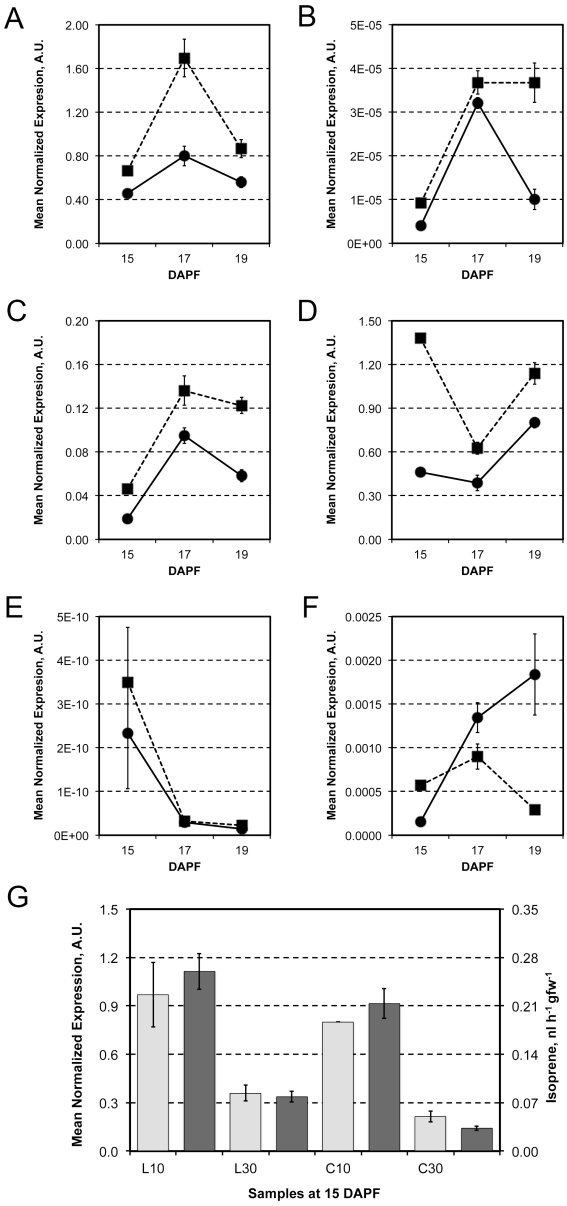

Figure 6. qPCR gene expression data of Md_9554:1:a (**A**), Md_29044:s (**B**), Md_11874:1:a (**C**), Md_1281:1:a (**D**), Md_246922:s (**E**), and Md_5069:1:a (**F**), in abscising (squares and dot-line) and non-abscising (circles and continuous line) fruitlets of cv Golden Delicious during early abscission induction. **G**. Transcript accumulation of Md_9554:1:a (light grey) and isoprene emission (dark grey) are also shown for the single fruit classes at the beginning of the experiments (15 DAPF). Samples are named as follows: L10 = small lateral fruitlets; L30 = big lateral fruitlets; C10 = small central fruitlets; C30 = big central fruitlets. Bars represent standard deviation.

Moreover, at the beginning of experiments, Md_9554:1:a expression, which was the highest among all candidates, already paralleled isoprene emissions of all fruitlet samples considered separately (Figure 6G). It is worthy to note that this gene pointed out a characteristic structure, with a very long 5'-UTR first intron (1,029 bp). This feature was previously investigated in Arabidopsis by Chung et al. (2006), indicating a correlation with high expression levels such as those herein reported for this gene. Indeed, its deduced amino acid sequence showed the typical conserved elements found in other ISPSs, such as the active site lid residues, particularly the two arginines at the N-terminal of the mature protein, and substrate and Mg^{2+} binding sites, particularly two aspartate-rich regions in the second half of the protein (Schnitzler et al., 2009, data not shown).

Expression of putative NCED-encoding genes

The same approach described above was adopted also with respect to genes coding for 9-cis-epoxycarotenoid dioxygenases (NCEDs), the key enzymes involved in the last step of the chloroplastic pathway of ABA biosynthesis cleaving either 9'-cis-neoxanthin or 9'-cis-violaxanthin (or both) to produce xanthoxin, the direct C_{15} precursor of the hormone. NCEDs are encoded in plants by multigene families and are chloroplast-targeted (Nambara and Marion-Poll, 2005).

Twelve candidate NCED-encoding genes were herein identified in apple, seven of which expressed at the cortex level. According to the subcellular localization prediction carried out with AtSubP, six of them are targeted to the chloroplast, whereas one is cytoplasmic (Table IV). Expression analyses pointed out a positive correlation with ABA levels for three of these six genes, namely Md_52168:s, Md_48078:s, and Md_711:3:a, showing in AFs a distinct peak at 17 DAPF followed by a decrease to the same levels as in NAFs (Figure 7A-C). However, at the beginning of the experiments, their transcript accumulation was not correlated to abscission potential. On the other hand, Md_48052:s expression, which was negatively correlated with the ABA peak at 17 DAPF, paralleled the hormone levels at 15 DAPF, especially when fruitlet classes were considered separately. In fact, its transcripts accumulated at the highest levels in L1 fruitlets, naturally destined to abscise, with a tenfold difference with respect to the expression levels found in the other samples (Figure 7G). After a clear drop of expression at 17 DAPF, its transcripts returned at higher levels in AFs than in NAFs at 19 DAPF (Figure 7D). Finally, Md_372:s resembled the same pattern,

although at different extents, whereas Md_9575:1:a was transiently up-regulated at 17 DAPF in NAFs (Figure 7E-F).

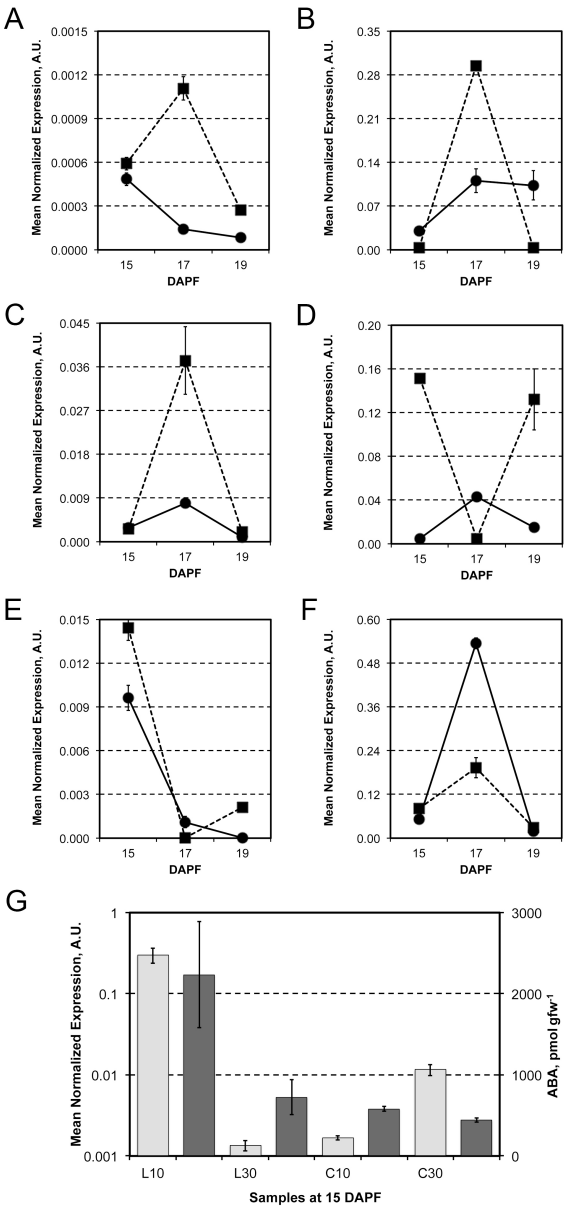

Figure 7. qPCR gene expression data of Md_52168:s (**A**), Md_48078:s (**B**), Md_711:3:a (**C**), Md_48052:s (**D**), Md_372:s (**E**), and Md_9575:1:a (**F**), in abscising (squares and dot-line) and non-abscising (circles and continuous line) fruitlets of cv Golden Delicious during early abscission induction. **G**. Transcript accumulation of Md_48052:s (light grey) and ABA content (dark grey) are also shown for the single fruit classes at the beginning of the experiments (15 DAPF). Samples are named as follows: L10 = small lateral fruitlets; L30 = big lateral fruitlets; C10 = small central fruitlets; C30 = big central fruitlets. Bars represent standard deviation.

