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Low temperature-induced leaf senescence and the expression of senescence-related genes in the panicles of *Litchi chinensis*

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Abstract

Litchi is one of the most important subtropical evergreen fruit trees in Southern Asia. Litchi floral buds are a mix of axillary or apical panicle primordia, leaf primordia, and rudimentary leaves. Under usual winter and early spring conditions, the axillary panicle primordia prevail, and the rudimentary leaves abscise when low temperatures reach a certain threshold. The floral buds ultimately develop into pure panicles. Understanding the regulatory mechanism of rudimentary leaf senescence is of great importance for litchi flowering. In this study, litchi potted trees at the floral differentiation stage were treated with low and high temperatures in order to induce senescence or development of leaves. The microstructure of the petiole base of the rudimentary leaves was determined. The results show several layers of flattened cells forming in the abscission zone of the rudimentary leaves that were treated with low temperatures as well as an obvious boundary regarded as the abscission layer zone. We also determined the gene expression in the leaves with different developmental fate. The results show that the *LcRboh*, *LcMC-1-like*, and *LcPirin* genes were significantly induced in the rudimentary leaves treated with low temperatures, and the expression increased with the proceeding of senescence. The expression of the genes encoding class I β-1,3-glucanase and β-xylosidase also increased with the senescence, suggesting their possible involvement in the low temperature-induced senescence of the rudimentary leaves.

Additional key words: abscission zone, flowering, litchi, rudimentary leaves.

Introduction

Leaf senescence is the final stage of leaf development (Jing *et al*. 2008, Wu *et al*. 2012). During senescence, the leaf cell is dismantled in a coordinated manner in order to remobilize nutrients and secure reproductive success. The process of senescence provides the plant with phenotypic plasticity that aids in adaptation to adverse environmental conditions (Schippers *et al*. 2015). Leaves may be artificially induced to senesce before maturity when they are exposed to drought or high or low temperatures (Shi *et al*. 2011, Allu *et al.* 2014, Naschitz *et al.* 2014). Young leaves in the leafy panicles of litchi are also induced to senesce by low temperature treatments (Zhou *et al*. 2008).

Litchi is an evergreen woody tree cultivated in subtropical and tropical regions (Liu *et al.* 2013). Previous studies indicate that low winter temperatures are indispensable for litchi flowering (Menzel and Simpson 1988, Chen and Huang 2005). Litchi floral buds are a mix of axillary or apical panicle primordia, leaf primordia, and rudimentary leaves. Under usual winter and early spring conditions, the axillary panicle primordia prevail, and the rudimentary leaves abscise when temperatures reach a certain low threshold. The floral buds ultimately develop into pure panicles. However, due to changes caused by global warming, the mixed buds might be exposed to relatively high temperatures causing the rudimentary leaves to develop into fully expanded leaves and the panicle primordia to stop developing and shrink (Zhou *et al*. 2008). The floral buds ultimately develop into leafy panicles or vegetative shoots. Regardless of the

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Abbreviations: AZ - abscission zone; PCD - programmed cell death; PI - propidium iodide; RT-qPCR - reverse transcription quantitative PCR; ROS - reactive oxygen species.

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exposure to high temperatures, growers can still encourage panicle development in order to get high-quality flowers. By spraying leaves with methyl viologen dichloride hydrate and ethephon (Zhou *et al.* 2008, 2012, 2013), growers can promote premature senescence. Therefore, understanding the regulatory mechanism of rudimentary leaf senescence is of great importance for litchi flowering.

In this study, potted litchi trees at the floral differentiation stage were treated with low and high temperatures in order to induce senescence and the development of leaves in the panicles, respectively. The microstructure and gene expression in leaves with varied developmental fate were determined. We aimed to manifest the morphological features of senescent and abscising panicle leaves in order to identify genes related to leaf senescence and abscission induced by low temperature and to provide basic tools for controlling the growth of rudimentary leaves, thus promoting floral development in litchi.

