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RESEARCH PAPER

Early gene expression events in the laminar abscission zone of abscission-promoted citrus leaves after a cycle of water stress/rehydration: involvement of CitbHLH1

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Abstract

Leaf abscission is a common response of plants to drought stress. Some species, such as citrus, have evolved a specific behaviour in this respect, keeping their leaves attached to the plant body during water stress until this is released by irrigation or rain. This study successfully reproduced this phenomenon under controlled conditions (24 h of water stress followed by 24 h of rehydration) and used it to construct a suppression subtractive hybridization cDNA library enriched in genes involved in the early stages of rehydration-promoted leaf abscission after water stress. Sequencing of the library yielded 314 unigenes, which were spotted onto nylon membranes. Membrane hybridization with petiole (Pet)- and laminar abscission zone (LAZ)-enriched RNA samples corresponding to early steps in leaf abscission revealed an almost exclusive preferential gene expression programme in the LAZ. The data identified major processes such as protein metabolism, cell-wall modification, signalling, control of transcription and vesicle production, and transport as the main biological processes activated in LAZs during the early steps of rehydration-promoted leaf abscission after water stress. Based on these findings, a model for the early steps of citrus leaf abscission is proposed. In addition, it is suggested that *CitbHLH1*, the putative citrus orthologue of *Arabidopsis BIGPETAL*, may play major roles in the control of abscission-related events in citrus abscission zones.

Key words: *Citrus clementina*, cDNA custom macroarray, expression profiling, laminar abscission zone, mandarin, petiole.

Introduction

Plants have developed sophisticated metabolic and physiological strategies to survive under adverse environmental conditions. To cope with stress, plants have evolved responses that recognize the stressful condition and subsequently activate counteractive reactions. In this respect, a common response is the shedding of physiologically damaged organs through the activation of the abscission programme.

Abscission of citrus organs is dependent on the activation of different abscission zones (AZs) (Tadeo *et al.*, 2008). Bud and

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AZ, abscission zone; AZ-A, pedicel abscission zone; AZ-C, calyx abscission zone; bHLH, basic helix–loop–helix; CSN, COP9 signalosome; EST, expressed sequence tag; GO, Gene Ontology; JA, jasmonic acid; LAZ, laminar abscission zone; Ped, pedicel; Pet, petiole; qRT-PCR, quantitative real-time RT-PCR; ROS, reactive oxygen species.

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flower abscission takes place at the AZ located within the pedicel (AZ-A), whereas fruitlet and fruit abscission activate the AZ located in the calyx between the pericarp and the nectary or floral disc (AZ-C). Mature citrus leaves drop by activation of the laminar AZ (LAZ) located at the interface between the petiole and the leaf blade, and aged leaves by activation of the branch AZ located at the branch-to-petiole junction. The shedding of citrus fruits and leaves is regulated by developmental, hormonal, and environmental cues (Tadeo *et al.*, 2008). A number of biotic and abiotic stresses such as fungus invasion (Li *et al.*, 2003), extreme temperatures (Young and Meredith, 1971), salinity (Gomez-Cadenas *et al.*, 1998), carbohydrate availability (Gomez-Cadenas *et al.*, 2000), and water stress (Tudela and Primo-Millo, 1992) have been reported to promote organ abscission, and a common pivotal role has been demonstrated for ethylene. Leaves in citrus trees under water stress get injured but not shed, remaining attached until the stress is released by rain or irrigation and, soon afterwards, the leaves abscise (Addicot, 1982). This behaviour has also been reported in a few other species, such as cotton (Jordan and Day, 1972). We have reported previously that citrus leaf abscission induced by rewatering after a period of water stress requires abscisic acid (ABA) accumulation in roots (Gomez-Cadenas *et al.*, 1996). Accordingly, this is paralleled by the pattern of expression of *CcNCED3*, a key regulatory gene for ABA biosynthesis (Agustí *et al.*, 2007*b*). Under these stressful conditions, xylem flow is arrested and ABA and 1-aminocyclopropane-1-carboxylic acid (ACC) accumulate sequentially in roots. In contrast, water-stressed leaves accumulate only ABA, while a transitory rise in both ACC content and ethylene emission is detected soon after rehydration (Gomez-Cadenas *et al.*, 1996).

Based on this knowledge, we took advantage of an experimental set-up used previously (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al.*, 1996) to induce leaf abscission in citrus seedlings after a drought and rehydration cycle. This system mimics natural conditions inducing abscission, allowing an *in vivo* study of the process. Importantly, the knowledge gained with this system predicted that the abscission machinery is triggered at the LAZ during the first 6 h of rewatering (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al.*, 1996).

To identify key regulatory genes at the onset of the process, we constructed a suppression subtractive hybridization cDNA library enriched in genes expressed within the LAZ during early time points in rehydration-promoted abscission. Following a differential expression strategy using a custom macroarray, we isolated a set of genes expressed differentially in the LAZ during the early events of the process. In addition, we performed detailed studies on CitbHLH1, a transcription factor identified previously in the LAZ during ethylene-promoted leaf abscission (Agustí *et al.*, 2008). The data presented here point to early and transitory preferential expression of *CitbHLH1* in LAZ during rehydration. Further analyses suggested that *CitbHLH1* is preferentially expressed in AZ-A and AZ-C during late abscission steps.

In summary, the results presented here expand previous transcriptome remodelling studies on abscission (Agustí *et al.*, 2008, 2009), defining the early molecular events that bring about the process in citrus. In addition, this work provides valuable information for future biotechnological applications.

Materials and methods

Plant material

For the drought/rehydration experiments, 1-year-old Clemenules mandarin (*Citrus clementina* Hort. Ex Tan.) grafted on the rootstock Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) seedlings were used. Each plant had around 70 leaves at this stage. Plants were grown in 2 l plastic pots filled with washed, inert sand. Greenhouse conditions were as described previously (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al.*, 1996; Agustí *et al.*, 2008). Plants were grown under constant temperature (26 °C), with a 16 h photoperiod and a relative humidity oscillating between 60 and 95%. Abscission zones and petioles were collected as described below. In the ethylene treatment experiments, leaves and developing ovaries were collected from adult Clemenules mandarin trees grafted on Cleopatra mandarin and grown in a homogeneous experimental orchard under standard cultural practices. Explants were sustained in 1% (w/v) agar in 9 cm Petri dishes (Sterilin) and incubated in sealed 10 l containers at 22 ºC with a 16 h light period under fluorescent lighting, as previously described (Agustí *et al.*, 2008). Treatments were performed with ethylene (10 μl l^{-1}) for different periods: 0, 2, 6, 12, and 24 h. In all cases, for each time point, three independent pools of 100 explants were each distributed in two Petri dishes. LAZ, petiole (Pet), AZ-A, pedicel (Ped), AZ-C, and ovary samples were obtained using a blade and stored at –80 ºC for future RNA extractions.

Water stress treatments and sampling

A drought/rehydration treatment was performed as described previously (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al.*, 1996). Drought was imposed by transplanting the plants to pots with dry sand. These conditions were maintained for 24 h. Water stress was then released by placing the stressed plants in 1 l jars filled with water. After 24 h of rehydration, leaf abscission was determined as the percentage of leaves that shed with a gentle touch. For additional experimental details, see the previous references given above. LAZ and Pet were collected at different periods of time during the drought/rehydration treatments. Samples for membrane hybridization and/or quantitative real-time RT-PCR (qRT-PCR) were collected in three different pools containing 100 LAZ or Pet. All samples were frozen with liquid nitrogen and stored at –80 ºC until used for the analyses described below.

RNA extraction

Total RNA was isolated from frozen plant material reduced to a fine powder using an RNeasy Plant Mini kit (Qiagen). RNase-free DNase (Qiagen) was used to treat RNA samples by column purification following the manufacturer's instructions. The quantity and quality of the samples was tested by UV absorption spectrophotometry and gel electrophoresis as described previously (Sambrook *et al.*, 1989).

Membrane printing and hybridization

A subtractive library was constructed with LAZ RNA from drought-stressed and rehydrated plants (1, 6, and 10 h after rehydration) subtracted with LAZ RNA from unstressed plant (Forment *et al.*, 2005; Terol *et al.*, 2007). Library sequencing yielded 314 genes. The clones were transferred in quadruplicate onto nylon membranes accompanied by four spikes from *Bacillus thuringiensis*, as previously described (Alberola *et al.*, 2004). To identify differentially expressed genes in citrus LAZ, the membranes were hybridized using ^{33}P-labelled RNAs of plants subjected to 24 h drought and 1 and 6 h rehydration from both AZ and Pet tissues. For each hybridized condition, we used three biological replicates. The hybridization data are shown in Supplementary Table S1 at *JXB* online.

