RESEARCH PAPER

The HD-Zip transcription factor LcHB2 regulates litchi fruit abscission through the activation of two cellulase genes

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Abstract

Cellulases play important roles in the shedding of plant organs; however, little is yet known about the functions of cellulase genes during the process of organ abscission. Abnormal fruitlet abscission is a serious problem in the production of litchi (Litchi chinensis), an economically important fruit widely grown in South Asia. In this study, two abscission-accelerating treatments (carbohydrate stress and application of ethephon) were evaluated in litchi fruitlets. Cell wall degradation and cell separation were clearly observed in the abscission zones of treated fruitlets, consistent with enhanced cellulase activities and reduced cellulose contents. The expression of two cellulase genes (LcCEL2 and LcCEL8) was strongly associated with abscission. Floral organs of transgenic Arabidopsis overexpressing LcCEL2 or LcCEL8 showed remarkably precocious abscission. Electrophoretic mobility shift assays and transient expression experiments demonstrated that a novel homeodomain-leucine zipper transcription factor, LcHB2, could directly bind to and activate HD-binding cis-elements in the LcCEL2 and LcCEL8 promoters. Our results provide new information regarding the transcriptional regulation of the cellulase genes responsible for cell wall degradation and cell separation during plant organ shedding, and raise the possibility of future manipulation of litchi fruitlet abscission by modulation of the activities of these two cellulases.

Keywords: Cell separation, cellulase, fruit abscission, HD-Zip, Litchi chinensis, lychee.

Introduction

Litchi (Litchi chinensis) is an important tropical fruit crop that is currently cultivated in over 20 countries. Under normal conditions, litchi produces many inflorescences with 100–250 female flowers per inflorescence. However, more than 95%
of the initial female flowers do not develop into mature fruit (Stern et al., 1995; Mitra et al., 2003). For normal-seeded litchi cultivars, three distinct waves of fruit abscission are observed (Yuan and Huang, 1988). The first occurs around the end of week 1 after full bloom with ~60% of fruitlet loss. The second wave occurs around the end of week 3 after full bloom, with about half of the remaining fruitlets being abscised. Soon after that, a small third wave begins. For aborted-seed cultivars, an additional pre-harvest abscission occurs 2–3 weeks before harvest and only ~60% of the remaining fruit survive (Yuan and Huang, 1988). In the period 2013–2015, the average yield of additional pre-harvest abscission occurs 2–3 weeks before harvest, with ~60% of fruitlet loss. The second cultivars, three distinct waves of fruit abscission are observed (Stern et al., 2015; Li et al., 2016). The excessive abscission of flowers/fruitlets is one of the main factors responsible for the universally low productivity in litchi (Yuan and Huang, 1988; Mitra et al., 2003).

An essential event during abscission is the separation of specialized cells within the abscission zone (AZ), which is located at the base of the organ that will be shed (Patterson, 2001; Estornell et al., 2013). The breakdown of cell wall components in AZ cells is dependent upon the activities of cell wall-specific hydrolases (Roberts et al., 2002). Endo-(1,4)-β-D-glucanases (or cellulases, CELs) are responsible for cellulose degradation and are thought to be important for organ abscission (Sexton and Roberts, 1982). In bean (Phaseolus vulgaris), the structural integrity of the pedicel AZ is lost during abscission owing to cellulase activity (Horton and Osborne, 1967), which is the result of de novo synthesis of a high-salt extractable cellulase named BEAN ABSCISSION CELLULASE (BAC) (Reid and Lewis, 1974; Tucker et al., 1991). In tomato (Solanum lycopersicum), three cellulase genes (SlCel1, SlCel2, and SlCel3) are found to be highly expressed in the pedicel AZs where cell separation occurs (Lashbrook et al., 1994; del Campillo and Bennett, 1996). Antisense suppression of SlCel1 mRNA accumulation reduces flower abscission by up to one third (Lashbrook et al., 1998), while knocking down the expression of SlCel2 results in greater force being required to remove fruits (Brummell et al., 1999). SlCel5 has been used as a cell wall-degradation marker gene for microarray analysis during ethylene-promoted tomato flower abscission (Wang et al., 2013). In Arabidopsis, there are 25 putative cellulase family members (Urbanowicz et al., 2007). A transcriptomic analysis using laser-capture microdissection has reported the induction of cellulase family members AtCel3 (At1g71380), AtCel5 (At1g22880), EGase10 (At1g75680), and EGase11 (At2g32990) in stamen AZs and their involvement in cell separation (Lashbrook and Cai, 2008). Recently, a newly discovered cellulase gene, AtCel6 (At4g39010), has been shown to promote silique dehiscence by promoting cell disintegration in the separation layer (He et al., 2018). Overall, these results suggest that enhancement of the expression and activity of cellulases is important for cell separation during organ abscission.

To date, at least 10 different abscission-associated cellulase genes have been found in fruit crops, including pEG1 and pEG4 in peach (Prunus persica) (Trainotti et al., 1997, 2006), PaCel1 in avocado (Persea americana) (Tonutti et al., 1995), MdEG1 in apple (Malus domestica) (Li and Yuan, 2008), and CitCEL3, CitCEL6 (CsCEL-a1), CitCEL10, CitCEL22, and CsCEL-b1 in citrus (Citrus sinensis) (Kazokas and Burns, 1998; Merele et al., 2017). However, the functions of these cellulases during abscission as well as their underlying mechanisms are still largely unknown.

