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...
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 rehydration 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., 2007b). Under these stressful conditions, xylem flow is arrested and ABA and 1-aminoacyclopropane-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). 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°C 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; Torol 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 35P-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

Materials and methods
Plant material
For the drought/rehydration experiments, 1-year-old Cleomenes 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 Cleomenes 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 (Sterlin) and incubated in sealed 10 l containers at 22°C 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°C 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°C until used for the analyses described below.

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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 (Genteman 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 log2=1 was imposed (Supplementary Table S2 at JXB online).

To perform a Gene Ontology (GO) analysis, the nearest homologous gene from Arabidopsis was assigned to each differentially expressed citrus gene (obtained from CGFP, Arabidopsis homologous gene from 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...
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.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Gene identification</th>
<th>Putative Ath orthologue</th>
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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.](https://academic.oup.com/jxb/article/63/17/6079/563536)
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).
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 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., 2007a). 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 lipoxygenase 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., 2008).
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
Early gene expression during leaf abscission in citrus

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.
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 CE1 (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
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](https://academic.oup.com/jxb/article-abstract/63/17/6079/563536). 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).

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