Normalization and statistical analysis

The spot signal values were estimated as the difference between the foreground and the background. We first applied the quartile

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normalization method between the three membranes for each condition and then normalized according to the expression of the spikes on the membrane. Based on this, we identified a putative secreted glycoprotein 3 (C21001G11) as a housekeeping gene for our conditions. We validated its expression by qRT-PCR as described below. The obtained values for this gene at different time points were then used to fine-tune the normalized membrane values (Supplementary Fig. S1 at *JXB* online). Statistics analyses were carried out using two different programs: maSigPro (Conesa *et al.*, 2006) and LIMMA (Gentleman *et al.*, 2004; Smith, 2004). We performed a contrast between the expressions associated with each tissue. A P value of 0.01 and a difference filter of $\log_2=1$ was imposed (Supplementary Table S2 at *JXB* online).

In order to perform a Gene Ontology (GO) analysis, the nearest homologous gene from *Arabidopsis* was assigned to each differentially expressed citrus gene (obtained from CGFP, http://bioinfo.ibmcp.upv.es/genomics/cfgpDB/) using the BLAST tool (Altschul *et al.*, 1990; E-value of less than $1.0E^{-3}$) at TAIR (The Arabidopsis Information Resource, www.arabidopsis.org). Citrus genes were grouped in functional categories using GO slims (Supplementary Table S2).

qRT-PCR analysis

RNA extractions were performed as described above and RNA concentration was determined by fluorometric assays in triplicate using RiboGreen dye (Molecular Probes) following the manufacturer's instructions. qRT-PCR was performed with a LightCycler 2.0 Instrument (Roche) equipped with LightCycler Software version 4.0 as described previously (Agustí *et al.*, 2008). Transformation of fluorescence intensity data into relative mRNA levels was carried out using a standard curve constructed with a tenfold dilution series of a single RNA sample. Relative mRNA levels were normalized to total RNA amounts as described previously (Bustin, 2002; Hashimoto *et al.*, 2004). We assessed the specificity of the amplification reactions by the post-amplification dissociation curves and product sequencing. We expressed the results as contrasts between abscission zone and petiole tissues. The sequences of the forward and reverse primers and the size of the resulting fragments are listed in Supplementary Table S3 at *JXB* online.

Results and discussion

Leaf abscission under cycles of drought and rehydration

We induced leaf abscission utilizing a cycle of drought and rehydration based on our previous findings (Gomez-Cadenas *et al*., 1996). This system presents clear advantages compared with others, especially when applied to perform molecular studies. Firstly, the treatments are performed on whole intact plants, avoiding secondary effects caused by the removal of organs from the plant. Secondly, our system emulates a natural environmental stressful condition that induces abscission in citrus and other species (Addicot, 1982). Combining this system with differential expression analyses allowed identification of fundamental regulatory genes for the onset of the process.

We used our *in vivo*-induced leaf abscission methodology in Clemenules seedlings grafted on Cleopatra mandarin, and abscission occurred in the LAZ, according to previous descriptions (Goren, 1993). Well-watered and 24 h water-stressed plants did not display any leaf abscission. After 24 h of rehydration, plants that had been stressed previously for 24 h displayed a high range of defoliation (~48%). Under these experimental conditions, mechanical wounding or hypoxia in roots had no influence on leaf abscission (Gomez-Cadenas *et al*., 1996).

Differential gene expression in the LAZ

Differential gene expression using a citrus custom macroarray (see Materials and Methods) was assessed, contrasting the hybridization signal between LAZ and Pet. The time points analysed in this study were selected based on previous physiological characterization of the experimental system (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al*., 1996; Agustí *et al*., 2008). For example, an ethylene peak in leaves, considered responsible for leaf abscission, was reported previously to occur only during the first hours after rehydration, and not during the stress period (Tudela and Primo-Millo, 1992). Therefore, we selected two early time points after rehydration (1 and 6 h) in order to detect genes responsible for the early abscission events. In addition, a 24 h water-stress time point was chosen because, at that stage, no ethylene is produced in leaves and no abscission occurs.

At 24 h of water stress, only eight genes were expressed differentially between LAZ and Pet samples, while at 1 and 6 h after rehydration, 195 and 196 genes displayed significant differential expression, respectively (Supplementary Table S2). Almost all regulated genes were expressed preferentially at the LAZ. This was expected, given the nature of the library. Therefore, the results presented here are exclusively those regarding genes expressed preferentially in the LAZ.

Clustering of genes into GO annotation highlighted the most activated functional categories during the onset of abscission that were involved in cell organization and biogenesis, and metabolism of fatty acid, nucleotide, and protein. Other important categories for the process were signal transduction, response to biotic and abiotic stimuli, carbohydrate metabolism, transcription control, development, and transport. Differential expression of selected genes and gene families of particular biological interest is illustrated in Table 1 and discussed in further detail in the next sections.

We validated our expression results by checking some genes by qRT-PCR (Fig. 2).

Protein biosynthesis and metabolism Among the genes related to protein biosynthesis and metabolism (degradation), 46 out of 59 (78%) were expressed preferentially in the LAZ during the rehydration period. Structural ribosomal proteins from both small (50S ribosomal protein L14) and large (60S ribosomal protein L7 and ribosomal protein L12) subunits were expressed preferentially in the LAZ (Table 1). The involvement of protein biosynthesis was also supported by the induction of genes encoding translation initiation and elongation factors. This is consistent with previous evidence of stimulation by ethylene of protein biosynthesis within the AZ in bean, cotton, and citrus (Abeles and Holm, 1966; Lewis and Bakhshi, 1968; Agustí *et al.*, 2008). Expression of the proteolytic machinery was also activated. This is in agreement with previous observations of protein degradation in the petal AZ of rose (Tripathi *et al.*, 2009). The combination of protein biosynthesis and degradation suggests a protein change context during the early steps of citrus leaf abscission. We found preferential expression in the LAZ for three putative E2 ligase proteins. One of them (C21002B05) is a putative citrus orthologue of the *Arabidopsis*

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Table 1. Relative gene expression values (LAZ versus Pet) of genes involved in different functional categories. –, No significant regulation. Extended biological information is described in Supplementary Table S2. Putative gene identifications are based on sequence homology with *Arabidopsis thaliana*.

Clone ID	Gene identification	Putative *Ath* orthologue	Relative expression (log_2) LAZ vs. Pet		
			Drought	Rehydration	
			24 h	1 h	6 h
Protein biosynthesis and metabolism					
C21003F07	50S ribosomal protein L14	AT5G46160	–	2.02	1.56
C21001B12	60S ribosomal protein L7	AT2G01250	–	2.61	2.13
C21001G04	60S ribosomal protein L12	AT5G60670	–	2.16	1.35
C21005C05	Translation initiation factor	AT4G27130	–	1.95	2.19
C21004F10	Translation elongation factor	AT1G57720	–	2.69	–
C21001B07	Ubiquitin-fold modifier	AT1G77710	–	2.20	1.84
C21002B05	Ubiquitin-conjugating enzyme	AT2G02760	–	2.43	1.79
C21005H08	Ubiquitin-conjugating enzyme	AT5G41340	–	–	2.41
C21001E07	Ubiquitin-conjugating enzyme	AT5G59300	–	1.38	0.88
C21007A04	RING/U-box domain-containing protein	AT2G47700	–	2.14	1.06
C21001C06	E3 ubiquitin-protein ligase	AT5G63970	–	–	2.72
C21005E02	Proteasome subunit α type	AT3G14290	–	2.64	2.16
C21004H02	COP9 signalosome complex subunit	AT3G61140	–	2.31	1.93
Carbohydrate biosynthesis and metabolism					
C21005A06	Xyloglucan endotransglucosylase/hydrolase	AT4G25810	–	3.35	1.55
C21005E09	UDP-glucose dehydrogenase	AT5G15490	–	3.43	2.69
C21001D09	Glycosyltransferase	AT3G57380	–	1.30	1.40
C21003F02	Pectate lyase	AT4G24780	–	2.77	1.65
C21008F11	Acidic cellulase	AT4G02800	3.37	3.20	1.92
Fatty acid biosynthesis and metabolism					
C21006C05	Acyl-CoA synthetase-like protein	AT3G23790	–	1.75	1.05
C21001F06	1-Acyl-sn-glycerol-3-phosphate acyltransferase	AT3G57650	–	1.53	–
C21002E09	Diacylglycerol kinase	AT5G07920	–	1.85	0.99
C21006E04	ω-3 Fatty acid desaturase	AT5G05580	1.86	1.63	1.23
C21006C01	Enoyl-CoA hydratase	AT1G76150	–	–	1.98
Purine and pirymidine nucleotide metabolism					
C21004H09	Adenosine kinase	AT2G37250	–	2.36	1.53
C21007D09	Nucleoside diphosphate kinase	AT4G09320	–	2.47	2.09
C21002H04	Uridylate kinase	AT5G26667	–	2.25	2.74
Transport					
C21008E12	Voltage-gated CLC-type chloride channel	AT1G55620	–	1.95	2.21
C21001C08	Metal transporter	AT1G05300	–	2.75	1.81
C21002B12	α-Soluble NSF attachment protein	AT3G56190	–	1.93	2.07
C21007A05	Transducin/WD-40 repeat family protein	AT2G30050	–	2.50	1.94
C21005C09	SNARE-like protein	AT1G15370	–	–	2.21
C21003C01	Syntaxin	AT5G06320	–	–	1.32
Response to abiotic and biotic stimulus					
C21001G09	Phospholipid hydroperoxide glutathione peroxidase-like protein	AT4G11600	–	2.26	1.44
C21007C11	Flavonoid 3-hydroxylase	AT1G33730	–	1.66	1.29
C21007B12	RAD23-like protein	AT3G02540	–	1.62	–
C21001D05	AMP-dependent CoA ligase	AT1G20510	–	1.90	1.38
C21007C07	Photoassimilate-responsive PAR-like protein	AT5G52390	3.39	3.23	3.01
C21001F11	Tubulin β–chain	AT5G62690	–	5.11	2.77
C21004D12	Glutathione *S*-transferase	AT2G02380	–	1.88	1.30
C21006E11	Putative respiratory burst oxidase-like protein B	AT1G09090	–	–	1.31
Signal transduction					
C21002D12	Sensor histidine kinase	AT4G16110	–	1.71	1.51
C21005A02	Leucine-rich repeat family protein	AT3G43740	–	2.53	2.65
C21005D04	Calmodulin	AT3G43810	–	3.15	2.97
C21008H05	Leucine-rich repeat receptor-like kinase	AT2G31880	–	1.83	–
Regulation of transcription					
C21001H11	bHLH transcription factor	AT1G59640	–	3.38	1.75
C21006F01	MYB transcription factor	AT1G68320	–	–	1.85