Promoter analysis of BAC gene during bean leaf abscission has shown that ethylene positively induces its expression, whereas auxin strongly suppresses expression (Koehler et al., 1996). However, because the core elements for ethylene and auxin responses have not been found in the BAC promoter (Koehler et al., 1996), these hormones probably affect BAC expression indirectly. Further analysis by Tucker et al. (2002) implicated three TGA-type basic leucine zipper (bZIP) transcription factors (TFs) in activating the BAC promoter. The Arabidopsis HD-Zip (homeodomain–leucine zipper) HDG11 can directly up-regulate the cellulase gene At2g32990 during the elongation of roots (Xu et al., 2014). Members of the HD-Zip gene family in higher plants encode TFs containing a DNA-binding homeodomain (HD) and an adjacent leucine zipper domain (bZIP, also known as an LZ domain) (Ariel et al., 2007). The HD-Zip family comprises four subfamilies (HD-Zip I–IV), each of which plays specific roles in plant development (Ariel et al., 2007); however, whether they are involved in the shedding of plant organs has yet not been studied.

Carbohydrate deficiency and hormone effects are the two main factors considered to affect fruit drop in litchi (Yuan and Huang, 1988; Hieke et al., 2002). Comprehensive transcriptome profiling studies under carbohydrate stress and ethylene-induced abscission have been documented in our previous studies (Li et al., 2015a, 2015b) that identified many genes potentially involved in litchi fruitlet abscission. These studies revealed several genes with putative functions related to cell wall modification, as well as TFs such as HD-Zip genes. However, which genes are essential for abscission and how they are regulated is not yet known. In the present study, two cellulase genes (LcCEL2 and LcCEL8) and a novel HD-Zip TF (LcHB2) were identified as key genes associated with fruitlet abscission in litchi. Ectopic expression of LcCEL2 and LcCEL8 in Arabidopsis showed that these genes could induce precocious floral organ abscission. Further, LcHB2 was shown to directly bind the promoters and activate expression of LcCEL2 and LcCEL8. These results add to our understanding of the transcriptional regulatory mechanisms by which cellulase genes are involved in plant organ shedding.

Materials and methods

Plant materials and treatments

Several 12-year-old litchi trees (Litchi chinensis Sonn. cvs. ‘Zhumuru’ and ‘Kulin’) were randomly chosen in an orchard at the South China Agricultural University (Guangzhou, China) in 2015. Thirty fruitlet-bearing shoots of similar diameter (about 5–8 mm) growing in different directions were tagged on each tree. At 30 d after anthesis, half of the fruitlet-bearing shoots of ‘Kulin’ were treated by girdling (removing a 0.5-cm-wide ring of bark and cambium from around the base of the branch) and by defoliation beyond the ring of girdling, hereafter referred to as the GPD treatment (‘girdling plus defoliation’; Supplementary Fig. S1A at JXB online). The remaining untreated shoots were used as the control. Half of the fruitlet-bearing shoots of ‘Kulin’ were dipped in a solution containing 250 mg l−1 ethephon and 0.05% Tween® 80 surfactant for 1 min (designated as the ETH treatment) and the remaining shoots...
were dipped in water and used as the control. Of the 15 treated shoots, three were used for monitoring the fruitlet abscission dynamics and the remainder were used for tissue sampling. We calculated the cumulative rate of fruitlet abscission according to our previous study (Kuang et al., 2012). Using a sharp razor blade, different regions of the fruitlet pedicels were sampled based around the fruitlet abscission zone (FAZ), which appears as a visible sunken ring of ~2 mm in length (Supplementary Fig. S1B). In addition to the FAZ, a region of the pedicel of ~2 mm in length between the FAZ and the fruitlet-bearing stem was cut and designated as the basal portion (BP). A region of similar length between the FAZ and the fruitlet was also excised and designated as the apical portion (AP).

After separation, the FAZ, BP, and AP tissues were quickly frozen in liquid nitrogen and stored at –80 °C. Each tree was considered a biological replicate and three replicates were performed for each treatment.

Microscopic and histochemical observations
Samples of the FAZs (1 mm length) were fixed in 4% paraformaldehyde with 1% glutaraldehyde at 4 °C for 24 h. Samples were then dehydrated in an ethanol series and embedded in paraffin prior to cutting 10-μm sections. Sections were stained for morphological observation using 1% (w/v) Safranin O (Amresco, Solon, USA) and 1% (w/v) Fast Green FCF (Merck, Overijse, Belgium) (Zou et al., 2011). Insoluble polysaccharides were visualized by periodic acid–Schiff (PAS) staining (Feder and O’Brien, 1968), and cellulose was stained using 0.01% Calcofluor White (Sigma-Aldrich) and the carbohydrate-binding module CBM3a (PlantProbes, Leeds, UK).

Measurement of cellulase activity and cellulose content
Cellulase activities were determined using the anthrone method (Viles and Silverman, 1949). Cellulase activities were measured using a tissue-blotting and gel-diffusion method (Bourgault and Bewley, 2002; Yang et al., 2015) for litchi FAZs and by the DNS (3,5-dinitrosalicylic acid) method for Arabidopsis leaves (Wang et al., 1998), respectively.