Expression and phylogenetic analyses of putative DXS-encoding genes

In order to assess the possible up-regulation of genes encoding elements of the MEP pathway, a search was carried out concerning DXS (1-deoxy-D-xylulose 5-phosphate

synthase, EC number 2.2.1.7), the enzyme involved in the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) by an acyloin condensation of hydroxyethylthiamine derived from the decarboxylation of pyruvate (PYR) with the C_1-aldehyde group of D-glyceraldehyde 3-phosphate (GAP) (Ghirardo et al., 2010). This enzyme is assumed to be important in regulating the metabolic flux within the pathway (Schnitzler et al., 2009; Estévez et al., 2001; Lois et al., 2000; Muñoz-Bertomeu et al., 2006; Walter et al., 2000) occurring in the plastid, which in turn supplies precursors for both isoprene and ABA biosynthesis (Rodríguez-Concepción, 2006).

Three genes putatively coding for DXSs were found in the apple database of expressed sequences, all of which were shown to be chloroplast-targeted according to the AtSubP predictions (Table IV). Accordingly, in many species, the number of genes coding for this enzyme was shown to vary between two and three (Cordoba et al., 2009). Concerning their expression patterns, no correlation was found with respect of either isoprene or ABA peaks found at 17 DAPF, or with the natural abscission potential at 15 DAPF. However, a clear up-regulation at 19 DAPF was pointed out for Md_191957:s in AFs (Figure 8).

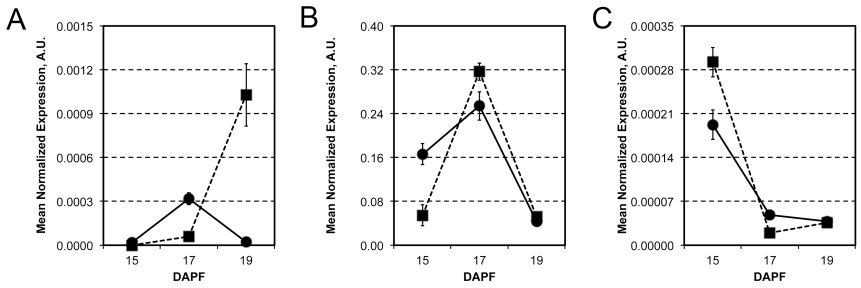

Figure 8. qPCR gene expression data of Md_191957:s (**A**), Md_17066:1:a (**B**), and Md_787:4:a (**C**), in abscising (squares and dot-line) and non-abscising (circles and continuous line) fruitlets of cv Golden Delicious during early abscission induction. Bars represent standard deviation.

Plant DXSs can belong to two different classes (class 1 and 2) according to the classification reported by Walter et al. (2002), with class 1 DXSs showing housekeeping functions and those belonging to class 2 participating in the biosynthesis of isoprenoids involved in secondary metabolism (Rodríguez-Concepción, 2006). The evolutionary relationships of the putative DXSs herein identified with respect to other plant DXSs were thus investigated by means of a phylogenetic analysis performed on the deduced amino acid sequences. The same sequences used by Zhang et al. (2009) were used as a reference. The phylogenetic analysis (Figure 9) revealed a relationship between the phylogeny of DXS

sequences and the phylogenetic distance, as pointed out by Zhang et al. (2009). Two of the putative apple DXSs (Md_191957:s and Md_17066:1:a) were shown to be closely related to *Medicago truncatula* DXS2, the latter belonging to class 2 plant DXSs (Walter et al., 2002), whereas only Md_787:4:a was comprised in the class 1.

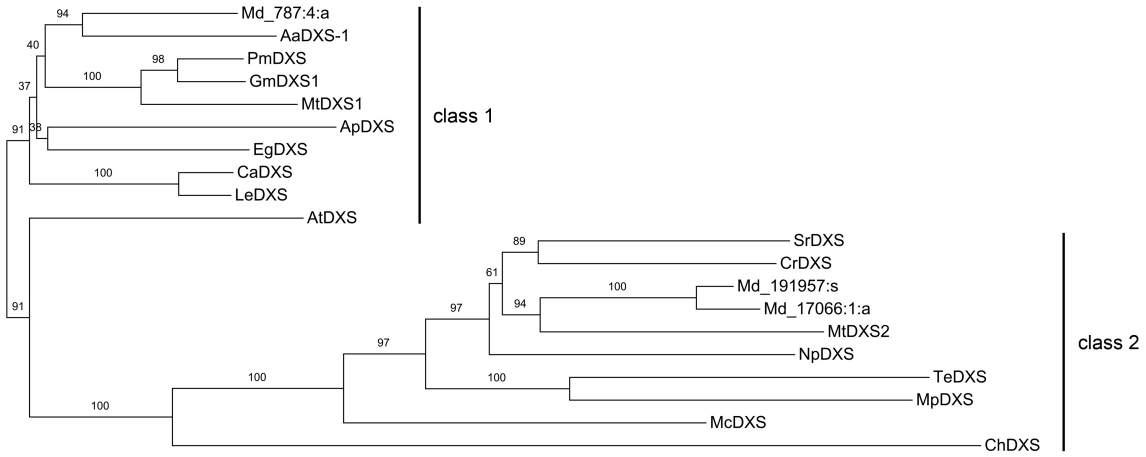

Figure 9. Phylogenetic tree of DXSs from different plants. The bootstrap value is indicated for each node. The deduced amino acid sequences of apple putative DXSs (Md_191957:s, Md_17066:1:a, and Md_787:4:a) were recovered from the IASMA (Istituto Agrario San Michele all'Adige) web site (http://genomics.research.iasma.it/) according to the respective sequence ID reported in Table IV, whereas the other proteins were deduced from the following nucleotide sequences recovered at the NCBI database (http://www.ncbi.nlm.nih.gov): AaDXS (*Artemisia annua*, AF182286), ApDXS (*Andrographis paniculataa*, AY254390), AtDXS (*Arabidopsis thaliana*, U27099), CaDXS (*Capsicum annuum*, Y15782), ChDXS (*Chlamydomonas reinhardtii*, AJ007559), CrDXS (*Catharanthus roseus*, AJ011840), EgDXS (*Elaeis guineensis*, AY583783), GmDXS1 (*Glycine max*, this work), LeDXS (*Lycopersicon esculentum*, AF143812), McDXS (*Morinda citrifolia*, AF443590), MpDXS (*Mentha* × *piperita*, AF019383), MtDXS1 (*Medicago truncatula*, AJ430047), MtDXS2 (*Medicago truncatula*, AJ430048), NpDXS (*Narcissus pseudonarcissus*, AJ279019), PmDXS (*Pueraria montana*, AY315652), SrDXS (*Stevia rebaudiana*, AJ429232), TeDXS (*Tagetes erecta*, AF251020).

Promoter analyses

In a recent research carried out by Botton et al. (2011), an overall transcriptomic investigation pointed out the abscission-related up-regulation of genes encoding transcription factors (TFs), occurring in AFs exclusively at 17 DAPF. The main groups of TFs involved in the early phases of abscission induction in the cortex were the WRKYs, MYB/MYCs, AP2/ERE-BP, and zinc-finger TFs. In order to investigate the possible involvement of these TFs and other *cis*-regulatory elements in the early phases of abscission induction, the proximal promoter sequences (2 Kb from the transcription start site, TSS) of Md_9554:1:a, Md_52168:s, Md_48078:s, Md_711:3:a, and Md_191957:s were recovered from the apple genome database and submitted to the PlantPan.