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ubiquitin-conjugating enzyme 2 (AtUBC2; Bartling *et al.*, 1993), recently associated with regulation of flowering time and other developmental events (Xu *et al.*, 2009). The other two (C21005H08 and C21001E07) are the putative citrus orthologues of the *Arabidopsis* ubiquitin-conjugating enzymes 4 and 7, associated with pollen germination as well as tube growth (Wang *et al.*, 2008). The *Arabidopsis* genome encodes 37 ubiquitin E2 proteins (Kraft *et al.*, 2005), and only a few of them have been characterized. It is known that plants can use the ubiquitin proteasome pathway to control the level and activity of their constituent proteins by performing a selective breakdown (Vierstra, 2003). We have already suggested that such regulation could be a major regulatory event during late stages of abscission (Agustí *et al.*, 2008), and the results presented here not only support this idea but also extend it to the onset of the process. In the same line of evidence, we also identified a nuclear RING/U-box domain-containing protein (C21007A04) whose closest orthologue in *Arabidopsis* is involved in red and far-red light signalling (Chen and Ni, 2006). In addition, a putative orthologue for the *Arabidopsis FUSCA6* (*FUS6*), which encodes the CSN1 subunit of the COP9 signalosome (CSN) was found to be preferentially expressed in the LAZ. In *Arabidopsis*, the CSN complex modulates a wide variety of cellular processes by regulating specific protein degradation events (Serino and Deng, 2003). These two genes could, therefore, be related not only to proteolysis but also to signalling events occurring within the abscission zone.

Taken together, our results suggested that the specific activation of the protein metabolism within the AZ is a consequence of remodelling of protein composition coupled with the activation of signalling events.

Metabolism of carbohydrates and lipids and membrane trafficking Sequencing of the library yielded 15 carbohydrate metabolism-related genes, 12 of which (80%) were expressed preferentially within the AZ (Fig. 1). Such regulation was expected, as it is well documented that the later steps of abscission involve degradation of the middle lamella and primary cell wall. Surprisingly, the citrus acidic cellulase (Burns *et al.*, 1998) was found to be already highly expressed at 24 h of drought treatment (Fig. 2, Table 1), a time period in which there is no abscission. Citrus acidic cellulase is triggered by ethylene (Burns *et al.*, 1998). However, previous reports on our experimental set-up indicated that even 48 h of drought did not trigger either ethylene production or abscission in citrus leaves, and that only after rehydration did ethylene production occur and abscission take place (Tudela and Primo-Millo, 1992; Gomez-Cadenas *et al.*, 1996). Our results suggested that, in addition to ethylene, another kind of regulation exists for the induction by drought stress of citrus acidic cellulase. It is known that ABA is able to stimulate abscission in citrus leaf explants (Sagee *et al.*, 1980) and that a continuous increase in ABA is detected in citrus leaves during water stress (Gomez-Cadenas *et al.*, 1996). These results support the idea that ABA could be involved in the regulation of the citrus acidic cellulase expression during the water-stress period. In other plant species such as *Arabidopsis thaliana*, a role for ABA in regulation of the expression of cell-wall hydrolases in AZs during abscission has been demonstrated (Ogawa *et al.*, 2009). It is possible that, after rehydration, ethylene or another endogenous signal may induce relatively high levels of acidic cellulase expression. The other genes selected from this category (xyloglucan endotransglycosylase, UDP-glucose dehydrogenase, pectate

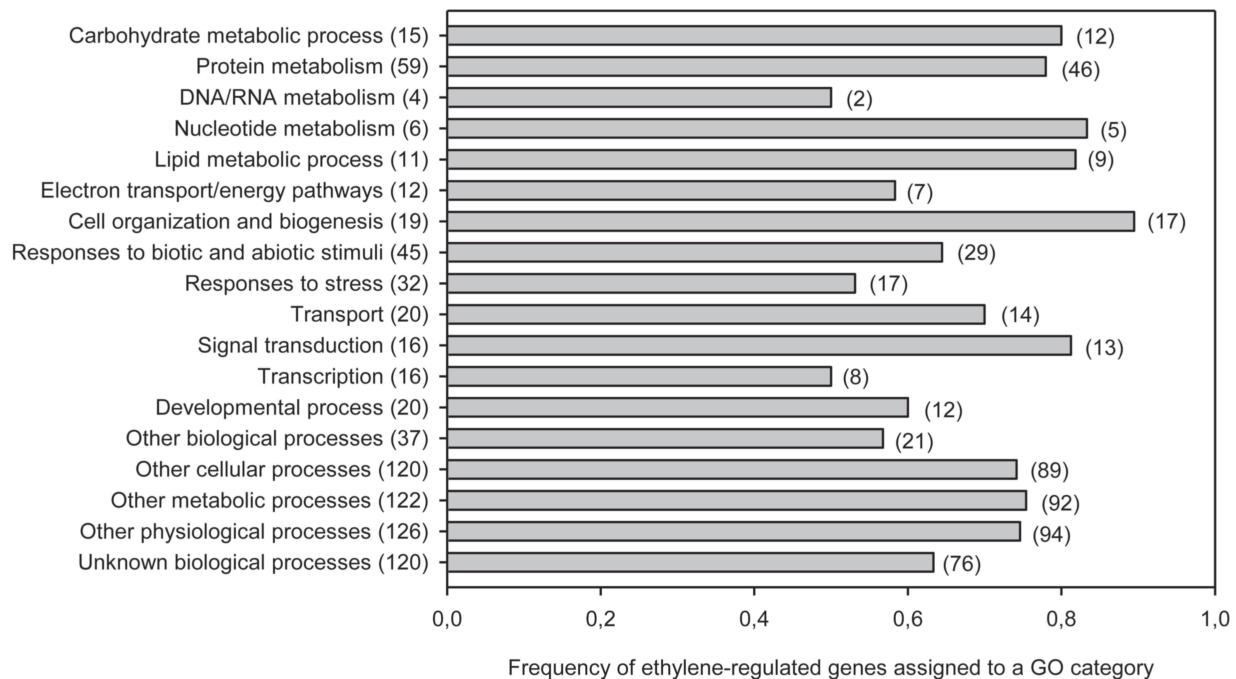

Fig. 1. Ratio and number of ethylene-regulated genes included in GO categories expressed in the LAZ subjected to a cycle of water stress/rehydration. The total number of genes included in the GO categories is shown in the vertical axis. Data are based on macroarray analyses.