Materials and methods

Plants and experimental procedures: Four-year-old trees (*Litchi chinenesis* Sonn. cv. Nuomici) were grown in 30-dm^3 pots with loam, mushroom cinder, and coconut chaff (v/v/v, 3:1:1). Twelve potted trees of 1 m height and with approximately 30 terminal shoots were selected for the experiment. The trees were treated with low temperatures in order to induce floral development in open fields during winter conditions. Once panicle primordia emerged, six potted trees were transferred to a chamber made from polycarbonate sheet and grown under natural irradiance (transmittance of 92 %), a 12-h photoperiod, and 28/23 °C day/night temperatures in order to encourage rudimentary leaf growth. Additionally, six trees were transferred to a similar chamber with 18/13 °C day/night temperatures in order to encourage panicle development and promote panicle leaf senescence. The stem sections with rudimentary leaf petiole bases treated to temperatures of 18/13 °C were excised every 10 d (Fig. 1*A-D*), and those of rudimentary leaves treated to temperatures of 28/23 °C were excised every 2 d (Fig. 1*E-H*).

Microscopy: The stem sections with rudimentary leaf petiole bases and the axillary buds from the new shoots were excised, vacuum penetrated, and fixed with 4 % (m/v) polyformaldehyde for 4 h. Furthermore, 4 % (v/v) ethylene-diamine was added to the buffer in order to soften the tissue. The samples were dehydrated in a graded ethanol series (30, 50, 70, 85, 95, and 100 %, v/v), treated with xylene, and lastly embedded in wax and cut into 6 - 8 μm sections using a microtome (*Leica RM2235*, Nussloch, Germany). The sections were stained with hematoxylin eosin and observed under a light microscope (*Leica DMLB*, Bensheim, Germany).

Nuclei were prepared according to the Dolezel *et al.* (2007) method. One gram of fresh rudimentary leaves were chopped into pieces and washed in ice cold water with 2 cm^3 of an ice cold nuclear extraction buffer. The buffer was composed of 20 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 2 mM Na_2EDTA, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % (m/v) polyvinylpyrrolidone (*PVP-10*; *Sigma*, St. Louis, USA), and 1 % (v/v) *TritonX-100* (Loureiro *et al*. 2007). The samples were kept in the ice cold buffer for 10 min and then filtered through a 50-μm nylon mesh. The filtrates were centrifuged at 800 *g* and 4 °C for 5 min. The supernatants were removed, and the residues were re-suspended in the nuclear extraction buffer. The suspension was filtered through a 38-μm nylon mesh. A buffer of RNase A and propidium iodide (PI) at a final concentration of 50 mg dm^{-3} was added to the filtrates. The solutions were then incubated at room temperature for 10 min, and the final suspensions were pipetted onto a microscope slide and covered with a coverslip. Images were obtained using fluorescence microscopy (*Leica DM1000*, excitation 535 nm, emission 615 nm).

Gene expression: The total rudimentary leaf RNA was isolated and used to generate first-strand cDNA using the method described by Lu *et al.* (2014). Expression analysis was performed at a transcript level which was determined by reverse transcription quantitative PCR (RT-qPCR) using single-stranded cDNA as a template. The primers are shown in Table 1 Suppl. The litchi homologue *Actin* (accession number HQ588865.1) was used as a reference gene due to its stable expression (Wei *et al.* 2012). The RT-qPCR was performed using a *CFX96* optical system (*Bio-Rad,* Hercules, USA) with a *SYBR Green* based RT-qPCR assay. The RT-qPCRs were run as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s, and 60 °C for 30 s in 96-well optical reaction plates (*Bio-rad*). Each RT-qPCR analysis was repeated three times. Amplification efficiencies were calculated as $10^{(-1/slope)} - 1$, where the slope is the value obtained from a standard curve. Transcript quantifications of the candidate genes were performed in relation to *Actin* and calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analysis: The data were analyzed by *ANOVA* using the *SPSS* program (*SPSS*, Chicago, IL, USA). Differences between two treatments were evaluated by Student's *t*-test.