Considering the whole 2 Kb promoter sequence, some distinctive elements were found among the five genes, especially concerning the presence of ABREs (ABA-responsive elements, Figure 10). This class of regulatory motifs were found in the *ISPS* and *NCED* genes, but not in the *DXS* gene, consistently with the correlation pointed out with the ABA peak only for the formers. Moreover, an ABRE element very close to the TSS (-80 bp) was found only in the promoter of the NCED-encoding gene Md_48078:s. This is consistent with its up-regulation measured from 15 to 17 DAPF in AFs, which is as high as about 80-fold, the highest among the four ABA-correlated genes. Indeed, a likely correlation seems to exist between the distance of the ABREs from the TSS and the extent of up-regulation at 17 DAPF. In fact, the *ISPS* gene and Md_52168:s showed about a two-fold up-regulation and in both genes the ABRE closest to the TSS was at about -800 bp. On the other hand, Md_711:3:a pointed out a 12-fold up-regulation from 15 to 17 DAPF in AFs, and the closest ABRE was located at about -700 bp from the TSS (Figures 7 and 10).

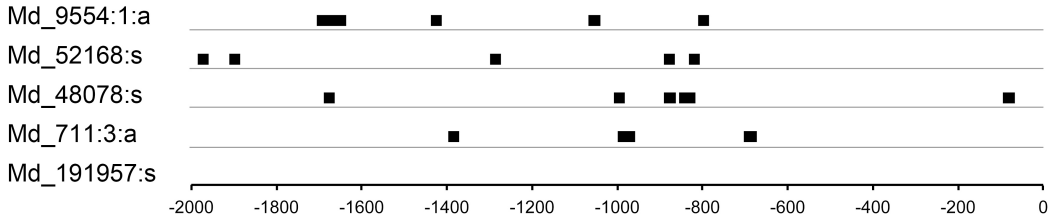

Figure 10. Position of abscisic acid responsive elements (ABREs) in the promoters of Md_9554:1:a, Md_52168:s, Md_48078:s, Md_711:3:a, and Md_191957:s genes. The distance in bp from the transcription start site is indicated above.

By carrying out the same analysis considering only a portion of 500 bp from the TSS, which is supposed to be more relevant for regulatory purposes, much more differences were pointed out. Specifically, the promoter of the *DXS* gene had some distinctive elements that were not found in the other genes such as three AGL3 (AGAMOUS-like 3; Huang et al., 1996) binding sites, only one copy of which was found in Md_52168:s promoter but not in the other genes, and two CPBCSPOR motifs, involved in the cytokinin response and functioning effectively when interacting with other cytokinin-related elements (Fusada et al., 2005). Moreover, besides lacking ABREs, the -500 bp region of Md_191957:s promoter did not contain any ARR1AT element, known to bind ARR transcriptional activators induced by exogenous cytokinin treatments (Sakai et al., 2000; To et al., 2004). Moreover, the same promoter lacked also the RAV1 motif, recognized by AP2-like TFs (Kagaya et al.,

1999), the ATHB-1, involved in light responses (Henriksson et al., 2005), MYB.ph3, which binds MYB TFs (Solano et al., 1995), and ATHB-5, recognized by leucine-zipper homeobox TFs that represent potential regulators of abscisic acid responsiveness in Arabidopsis (Johannesson et al., 2003). Concerning the latter TFBS, only one copy was present in the -500 bp region of Md_191957:s promoter, whereas from 5 to 8 of these motifs were pointed out in the other genes. Results are summarized in Table V.

Table V Number and types of transcription factor binding sites (TFBSs) found in the -500 bp (from TSS) promoter region of the corresponding genes. The name of the TFBS is given, along with the corresponding database, the TF that binds to it, and the physiological factors involved.

TFBS	Database	TF (factor/s)	Number of TFBSs				
			Md_9554:1:a	Md_52168:s	Md_48078:s	Md_711:3:a	Md_191957:s
CPBCSPOR	PLACE	na (cytokinins)	0	0	0	0	2
AMYBOX2	PLACE	na	0	0	0	0	1
P1BS	PLACE	MYB (phosphate starvation)	0	0	0	0	1
S1FBOXSORPS1L21	PLACE	S1F	0	0	0	0	1
S2FSORPL21	PLACE	S2F	0	0	0	0	1
TATCCAOSAMY	PLACE	MYB (gibberellin, sugar starvation)	0	0	0	0	1
TATCCAYMOTIFOSRAMY3D	PLACE	MYB (sugar repression)	0	0	0	0	1
WBOXNTCHN48	PLACE	WRKY (pathogens)	0	0	0	0	1
AGL3	TRANSFAC	AGL3	0	1	0	0	3
ATHB-5	TRANSFAC	HDZip (ABA)	7	5	6	8	1
Athb-1	TRANSFAC	HDZip (light)	4	2	3	5	0
RAV1	TRANSFAC	RAV1/AP2 (senescence)	1	2	1	4	0
RAV1-A	AGRIS	RAV1/AP2 (senescence)	1	2	1	4	0
RAV1AAT	PLACE	RAV1/AP2 (senescence)	1	2	1	4	0
ARR1AT	PLACE	ARR (cytokinins)	9	2	6	3	0
HMG-IY	JASPER	HMG	4	8	1	2	0
Bellringer	AGRIS	BELL	2	1	1	1	0
MYB.ph3	JASPER	MYB	3	1	1	1	0

Discussion

The apple fruit cluster represents a unique model system for studying correlatively-driven abscission, and the availability of chemicals able to selectively induce fruit shedding allows to set up controlled field experimental plans, aimed at magnifying the natural physiological drop in order to obtain fruitlet populations with clearly predictable abscission potentials. Botton et al. (2011) took advantage of these features for a massive transcriptomic analysis of abscission induction, and suggested a hypothetical model describing the early phases of the process at both the cortex and seed level. According to this model, sugar signalling would primarily transduce the nutritional stress imposed to abscising fruitlets to secondary signalling pathways at the cortex level, involving ABA, ROS, and ethylene. One of the earliest metabolic markers of abscission induction was hydrogen peroxide, whose levels increased already 2 days after the thinning treatment (17 DAPF). The present research was mainly focused on this temporal window (*i.e.* within maximum 4 days after treatment), during which the first tentative reaction of the fruits to stress occurs along with a likely metabolic rearrangement, both highly regulated and coordinated, presumably by ABA.

Experiments were carried out according to a well-tested strategy (Botton et al., 2011; Dal Cin et al., 2009a; Dal Cin et al., 2009b; Dal Cin et al., 2007; Dal Cin et al., 2005), consisting in making up fruitlet populations with different abscission potentials, by enlarging the naturally available biological variation with the use of thinning chemicals. The whole experimental strategy is already described by Botton et al. (2011), with the exception of the use of one additional chemical (MET). Moreover, both BA and MET were applied on Golden Delicious (an easy-to-thin cultivar) and Red Chief (a difficult-to-thin spur cultivar).

Fruit drop dynamics confirmed that an effective magnification of the natural abscission potential was achieved in both cultivars, although at different extents and depending on the chemical. Therefore, fruit samples may give an overall representation of the biological variation due both to genotypes and modes of action of the thinning agents.