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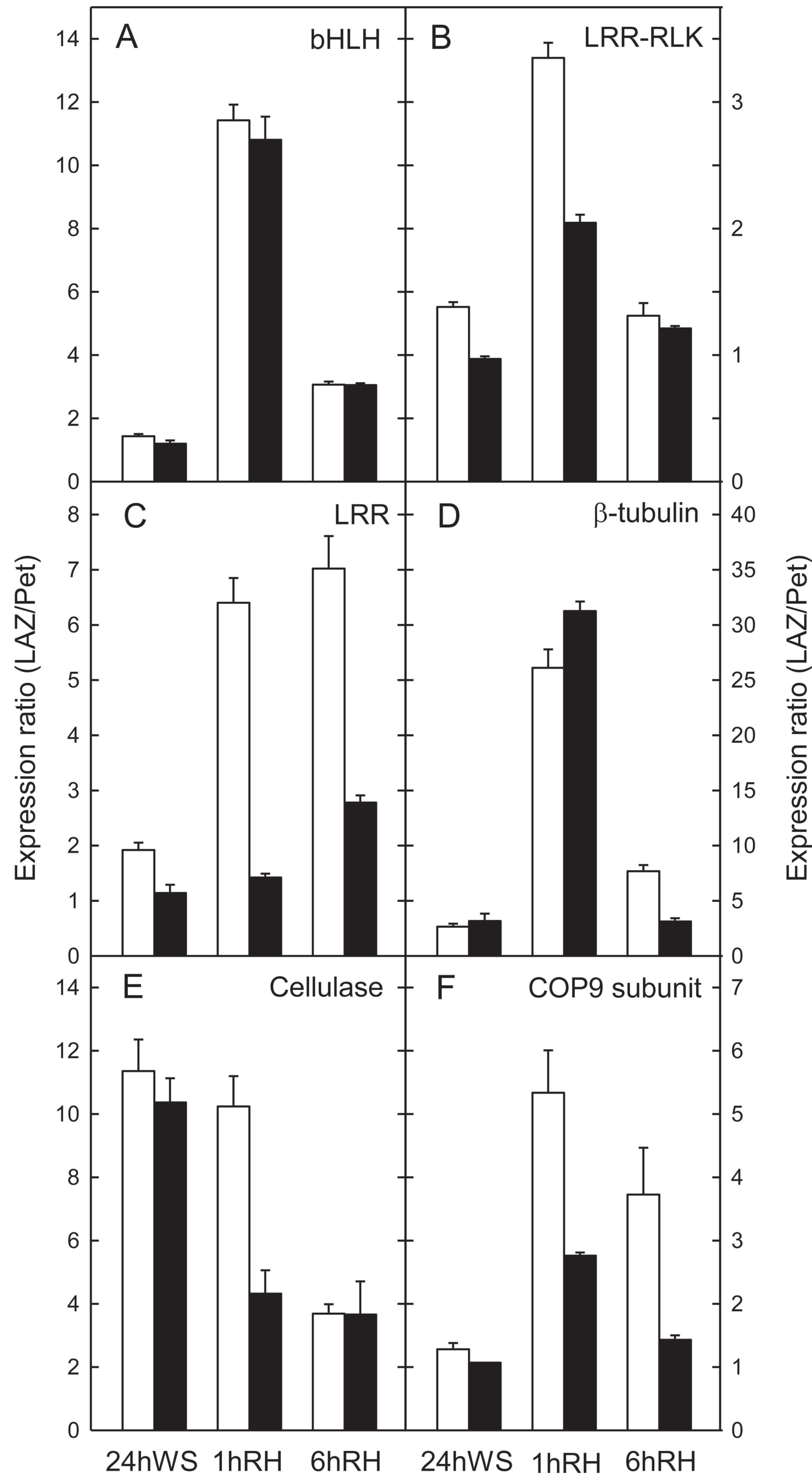

Fig. 2. Macroarray validation by qRT-PCR. Expression ratio (linear) between the LAZ and Pet at 24 h of drought (24 WS), 1 h rehydration (1HRH) and 6 h rehydration (6HRH). (A) C21001H11 (CitbHLH1); (B) C21008H05 (putative LRR-RLK protein, CitEVR); (C) C21005A02 (putative LRR protein); (D) C21001F11 (β1-tubulin); (E) C21008F11/C21006H12 (acidic cellulase); (F) C21004H02 (putative proteasome component domain PCI protein). Open bars, macroarray data; filled bars, qRT-PCR data.

lyase, and glycosyltransferase) followed a pattern of expression in accordance with previously reported ethylene production in the experimental system (Gomez-Cadenas *et al.*, 1996). This is in agreement with the current knowledge about these genes, which are well documented to be active in cell-wall metabolism (Fry *et al.*, 1992).

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A number of purine and pyrimidine metabolism-related genes were expressed preferentially in the LAZ (Table 1). Cell-wall degradation and production are usually coupled, and pyrimidine metabolism, apart from being crucial for cell division, is very important in the production of the cell-wall polysaccharides and carbohydrates, providing UDP/GDP to form sugar-activated blocks (Schroder *et al.*, 2005).

Considering that under our experimental set-up abscission only started after 24 h of rehydration, it is remarkable that some of the crucial genes taking part in cell-wall remodelling already exhibited high expression levels at early rehydration time points (i.e. 1 h; Table 1). This could mean that several steps need to be taken from abscission-related gene expression to leaf detachment, including protein translation and degradation (reflected in the activation of the protein metabolism machinery described above) and probably also transport to the extracellular matrix. Indeed, our results also identified the induction of several genes involved in vesicle trafficking (α-soluble attachment protein, transducin/WD-40 repeat family protein, SNARE-like protein, and syntaxin; Table 1), a process that has recently been indicated as crucial for abscission (Liljegren *et al.*, 2009). In the same line of argumentation, the lipid metabolism category displayed a high frequency of preferential expression within the AZ. Early molecular events in leaf abscission were associated with the activation of both glycerolipid (1-acyl-sn-glycerol-3-phosphate acyltransferase, C21001F06, and diacylglycerol kinase, C21002E09) and fatty acid metabolism (acyl-CoA synthetase, C21006C05, and enoyl-CoA hydratase, C21006C01; Table 1). In general, engaged lipid metabolism usually involves an extensive network of endoplasmic reticulum and Golgi (Speels, 1980), a characteristic of cells undergoing abscission (Iwahori and van Steveninck, 1976; Addicot, 1982). Therefore, the fatty acid metabolism enhancement during abscission could be due partially to the production of new endoplasmic reticulum profiles and Golgi bodies, generated to assist the required membrane trafficking. This scenario would fit the high frequency of induction of genes related to cell organization and biogenesis (89%; Fig. 1). We suggest that this category is also linked with endomembrane system production.

Signal transduction The signalling category displayed induction of 81% of the genes (Fig. 1). A putative calmodulin gene displayed high expression levels within the AZ during the rehydration period. Although several studies have found changes in calcium levels within the middle lamella and primary cell wall during abscission (Sampson, 1918; Stösser *et al.*, 1969; Poovaiah and Rasmusen, 1973), none provided results concluding whether these changes were due to the onset of the process or a consequence of cell-wall breakdown (Sexton and Roberts, 1982). The rapid and continuous preferential expression of the putative calmodulin gene identified in the present work would rather argue for a role of calcium as a second messenger and, in this sense, a role more related to signalling events, as previously suggested (Agustí *et al.*, 2007*a*). Our results also identified the putative orthologue of the *Arabidopsis* response regulator 2 (sensor histidine kinase, C21002D12), a gene that contributes to both cytokinin and ethylene signalling, acting downstream of ETR1 to increase ethylene sensitivity (Hass *et al.*, 2004). We consider the preferential expression of this gene within the AZ (see Supplementary Table S2) as a direct response to the high level of ethylene production during the rehydration period (Gomez-Cadenas *et al.*, 1996). A putative LRR family protein (C21005A02) upregulated in the LAZ has an orthologue in *Arabidopsis* that localizes within the endomembrane system. Interestingly, we identified a leucine-rich repeat-receptor-like kinase (LRR-RLK; C21008H05), which is likely to be the citrus orthologue of *EVERSHED* (CitEVR; Fig. S2 at *JXB* online), a gene that has been shown to regulate membrane trafficking during floral organ abscission in *Arabidopsis* (Leslie *et al.*, 2010). Based on GUS assays as well as RT-PCR and genome wide transcriptional analyses, *EVERSHED* was demonstrated to be expressed in the abscission AZ prior to abscission (Leslie *et al.*, 2010) in a very similar fashion to *HAESA* and *HAESA-like2* (Cho *et al.*, 2008; Jinn *et al.*, 2000). The authors concluded that this expression profile is consequent with a role for *EVR* in modulating the timing of organ shedding, possibly by regulating membrane trafficking. Our results showed a transient differential expression of the putative citrus orthologue for *EVR* in the early periods after rehydration (Fig. 2 and Table 1), suggesting a similar role for this gene in citrus and *Arabidopsis*. Thus, these results would be in concordance with the activation and organization of membrane trafficking during the first steps of citrus leaf abscission discussed above.