Sequence analysis and quantitative real-time PCR (qRT-PCR)
Protein sequences of all candidate genes were retrieved from the litchi genome database (http://litchidb.genomics.cn/page/species/index.jsp), the Arabidopsis genome database (https://www.arabidopsis.org/index.jsp), and the plant genomics resource (Phytozome version 12.1; https://phytozome.jgi.doe.gov/pz/portal.html). Multiple alignments were performed using the ClustalW 1.83 (Thompson et al., 1994) and GeneDoc software (Nicholas, 1997). Phylogenetic trees were constructed using neighbor-joining analysis in MEGA 7 (Tanura et al., 2011) with the Poisson correction model, pairwise deletion method, and bootstrapping with 1000 replicates. Total RNA was isolated using a Column Plant RNAout 2.0 Kit (Tiandz, Beijing, China). qRT-PCR was performed using GoTaq® qPCR Master Mix (Promega) on a CFX96 Real-Time PCR System (Bio-Rad). Gene expression levels were normalized using LeEF-1α and LcGAPDH as the internal reference genes for litchi (Zhong et al., 2011) and AtUBQ for Arabidopsis (Ying et al., 2016). Three biological replicates were performed. All primers used in this study are listed in Supplementary Table S1.

Subcellular localization analysis
Coding sequences of LcCEL2 (1863 bp), LcCEL8 (1491 bp), and LcHB2 (684 bp), each lacking a stop codon, were subcloned into the pEAQ-HT-GFP vector (Ye et al., 2016) and fused in-frame with the green fluorescent protein (GFP) sequence under the control of the CaMV 35S promoter using a ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). Agrobacterium tumefaciens strain GV3101 harboring LcCEL2-GFP, LcCEL8-GFP, LcHB2-GFP, or the positive control were individually infiltrated into the abaxial side of leaves of tobacco (Nicotiana benthamiana) or inoculated into placental epidermal cells of onion (Allium cepa). GFP fluorescence signals were visualized using an Axioskop 2 Plus fluorescence microscope (Zeiss). All assays were performed with at least three replications.

Generation of transgenic plants and BCECF fluorescence analyses
Coding sequences of LcCEL2 and LcCEL8, each lacking a stop codon, were subcloned under the control of the CaMV 35S promoter into the pCAMBIA1302 vector, and then these constructs were individually transformed into Arabidopsis Columbia-0 (Col-0) ecotype plants using the floral dip method (Clough and Bent, 1998). Further phenotypic analysis and BCECF [2′,7′-Bis(2-carboxyethyl)-5′-(and-6′)-carboxyfluorescein] fluorescence assays were conducted as described by Ying et al. (2016). Flower position numbers were counted from the first flower with visible white petals at the top of the inflorescence.

Histochemical GUS assays
Genomic DNA was extracted from litchi leaves following the CTAB protocol (Puchooa, 2004). The promoter fragments of LcCEL2 (2215 bp), LcCEL8 (2192 bp), and LcHB2 (2048 bp) were subcloned into the pCAMBIA1391 vector upstream of the GUS (β-glucuronidase)-coding region (Supplementary Fig. S2A). These constructs were then individually transformed into Arabidopsis Col-0 plants. Inflorescences, siliques, and cauline leaves were incubated in GUS staining buffer (Waryong, Beijing, China) for 2–4 h at 37 °C in the dark. The samples were then de-colored in 100% ethanol, cleared in transparent solution (30 g Chloral hydrate, 10 ml glycerol, 30 ml water) overnight, and visualized using a Zeiss SV11 stereoscope.

Dual-luciferase reporter assays
Diagrams of the effector and reporter vectors used for dual-luciferase reporter assays are shown in Supplementary Fig. S2B). The effector and reporter plasmid constructs were co-transformed into tobacco leaves using Agrobacterium infiltration. After 2–3 d, activities of the LUC and REN luciferases were measured using the Dual-Luciferase® Reporter Assay System (Promega) on a Luminoskan Ascent Microplate Luminometer (ThermoFisher Scientific). The results were calculated as the ratio of fluorescence of LUC to that of REN. At least six biological replications were performed for each combination of effector and reporter.

Electrophoretic mobility shift assays (EMSA)
LcHB2 was cloned into the pET-28a(+) vector and expressed in E. coli BL21 (DE3) cells. Recombinant proteins were purified and used for EMSAs along with biotin-labeled fragments (~50 bp) of the LcCEL2 and LcCEL8 promoters. The same fragment, but unlabeled, was used as a competitor, while a probe within the mutant HD-binding cis-elements (interchanging A with G or T with C) was used as a mutant competitor in the assay. EMSAs were performed using a LightShift™ Chemiluminescent EMSA Kit (ThermoFisher Scientific). After cross-linking, the membrane was detected by the chemiluminescence method on a ChemiDoc™ MP Imaging System (Bio-Rad).

Accession numbers
Gene accession numbers are listed in Supplementary Tables S2–S4.

Results
Effects of carbohydrate stress and ethephon treatments on litchi fruitlet abscission
To determine the effect of girdling plus defoliation (GPD), which leads to carbohydrate stress, and application of ethephon
(ETH) on fruitlet abscission, the rate of fruitlet drop under each treatment was recorded each day for 5 d after the treatments. Compared to the control, GPD dramatically accelerated fruitlet drop, with a rate of 77.27% at day 2 and up to 96.84% at day 3 (Fig. 1A). At day 3, a clear white ring (the abscission ring) could be seen to have formed across the FAZ, which was caused by fracturing of the epidermis (Fig. 1B). Although the fruitlets remain attached to the branch, they could easily be detached with only a light external force. Under the ETH treatment, the rate of abscission gradually increased and reached a maximum of 92.1% at day 5, when visible abscission rings were again observed (Supplementary Fig. S3A, B). In contrast, fruitlet drop was much slower in the controls, with a cumulative abscission rate of only 18.99% at day 3 in the control for GPD and of 14.5% at day 5 in the control for the ETH treatment.