The association analyses pointed out a strong correlation between the emission of some VOCs and abscission potential. However, among the most discriminating masses measured by the PTR-MS system, only m/z 69 was univocally identified as the isoprene (Barta and Loreto, 2006; Cinege et al., 2009; Behnke et al., 2010; Rasulov et al., 2010). Therefore, detailed investigations were herein carried out only concerning this volatile, whereas the

unidentified masses will undergo future studies. The diagnostic value of isoprene was shown to be very high in both genotypes, although the most specific emission profile was observed in Golden Delicious, herein taken as a model cultivar. Besides being emitted at higher levels by abscising fruitlets taken as a whole (Figure 2), its emission was already enhanced also in naturally abscising fruitlets. Moreover, it is worthy to note that the peak of isoprene emission shown by AFs at 17 DAPF was due mainly to L3 fruitlets treated either with BA or MET (Figure 3). These fruits are suffering from a tough nutritional stress, indirectly caused by the chemical thinner, which triggers abscission thereafter. 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., 2009). It is here demonstrated that isoprene emission is strictly and specifically associated with abscission induction, thus representing a potential marker to predict fruit's destiny. It is not surprising that isoprene is highly emitted by organs, such as the abscising fruitlets, undergoing assimilates shortage. In fact, isoprenoid emissions are often sustained even when carbon supply becomes scarce under stress conditions or null photosynthetic rates (Brilli et al., 2007; Loreto and Sharkey, 1990; Monson and Fall, 1989). This would mean that the function of isoprene is considered 'vital' by the plant and, since the oxidative status of AFs was disrupted by high levels of ROS measured in the cortex (Botton et al., 2011), it may be speculated that this volatile may behave directly as an antioxidant, as recently suggested (Sharkey et al., 2008; Vickers et al., 2009). Therefore, the fruit stimulated to abscise tries to recover a non-cytotoxic oxidative status by scavenging ROS by means of a non-enzymatic system. This early reaction would fall within the homeostatic mechanisms set up by the fruits during abscission induction, as recently demonstrated (Botton et al., 2011).

The determination of ABA levels in the cortex allowed to point out a strong correlation between the hormone content and isoprene emission (Figure 5), as demonstrated by Barta and Loreto (2006) in *Populus* leaves. A pivotal role for ABA in abscission was largely studied and discussed in different species (Vernieri et al., 1992; Guinn and Brummett, 1988; Yuan and Huang, 1988; Gomez-Cadenas et al., 2000; Sagee and Erner, 1991; Talon et al., 1990), especially concerning its cross-talk with ethylene. More specifically, Talon et al. (1997) found a close relationship between ABA concentration and abscission in citrus fruit,

hypothesizing a specific stimulatory effect of ABA on *ACC synthase* (*ACS*) transcription and ethylene biosynthesis. Besides this late involvement, ABA signalling was shown to play a major role also in the early phases of abscission induction in apple, already at 2 days after the thinning treatment (Botton et al., 2011). Accordingly, ABA content was shown to be higher in AFs than NAFs at the same date. Indeed, as reported for isoprene, the major contribution to this increased level is given by those fruitlets whose destiny is changed by the application of the treatments, *i.e.* the bigger laterals that would naturally persist (Figure 4D). The fact that several ABA signalling elements, such as transcription factors (*i.e.* WRKYs, bZIPs, MYC/MYBs, AP2/ERFs) and other regulatory proteins (*i.e.* MAPKs) know to be involved in ABA signal transduction, were previously shown to be triggered during abscission induction (Botton et al., 2011) indicates that the increased levels of the hormone do have a 'signalling value'. This would mean that the hormone is perceived and its signal transduced, leading to the transcriptional activation of downstream target processes and secondary messengers, such as the ROS, whose increase was previously observed in AFs (Botton et al., 2011). Also isoprene biosynthesis may fall within the ABA target processes. Therefore, a detailed investigation was carried out at the transcriptomic level, in order to identify the putative target genes playing a role in both isoprene emission and increased biosynthetic rates of the hormone. Putative isoprene synthase (ISPS)- and 9-cis-epoxycarotenoid dioxygenase (NCED)-encoding genes were searched for this purpose, among which only those encoding chloroplast-targeted proteins were further investigated. A significant correlation was found between isoprene emission and the expression of Md_9554:1:a, whose gene product shows high structural homology and sequence similarity with isoprene synthases of other species (data not shown). Concerning ABA, three putative *NCED* genes (Md_52168:s, Md_48078:s, and Md_711:3:a) were found correlated with the hormone content in fruit cortex, especially with respect to the peak observed at 17 DAPF. The presence of several ABRE motifs in the promoter sequences of all these four genes (Figure 10) indicates that they may represent potential ABA targets, likely justifying both the co-regulation pattern and the correlation between their transcription patterns and the ABA peak. Moreover, the closer is the ABRE to the transcription start site the higher and more specific is the ABA-dependent up-regulation of the corresponding gene, indicating the

relevance of these regulatory motifs in determining the specificity and extent of ABA responsiveness in the genes analysed.

The precursors for both ABA and isoprene biosynthesis are supplied by the MEP pathway, whose multilevel regulation in plants was shown to be very complex (Wolfertz et al., 2004; Rodríguez-Concepción, 2006). In bacteria, it was shown that isoprenoid biosynthesis was limited by the activity of DXS (1-deoxy-D-xylulose 5-phosphate synthase), shown to be a critical enzyme of the pathway (Cordoba et al., 2009). Moreover, a high level of DXS regulation during isoprene emission was demonstrated in *Eucalyptus globulus* (Wolfertz et al., 2004). Briefly, a negative feedback on DXS would normally limit the supply of 1-deoxy-D-xylulose 5-phosphate (DXP) along the MEP pathways. When the activity of the downstream processes increases, the feedback is relieved to divert more carbon to these pathways and the activity of DXS becomes substrate-limited (Wolfertz et al., 2004). In agreement, the expression of MEP pathway genes was shown to either precede or parallel the biosynthesis of isoprenoid end-products, such as the carotenoids in pepper and tomato fruit (Bouvier et al., 1998; Lois et al., 2000; Botella-Pavía et al., 2004). This co-regulation may be partially due to the presence of common regulatory motifs (*i.e.* the ATCTA element) in the promoter of *DXS*, *HDR* (*hydroxymethylbutenyl diphosphate reductase*), and *PSY* (*phytoene synthase*) genes (Rodríguez-Concepción, 2006). However, concerning the genes herein studied, profound differences were pointed out with respect to both the promoters and the expression profiles. In fact, while *ISPS* and *NCEDs* are co-regulated and correlated with both isoprene and ABA, the differential expression of the *DXS* gene (Md_191957:s) seemed to be unrelated to the latters, showing an initial down-regulation at 17 DAPF followed by a delayed up-regulation 2 days later (19 DAPF). Moreover, since no ABRE motif was found in the promoter of this gene, a likely ABA-independent regulation of its expression can be hypothesised. As pointed out above, plant DXSs can belong to two different classes, with class 1 DXSs showing housekeeping functions and class 2 isozymes participating in the biosynthesis of isoprenoids involved in secondary metabolism (Walter et al., 2002; Rodríguez-Concepción, 2006). Phylogenetic analyses revealed that the protein encoded by Md_191957:s is a class 2 DXS. Besides the coordinated regulation of MEP pathway and downstream biosynthetic processes reported above for class 1 DXSs, a time-shift of up-regulation was demonstrated for a class 2 *DXS* with respect to other isoforms in

Norway spruce following wounding (Phillips et al., 2007), indicating that the kinetics of expression may change according to the gene, the class to which it belongs, and the stimulus controlling its expression. Therefore, the expression profile of the class 2 *DXS* Md_191957:s might be justified by both the specific function of the enzyme it codes for and the control exerted by the downstream processes on its regulation. It may be speculated that the up-regulation of the *DXS* gene is delayed because in the first phase (until 17 DAPF) a negative feedback keeps its transcription at basal levels, and class 1 DXSs with housekeeping functions are able to supply enough carbon for isoprene and ABA biosynthesis peaks to occur. Moreover, since post-transcriptional control mechanisms may modulate the levels of functional DXSs (Cordoba et al., 2009), an increased activity could be initially guaranteed in this way. When constitutive DXSs become unable to guarantee an extra-supply of precursors, as needed, the specific up-regulation of the class 2 *DXS* gene provides an adequate amount of carbon to sustain both isoprene and ABA biosynthesis. This up-regulation might be controlled by *de novo* synthesized TFs, different from those controlling the transcription of ISPS and NCED genes.