We have proposed a role for lipid membrane trafficking in abscission (see above). However, it has been demonstrated that the conversion of certain fatty acids yields potent secondary messengers. For instance, diacylglycerol can be further phosphorylated by diacylglycerol kinase to form phosphatidic acid, a lipid with many regulatory functions in plants (Meijer and Munnik, 2003). In our survey, we found a putative diacylglycerol kinase (C21002E09) to be expressed preferentially in the LAZ during the rehydration period. Hence, we propose that, in addition to playing a role in membrane systems production, fatty acids could partly contribute to signalling events. The abscission-stimulating effect of the pyrazole derivative 5-chloro-3-methyl-4-nitro-1H-pyrazole on citrus plants has been associated with increases in phospholipase A2 and lipooxygenase protein activities and in the levels of lipid hydroperoxide, suggesting the involvement of lipid signalling in abscission (Alferez *et al.*, 2005).

In summary, these results highlight the activation of certain signalling cascades that could be involved in the regulation of several abscission aspects, in particular membrane trafficking. This last idea is linked with results discussed above for other functional categories such as cell organization and biogenesis, and carbohydrate and protein metabolism.

Stress and biotic and abiotic responses A large number of genes belonging to the categories of stress and biotic and abiotic responses were found to be expressed preferentially within the AZ (Table 1). These included genes involved in reactive oxygen species (ROS) detoxification (i.e. glutathione *S*-transferase). Our previous studies associated ROS activation with defence events taking place in the petiole during the last stages of abscission (Agustí *et al.*, 2008, 2009). However, although physiological roles remain to be determined, increases in peroxidase activity in the AZ during the onset of abscission are well documented (Hinman and Lang, 1965; Gahagan *et al.*,

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1968; Henry, 1975; McManus, 1994). Oxidative reactions are thought to be essential for abscission (Marynick, 1977) and antioxidant treatments are able to reduce significantly abscission rates under certain conditions (Michaeli *et al.*, 1999). In addition, peroxidases may be involved in the coordination of gene expression in response to pathogens (Sexton and Roberts, 1982). Our results are in agreement and complement our previous findings (Agustí *et al.*, 2008, 2009). ROS are versatile molecules associated with diverse cellular processes, such as programmed cell death, development, tropisms, hormonal signalling, and abscission (Kwak *et al.*, 2006; Sakamoto *et al.*, 2008). Based on our previous studies and the present work, we suggest a balance of preferential expression of ROS-related genes between the LAZ and Pet during abscission. This balance would be biased towards the LAZ during the early events prior to detachment and to the Pet once cell separation has already started. Hence, ROS could be involved in signalling events taking part during the onset of the process (Agustí *et al.*, 2008). A very interesting gene within this category is the putative AMP-dependent Acyl-CoA ligase. The putative orthologue for this protein in *Arabidopsis* has been reported to increase a range of jasmonic acid (JA) precursors (Kienow *et al.*, 2008). Remarkably, JA has been shown to induce abscission in bean leaves (Ueda *et al.*, 1996) and citrus fruits (Hartmond *et al.*, 2000). It is well established that JA and its precursors (jasmonates) constitute a family of bioactive oxylipins derived from oxygen-containing fatty acids. This would represent a connection between the activation of fatty acid metabolism and abscission, in this case via JA production.

Concerning other genes in this section, they could be in charge of activation of defence programmes during the onset of the process.

Regulation of transcription Overall, 50% of the transcription factors identified in this study (eight out of 16) showed preferential expression in the LAZ. An interesting example is the MYB factor (Table 1). Our previous studies identified other MYB factors preferentially expressed in LAZ during the late stages of the process (Agustí *et al.*, 2008, 2009). We propose that different members of the MYB family could be controlling different aspects of abscission at different stages of the process. The MYB family is very large, and different members are specialized in different biological processes, including cell separation. For example, an essential role for AtMYB26 has been demonstrated in the regulation of the swelling and lignification of the endothecium cell layer in the anther, which is essential to force proper opening of the stomium and pollen release (Steiner-Lange *et al.*, 2003). In addition, our data indicated the preferential expression in the LAZ of *CitbHLH1*, a gene that we had already identified as preferentially expressed in the LAZ during late stages of ethylene-promoted abscission (Agustí *et al.*, 2009). Our results demonstrated a quick induction of the gene after rehydration, coinciding with the ethylene production peak reported previously (Gomez-Cadenas *et al.*, 1996). These data in combination with our previous reports suggest a preferential expression of *CitbHLH1* in the LAZ during the entire process.

To gather general information on the regulation of gene expression during abscission, comparison of genes expressed differentially in water stress/rehydrated LAZ of citrus leaves against available gene expression data during stamen abscission in *Arabidopsis* (Cai and Lashbroock, 2008) was performed (Supplementary Table S4 at *JXB* online). Moreover, another round of comparison was performed against available gene expression data during ethylene-promoted citrus leaf abscission (Agustí *et al.*, 2008, 2009) to uncover similarities between environmental and hormonal cues that promote citrus leaf abscission.

Among the 199 citrus putative orthologues to *Arabidopsis* genes that were expressed differentially in water stress/rehydrated LAZs, only six genes matched those regulated during *Arabidopsis* stamen abscission (Cai and Lashbroock, 2008; Supplementary Table S4). These genes corresponded to those encoding a hypothetical protein (AT5G23850), a poly(A)-binding protein (AT1G49760), a pathogenesis-related protein (AT3G04720), an auxin-responsive protein (AT5G35735), a photoassimilate-responsive protein (AT5G52390), and a MYB transcription factor (MYB116, AT1G25340). Interestingly, AtMYB116 expression is also located in the fruit dehiscence zone according to TAIR locus detail information. This suggests that this gene could be associated with several plant cell separation processes.

Several cellular processes such as protein biosynthesis and metabolism, purine and pyrimidine metabolism, and carbohydrate metabolism accounted for most of the genes regulated in the LAZ during stress- and ethylene-promoted citrus leaf abscission (Supplementary Table S4). In addition, a calcium-related signalling transducer (calmodulin) and a member of the basic helix–loop–helix (bHLH) transcription factor family (CitbHLH1) were also expressed preferentially in stress- and ethylene-activated LAZs. These common molecular elements could support the generalization of the model for molecular events occurring in citrus LAZ during leaf abscission.

CitbHLH1, the putative orthologue of Arabidopsis BIGPETAL, is expressed in citrus AZs

The nucleotide sequence of *CitbHLH1* was reconstructed from three citrus expressed sequence tags (ESTs) derived from different leaf and fruit AZ libraries and four ESTs derived from libraries constructed with transcripts of flower, fruit rind, leaf, and root tissues, respectively (CitbHLH1 supplementary data at *JXB* online). Sequence homology suggested *CitbHLH1* to be a putative citrus orthologue of *Arabidopsis BIGPETAL* (*BPE*). In fact, CitbHLH1 and BPE share 58/71% sequence identity/similarity (Fig 3; CitbHLH1_Supplementary Data). In *Arabidopsis*, *BPE* is expressed via two mRNA transcripts derived from an alternative splicing event (Szécsi *et al.*, 2006). The *BPEub* (AT1G59640.1) transcript is expressed ubiquitously, whereas the *BPEp* (AT1G59640.2) transcript is derived from *BPEub* and expressed preferentially in petals. *BPEp* acts downstream of petal organ identity genes and regulates petal size by restricting cell expansion. CitbHLH1, BPEub, and BPEp share sequence similarities with CrMYC1 (Fig. 3; CitbHLH1_Supplementary Data), a bHLH transcription factor that has been reported to control gene expression in response to jasmonate in *Catharanthus*