**Cell disruption and separation initiate in the FAZs during abscission**

Microscopic observations revealed clear evidence of cell breakdown in the FAZ during litchi fruitlet abscission. The FAZ usually included 7–10 layers of small, dense, oblong cells (Fig. 1C–E; Supplementary Fig. S3C–E). At 2 d following GPD treatment, cortical cells in the FAZ became enlarged and less Safranin O/Fast Green staining was absorbed. Separation of cells in the epidermis and cortex then occurred at the abscission site (Fig. 1D). Soon afterwards, the FAZ cells expanded cells in the epidermis and cortex then occurred at the abscission site (Fig. 1D). Soon afterwards, the FAZ cells expanded and disintegrated, leading to the formation of an intercellular space. As a result, a fracture appeared in the FAZ and further progressed toward the vascular bundle and pith, indicating that cell separation occurred at the FAZ after GPD treatment (Fig. 1D, E). In the FAZ from ETH-treated branches, enlarged and distorted cells appeared on day 3, and the separation layer was visible on day 5 (Supplementary Fig. S3D, E). In contrast, the FAZ of the controls showed no cell expansion and separation at day 3 after GPD and day 5 after ETH (Fig. 1C, E). Both treatments significantly decreased the number of FAZ cells (only 29% of the control at day 3 after GPD and 49% of the control at day 5 after ETH treatment), and the cells were longer and wider than those in the controls (Fig. 1F; Supplementary Fig. S3F). All the results indicated that the enlargement of FAZ cells was involved in the processes of cell disruption and separation during fruitlet abscission.

**Cellulose degradation occurs upon disruption of FAZ cells during abscission**

PAS staining was used to characterize the anatomical locations of insoluble polysaccharides in the FAZs after the GPD and ETH treatments. The walls of the separation layer cells in the FAZs showed a decreased affinity for PAS compared to those of the controls, indicating lower contents of insoluble cell wall polysaccharides in the FAZ cells (Fig. 2A). Calcofluor White staining and CBM3a labeling were further used to investigate the changes in cellulose contents. In control the FAZs, Calcofluor White staining of the walls of cells in the separation layer remained high (Fig. 2B), and no clear differences in CBM3a labeling were detected throughout the experiment (Fig. 2C). In contrast, at later stages following the GPD and ETH treatments, remarkably low intensities of Calcofluor White and CBM3a labeling signals were observed in FAZ cells, specifically where cell separation occurred (Fig. 2B, C).

Cellulose concentrations in the FAZs at day 3 after the GPD treatment and at day 5 after the ETH treatment were much less than those of the controls (Fig. 3A, B). Further, the concentration of cellulose was significantly negatively correlated with fruitlet abscission rates (r = −0.51) (Fig. 3C). Compared with the control, cellulase activities in the FAZs were significantly higher at day 2 after the GPD treatment and at day 4 after the ETH treatment, and reached their highest levels at day 3 (~7.2-fold) after GPD and at day 5 (~3.2-fold) after ETH treatment (Fig. 3D, E). Cellulase activities were significantly and positively correlated with fruitlet abscission rates (r = 0.849) (Fig. 3F). In contrast, control FAZs exhibited extremely low or non-detectable cellulase activity. Collectively, these results indicated that the increase in cellulase activity in the FAZ cells caused loss of cellulose content in cell walls, and directly resulted in the enhancement of fruitlet abscission.

**Two cellulase genes are associated with fruitlet abscission**

A total of 20 cellulase genes, designated as *LcCEL1–LcCEL20*, were identified in the litchi genome (Supplementary Table S2). The expression of three putative target genes (*LcCEL2, LcCEL8, and LcCEL9*) was found to be significantly higher during fruitlet abscission according to our previous RNA-Seq transcriptome analysis (Supplementary Fig. S4, Supplementary Table S5; Li et al., 2015b). To further confirm the expression of these genes, we performed qRT-PCR analysis and found that the expression of *LcCEL2* and *LcCEL8* was induced in FAZ tissues during the abscission process. Compared with the control, the expression of *LcCEL2* in FAZs after GPD treatment significantly increased from day 2 and reached its highest level at day 3 (~3.5-fold) (Fig. 3G). Expression of *LcCEL8* in FAZs increased more dramatically, by ~70-fold at day 2 and ~40-fold at day 3 after the GPD treatment (Fig. 3J). During ETH-induced abscission, both *LcCEL8* and *LcCEL2* expression in FAZs strongly increased from day 4 onward and reached their highest levels (~23-fold and ~4.7-fold, respectively) at day 5 (Fig. 3H, K). Thus, *LcCEL2* and *LcCEL8* expression in FAZs was significantly correlated with fruitlet abscission rates (r = 0.924 and 0.813, respectively) (Fig. 3I, L). Moreover, when the expressions of *LcCEL8* and *LcCEL2* were examined in the two pedicel regions flanking the FAZs, the apical portion (AP) at the fruitlet side and the basal portion (BP) at the proximal pedicel side (Supplementary Fig. S1B), *LcCEL2* and *LcCEL8* transcripts accumulated exclusively in the FAZ under both GPD and ETH treatments (Fig. 3G, H, J, K). Although transcriptomic data from abscising fruitlets showed higher RPKM values for *LcCEL9* under GPD and ETH treatments than in the control group (Supplementary Fig. S4, Supplementary Table S5), the accumulation of *LcCEL9* transcripts could not be detected by qRT-PCR. We therefore
Fig. 1. Effects of carbohydrate stress treatment (girdling plus defoliation, GPD) on the cumulative fruitlet abscission rate, phenotypic performance, and cell separation in the fruitlet abscission zone (FAZ) of litchi. (A) Cumulative fruitlet abscission rate. Data are means (±SE) from three replicates. Significant differences between GPD and control branches were determined using Student’s t-test: *P<0.05. (B) Phenotypic characteristics of FAZs during the fruitlet abscission process. The images on the right show magnifications of the FAZs at 3 d after GPD treatment. Arrows indicate the location of the abscission layers. (C, D) Longitudinal sections of the FAZs from fruitlets from control (C) and GPD-treated (D) branches stained with Safranin O and Fast Green at 4× and 20× magnification, respectively. Arrows indicate the location of the abscission layers. CT, cortex; EC, expanding cells; PI, pith; VB, vascular bundle. Scale bars are 200 μm at 4× magnification and 50 μm at 20× magnification. (E) Longitudinal sections of FAZs from control and GPD-treated branches at 1 d and 3 d after treatment. Scale bars are 50 μm. (F) Cell number, cell length, and cell width of the FAZs. A vision field of ~10 000 μm² (shown by the dashed lines in E) were used to count cell numbers. Up to 90 cells were used to measure the length and width. Significant differences between GPD and control branches were determined using Student’s t-test: **P<0.01, for at least three longitudinal sections.
chose LcCEL2 and LcCEL8 for further analysis as these two genes could be strongly associated with fruitlet abscission.