Conclusion

A hypothetical model was set up based upon the main findings and existing knowledge, in order to explain the regulatory action exerted by ABA in apple cortex during the early phases of abscission induction (Figure 11). According to this model, the nutritional stress perceived by the AFs is primarily transduced by means of sugar signalling, as hypothesized by Botton et al. (2011) based upon transcriptomic data. ABA perception and the following activation of its transduction pathway, paralleled by increased levels of the hormone, would lead to a burst of ROS, which is a typical secondary messenger (Hubbard et al., 2010), and the subsequent activation of the specific detoxifying machinery at the transcriptional level. Isoprene is part of this mechanism of detoxification, and the up-regulation of its biosynthetic gene *ISPS* may be due to the presence of ABA-responsive elements (ABREs) in its promoter. A concurrent positive feedback, involving *NCED* genes, would ensure the higher levels of abscisic acid measured thereafter in AFs and the cross-talk with ethylene, whose physiological consequences are observed in the following days. The direct players (*i.e.* TFs) involved in the transcriptional regulation of ABA target genes in the cortex remain to be identified, although a list of candidates, whose actual role will be investigated in future researches, was already done by Botton et al. (2011), including WRKYs, bZIPs, MYC/MYBs, AP2/ERFs, and other TF families. The processes described by this model, however, are just a 'tessera' of the mosaic of abscission signalling. The present research represents a further progress towards the full comprehension of abscission induction and, prominently, of its primary determinant, which is thought to be triggered at the fruit level (Botton et al., 2011). Further investigations are needed, also concerning the seed, to shed light on the transduction of the nutritional stress into abscission signal, upstream the ABA signalling.

As a concluding remark, 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. Therefore, a high-resolution dedicated system is being developed in order

to test its actual efficacy in predicting fruit load and, consequently, tune the thinning treatments according to a modern environment-friendly agriculture.

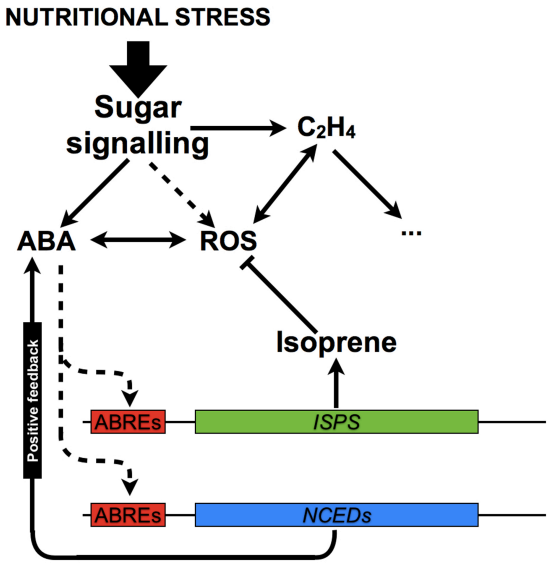

Figure 11. Hypothetical model showing the regulatory action of abscisic acid (ABA) during the early phases of abscission induction in the cortex. Besides a positive feedback of its biosynthesis by stimulating the expression of 9-cis-epoxycarotenoid dioxygenase (NCED) genes, ABA would up-regulate the transcription of a specific isoprene synthase (ISPS) gene involved in the biosynthesis of the volatile from dimethylallyl pyrophosphate, in order to detoxify the cell from reactive oxygen species (ROS).

Materials and Methods

Plant material and treatments

Experiments were carried out in 2008 and 2009 on apple trees of cvs Golden Delicious/M9 and Red Chief/M26 trained with standard horticultural practices at the experimental farm of the IASMA (Trento, Italy). Populations of fruits with different abscission potentials (abscising fruitlets versus persisting fruitlets) were established as described by Botton et al. (2011), except for the use of an additional thinning chemical, the Metamitron (MET, a photosystem II inhibitor; Schmidt and Fedtke, 1977), sprayed at 350 $\mu L \cdot L^{-1}$at the same time of benzyladenine (BA).

PTR-MS measurements

Volatile organic compounds (VOCs) emissions were measured with a proton transfer reaction mass spectrometer (PTR-MS) on at least eight representative fruitlets for class, placed in a 100 ml glass bottle (Kavalier, Sázava, CZ), topped with Teflon® caps, and kept at a constant room temperature of 20°C along the whole 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 sccm/min (standard cubic centimeters per minute). Mass data were collected in a range from 20 to 200 amu (m/z) with a dwell time of 0.5 s per mass under drift tube conditions of 120 Td (Townsend, where 1 Td = 10-17 V cm^2 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 stationary conditions. Then, blank subtraction was carried out and values converted into nanoliters per hour per gram of fresh weight (nl h^{-1} gfw^{-1}).

Quantification of abscisic acid

Fifty milligrams of fruitlet cortex, previously frozen in liquid nitrogen and ground with a mortar, in 1.5 mL of water were boiled at 100°C for 5 min, in order to prevent from hydrolysis of endogenous ABA-conjugates which would have caused an overestimation of free ABA (Loveys and van Dijk, 1988). Samples were then extracted for 12 hours in complete darkness at 4°C on a shaker. The extracts were centrifuged at 10,000 g 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., Elkhart, USA) according to the indications of the manufacturer. The monoclonal antibody raised against ABA (ABA-15-I-C-5) was previously shown to have high specificity for 2-cis-(S)-ABA and cross-reactivity of less than 1 or 0 against 12 different structurally ABA-related compounds (Weiler, 1982).

Quantitative (q)PCR expression analyses

For qPCR 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 few adaptations as described by Botton et al. (2011). Total RNA was quantified spectrophotometrically and its integrity checked by running 1 µg in a 1% agarose gel stained with SYBR® Safe (Invitrogen, Carlsbad, CA).

cDNA was synthesized from 2 µg of DNA-free total RNA in a final volume of 25 µL containing 200 Units of MMLV Reverse Transcriptase (Promega, Madison, WI), 1X MMLV Buffer, 25 Units of RNasin (RNase inhibitor, Amersham Biosciences, Piscataway, NJ), 1 µg of Random Hexamers (Invitrogen, Carlsbad, CA) and 2mM dNTPs. The reaction was carried out for 1 h at 37°C in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

Real-time PCR relative quantification was performed in triplicate on two biological replicates, as described by Botton et al. (2011). Primers for both the target and reference genes are reported in Table VI. Data were acquired, elaborated and exported with the StepOne Software v2.1 (Applied Biosystems, Foster City, CA), whereas all the final calculations were carried out with the automated Excel spreadsheet Q-Gene designed by Simon (2003), using the modifications of the delta Ct method suggested by Pfaffl (2001). Gene expression values were normalized to the housekeeping genes identified above and reported as arbitrary units (A.U.) of Mean Normalized Expression, using the equation 2 of Q-Gene.

Table VI Primers used in qPCR experiments given in 5'-3' orientation. The last five genes were used as reference for qPCR data normalization. More information is available in the references reported in the last column.