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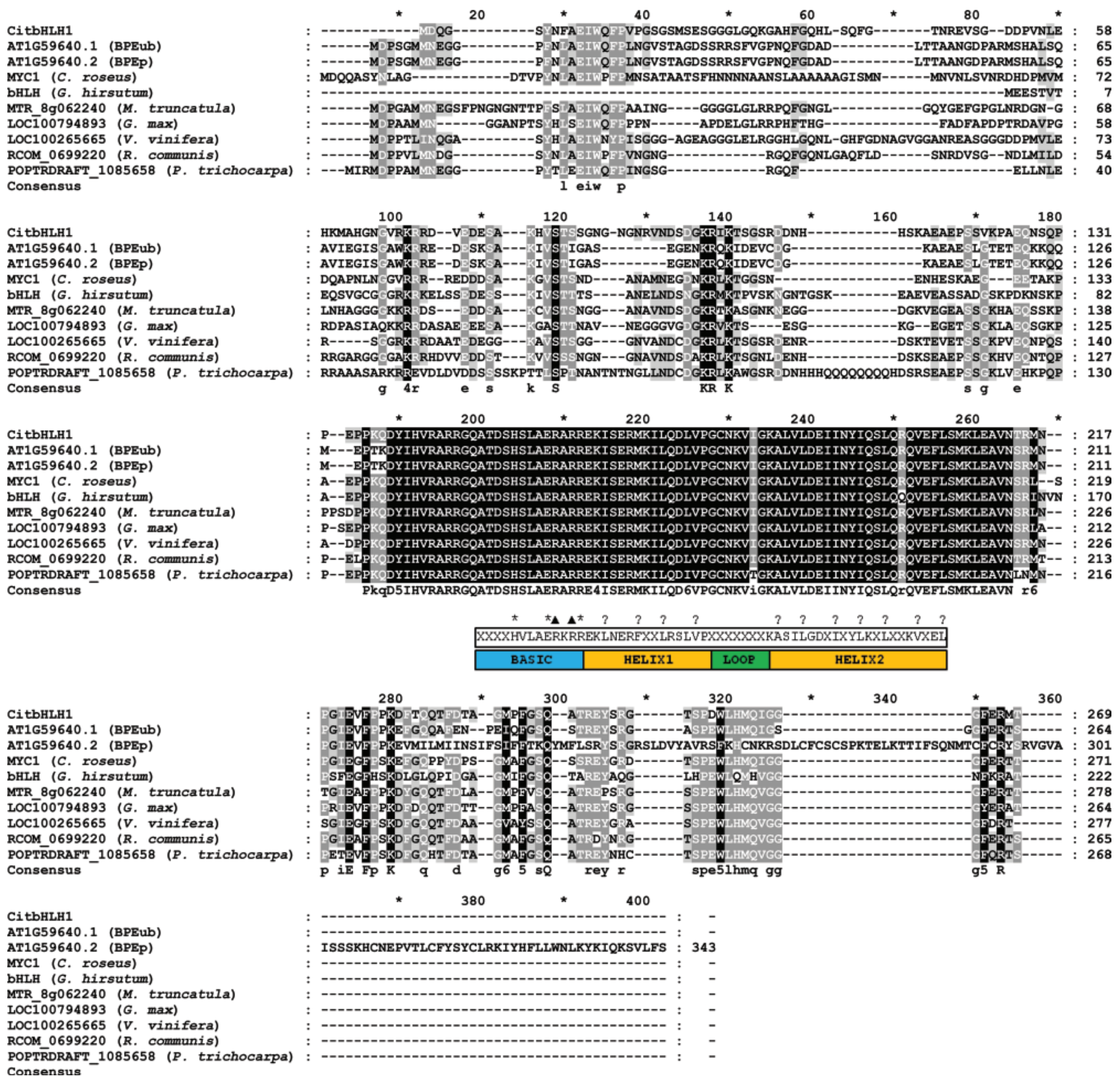

Fig. 3. Protein sequence alignment of CitbHLH1 with potential orthologues from *Ricinus comunis* [RCOM_0699220 (EEF42232), E-value 4e[−127]], *Vitis vinifera* [LOC100265665 (XP_002272776), E-value 2e[−115]], *Medicago truncatula* [MTR_8g062240 (XP_003628585), E-value 1e[−112]], *Glycine max* [LOC100794893 (XP_003517894), E-value 7e[−104]], *Populus trichocarpa* [POPTRDRAFT_1085658 (XP_002311780), E-value 3e[−102]], *Catharantus roseus* [CrMYC1 [(AAQ14331), E-value 1e[−97]], *Gossypium hirsutum* [bHLH (AAV51936), E-value 1e[−83]], and *Arabidopsis thaliana* (AT1G59640.1, BPEub, and AT1G59640.2, BPEp). Note that the grey scale indicates the degree of similarity between all aligned protein sequences. *, Amino acid contacts with nucleotide bases; filled triangle, amino acid contacts with DNA backbone;?, non-polar residues in protein-protein interactions; X, consensus sequence (Heim *et al.*, 2003). (This figure is available in colour at *JXB* online.)

roseus cells (Chatel *et al.*, 2003). The expression of *BPEub* and *BPEp* in petals of *Arabidopsis* is not affected by jasmonate, whereas in flowers of the jasmonate biosynthesis mutant *opr3* (defective in 12-oxophytodienoate reductase), treatments with jasmonate triggered the accumulation of *BPEp* but did not affect that of *BPEub* (Brioudes *et al.*, 2009), suggesting that the regulation of expression of *BPEp* by jasmonate must occur at the post-transcriptional level.

To investigate whether the potential role of *CitbHLH1* could be general for citrus organ abscission or exclusive for the LAZ, we analysed its expression in different AZs. To that end, we performed an *in vitro* ethylene-induced abscission time-course assay. We treated debladed leaves and detached fruitlets with 10 μl l^{-1} of ethylene. This treatment has been reported to cause abscission in citrus leaves after 24 h (Agustí *et al.*, 2008). We analysed the expression rates of *CitbHLH1* at 0, 6, 12, and 24 h

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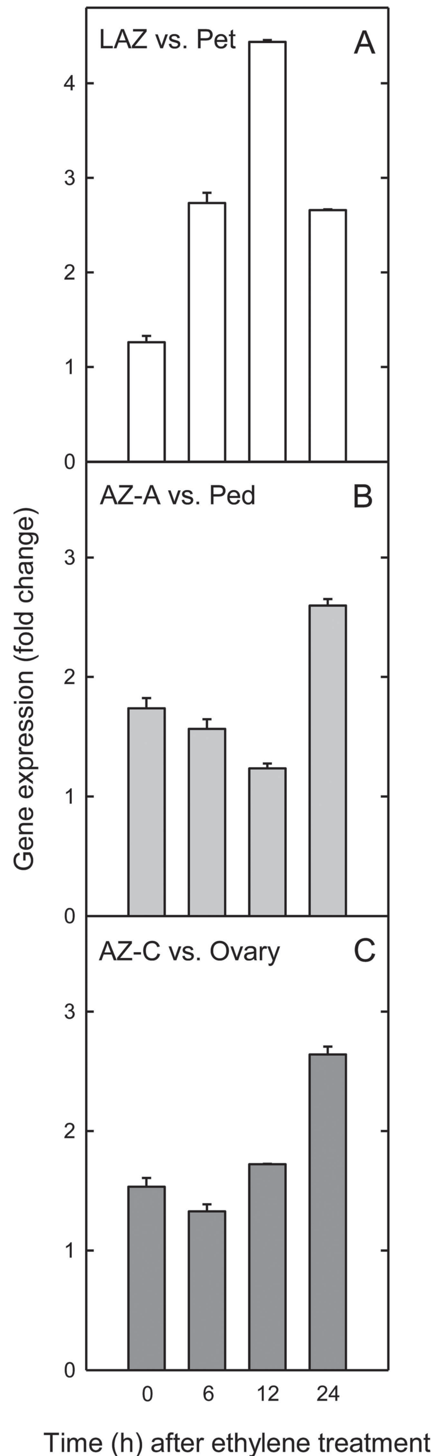

Fig. 4. Relative qRT-PCR expression of *CitbHLH1* under *in vitro* ethylene treatments (10 μl l^{-1}). (A) Comparison of expression between the LAZ and Pet (A), the AZ-A and Ped (B), and AZ-C and ovary tissue (C). In all cases, *n*=4.

using qRT-PCR on ethylene-treated LAZ versus Pet, AZ-A versus Ped, and AZ-C versus ovary. Before ethylene treatment, LAZ and Pet showed an expression ratio very close to 1 (Fig. 4). However, 6 h after ethylene treatment, *CitbHLH1* displayed an evident preferential expression in LAZ (2.73-fold), which increased until reaching a peak at 12 h treatment (4.43-fold). At 24 h, the differential expression was 2.65. Taking this together with the induction of *CitbHLH1* during the rehydration period in the *in vivo* experiment (Table 1, Fig. 2), we suggest an ethylene-induced preferential expression in the LAZ. In this scenario, *CitbHLH1* could regulate either a single crucial aspect during the entire process or several different ones at different stages.

In the case of AZ-A and AZ-C, ethylene did not induce differential expression between the AZ and control tissues until 24 h of treatment (Fig. 4). This suggests that, in these AZs, the activity of *CitbHLH1* may be restricted to the later stages of the process. In summary, these results would argue for *CitbHLH1* regulating common events for all AZs during the last stages of the process and specific ones in the LAZ during the early stages of leaf abscission.

The coordinated crosstalk between ethylene and JA converges at the transcriptional activation of *ERF1* (Lorenzo *et al.*, 2003) or *CEJ1* (Nakano *et al.*, 2006). Given the homology between CitbHLH1 and AtBPE and the positive regulation of BPE by JA, it is tempting to speculate that *CitbHLH1* is placed downstream of the JA and ethylene signalling convergence points. However, further experiments are necessary to confirm this point.