**Ectopic expression of LcCEL2 and LcCEL8 activate abscission of floral organs in Arabidopsis**

To further investigate the functions of LcCEL2 and LcCEL8, the in vivo subcellular localizations of the LcCEL2 (621 aa) and LcCEL8 (497 aa) proteins were examined. We found that LcCEL2-GFP and LcCEL8-GFP were exclusively localized to cell walls when transiently expressed in tobacco leaves and plasmolytic onion epidermal cells (Fig. 4A). This suggested that LcCEL2 and LcCEL8 are cell wall-localized proteins, which agrees with their potential functions in cell wall degradation. We then transgenically expressed LcCEL2 and LcCEL8 under the control of the CaMV 35S promoter in Arabidopsis. Homozygous 35S::LcCEL2 and 35S::LcCEL8 transgenic lines were obtained for further experiments. Both cellulase activities and expression of LcCEL2 and LcCEL8 in the transgenic lines were significantly higher than those of the corresponding wild-type (Col-0) (Fig. 4B, C). The phenotypes of the flowers or siliques at specific flower positions on Col-0, 35S::LcCEL8, and 35S::LcCEL2 transgenic plants were examined. Both 35S::LcCEL2 and 35S::LcCEL8 plants dropped their flowers first at position 5 or 6 (Fig. 4D; Supplementary Fig. 5A, B). In contrast, Col-0 plants first abscised their flowers at position 8 (Fig. 4D). This suggested that transgenic expression of LcCEL2 and LcCEL8 in Arabidopsis could induce precocious abscission of floral organs.

Sundaresan et al. (2014) demonstrated that cytosolic pH increases in AZ cells concomitant with organ abscission. We also observed an early increase in the cytosolic pH of AZ cells of the floral organs using the pH-sensitive indicator BCECF. Green fluorescence from BCECF was normally observed in the AZ of flowers beginning at position 5 in the wild-type plants (Fig. 4E). In contrast, the lines expressing 35S::LcCEL2 and 35S::LcCEL8 showed much earlier fluorescence signals that could be detected first in flowers at position 3 (Fig. 4E; Supplementary Fig. 5C), consistent with the precocious abscission phenotypes of these transgenic lines.

Next, the 5’ sequences upstream of the transcription start sites of LcCEL2 and LcCEL8 genes were amplified from the genome of litchi. The sizes of fragments amplified from these genomic regions were 2215 bp and 2192 bp for LcCEL2 and LcCEL8, respectively (Supplementary Dataset S1). We then transgenically expressed a GUS reporter gene fused downstream of the native LcCEL2 and LcCEL8 promoters to assess the strength and specificity of their expression in Arabidopsis. Both LcCEL2 and LcCEL8 were successfully expressed in Arabidopsis floral AZs during organ abscission (Fig. 5). GUS signals in proCEL2::GUS plants were strongly detected in AZs of flowers from position 5 to 9, and were also apparent in stigma tissues, anther filaments, and petals, and in the dehiscence and seed-shedding zones of the siliques (turning yellow),...
encompassing the replum, the valve margins, and the funiculi (Fig. 5A, C, D). GUS signals in proCEL8::GUS plants were exclusively in the abscission regions, such as the flower AZs from position 3 onward, the dehiscence and seed-shedding zones of the siliques, and the base of cauline leaves (Fig. 5B, E–G). Collectively, these data demonstrated that LcCEL2 and LcCEL8 probably have important functions during the abscission of plant organs.

**LcHB2 activates the expression of LcCEL2 and LcCEL8 by directly binding to their promoters**

To characterize potential regulators of LcCEL2 and LcCEL8, their promoter sequences were further analyzed for core cis-elements and other motifs. We found that the promoters of both genes contained two HD-binding cis-elements (Supplementary Dataset S1). The LcCEL2 promoter had two HD-binding cis-elements: AAATTAAA at position −95 to −46 relative to the start of transcription, and AAATTAGT at position −725 to −676 relative to the start of transcription. Interestingly, the LcCEL8 gene promoter contained binding sites for HD-Zip TFs at position −1589 to −1540 (TAAATGCA) relative to the transcription start site and at position −2076 to −2027 (AAATTAGT) relative to the transcription start site. The promoter regions of both LcCEL2 and LcCEL8 probably have important functions during the abscission of plant organs.

