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RESEARCH PAPER

KNOX protein KNAT1 regulates fruitlet abscission in litchi by repressing ethylene biosynthetic genes

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Abstract

Abscission is triggered by multiple environmental and developmental cues, including endogenous plant hormones. KNOTTED-LIKE HOMEOBOX (KNOX) transcription factors (TFs) play an important role in controlling abscission in plants. However, the underlying molecular mechanism of KNOX TFs in abscission is largely unknown. Here, we identified LcKNAT1, a KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1)-like protein from litchi, which regulates abscission by modulating ethylene biosynthesis. *LcKNAT1* is expressed in the fruit abscission zone and its expression decreases during fruitlet abscission. Furthermore, the expression of the ethylene biosynthetic genes *LcACS1*, *LcACS7*, and *LcACO2* increases in the fruit abscission zone, in parallel with the emission of ethylene in fruitlets. *In vitro* and *in vivo* assays revealed that LcKNAT1 inhibits the expression of *LcACS*/*ACO* genes by directly binding to their promoters. Moreover, ectopic expression of *LcKNAT1* represses flower abscission in tomatoes. Transgenic plants expressing *LcKNAT1* also showed consistently decreased expression of *ACS*/*ACO* genes. Collectively, these results indicate that LcKNAT1 represses abscission via the negative regulation of ethylene biosynthesis.

Keywords: ACS/ACO, ethylene biosynthesis, fruitlet abscission, KNAT1, litchi (*Litchi chinensis* Sonn.), transcription.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACO, 1-aminocyclopropane-1-carbo-xylate oxidase; ACS, 1-aminocyclopropane-1-carboxylate synthase; AZ, abscission zone; BCECF, 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; CaMV: Cauliflower Mosaic Virus; ChIP, chromatin immunoprecipitation; Col, Columbia; DAT, days after treatment; DEG, differentially expressed gene; dpa, days post-anthesis; EMSA, electrophoretic mobility shift assay; FAZ, fruit abscission zone; GA, gibberellin; GO, Gene Ontology; GPD, girdling plus defoliation; GUS, β-glucuronidase; HD, homeodomain; KD1, KNOTTED1-LIKE HOMEOBOX PROTEIN1; KNAT1, KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1; KNOX, KNOTTED-LIKE HOMEOBOX; TF, transcription factor.

Introduction

Abscission is the process by which plants shed organs such as seedpods, leaves, floral organs, and fruits by separating them at the abscission zone (AZ) (Patharkar and Walker, 2018). Abscission can be initiated by either developmental or environmental factors and is mainly controlled by an interaction between two plant hormones, ethylene and auxin (Roberts *et al.*, 2002; Estornell *et al.*, 2013; Botton and Ruperti, 2019; Meir *et al.*, 2019; Kucko *et al.*, 2019). Before abscission, auxin is directionally transported through the AZ to repress the ethylene sensitivity of AZ cells (Dhanalakshmi *et al.*, 2003; Blanusa *et al.*, 2005; Meir *et al.*, 2006, 2010; Shi *et al.*, 2017). For example, decreasing polar auxin transport in sweet cherry pedicels treated with the inhibitors of auxin transport activates fruit abscission due to increased ethylene sensitivity in AZ cells (Blanusa *et al.*, 2005). In cultivated crops, the application of ethylene biosynthesis inhibitors can delay abscission, while treatment with exogenous ethylene (ethephon) promotes abscission (Williams and Flook, 1980; Bessis *et al.*, 2010; Zhu *et al.*, 2010; Li *et al.*, 2015*b*). Ethylene biosynthesis increases just before abscission, which activates the genes encoding the cell wall remodeling enzymes (Goren, 1993; Bonghi *et al.*, 2000; Taylor and Whitelaw, 2010; Zhu *et al.*, 2010). In apple fruits, chemical thinner-induced abscission promotes the up-regulation of key regulatory genes simultaneously with ethylene biosynthesis (Dal Cin *et al.*, 2005, 2007, 2009). Similar processes have also been reported during flower and fruit abscission in grapevine (Hilt and Bessis, 2015). Moreover, increased expression of key enzymes involved in the ethylene biosynthetic pathway such as 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) and ACC oxidase (ACO) has also been observed during organ abscission (Dal Cin *et al.*, 2005, 2007, 2009; Ruperti *et al.*, 2001). However, the mechanism by which ethylene biosynthesis is modulated during abscission in these crops remains elusive.