Taking together the *CitbHLH1* expression behaviour (constant in LAZ and restricted to late stages in AZ-A and AZ-C) and our previous description of cell elongation during the last stages of the LAZ abscission process (Agustí *et al.*, 2009), we propose two complementary roles for *CitbHLH1*. On the one hand, *CitbHLH1* could be involved in the cell expansion associated with the late stages of abscission. This would be a general role for all the different types of AZs in citrus and would fit with the role of *BIGPETAL* in *Arabidopsis* and with the preferential expression of *CitbHLH1* in the last stages in all studied abscission zones (Fig. 4). In addition, we propose a tissue-specific role during the onset of the process in leaves. This second role would fit with the rapid response of *CitbHLH1* under ethylene stimulation, either *in vivo* or *in vitro* (Figs 2 and 4). This would be of great relevance not only for the study of basic aspects of the process of abscission but also for biotechnological applications. From a biological point of view, unravelling molecular mechanisms for the onset of the process in different organs would represent a novel aspect in abscission. On the other hand, identifying tissue-specific markers for abscission in different organs of the same organism is highly desirable, especially for those at the onset of the process. This would open up the possibility of studying and manipulating one or other AZs independently. In the case of citrus, this would have special relevance. For example, in citrus varieties cropped for juice production, coordinating fruit abscission is a highly desirable trait because it would allow the use of mechanical harvesting methodologies such as stem vibration, increasing cropping efficiency.

Conclusion

The data provided in this work suggest a model for the initial steps of abscission (Fig. 5). After 1 h rehydration, ethylene is produced in leaves, triggering early abscission signalling events and expression of genes involved in cell-wall metabolism. At the same time, a rise in lipid metabolism is detected in the LAZs, probably related to increases in both endoplasmic reticulum profiles and Golgi bodies. The generation of Golgi-derived vesicles

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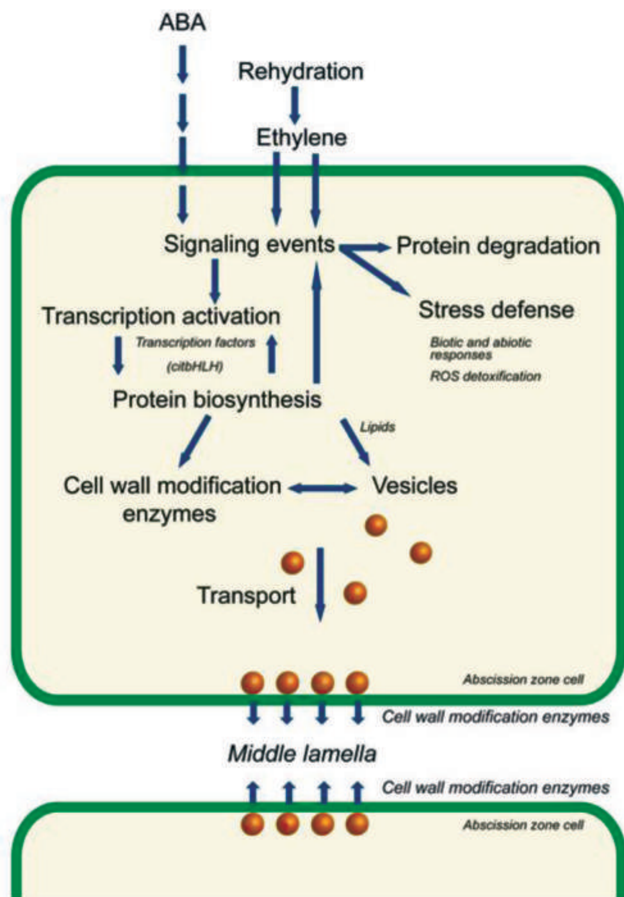

Fig. 5. Proposed model for molecular events occurring in the citrus LAZ during water stress/rehydration-induced leaf abscission based on expression data obtained from macroarray hybridization. (This figure is available in colour at *JXB* online.)

containing cell-wall metabolism-related enzymes would be responsible of the transport of these enzymes to the extracellular matrix, facilitating degradation of the middle lamella and primary cell wall and the biosynthesis of new cell wall. Throughout the entire process, the protein metabolism machinery appears to be activated to coordinate new protein scenarios. Specific signalling events and transcription factors are activated to regulate the steps of the process. Among the isolated transcription factors, *CitbHLH1* appears to play a pivotal role during the onset of the abscission process in leaves in a tissue-specific manner. The results presented here cover the initial events of citrus leaf abscission, complementing and expanding previous reports describing the late stages.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Table S1. Raw and normalized data of citrus custom macroarray hybridizations.

Supplementary Table S2. Annotation of genes spotted on the custom microarray and a list of genes expressed differentially between the LAZ and Pet (after 24 h of water stress and 1 h and 6 h after rehydration).

Supplementary Table S3. Specific primers used for qRT-PCR.

Supplementary Table S4. Comparison of genes regulated in the LAZ of citrus leaves and the stamen AZ of Arabidopsis.

Supplementary Fig. S1. qRT-PCR-based relative expression of the putative secreted glycoprotein 3 gene (C21001G11) in LAZ and Pet.

Supplementary Fig. S2. Protein sequence alignment of CitEVR with potential orthologues from Populus trichocarpa, Ricinus communis, Arabidopsis thaliana, and Nicotiana tabacum.

CitbHLH1 supplementary data. ESTs assembled to reconstruct CitbHLH1, in silico analysis of the preferential expression of CitbHLH1 in different citrus tissues and organs, and gene structure of CitbHLH1 (intron number and exon length) in Citrus clementina in comparison with AtBPE.

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References

Abeles FB, Holm RE. 1966. Enhancement of RNA synthesis, protein synthesis and abscission by ethylene. *Plant Physiology* **41,** 1337–1342.

Addicot F. 1982. *Abscission*. CA, USA: University of California Press.

Agustí J, Conesa A, Cercós M, Talón M, Tadeo FR. 2007*a*. Calcium signaling in water stress-induced leaf abscission in citrus plants. In: Ramina A, Chang C, Giovannoni J, Klee H, Perata P, Woltering E, eds. *Advances in plant ethylene research. Proceedings of the 7th International Symposium of the Plant Hormone Ethylene*. Dordrecht, The Netherlands: Springer, 303–304.

Agustí J, Merelo P, Cercós M, Tadeo FR, Talón M. 2008. Ethylene-induced differential gene expression during abscission of citrus leaves. *Journal of Experimental Botany* **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 Biology* **9,** 127.

Agustí J, Zapater M, Iglesias DJ, Cercós M, Tadeo FR, Talón M. 2007*b*. Differential expression of putative 9-*cis*-epoxycarotenoid dioxygenases and abscisic acid accumulation in water stressed vegetative and reproductive tissues of citrus. *Plant Science* **172,** 85–94.

Alberola TM, Garcia–Martinez J, Antunez O, Viladevall L, Barcelo A, Arino J, Perez–Ortin JE. 2004. A new set of DNA macrochips for the yeast *Saccharomyces cerevisiae*: features and uses. *International Microbiology* **7,** 199–206.

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Alferez F, Singh S, Umbach A, Hockema B, Burns JK. 2005. Citrus abscission and *Arabidopsis* plant decline in response to 5-chloro-3-methyl-4-nitro-1*H*-pyrazole are mediated by lipid signalling. *Plant, Cell & Environment* **28,** 1436–1449.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215,** 403–410.

Bartling D, Rehling P, Weiler EW. 1993. Functional expression and molecular characterization of AtUBC2-1, a novel ubiquitin-conjugating enzyme (E2) from *Arabidopsis thaliana*. *Plant Molecular Biology* **23,** 387–396.

Brioudes F, Joly C, Szecsi J, Varaud E, Leroux J, Bellvert F, Bertrand C, Bendahmane M. 2009. Jasmonate controls late development stages of petal growth in *Arabidopsis thaliana*. *The Plant Journal* **60,** 1070–1080.

Burns JK, Lewandowski DJ, Nairn CJ, Brown GE. 1998. Endo-1,4-β-glucanase gene expression and cell wall hydrolase activities during abscission in Valencia orange. *Physiologia Plantarum* **102,** 217–225.

Bustin SA.. 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology* **29,** 23–39.

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 Physiology* **146,** 1305–1321.

Chatel G, Montiel G, Pre M, Memelink J, Thiersault M, Saint–Pierre B, Doireau P, Gantet P. 2003. *CrMYC1*, a *Catharanthus roseus* elicitor and jasmonate-responsive bHLH transcription factor that binds the G-box element of the strictosidine synthase gene promoter. *Journal of Experimental Botany* **54,** 2587–2588.

Chen M, Ni M. 2006. RED AND FAR-RED INSENSITIVE 2, a RING-domain zinc finger protein, mediates phytochrome-controlled seedling deetiolation responses. *Plant Physiology* **140,** 457–465.

Cho SK, Larue CT, Chevalier D, Wang H, Jinn TL, Zhang S, Walker JC. 2008. Regulation of floral organ abscission in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **105,** 15629–15634.

Conesa A, Nueda MJ, Ferrer A, Talón M. 2006. maSigPro: a method to identify significantly differential expression profiles in time-course microarray experiments. *Bioinformatics* **22,** 1096–1102.

Forment J, Gadea J, Huerta L, *et al.* 2005. Development of a citrus genome-wide EST collection and cDNA microarray as resources for genomic studies. *Plant Molecular Biology* **57,** 375–391.