**Fig. 3.** Effects of two abscission-accelerating treatments on cellulose content (A–C), cellulase activity (D–F), and gene expression of LcCEL2 (G–I) and LcCEL8 (J–L) during fruitlet abscission in litchi. (A, D, G, J) Samples collected after girdling plus defoliation (GPD). (B, E, H, K) Samples collected after treatment with ethephon (ETH). (C, F, I, L) Correlations between the cumulative fruitlet abscission rate and (C) cellulose content, (F) cellulase activity, and expression of (I) LcCEL2 and (L) LcCEL8 in the fruitlet abscission zone. Data are means (±SE) from three replicates. Significant differences between treated and control branches in (A, B, D, E) were determined using Student’s t-test: **P<0.01. Different letters in (G, H, J, K) indicate significant differences as determined using Duncan’s multiple range test (P<0.05). The correlation coefficients (r) are significant at *P<0.05 (C, F, I, L). FAZ, fruitlet abscission zone; AP, apical portion of pedicel; BP, basal portion of pedicel (see Methods and Supplementary Fig. S1B).
Fig. 4. Functional analysis of litchi LcCEL2 and LcCEL8. (A) Subcellular localization of LcCEL2-GFP and LcCEL8-GFP fusion proteins in tobacco leaves and plasmolytic onion epidermal cells. (B) Expression of LcCEL2 and LcCEL8 in leaves of different transgenic Arabidopsis plants. The 35S::LcCEL2-1, 35S::LcCEL2-2, and 35S::LcCEL2-3 lines ectopically expressed LcCEL2 under the control of the CaMV 35S promoter in wild-type plants (Col-0). The 35S::LcCEL8-1, 35S::LcCEL8-2, and 35S::LcCEL8-3 lines ectopically expressed LcCEL8 under the control of the CaMV 35S promoter in Col-0. AtUBQ was used as an internal control for qRT-PCR analysis. The y-axis represents the fold-change in the expression levels relative to Col-0. Data are means (±SE) from three replicates. (C) Cellulase activity in leaves of different transgenic Arabidopsis plants. Data are means (±SE) from three replicates. (D) Phenotypes of floral organ abscission in transgenic Arabidopsis lines. Position numbers were counted from the first flower with visible white petals at the top of the inflorescence. (E) BCECF fluorescence micrographs of floral organ abscission zones in transgenic Arabidopsis lines. The images represent BCECF fluorescence merged with chlorophyll autofluorescence. An increase in pH is indicated by green fluorescence; chlorophyll autofluorescence is in red. Representative images are shown from 3–4 replicates in total. Scale bars are 25 μm (A), 3 mm (D), and 100 μm (E).
et al., 2015b). In the present study, we cloned the full-length cDNA and designated this gene as LcHB2. Phylogenetic analysis showed that LcHB2 clustered with ATHB7, ATHB12, and CsHB18 in the HD-Zip I subfamily (Supplementary Fig. S6). LcHB2 was expressed exclusively and strongly in the FAZs, with expression highest at day 1 after GPD (~8.5-fold) (Fig. 6A) and at day 4 after ETH treatment (~44-fold) (Fig. 6B). The expression of LcHB2 in the FAZs was significantly positively correlated with the cumulative fruitlet abscission rate (Fig. 6C). In vivo subcellular localization analysis revealed that the LcHB2 protein was located in the nucleus (Fig. 6D). Furthermore, dual-luciferase assays showed that the LcHB2 protein acted as a transcriptional activator in vivo (Fig. 6E). These results demonstrated that LcHB2 was a genuine TF that could activate the expression of downstream genes. In addition, LcHB2 promoter activity was examined using a promoter::GUS transgene (proLcHB2::GUS). The GUS signals were strongly detected in the abscission regions, such as the flower AZs from position 5 onward, the dehiscence zones of the siliques (replum and valve margins), the seed-shedding zone (funiculi), and the base of cauline leaves (Fig. 6F–H). The GUS signal also appeared in anther filaments and petals (Fig. 6F).

To examine the function of LcHB2 in the regulation of expression of LcCEL2 and LcCEL8, we performed transient expression assays using a dual-luciferase system. Compared with the empty-vector control, the ratio of LUC/REN expression was significantly increased when the pEAQ-LcHB2 effector construct was co-transfected with either the proLcCEL2-LUC or the proLcCEL8-LUC reporter construct (Fig. 7A, B), confirming that LcHB2 is a transcriptional activator of LcCEL2 and LcCEL8. EMSAs were performed to test whether LcHB2 could bind to the HD-binding cis-elements in the LcCEL2 and LcCEL8 promoters. Four DNA fragments (~50 bp) containing HD-binding cis-elements in the promoter regions of LcCEL2 and LcCEL8 were synthesized and labeled with biotin. The recombinant histidine-tagged (His)-LcHB2 fusion protein was expressed in E. coli BL21 (DE3) cells and purified (Fig. 7C). We found that recombinant LcHB2 protein could bind strongly to the LcCEL2 and LcCEL8 promoter fragments, and that this binding could be abolished by high amounts of competitive unlabeled sequences that were otherwise identical, but not by promoter sequences containing mutant binding sites (Fig. 7D, E). Mobility shifts were not observed if the LcCEL2 and LcCEL8 promoter fragments were incubated with poly-His alone (Fig. 7D, E). Thus, LcCEL2 and LcCEL8 are direct targets of LcHB2, which activates the expression of these two genes by directly binding to their promoters.