Gene[a]	Forward primer	Reverse primer	Reference
Md_17066:1:a (CN851542)	ACAGTAGCCGATGCACGATTT	CCTCCGATTGAACCTTCTTCC	-
Md_191957:s (CN900023)	GACCCACAAACCGGCAAG	TCTTGTCATCTATTTCAGCTTCC	-
Md_787:4:a (CN883461)	TCACAGTTCCACCACCATCTC	CTTCATACGCTTGCCCTGCT	-
Md_372:s (AY347797)	TGAAGAATCAATGCACCCTGA	CCCGTTGGACGACACATCT	-
Md_48052:s (EB114699)	CCCAACTCGGAGAATGAGGA	TTCCACTCCTTCTCGTCGTG	-
Md_48078:s (EB114673	ACAAGACACCGCCACCTTTC	TTGGGATTTGGATTACAGAAGG	-
Md_52168:s (EB110583)	TGCTTCTGCTTCCACCTCTG	AAGCCGAATTTCTGACAAGAC	-
Md_629:3:a (EB121925)	GCTGAACCAGAGGTCGGAAA	GCCAGGAATACGAGGGACAA	-
Md_711:3:a (CV986431)	ACAACGTACATGCTATCAATGC	GCTCCATTCTCTCCATTGTGTG	-
Md_9575:1:a (CN995338)	TGGTGGGTTAAATTACGGAAGG	CTGTCTGCCTGCCTAACAAA	-
Md_11874:1:a (EH034629)	CAACGATTCTTCGGCTATGG	GCTCGTGCATCTTCGTCTGA	-
Md_1281:1:a (EG631361)	GATGGAGATGGGTTTGGTGA	ATGACGAACTGAACCGCAAA	-
Md_246922:s (CN491852)	GGAATAGATGGTCAAGGAAGGA	CAATAGCAACAGGCAAATACTCC	-
Md_29044:s (EB133707)	TTGCTCGGATTTCCCAATG	AATGGGATCGACAATCACTG	-
Md_5069:1:a (EG631334)	GTCCAGTCCCTTCACCAGAAA	AATCCCAGCTCTTTCCACCA	-
Md_67394:s (DR994337))	CATTCTTTGAGAGTTTCGTGGA	AACCCTACGGCAGTTCAATC	-
Md_9554:1:a (DR992924)	AAACAAACGCACAACCCAAA	CGCCCAAGTTGCCTACAAATA	-
MdUBI	CATCCCCCCAGACCAGCAGA	ACCACGGAGACGCAACACCAA	Dal Cin et al., 2005
Md18S	GTTACTTTTAGGACTCCGCC	TTCCTTTAAGTTTCAGCCTTG	Dal Cin et al., 2005
MdACT	TGACCGAATGAGCAAGGAAATTACT	TACTCAGCTTTGGCAATCCACATC	Li and Yuan, 2008
Md_8283:1:a (CN940765)	CTCGTCGTCTTGTTCCCTGA	GCCTAAGGACAGGTGGTCTATG	Botton et al., 2011
Md_4592:1:a (EB107049)	GTCGAAATGGTCAGCGGTAG	GCAATGGCAAACTCCACCTT	Botton et al., 2011

[a] The Apple ID is given according to the collection described by Botton et al. (2011). The dbEST (http://www.ncbi.nlm.nih.gov/projects/dbEST/) accession number of the sequence used for primer design is given between brackets. Alternatively the gene name is indicated along with the related reference for more information.

Statistical analyses

The Globaltest package v 4.14.4 (Goeman et al., 2004) of R software v 2.9.1 (http://www.r-project.org/) was used to test the overall volatile emissions for significant association with the abscission potential of the samples analysed. A Z score, calculated for each volatile, gives the strength of these associations.

Common statistics (Student's t-tests, LSD tests, and Pearson correlations) were calculated with the StatPlus:mac LE.2009 package (AnalystSoft Inc.) for Microsoft Excel.

Bioinformatic analyses

Genomic sequences for bioinformatics analyses were recovered by matching the target ESTs against the apple genome sequence available for blast analysis at the IASMA (Istituto Agrario San Michele all'Adige) web site (http://genomics.research.iasma.it/). The gene predictions were used to deduce the corresponding amino acid sequences for the subcellular localization predictions, whereas the promoter sequences were recovered based upon an *ex novo* prediction of the TSS carried out with the NNPP (Neural Network Promoter Prediction; Reese, 2001) tool available at http://www.fruitfly.org/seq_tools/promoter.html, by setting the minimum score at 0.8.

The subcellular localization prediction was carried out with the AtSubP tool (http://bioinfo3.noble.org/AtSubP/), recently made available by Kaundal et al. (2010) and demonstrated to be reliable also for species other than Arabidopsis. The best hybrid AA+NCC+PSI-BLAST+PSSM-based prediction module was selected.

Alignments and phylogenetic analyses were performed with CLC Sequence Viewer 6 (http://www.clcbio.com), using the Clustal algorithm and the neighbour-joining method, respectively, both with default parameters. The phylogenetic tree was edited with TreeGraph v2.0.45-197 beta (Stöver and Müller, 2010).

The promoter analyses were performed with the PlantPan tool (Chang et al., 2008; http://plantpan.mbc.nctu.edu.tw/seq_analysis.php), by selecting all the available libraries.

References

Bangerth F (2000) Abscission and thinning of young fruit and thier regulation by plant hormones and bioregulators. Plant Growth Regul **31:** 43–59

Barta C, Loreto F (2006) The relationship between the methyl-erythritol phosphate pathway leading to emission of volatile isoprenoids and abscisic acid content in leaves. Plant Physiol **141:** 1676–1683

Behnke K, Kaiser A, Zimmer I, Brüggemann N, Janz D, Polle A, Hampp R, Hänsch R, Popko J, Schmitt-Kopplin P, Ehlting B, Rennenberg H, Barta C, Loreto F, Schnitzler JP (2010) RNAi-mediated suppression of isoprene emission in poplar transiently impacts phenolic metabolism under high temperature and high light intensities: a transcriptomic and metabolomic analysis. Plant Mol Biol **74**: 61–75

Botella-Pavía P, Besumbes O, Phillips MA, Carretero-Paulet L, Boronat A, Rodríguez-Concepción M (2004) Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. Plant J **40:** 188–199

Botton A, Eccher G, Forcato C, Ferrarini A, Begheldo M, Zermiani M, Moscatello S, Battistelli A, Velasco R, Ruperti B, Ramina A (2010) Signalling pathways mediating the induction of apple fruitlet abscission. Plant Physiol **155**: 185-208.

Brilli F, Barta C, Fortunati A, Lerdau M, Loreto F, Centritto M (2007) Response of isoprene emission and carbon metabolism to drought in white poplar (*Populus alba*) saplings. New Phytol **175:** 244–254

Buban T (2000) The use of benzyladenine in orchard fruit growing: a mini review. Plant Growth Regul **32:** 381–390

Chang W, Lee T, Huang H, Huang H, Pan R (2008) PlantPAN: Plant promoter analysis navigator, for identifying combinatorial cis-regulatory elements with distance constraint in plant gene groups. BMC Genomics 9: 561-575

Chung B, Simons C, Firth A, Brown C, Hellens R (2006) Effect of 5'UTR introns on gene expression in *Arabidopsis thaliana*. BMC Genomics **7:** 120-133

Cinege G, Louis S, Hänsch R, Schnitzler JP (2009) Regulation of isoprene synthase promoter by environmental and internal factors. Plant Mol Biol **69:** 593–604

Cordoba E, Salmi M, León P (2009) Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. J Exp Bot **60:** 2933–2943

Dal Cin V, Barbaro E, Danesin M, Murayama H, Velasco R, Ramina A (2009) Fruitlet abscission: A cDNA-AFLP approach to study genes differentially expressed during shedding of immature fruits reveals the involvement of a putative auxin hydrogen symporter in apple (*Malus domestica* L. Borkh). Gene **442:** 26–36

Dal Cin V, Boschetti A, Dorigoni A, Ramina A (2007) Benzylaminopurine application on two different apple cultivars (*Malus domestica*) displays new and unexpected fruitlet abscission features. Ann Bot **99:** 1195–1202

Dal Cin V, Danesin M, Boschetti A, Dorigoni A, Ramina A (2005) Ethylene biosynthesis and perception in apple fruitlet abscission (*Malus domestica* L. Borck). J Exp Bot **56:** 2995–3005