Results

Under the low temperature, the rudimentary leaves underwent four stages of development and were later abscised with a gentle touch (Fig. 1*A-D*). At stage 1, the rudimentary leaves were at their primary stage of development, where the leaflets of the compound leaves were still conglutinated (Fig. 1*A*). At stage 2, the petiole of the rudimentary leaf began to elongate and each individual leaflet was possible to identify (Fig. 1*B*). At stage 3, the petiole of the rudimentary leaf began to bend (Fig. 1*C*) and showed a downward curvature and early signs of abortion (Zhou *et al.* 2008), which are typical signs of epinasty. At stage 4, the petiole of the rudimentary leaf continued to curve and could be abscised with a gentle touch (Fig. 1*D*). Panicle primordia developed during the four stages and ultimately developed into axillary panicles (Fig. 1*A-D*).

The four developmental stages of the rudimentary leaves under the high temperature conditions are shown in Fig. 1*E-H*. At stage 1, the rudimentary leaves were at their primary stage of development, and the leaflets were conglutinated (Fig. 1*E*). At stage 2, the petiole of the rudimentary leaf began to elongate, and each individual leaflet was possible to identify (Fig. 1*F*). At stages 3 and 4, the petioles continued to elongate, and the leaflets began to expand (Fig. 1*G,H*). Panicle primordia did not develop during the four stages and ultimately shrank (Fig. 1*E-H*).

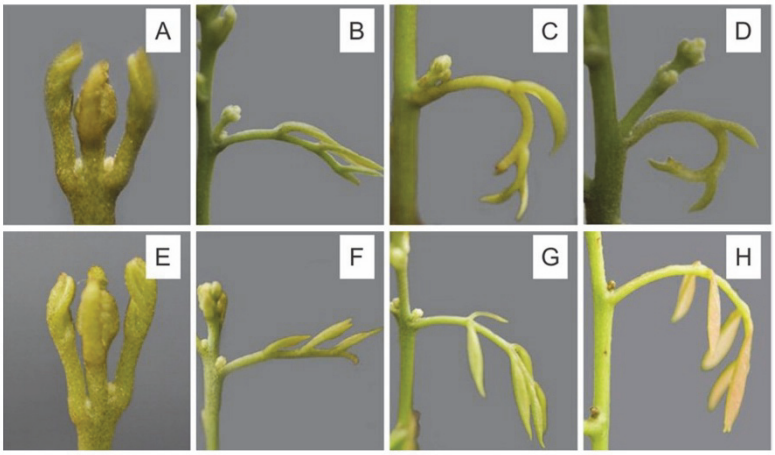

Fig. 1. The phenotype of four developmental stages of rudimentary leaves in litchi trees subjected to low (18/13 °C; *A-D*) or high (28/23 °C; *E-H*) day/night temperatures. *A*, *E* - stage 1; *B*, *F* - stage 2; *C*, *G* - stage 3; *D*, *H* - stage 4.

Under the low temperature, the morphology of the cortex cells in the abscission zone (AZ) and the adjacent region were equal at stage 1, and all of the cells were orderly arranged (Fig. 2*A-C*). At stage 2, some swollen cells were present near to or in the AZ (Fig. 2*D-F*). At stage 3, swollen cells were still present. Additionally, several layers of flattened, condensed, and orderly arranged cells were formed in the AZ, and an obvious boundary regarded as the abscission layer was identified (Fig. 2*G-I*). Rupture occurred at stage 4, where an abscission layer was observed, and several layers of flattened and well-organized cells were identified at the proximal side (Fig. 2*J-K*).

Under the high temperature, the morphology of the cortex cells in the potential AZ and the adjacent region were equal at all the stages. All cells were orderly arranged. No abscission layers could be identified in the potential AZ.

Figure 4 shows the morphology of the nuclei stained with PI. The intensity of fluorescence was still high at stage 1 in cells treated with the low temperatures, but decreased thereafter. Furthermore, nuclei shape started to become irregular at stage 2. However, in the high temperature treated cells, nuclei maintained a clear shape and high fluorescence.