**Discussion**

The fruitlet abscission zone (FAZ) in litchi where fruitlets detach from maternal plants is located at the pedicel–fruitlet junction (Supplementary Fig. S1B) and consists of 7–10 layers of small cells with dense cytoplasm that are histologically distinct from their surrounding cells (Fig. 1). We explored the changes
in the microscopic structure of FAZs during fruitlet abscission under two abscission-accelerating treatments, namely carbohydrate deficiency (caused by girdling plus defoliation, GPD) and application of ethephon (ETH). GPD completely blocks the carbohydrate transport to fruits and leads to a serious carbohydrate stress for fruit development (Obeso, 1998; Gómez-Cadenas et al., 2000), while ethylene is known to accelerate abscission of plant organs (Taylor and Whitelaw, 2001). Cell separation in FAZs was clearly observed following these two treatments in our study. The disruption of cells initiated in the stem cortex and epidermis and spread toward the vascular bundle and pith regions in the FAZ. Significant increases in the sizes of FAZ cells were also observed (Fig. 1; Supplementary Fig. S3). Similar enlargement in abscission zone cells has previously been observed in flower AZs of soybean (Glycine max; Oberholster et al., 1991) and tomato (Tabuchi et al., 2001), in floral AZs of Arabidopsis (Shi et al., 2011), in leaf AZs of olive (Olea europaea; Kitsaki et al., 1999), and in fruit AZs of apple (Pandita and Jindal, 1991) and citrus (cv. ‘Shamouti’, Huberman et al., 1988; cv. ‘Satsuma Mandarin’ and ‘Kiyomi’, Li et al., 2017). Enlargement of separation zone cells is the result of tension across intact walls that is released during separation (Sexton et al., 1980; Brummell et al., 1999) and pod dehiscence (Chauvaux et al., 1997). Thus, it was not surprising to find cellulase activity associated with litchi fruitlet abscission (Figs 3, 4), because increased activity seems to be a feature of separating AZs (Sexton et al., 1980; Trainotti et al., 1998a, 1998b;
HD-Zip transcription factor activates cellulases in litchi fruit abscission (Brummell et al., 1999). We have assumed that the natural substrate for the cellulase that we measured is cellulose, the predominant insoluble cell wall polysaccharide that is composed of D-glucose moieties joined by β(1→4) linkages into a linear molecule (O’Brien et al., 1964). In our present study, the strong loss in intensity of PAS, Calcofluor White, and CBM3a staining in walls of abscising FAZ cells that we observed after GPD or ETH treatment suggested that the polysaccharide constituents, or to be more exact, cellulose, decreased exclusively at the onset of cell separation (Fig. 2). We also found increases in cellulase activities and corresponding decreases in cellulose contents in abscising FAZs (Fig. 3A, B), confirming that cellulases are involved in degrading cellulose during cell separation of in the abscission zone of litchi fruitlets.

The accumulation of a cellulase-encoding mRNA induced by ethylene treatment was first reported in abscising bean leaves (Tucker et al., 1988). Since then, several studies have demonstrated that cellulase activity and gene expression are correlated with events in AZs during shedding of plant organs (in avocado, Tonutti et al., 1995; in tomato, del Campillo and Bennett, 1996; in pepper, Capsicum annuum, Trainotti et al., 1997; and in citrus, Kazokas and Burns, 1998). Cellulases are encoded by a multigene family, as confirmed in our study that identified 20 cellulase genes in the litchi genome. Among these, the abscission-specific expression of LcCEL2 and LcCEL8 were followed by enhanced cellulase activities and subsequently by decreased cellulose contents in FAZ cells (Fig. 3). Although many abscission-related cellulase genes have also been identified in the FAZ tissues of several other fruit crop species (Trainotti et al., 1997; Li et al., 2010; Merelo et al., 2017), our understanding of the functions of these cellulases during fruit abscission is limited. Here, overexpression of LcCEL2 and LcCEL8 in Arabidopsis was able to accelerate floral organ abscission, and analysis of GUS-promoter fusion lines further showed that these genes were expressed in cells in the AZ during floral organ abscission and silique dehiscence (Figs 4D, E, 5; Supplementary Fig. S5), suggesting that LcCEL2 and LcCEL8 are functionally involved in abscission.

Interestingly, the LcCEL2 and LcCEL8 proteins were not highly conserved with each other and showed only 49% similarity. Compared to cellulases in other plants (Supplementary Fig. S7), LcCEL8 had higher similarity to abscission-related cellulases in citrus (CitCEL6; Merelo et al., 2017), peach (PpEG1; Trainotti et al., 1997), tomato (SiCel5; Kalaitzis et al., 1999), and pepper (cCel2; Trainotti et al., 1998b), while LcCEL2 was more closely related to cotton (Gossypium hirsutum) GhCel1 (Mishra et al., 2008) and peach PpEG4 (Trainotti et al., 2006). These data might not seem surprising if the comparisons refer only to the structure of the polypeptides rather than to their functions in vivo. Still, proteins from the subfamilies to which LcCEL2 and LcCEL8 belong seem more likely to be involved in organ abscission than do members of other cellulase families. Notably,
the *LcCEL2* and *LcCEL8* promoters could drive GUS expression not only in the floral AZ but also in the dehiscence zones of the siliques in Arabidopsis, encompassing the replum, the valve margins, and the funiculi. (Fig. 5), indicating that *LcCEL2* and *LcCEL8* might also affect silique dehiscence in transgenic Arabidopsis. In addition, the GUS signal in *proLcCEL2::GUS* plants was present in the stigmatic tissues, anther filaments, and petals, suggesting that *LcCEL2* might also affect floral organ development.