Dal Cin V, Velasco R, Ramina A (2009) Dominance induction of fruitlet shedding in *Malus* X *domestica* (L. Borkh): molecular changes associated with polar auxin transport. Bmc Plant Biol **9:** 139-153

Estévez JM, Cantero A, Reindl A, Reichler S, León P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. J Biol Chem **276:** 22901–22909

Fusada N, Masuda T, Kuroda H, Shimada H, Ohta H, Takamiya K (2005) Identification of a novel cis-element exhibiting cytokinin-dependent protein binding *in vitro* in the 5'-region of NADPH-protochlorophyllide oxidoreductase gene in cucumber. Plant Mol Biol **59:** 631–645

Ghirardo A, Zimmer I, Brüggemann N, Schnitzler JP (2010) Analysis of 1-deoxy-D-xylulose 5-phosphate synthase activity in Grey poplar leaves using isotope ratio mass spectrometry. Phytochemistry **71:** 918–922

Goeman JJ, van de Geer SA, De Kort F, van Houwelingen HC (2004) A global test for groups of genes: testing association with a clinical outcome. Bioinformatics **20:** 93–99

Gomez-Cadenas A, Mehouachi J, Tadeo FR, Primo-Millo E, Talòn M (2000) Hormonal regulation of fruitlet abscission induced by carbohydrate shortage in citrus. Planta **210**: 636-643

Greene DW, Autio WR, Erf JA, Mao ZY (1992) Mode of action of benzyladenine when used as a chemical thinner on apples. J Am Soc Hortic Sci **117:** 775–779

Guinn G, Brummett DL (1988) Changes in free and conjugated indole 3-acetic acid and abscisic acid in young cotton fruits and their abscission zones in relation to fruit retention during and after moisture Stress. Plant Physiol **86**: 28-31

Henriksson E, Olsson ASB, Johannesson H, Johansson H, Hanson J, Engstrom P, Soderman E (2005) Homeodomain leucine zipper class I genes in Arabidopsis. Expression patterns and phylogenetic relationships. Plant Physiol **139:** 509

Huang H, Tudor M, Su T, Zhang Y, Hu Y, Ma H (1996) DNA binding properties of two Arabidopsis MADS domain proteins: binding consensus and dimer formation. Plant Cell **8:** 81–94

Hubbard KE, Nishimura N, Hitomi K, Getzoff ED, Schroeder JI (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. Genes Dev **24:** 1695–1708

Johannesson H, Wang Y, Hanson J, Engström P (2003) The *Arabidopsis thaliana* homeobox gene ATHB5 is a potential regulator of abscisic acid responsiveness in developing seedlings. Plant Mol Biol **51:** 719–729

Kagaya Y, Ohmiya K, Hattori T (1999) RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Res **27:** 470–478

Kaundal R, Saini R, Zhao PX (2010) Combining machine learning and homology-based approaches to accurately predict subcellular localization in Arabidopsis. Plant Physiol **154:** 36–54

Lindinger W, Hansel A, Jordan A (1998) On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) - Medical applications, food control and environmental research. Int J Mass Spectrom **173:** 191–241

Lois LM, Rodríguez-Concepción M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. Plant J **22:** 503–513

Loreto F, SHARKEY TD (1990) A gas-exchange study of photosynthesis and isoprene emission in *Quercus rubra* L. Planta **182:** 523–531

Loveys B, Van Dijk H (1988) Improved extraction of abscisic acid from plant tissue. Functional Plant Biology **15:** 421–427

Monson RK, Fall R (1989) Isoprene emission from aspen leaves: influence of environment and relation to photosynthesis and photorespiration. Plant Physiol **90:** 267–274

Muñoz-Bertomeu J, Arrillaga I, Ros R, Segura J (2006) Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. Plant Physiol **142:** 890–900

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Ann Rev Plant Biol **56:** 165–185

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:** e45

Phillips MA, Walter MH, Ralph SG, Dabrowska P, Luck K, Urós EM, Boland W, Strack D, Rodríguez-Concepción M, Bohlmann J, Gershenzon J (2007) Functional identification and differential expression of *1-deoxy-D-xylulose 5-phosphate synthase* in induced terpenoid resin formation of Norway spruce (Picea abies). Plant Mol Biol **65:** 243–257

Rasulov B, Huve K, Bichele I, Laisk A, Niinemets U (2010) Temperature response of isoprene emission in vivo reflects a combined effect of substrate limitations and isoprene synthase activity: a kinetic analysis. Plant Physiol **154**: 1550-1570

Rodríguez-Concepción M (2006) Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. Phytochemistry Reviews **5:** 1–15

Ruperti B, Bonghi C, Rasori A, Ramina A, Tonutti P (2001) Characterization and expression of two members of the peach 1-aminocyclopropane-1-carboxylate oxidase gene family. Physiol Plantarum **111:** 336–344

Sakai H, Aoyama T, Oka A (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J **24:** 703–711

Sakai H, Aoyama T, Oka A (2000) Arabidopsis ARR1 and ARR2 response regulators operate as transcriptional activators. Plant J **24:** 703–711

Schmidt R, Fedtke C (1977) Metamitron activity in tolerant and susceptible plants. Pestic Sci **8:** 611–617

Schnitzler J, Louis S, Behnke K, Loivamäki M (2009) Poplar volatiles–biosynthesis, regulation and (eco) physiology of isoprene and stress-induced isoprenoids. Plant Biology **12:** 302–316

Sharkey TD, Wiberley AE, Donohue AR (2008) Isoprene emission from plants: why and how. Ann Bot **101:** 5–18

SHARKEY TD, Yeh SS (2001) Isoprene emission from plants. Annu Rev Plant Phys **52:** 407–436

Solano R, Nieto C, Avila J, CANAS L, Diaz I, PAZARES J (1995) Dual DNA-binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. Embo J **14:** 1773–1784

Stöver B, Müller K (2010) TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics 2010 **11**:7-16

Talòn M, Hedden P, Primo-Millo E (1990) Hormonal changes associated with fruit set and development in mandarins differing in their parthenocarpic ability. Physiol Plant **79**: 400-406

Talòn M, Tadeo FR, Ben-Cheikh W, Gomez-Cadenas A, Mehouachi J, Perez-Botella J, Primo-Millo E (1997) Hormonal regulation of fruit set and abscission in Citrus: Classical concepts and new evidence. Acta Hort **463**: 209–217

To JP, Haberer G, Ferreira FJ, Deruère J, Mason MG, Schaller GE, Alonso JM, Ecker JR, Kieber JJ (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. Plant Cell **16:** 658–671

Velasco R, Zharkikh A, Affourtit J, Dhingra A, Cestaro A, Kalyanaraman A, Fontana P, Bhatnagar SK, Troggio M, Pruss D et al. (2010) The genome of the domesticated apple (*Malus* x *domestica* Borkh.). Nat Genet **42**: 833-839

Vernieri P, Tagliasacchi AM, Forino L, Lanfranchi A, Lorenzi R, Avanzi S (1992) Abscisic acid levels and cell structure in single seed tissues of shedding affected fruits of *Malus domestica* Borkh. Plant Physiol **140**: 699-706

Vezzaro A, Boschetti A, Dell'anna R, Canteri R, Dimauro M, Ramina A, Ferasin M, Giulivo C, Ruperti B (2011) Influence of olive (cv Grignano) fruit ripening and oil

extraction under different nitrogen regimes on volatile organic compound emissions studied by PTR-MS technique. Analytical and bioanalytical chemistry (online). DOI: 10.1007/s00216-010-4636-1.