To determine whether any senescence-related genes were involved in the low temperature-induced senescence and abscission of the rudimentary leaves, we determined the expression levels of *LcMC-1-like, LcBAD, LcBI-1, LcDAD-1, LcPirin, LcS-like, LcWIP,* and *LcRboh,* genes regarded as programmed cell death (PCD) promoters or suppressors. We also assayed expression of genes encoding class I enzymes β-1,3-glucanase, chitinase, polygalacturonase, phenylalanine ammonia lyase, cellulose synthase, and β-xylosidase related to cell wall metabolism. All the genes were screened from our RNA-seq data set of reactive oxygen species (ROS)-induced senescent rudimentary leaves (Lu *et al.* 2014). Relative expressions of *LcMC-1-like* and *LcPirin* in the low temperature-treated rudimentary leaves were significantly increased at stages 2 and 4 (Fig. 5). For example, the expression level of *LcMC-1-like* was

804.6 % higher in the low temperature treatment when compared to the high temperature treatment at stage 4. Expression of *LcRboh* significantly increased at stage 2 and continued to increase with development of senescence. Expressions of the remaining genes, such as *LcBI-1, LcDAB*, *LcDAD-1*, *LcS-like*, and *LcWIP* did not show increasing or decreasing profiles with development of senescence. Expressions of genes encoding the class I enzymes encoding β-1,3-glucanase and β-xylosidase increased with development of senescence. This suggests a relation to leaf senescence induced by the low temperatures. Others, such as the chitinase, polygalacturonase, phenylalanine ammonia lyase, and cellulose synthase encoding genes, did not show this trend.