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

Gentleman RC, Carey VJ, Bates DM, *et al.* 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biology* **5,** R80.

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.

Gomez–Cadenas A, Tadeo FR, Primo–Millo E, Talon M. 1996. Leaf abscission induced by ethylene in water-stressed intact seedlings of Cleopatra mandarin requires previous abscisic acid accumulation in roots. *Plant Physiology* **112,** 401–408.

Gomez–Cadenas A, Tadeo FR, Primo–Millo E, Talon M. 1998. Involvement of abscisic acid and ethylene in the responses of citrus seedlings to salt shock. *Physiologia Plantarum* **103,** 475–484.

Goren R. 1993. Anatomical, physiological, and hormonal aspects of abscission in citrus. *Horticultural Reviews* **15,** 145–182.

Hartmond U, Yuan R, Burns JK, Grant A, Kender WJ. 2000. Citrus fruit abscission induced by methyl-jasmonate. *Journal of the American Society of Horticultural Science* **125,** 547–552.

Hashimoto JG, Beadles–Bohling AS, Wiren KM. (2004) Comparison of RiboGreen and 18S rRNA quantitation for normalizing real-time RT-PCR expression analysis. *Biotechnology* **36,** 54–56.

Hass C, Lohrmann J, Albrecht V, *et al.* 2004. The response regulator 2 mediates ethylene signalling and hormone signal integration in *Arabidopsis*. *EMBO Journal* **23,** 3290–3302.

Heim MA, Jakoby M, Werber M, Martin C, Weisshaar B, Bailey PC. 2003. The basic helix-loop-helix transcription factor family in plants: a genome-wide study of protein structure and functional diversity. *Molecular Biology & Evolution* **20,** 735–747.

Henry EW. 1975. Peroxidases in tobacco abscission zone tissue. III. Ultrastructural localization in thylakoids and membranebound bodies of chloroplasts. *Journal of Ultrastructural Research* **52,** 289–298.

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

Iwahori S, van Stevenink RFM. 1976. Ultrastructural observation of lemon fruit abscission. *Scientia Horticulturae* **4,** 235–246.

Jinn TL, Stone JM, Walker JC. 2000. *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes & Development* **14,** 108–117.

Jordan L, Day B.. 1972. Weed control. In: W Reuther, ed., *The citrus industry*, vol **3**. CA, USA: University of California, 82–97.

Kienow L, Schneider K, Bartsch M, Stuible HP, Weng H, Miersch O, Wasternack C, Kombrink E. 2008. Jasmonates meet fatty acids: functional analysis of a new acyl-coenzyme A synthetase family from *Arabidopsis thaliana*. *Journal of Experimental Botany* **59,** 403–419.

Kraft E, Stone SL, Ma L, Su N, Gao Y, Lau OS, Deng XW, Callis J. 2005. Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. *Plant Physiology* **139,** 1597–1611.

Kwak JM, Nguyen V, Schroeder JI. 2006. The role of reactive oxygen species in hormonal responses. *Plant Physiology* **141,** 323–329.

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.

Lewis LN, Bakhshi JC. 1968. Protein synthesis in abscission: the distinctiveness of the abscission zone and its response to gibberellic acid and indoleacetic acid. *Plant Physiology* **43,** 359–364.

Li W, Yuan R, Burns JK, Timmer L, Chung KR. 2003. Genes for hormone biosynthesis and regulation are highly expressed in citrus flowers infected with the fungus *Colletorichum acutatum*, causal

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agent of postbloom fruit drop. *Journal of the American Society of Horticultural Science* **128,** 578–583.

Liljegren SJ, Leslie ME, Darnielle L, *et al.* 2009. Regulation of membrane trafficking and organ separation by the NEVERSHED ARF-GAP protein. *Development* **136,** 1909–1918.

Lorenzo O, Piqueras R, Sanchez–Serrano JJ, Solano R. 2003. ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *Plant Cell* **15,** 165–178.

Marynick MC. 1977. Patterns of ethylene and carbon dioxide evolution during cotton explant abscission. *Plant Physiology* **59,** 484–489.

McManus MT. 1994. Peroxidases in the separation zone during ethylene induced bean leaf abscission. *Phytochemistry* **35,** 567–572.

Meijer HJ, Munnik T. 2003. Phospholipid-based signaling in plants. *Annual Review of Plant Biology* **54,** 265–306.

Michaeli R, Riov J, Philosoph–Hadas S, Meir S. 1999. Chilling induced leaf abscission of *Ixora coccinea* plants. II. Alteration of auxin economy by oxidative stress. *Physiologia Plantarum* **107,** 174–180.

Nakano T, Suzuki K, Ohtsuki N, Tsujimoto Y, Fujimura T, Shinshi H. 2006. Identification of genes of the plant-specific transcription-factor families cooperatively regulated by ethylene and jasmonate in *Arabidopsis thaliana*. *Journal of Plant Research* **119,** 407–413.

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.

Poovaiah BW, Rasmusen HP. 1973. Calcium distribution in the abscission zone of bean leaves: electron microprobe X-ray analysis. *Plant Physiology* **52,** 683–684.

Sagee O, Goren R, Riov J. 1980. Abscission of citrus leaf explants. Interrelationships of abscisic acid, ethylene, and hydrolytic enzymes. *Plant Physiology* **66,** 750–753.

Sakamoto M, Munemura I, Tomita R, Kobayashi K. 2008. Reactive oxygen species in leaf abscission signaling. *Plant Signaling & Behavior* **3,** 1014–1015.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sampson HC. 1918. Chemical changes accompanying abscission in *Coleus blumei*. *Botanical Gazette* **66,** 32–53.

Schroder M, Giermann N, Zrenner R. 2005. Functional analysis of the pyrimidine de novo synthesis pathway in solanaceous species. *Plant Physiology* **138,** 1926–1938.

Serino, G, Deng XW. 2003. The COP9 signalosome: regulating plant development through the control of proteolysis. *Annual Review of Plant Biology* **54,** 165–182.

Sexton R, Roberts JA. 1982. Cell biology of abscission. *Annual Review of Plant Physiology* **33,** 133–162.

Smith GK. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology **3**, Article 3.

Speels M. 1980. The endoplasmic reticulum. In: Stumpf PK, Conn EE, eds. *The biochemistry of plants*. New York: Academic Press, 389–412.

Steiner–Lange S, Unte US, Eckstein L, Yang C, Wilson ZA, Schmelzer E, Dekker K, Saedler H. 2003. Disruption of *Arabidopsis thaliana* MYB26 results in male sterility due to non-dehiscent anthers. *The Plant Journal* **34,** 519–528.

Stösser R, Rasmussen HP, Bukovac MJ. 1969. A histological study of abscission layer formation in cherry fruits during maturation. *Journal of the American Society for Horticultural Science* **94,** 239–243.

Szécsi J, Joly C, Bordji K, Varaud E, Cock JM, Dumas C, Bendahmane M. 2006. *BIGPETALp*, a *bHLH* transcription factor is involved in the control of *Arabidopsis* petal size. *EMBO Journal* **25,** 3912–3920.

Tadeo FR, Cercós M, Colmenero–Flores JM, *et al.* 2008. Molecular physiology of development and quality of citrus. *Advances in Botanical Research* **47,** 148–202.

Terol J, Conesa A, Colmenero JM, *et al.* 2007. Analysis of 13000 unique Citrus clusters associated with fruit quality, production and salinity tolerance. *BMC Genomics* **8,** 31.

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. *Journal of Experimental Botany* **60,** 2035–2044.

Tudela D, Primo–Millo E. 1992. 1-Aminocyclopropane-1-carboxylic acid transported from roots to shoots promotes leaf abscission in Cleopatra mandarin (*Citrus reshni* Hort. Ex Tan.) seedlings rehydrated after water stress. *Plant Physiology* **100,** 131–137.

Ueda J, Miyamoto K, Hashimoto M. 1996. Jasmonates promote abscission in bean petiole explants: its relationship to the metabolism of cell wall polysaccharides and cellulase activity. *Journal of Plant Growth Regulation* **15,** 189–195.

Vierstra RD. 2003. The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. *Trends in Plant Sciences* **8,** 135–142.

Wang Y, Zhang WZ, Song LF, Zou JJ, Su Z, Wu WH. 2008. Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. *Plant Physiology* **148,** 1201–1211.

Xu L, Menard R, Berr A, Fuchs J, Cognat V, Meyer D, Shen WH. 2009. The E2 ubiquitin-conjugating enzymes, AtUBC1 and AtUBC2, play redundant roles and are involved in activation of FLC expression and repression of flowering in *Arabidopsis thaliana*. *The Plant Journal* **57,** 279–288.

Young R, Meredith F. 1971. Effect of exposure to subfreezing temperatures on ethylene evolution and leaf abscission in citrus. *Plant Physiology* **48,** 724–727.

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