Studies in the model plant Arabidopsis have revealed that three KNOTTED-LIKE HOMEOBOX (KNOX) transcription factors (TFs), which act as downstream factors of the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)–IDA-LIKE (IDL) pathway, play critical roles in floral organ abscission (Jinn *et al.*, 2000; Butenko *et al.*, 2003; Cho *et al.*, 2008; Stenvik *et al.*, 2008; Liljegren *et al.*, 2009; Shi *et al.*, 2011; Meng *et al.*, 2016; Santiago *et al.*, 2016). KNOX proteins comprise a subfamily of three amino acid loop extension (TALE) homeobox TFs that can bind *cis*-elements containing a TGAC core (TGACTGAC or TGACAGG/CT) (Krusell *et al.*, 1997; Smith *et al.*, 2002; Tioni *et al.*, 2005; Viola and Gonzalez, 2006; Bolduc *et al.*, 2012). KNOX proteins are primarily known to play central roles in the development and maintenance of the floral and shoot apical meristems (Hake *et al.*, 1995; Long *et al.*, 1996; Endrizzi *et al.*, 1996; Reiser *et al.*, 2000; Brand *et al.*, 2002; Hake and Ori, 2002; Ito *et al.*, 2002; Tsuda *et al.*, 2011). Recently, chromatin immunoprecipitation followed by next-generation sequencing (ChIP-seq) analysis in maize and rice revealed the binding of KNOX to the promoters of several target genes associated with auxin and ethylene pathways (Bolduc *et al.*, 2012; Tsuda *et al.*, 2014), suggesting that the function of KNOX TFs may be dependent on plant hormones. Thus, it will be of interest to investigate whether KNOX proteins could regulate plant hormone actions in the control of abscission. In tomato pedicel AZs, auxin depletion, by the removal of flowers or application of auxin transport inhibitors, activates pedicel abscission, while treatment with an ethylene inhibitor delays abscission (Meir *et al.*, 2010). Furthermore, knockdown of *KNOTTED1-LIKE HOMEOBOX PROTEIN1* (*KD1*) in tomatoes significantly delays pedicel and petiole abscission by regulating the genes involved in auxin transport and signaling (Ma *et al.*, 2015), indicating that KD1 can regulate abscission by modulating the auxin pathway in tomatoes. Ethylene is another plant hormone involved in abscission; however, whether KNOX proteins can regulate abscission via ethylene is largely unknown.

Litchi (*Litchi chinensis* Sonn.), a tropical and subtropical fruit species that originated in south China, is widely cultivated in Southeast Asia. Litchi crops are subject to three to four waves of fruit drop throughout fruit development depending on the cultivar, which leads to great economic losses (Yuan and Huang, 1988). Since the AZ at the pedicel is clearly visible and fruitlet abscission can be easily induced by ethylene application or via carbohydrate stress by girdling plus defoliation (GPD) treatments (Li *et al.*, 2015*a*, *b*), litchi may serve as a representative crop for the study of fruit abscission. Understanding the mechanisms underlying litchi fruitlet abscission may be useful, not only for managing litchi fruit production, but also for improving the management of other cultivated crops. Our previous studies have shown that ethylene emission is induced prior to litchi fruitlet abscission (Li *et al.*, 2015*b*). In addition, *KNOX* genes and the ethylene biosynthetic genes *ACS* and *ACO* are antagonistically regulated (Li *et al.*, 2015*b*). Given that both ethylene and KNOX TFs play key roles in abscission, we hypothesize that a link might exist between ethylene biosynthesis and KNOX TF functions during fruitlet abscission in litchi. Here, our in-depth studies revealed that the regulation of ethylene biosynthesis by a KNAT1-like protein, LcKNAT1, is involved in litchi fruitlet abscission.

Material and methods

Plant materials and treatments

For fruitlet abscission-induced treatment, three 14-year-old 'Huaizhi' trees (*Litchi chinensis* Sonn. cv. 'Huaizhi') and three 15-year-old 'Feizixiao' trees (*Litchi chinensis* Sonn. cv. 'Feizixiao') grown in an orchard in the South China Agricultural University in Guangzhou, China, were randomly chosen in 2017 and 2018, respectively. Briefly, at 35 d after anthesis, 30 fruit-bearing shoots of a similar diameter (about 5–8 mm), growing in different directions on each tree, were tagged. Ten shoots were treated with girdling (a ring of bark about 0.5 cm in width along with cambium was removed from the branch base), followed by defoliation (removal of all leaves above the girdle) (GPD treatment); 10 shoots were then dipped in 250 mg l^{-1} ethephon solution (containing 0.05% Tween-80 surfactant) for 1 min (ethylene treatment), and the remaining untreated shoots were used as controls.