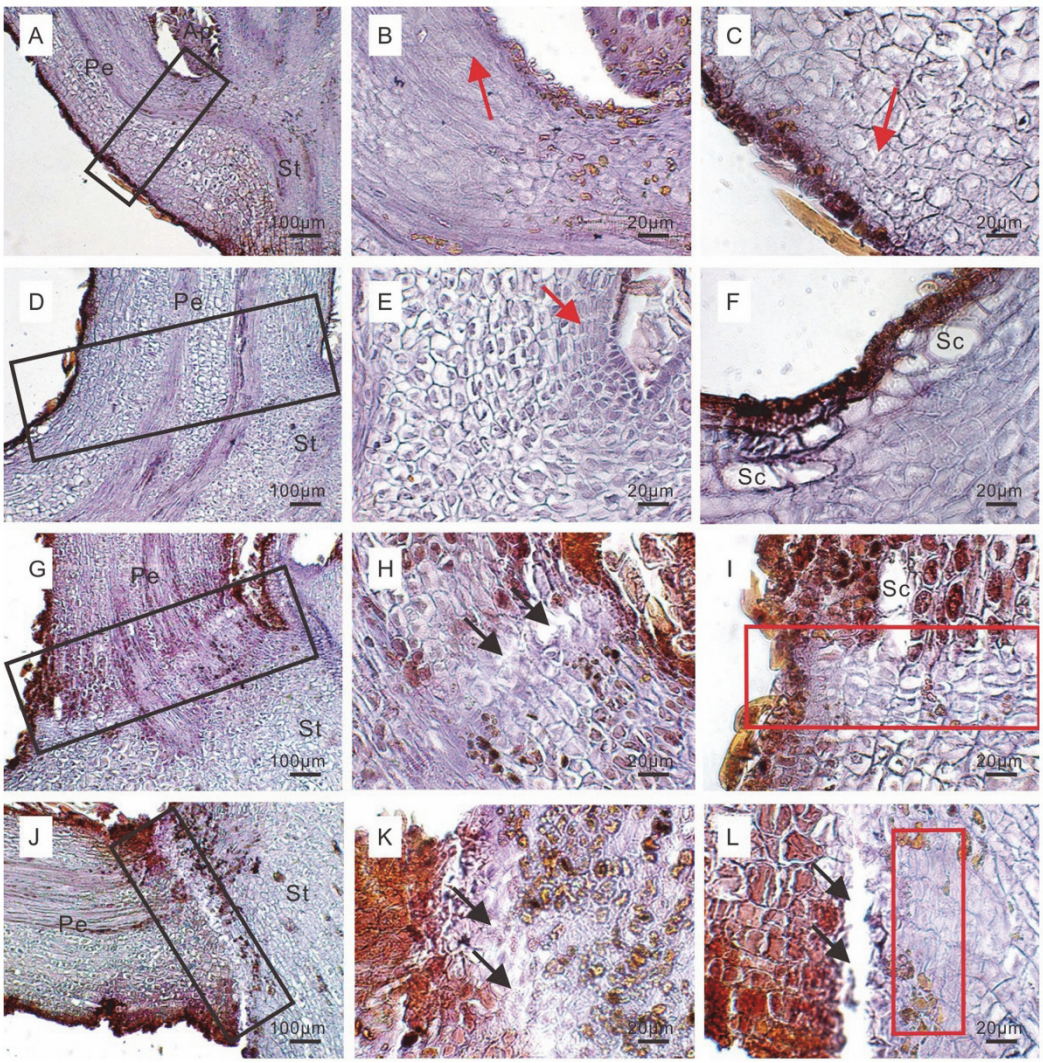

Fig. 2. The microstructure of the petiole bases of rudimentary leaves at four developmental stages under a low temperature. Leaf petiole bases were collected at stage 1 (*A-C*), stage 2 (*D-F*), stage 3 (*G-I*), and stage 4 (*J-L*) of development. The micrographs in the second column are enlarged images of the upper part of the *black rectangular regions* marked in the same line of the first column. The micrographs in the third column are enlarged images of the lower part of the *black rectangular regions* marked in the same line of the first column. The *red rectangular regions* indicate flattened, condensed, and orderly arranged cells at the proximal side. The *red arrows* indicate cortex cells. The *black arrows* indicate longitudinal splitting cortical cells. Ap - axillary panicle primordium; Pe - petiole; Sc - swollen cell; St - stem.

Discussion

Abscission is the last stage of senescence through which plants shed unnecessary segments. The process of abscission occurs inside a group of functionally specialized cells known as the AZ. It consists of several layers of cells that form well before organ separation occurs and are distinct from surrounding cells (Roberts

et al. 2002). Leaf abscission is propelled by developmental and environmental cues (Taylor and Whitelaw 2001). In litchi, panicle rudimentary leaves may easily abscise under low temperature at a specific