Vickers CE, Gershenzon J, Lerdau MT, Loreto F (2009) A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nat Chem Biol **5:** 283–291

Walter MH, Fester T, Strack D (2000) Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the 'yellow pigment' and other apocarotenoids. Plant J **21:** 571–578

Walter MH, Hans J, Strack D (2002) Two distantly related genes encoding 1-deoxy-d-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. Plant J **31:** 243–254

Weiler EW (1982) An enzyme-immunoassay for cis-(+)-abscisic acid. Physiol Plant **54**: 510-514

Wolfertz M, Sharkey TD, Boland W, Kühnemann F (2004) Rapid regulation of the methylerythritol 4-phosphate pathway during isoprene synthesis. Plant Physiol **135:** 1939–1945

Xu YW, Wesely ML, Pierce TE (2002) Estimates of biogenic emissions using satellite observations and influence of isoprene emission on O^3 formation over the eastern United States. Atmos Environ **36:** 5819–5829

Yuan R, Huang H (1988) Litchi fruit abscission: its patterns, effect of shading and relation to endogenous abscisic acid. Sci Hort **36**: 281-292

Zhang M, Li K, Zhang C, Gai J, Yu D (2009) Identification and characterization of class 1 DXS gene encoding 1-deoxy-d-xylulose-5-phosphate synthase, the first committed enzyme of the MEP pathway from soybean. Mol Biol Rep **36:** 879–887

Chapter 4 – General conclusions and future perspectives

General conclusions

Besides its obvious practical implications, the full comprehension of the fruitlet abscission process may represent an intriguing challenge also from a basic research point of view. The identification of the primary signal that triggers abscission relies upon the ability of selectively induce the process in an easily identifiable subpopulation of fruitlets. This is the key experimental strategy allowing researchers to observe the changes occurring in those fruitlets whose destiny can be predicted with good approximation. In this context, the use of thinning chemicals with known effects at the field level represents an indefeasible tool for research in fruit abscission. Among these compounds, those magnifying the physiological drop by mimicking its natural causes are particularly powerful, although the mechanism through which most of them act still remains partially unknown.

Benzyladenine (BA) was used in this study to selectively induce apple fruitlet abscission and allow to point out the changes detectable at the transcriptional and metabolomic levels during the early phases of shedding event. Metamitron (MET) was also used as an additional thinning agent. In the present dissertation, a model was prompted out based upon a massive transcriptomic study carried out on abscising (AFs) and non-abscising (NAFs) fruitlets. The main regulatory networks triggering abscission induction were identified in cortex and seed, by means of a newly released 30K microarray. Concurrently, the volatile organic compounds (VOCs) emitted by AFs and NAFs were monitored by means of proton-transfer reaction mass spectrometer, allowing the identification of metabolic abscission markers.

According to the main model, apple fruitlet abscission takes place in four main steps, corresponding to the four structural levels where the key events may occur (*i.e.* the tree, the fruit cortex, the seed, and the abscission zone). The initial steps arise at the tree level, where a nutritional stress is established either naturally or upon a thinning treatment with BA. This condition is plausible since at the beginning of the vegetative and reproductive season the growth of shoots and fruitlets is supported at a large extent by stored assimilates. As a consequence, a strong competition for storage assimilate reallocation exists among shoots, between shoots and fruits, among fruits of different clusters, and fruits of the same cluster. Since young growing shoots act as stronger sinks than fruits, the tree is unable to support all the growing fruitlets, causing the ‘weaker’ ones to naturally abscise thus generating the

fruitlet physiological drop. When BA is used as a thinner, a magnification of the already existing nutritional stress occurs, since this cytokinin is known to induce lateral bud outgrowth, thus enhancing the shoot sink activity and, consequently, the competition for assimilates. Sugar starvation would not directly trigger senescence-associated gene expression in the cortex of AFs, but rather contribute to the installation of the sugar signalling causing in turn the transcriptomic reaction associated to abscission induction. This reaction would be most likely mediated by ROS accumulation and, in this context, ABA signalling concurrently orchestrates sugars-ROS cross-talk, as pointed out by transcriptomic data showing typical signatures of ABA action that are differentially expressed during abscission induction. During the early phases of abscission induction, an active resource mobilization is already established in the cortex according to gene expression data. Moreover, persisting fruitlets show transcriptomic profiles typical of actively growing organs, in contrast with the abscising ones displaying expression levels for the same genes compatible with a block of their growth, especially in terms of transcripts encoding structural and metabolic elements. Gene expression data indicate not only an active resource mobilization, but also active protein degradation and vesicular trafficking, all of which are most likely triggered later on when the abscission signal is fully installed. Finally, ethylene (ET) signalling was also found to be triggered, but only downstream the abscission signal generation and as a consequence of the cross-talk with ABA and ROS. In this context, it is worthy to note that hormone-related transcriptomic signatures assessed 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. In fact, our data show that ABA and ET signalling are strongly up-regulated concurrently with a down-regulation of GA signalling specifically in fruits induced to abscise. After the early reaction of the cortex, it may be hypothesized that a link is established with the seed when the abscission inductive process reaches an irreversible status. The seed is a structure with a stronger homeostasis than the cortex, since it represents the reproductive endeavour carried out by the tree, and thus protected until the abscission process may become unrecoverable. Ethylene may function as the signal generated within the cortex and, through diffusion, carrying to the seed the abscission signal, as suggested by the transcription rates of several elements of its transductive pathway. In the seed, the signalling cascade activated by the

abscission induction causes a block of the embryogenesis. Reactive oxygen species may play an important role also in this case, as some typical transcriptional signatures of high ROS levels were found also in the seed. Therefore, the oxidative atmosphere where the seed is constricted at this stage along with the nutritional stress and the signals coming from the cortex may contribute to the increase of ROS production, which in turn would disrupt metabolism and suppress the synthesis of IAA as previously described. The reduced supply of auxin to the AZ concurrently with a likely depolarization of its transport would enhance its sensitivity to ethylene and the consequent activation of cell wall degrading enzymes.

The analysis of VOCs emitted by AFs and NAFs allowed to further implement this model, especially concerning the regulatory role played by abscisic acid at the cortex level. According to the main findings, AFs emitted more isoprene than NAFs, and this emission was strongly correlated with the content of ABA of the cortex, showing a peak 2 days after the thinning treatments such as isoprene. ABA perception and the following activation of its transduction pathway, paralleled by increased levels of the hormone, would lead to a burst of ROS, which is a typical secondary messenger, and the subsequent activation of the specific detoxifying machinery at the transcriptional level. Isoprene is part of this mechanism of detoxification, and the up-regulation found for its biosynthetic gene ISPS may be due to the presence of ABA-responsive elements (ABREs) pointed out in its promoter. A concurrent positive feedback, involving NCED genes, would ensure the higher levels of abscisic acid measured thereafter in AFs and the cross-talk with ethylene, whose physiological consequences are observed in the following days. Interesting findings were also found with respect to the upstream pathway (i.e. the MEP pathway), whose regulation at the transcriptional level was demonstrated. A novel feedback control was also pointed out for a gene encoding a key enzyme of the MEP pathway, the DXS.

Future perspectives

The model herein proposed for apple fruitlet abscission represents with good approximation the main signalling pathways involved in the early phases of the process. However, the primary signal that triggers abscission is still unknown. It is likely that this ‘determinant’ can be found among the actors involved in sugar signalling, as they are supposed to be the initial transducers of the nutritional stress to the fruit. Therefore, future investigation will be focused on this promising part of the model, which is strictly linked to ABA and ROS

signallings. Particular attention will be paid to the transcription factors and to the kinases. Moreover, new candidates may also be supposed to play a relevant role during abscission induction: the micro RNAs. miRNAs would represent the ideal candidates because they could be involved both in the cross-talk between cortex and seed, and in the transmission of the signal to the abscission zone. Moreover, their role is also strictly connected to the transcription factors, which often represent a miRNA target.

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. However, the resolution of these imaging systems is not sufficient to provide precise data at the orchard level. Therefore, a high-resolution dedicated system is being developed in order to test its actual efficacy in predicting fruit load and, consequently, tune the thinning treatments according to a modern environment-friendly agriculture.

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