HD-Zip TFs are a large class of plant TFs that are widely involved in the regulation of different growth and developmental processes. Although no study has directly confirmed whether HD-Zip TFs control plant organ shedding, high-throughput gene expression analyses have shown that HD-Zip genes encode differentially expressed TFs that might have central roles in the shedding of organs (e.g. in tomato flowers, Meir et al., 2010; apple fruitlets, Zhu et al., 2011; Heo et al., 2016; olive fruit, Gil-Amado and Gomez-Jimenez, 2013; soybean leaves, Kim et al., 2016; and rose petals, Gao et al., 2016). Indeed, transcripts of a putative HD-Zip gene (*LcHB2*) did show significantly increased abundance during the abscission of litchi fruitlets upon carbohydrate stress or application of ethylene (Fig. 6). *LcHB2* belongs to one of the four subfamilies (I–IV) of HD-Zip TFs, which show binding preferences for variant HD-binding motifs (Sessa et al., 1993, 1998; Palena et al., 2001; Lin et al., 2008; Tominaga-Wada et al., 2009; Xu et al., 2014).

The Arabidopsis cellulase gene At2g32990 contains three such HD-binding motifs in its promoter that allow its transcription to be directly regulated by an HD-Zip IV TF (*HDG11*) to promote root elongation and enhance drought tolerance (Xu et al., 2014). It is noteworthy that At2g32990 has also been reported to be involved in cell wall remodeling in Arabidopsis stamen AZs (Lashbrook and Cai, 2008).

In our study, *LcCEL2* and *LcCEL8* associated with fruitlet abscission in litchi also contained two HD-binding cis-elements in their promoters. Further experiments confirmed that LcHB2 could directly bind with promoter segments from *LcCEL2* and *LcCEL8* that contained an HD-binding cis-element and hence serve as a transcriptional activator for these genes (Fig. 7). Notably, accumulation of *LcHB2* transcripts occurred earlier than *LcCEL2* and *LcCEL8* transcripts, which further supports the hypothesized role of LcHB2 as an upstream regulator of these two cellulase genes. HD-Zip TFs participate in responses to environmental cues including abiotic stress and stresses mediated by ABA (Johannesson et al., 2003; Olsson et al., 2004; Ariel et al., 2007, 2010; Romani et al., 2016), in leaf or floral development (Kim et al., 2007; Lin et al., 2008), root elongation (Miao et al., 2018), flower senescence (Chang et al., 2014; Lü et al., 2014), and fruit ripening (Lin et al., 2008; Jiang et al., 2017). Here, we demonstrated that an HD-Zip TF is also involved in organ shedding through regulation of the expression of cellulase genes. Interestingly, the regulation of abscission- or dehiscence-related cellulase genes by HD-Zip TFs is probably conserved among plant species, as at least one HD-binding cis-element was found in the promoter regions of 17 cellulase-encoding genes from six plant species (Supplementary Fig. S8, Supplementary Dataset S1).

Taken our results together, we propose that LcHB2 may act as a positive regulator of fruitlet abscission through directly activating *LcCEL2* and *LcCEL8* in litchi. When fruitlets sense abscission signals such as carbohydrate deficiency or ethylene stimuli, the expression levels of *LcHB2* are up-regulated. *LcCEL2* and *LcCEL8* expressed specifically in the FAZ are induced by LcHB2 via direct binding to their promoters, and cellulase activities are increased. Cellulose contents are therefore reduced, and ultimately fruitlets absicde due to cell wall degradation and cell separation in the FAZ.

**Supplementary data**

Supplementary data are available at *JXB* online.

Fig. S1. Illustration of girdling and the pedicel samples examined in this study.

Fig. S2. Constructs used in this study.

Fig. S3. Effects of ethephon treatment on cumulative fruitlet abscission rate, phenotypic performance, and cell separation in the FAZ.

Fig. S4. Expression profiles of 20 cellulase genes from litchi after GPD and ETH treatments.

Fig. S5. Overexpression of *LcCEL2* and *LcCEL8* causes earlier floral organ abscission in Arabidopsis.

Fig. S6. Multiple sequence alignments and phylogenetic analysis of LcHB2 with other plant HD-Zip proteins.

Fig. S7. Multiple sequence alignments and phylogenetic analysis of LcCEL2 and LcCEL8.

Fig. S8. Diagram of 17 promoters of abscission-related cellulase genes from various plant species.

Table S1. List of primers used in this study.

Table S2. Characteristics of cellulase and HD-Zip proteins from litchi.

Table S3. Presence of cellulase genes identified in this study in other plant species.

Table S4. Presence of HD-Zip genes identified in this study in other plant species.

Table S5. RPKM values of 20 cellulase genes of litchi identified in the transcriptomes of plants in the GPD and ETH treatments.

Dataset S1. Nucleotide sequences of the promoters of LcHB2 and 19 cellulase genes.

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Author contributions
CL, JL, HW, and MZ designed the study; CL, XM, ZW, PY, and MP performed the experiments; MZ and XN provided critical technical assistance; CL and JL wrote the manuscript with contributions from all the authors; CL, JL, MZ, HW, and RX supervised the project, interpreted the data, and revised the manuscript. All authors read and approved the final manuscript.

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