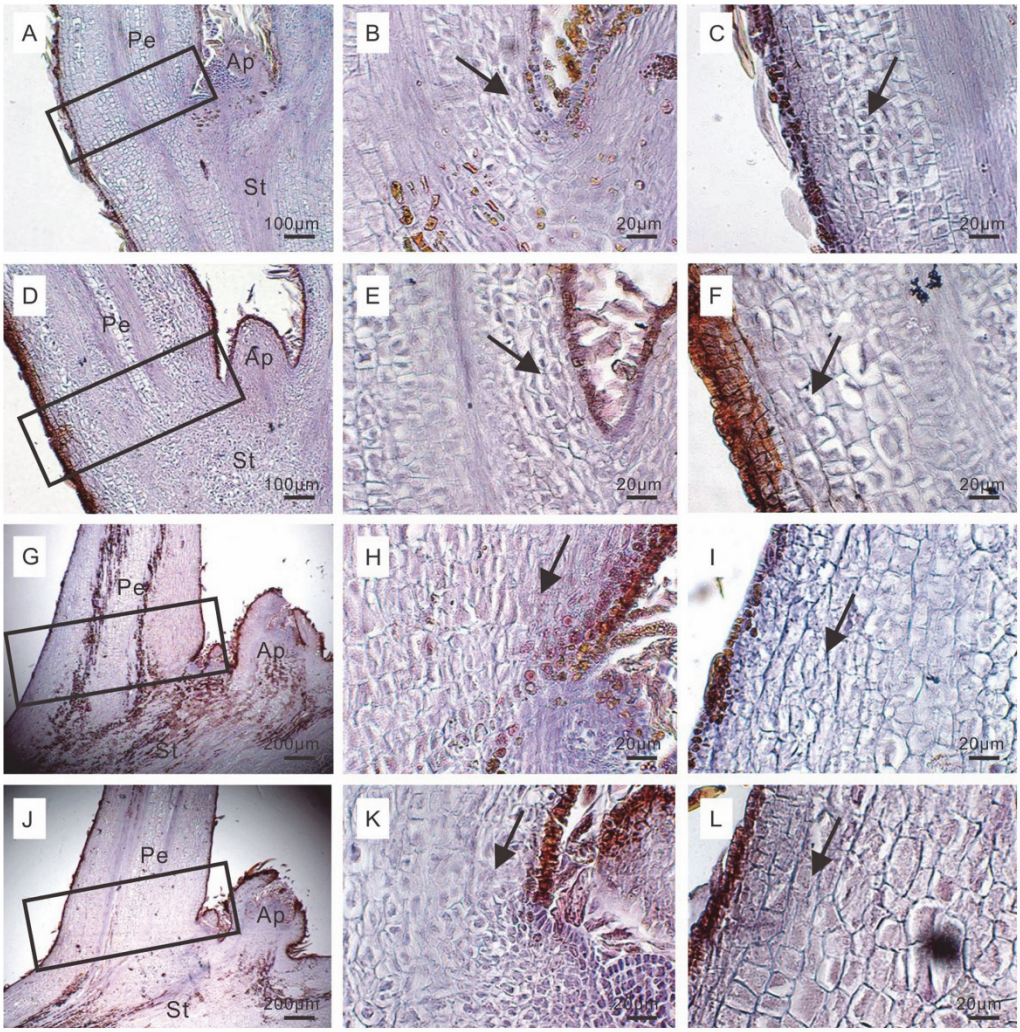

Fig. 3. The microstructure of the petiole bases of rudimentary leaves at four developmental stages under a high temperature. Leaf petiole bases were collected at stage 1 (*A-C*), stage 2 (*D-F*), stage 3 (*G-I*), and stage 4 (*J-L*) of development. The micrographs in the second column are enlarged images of the upper part of the *black rectangular regions* marked in the same line of the first column. The micrographs in the third column are enlarged images of the lower part of the *black rectangular regions* marked in the same line of the first column. The *arrows* indicate cortex cells. Ap - axillary panicle primordium; Pe - petiole; St - stem.

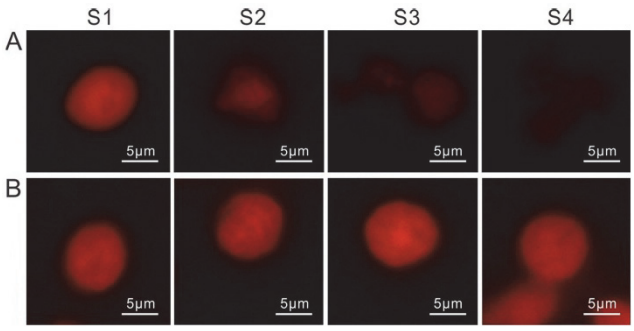

Fig. 4. Images of nuclei in low temperature-treated cells (*A*) and high temperature-treated cells (*B*) in rudimentary leaves at stage 1 (S1), stage 2 (S2), stage 3 (S3), and stage 4 (S4). The images were observed using fluorescence microscopy (*Leica DM1000*, excitation 535 nm, emission 615 nm).

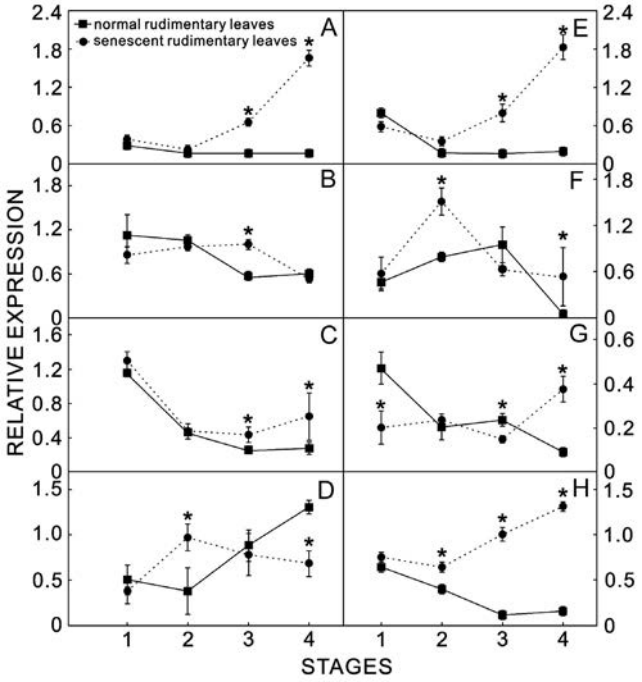

Fig. 5. Relative expressions of *LcMC-1-like* (*A*), *LcBAD* (*B*), *LcBI-1* (*C*), *LcDAD-1* (*D*), *LcPirin* (*E*), *LcS-like* (*F*), *LcWIP* (*G*), and *LcRboh* (*H*) in rudimentary leaves under low (*circles*) and high (*squares*) day/night temperatures at stage 1, stage 2, stage 3, and stage 4. Relative transcription was measured by reverse transcription quantitative PCR and calculated using the $2^{-\Delta\Delta CT}$ method with *Actin* as a reference gene. Means ± SEs of three replicates. Significant differences ($P \leq 0.05$) according to Student's *t*-test between the low and high temperature treated leaves are indicated by *asterisks*.

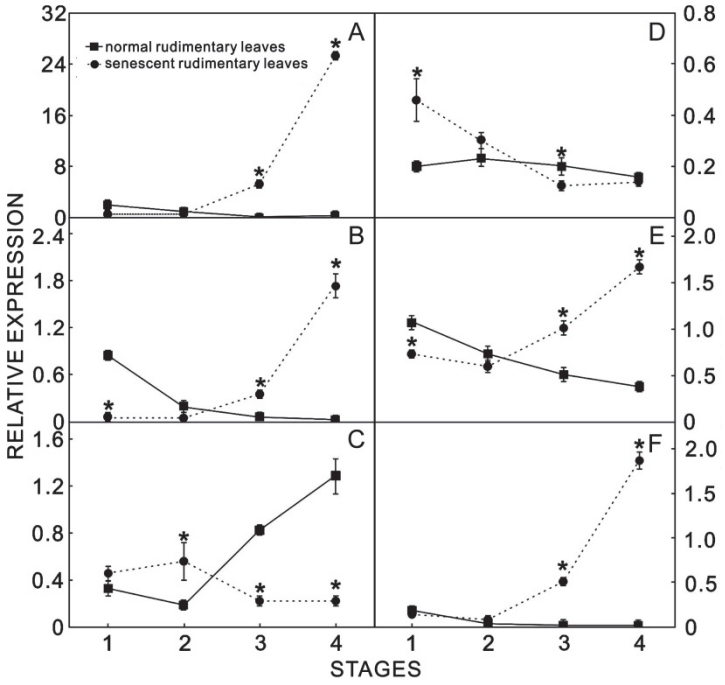

Fig. 6. Relative expressions of genes encoding class I β-1,3-glucanase (*A*), chitinase (*B*), polygalacturonase (*C*), phenylalanine ammonia-lyase (*D*), cellulose synthase (*E*), and β-xylosidase (*F*) encoding class I enzymes in rudimentary leaves treated with low (*circles*) and high (*squares*) day/night temperatures at stage 1, stage 2, stage 3, and stage 4. Relative transcription was measured by reverse transcription quantitative PCR and calculated using the $2^{-\Delta\Delta Ct}$ method with *Actin* as a reference gene. Means ± SEs of three replicates. Significant differences ($P \leq 0.05$) according to Student's *t*-test between the low and high temperature treated leaves are indicated by *asterisks*.

developmental stage. The features of the separation event in the leaves remain unknown.

In this study, the litchi trees were treated with low and high temperatures in order to induce senescence and development of leaves, respectively. The results show several layers of flattened cells formed in the AZ at stage 3 of the low temperature-treated rudimentary leaves when the petiole began to bend (Fig. 1*C*). In addition, an obvious boundary regarded as an abscission layer was identified in the AZ at this stage. A rupture occurred at stage 4 when separation occurred with a gentle touch (Fig. 2). Interestingly, swollen cells were found in the AZ at stages 2 and 3 suggesting that their formation might be related to a decrease in osmotic potential in the AZ and might be beneficial to the rupture of the vascular bundle (Roberts *et al.* 1984, Kou 2012). Our results suggest that abscission of rudimentary leaves undergo the AZ formation process.

Changes in nucleus morphology, which include chromatin condensation and nuclear DNA fragmentation, are PCD hallmarks (Vanyushin *et al.* 2004, Huang *et al.* 2014). Staining cell nuclei with PI reveal a characteristic apoptotic nuclear fragmentation (Rina *et al.* 1997). The results of this study show that the shape of the nuclei became irregular before the abscission layer could be found (Fig. 2) in stage 2 (Fig. 4) indicating chromatin disorganization and condensation. Moreover, the results suggest that cell death occurred before formation of the abscission layer in the rudimentary leaves. Our previous study determined PCD features, such as chromatin condensation, revealed by ultrastructural observation, and DNA breakdown with production of fragments in 180 or 360 bp nucleosomal units (Zhou *et al.* 2008). The results from this study further suggest that abortion of the rudimentary leaves caused by panicle development might involve PCD.

Induction of different protease activities has been associated with the occurrence of PCD (Reape and McCabe 2010, Woltering 2010). A caspase-like gene is involved in protein degradation (Grudkowska and Zagdanska 2004). This study identified a litchi *Caspase-like* homologue (*LcMC-1-like*) in our RNA-seq data set (Lu *et al.* 2014). *LcMC-1-like* was significantly induced by the low temperature treatment and increased with proceeding of senescence suggesting its role in controlling low temperature-induced senescence and abscission (Fig. 5*A*). A lepirin-deduced protein is homologous to the human protein Pirin, a nuclear factor reported to form quaternary complexes with target sequences in the promoter regions of anti-apoptotic genes and the transcription factors NF-κB and Bcl-3. *LcPirin* was significantly induced by the low temperatures and increased with proceeding of senescence in the low temperature-treated rudimentary leaves. This suggests a relation to low temperature-induced senescence (Fig. 5*E*).

It was established that ROS are involved in plant PCD and therefore associated with developmental processes and environmental stress responses (Van Breusegem and Dat 2006). The NADPH oxidase is a key enzyme for ROS production in response to biotic and abiotic stresses in plants as well as during PCD (Torres and Dangl 2005). In our previous study, we found that ROS accumulate in senescent rudimentary leaves (Zhou *et al.* 2008). Application of the ROS inducer methyl viologen dichloride hydrate increases ROS content and promotes senescence and abscission in litchi rudimentary leaves (Zhou *et al.* 2012). In this study, the low temperature induction of *LcRboh* occurred sooner than of *LcMC-1-like* and *LcPirin* suggesting that *LcRboh* and ROS might play a key role in the low temperature-induced senescence in the rudimentary leaves (Fig. 5*H*).

The plant cell wall is mainly composed of cellulose, hemicellulose, and lignin. Cell wall degradation occurs before organ abscission. The β-1,3-glucanases typically hydrolyze β-glucan chains by cleaving glucose residues from the non-reducing end (Martin *et al.* 2007) and by degrading β-glucan in the cell wall (Stubbs *et al.* 1999). Xylan is a major component of hemicellulose. The β-xylosidase is involved in xylan degradation (Benassi *et al.* 2014). In this study, expression of the genes encoding class I β-1,3-glucanase and β-xylosidase increased with development of senescence (Fig. 6*A*,*F*) suggesting that they might be related to the low temperature-induced senescence of the rudimentary leaves. However, the role of the senescence-related genes requires further investigation.

In summary, this study revealed the morphological features of low temperature-induced senescence of the rudimentary leaves in the litchi panicles. We also identified genes with a possible relation to senescence of rudimentary leaves that underlay panicle development.

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