For the natural fruitlet abscission assays, three 21-year-old 'Huaizhi' trees (*Litchi chinensis* Sonn. cv. 'Huaizhi') grown in Xili orchard in Shenzhen, China, were randomly chosen in 2018. Twenty fruit-bearing

shoots of a similar diameter (about 5–8 mm), growing in different directions on each tree, were tagged. Ten shoots were used for fruitlet sampling, which was conducted at 0, 7, 14, and 21 d post-anthesis (dpa), and the remaining 10 shoots were used for the evaluation of the relative abscission rate. The relative fruitlet abscission rate was calculated by subtracting the number of remaining fruitlets from the last recorded number, dividing it by the last number, and multiplying it by 100.

To generate *LcKNAT1* overexpression lines in Arabidopsis, the full-length open reading frame (ORF) of *LcKNAT1* was subcloned into vector pCAMBIA1302, driven by the *35S* promoter using the ClonExpress® Ultra One Step Cloning Kit (Vazyme Biotech Co., Ltd), and then the construct was transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998). To generate *LcKNAT1* overexpression lines in tomatoes, the ORF of *LcKNAT1* was subcloned into vector pPZP600. Then, the constructed plasmid was introduced into the *Agrobacterium tumefaciens* strain GV3101 by electroporation. Tomato plants (*Solanum lycopersicum*, cv. Micro-Tom) were transformed according to a method described previously (Wang *et al.*, 2005). The positive transformants were selected by kanamycin resistance (50 mg l^{-1}) and confirmed by the transcript levels of *LcKNAT1*. T_3 homozygous transgenic tomato plants were used for phenotypic analysis. All of the plants were grown at 22 °C under long-day (16 h light/8 h dark) conditions. The primers used here are listed in Supplementary Table S1 at *JXB* online.

Quantitative RT-PCR analysis

Tissue samples for total RNA extraction were taken from the AZ (50 segments of less than 1 mm thickness for each time point, excised less than 0.5 mm from each side of the visible AZ of litchi peduncles and tomato pedicels). Total RNA was isolated from each sample using 1 ml Trizol reagent (Thermo Fisher Scientific). qRT-PCR was performed, as described previously (Ying *et al.*, 2016). Each sample was quantified in at least triplicate and the expression was normalized against that of *SAND* (Expósito-Rodríguez *et al.*, 2008) and *EF-1a* as an internal control for tomato and litchi, respectively. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The values represent the mean of three biological replicates. The primers used for qRT-PCR are listed in Supplementary Table S1.

2,7-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein fluorescence analyses

2,7-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescence analysis was performed according to a previous study with some modifications (Srivignesh *et al.*, 2015). Images were taken using a confocal laser scanning microscope (LSM 7 DUO, Zeiss, Germany), with BCECF fluorescence and chlorophyll autofluorescence detected through 494–598 and 647–721 filters, respectively. All BCECF images presented here are representative examples from an experiment repeated three times with different biological samples. BCECF fluorescence quantification was performed using ImageJ software.

Sequence alignments and phylogenetic analysis

Multiple alignments of KNOX and ACS/ACO proteins from *Litchi chinensis* Sonn. with homologous proteins from other plant species were performed using ClustalW with default options (Thompson *et al.*, 1994). Maximum-likelihood phylogenetic trees were obtained using MEGA 5.2 (Thompson *et al.*, 1994), with the following parameters: Poisson correction, pairwise deletion, and bootstrap analysis with 1000 replicates.

Histochemical β-glucuronidase assays

The *LcKNAT1* promoter region (−1 to −2079 bp) was subcloned into vector pCAMBIA1391 to generate the *LcKNAT1pro:GUS* construct to determine the localization of the expression controlled by the *LcKNAT1* promoter. The *LcKNAT1pro:GUS* construct was delivered into Micro-Tom tomato using *Agrobacterium* GV3101 (Wang *et al.*, 2005) and T_1 transgenic plants were used for β-glucuronidase (GUS) assays. To assay the promoter activity, the *LcKNAT1pro:GUS* construct was delivered into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and injected into the abaxial side of 4- to 6-week-old tobacco (*Nicotiana benthamiana*) leaves, followed by treating leaves with (50 μl l^{-1}) or without ethylene for 48 h. Infected leaf tissues were stained in GUS solution for 6 h at 37 °C and then cleared in 70% ethanol. GUS expression was observed using a Nikon 5300 camera. The primers used here are listed in Supplementary Table S1.

Electrophoretic mobility shift assay

The putative DNA-binding domain of LcKNAT1 (encoded from 532 to 981 bp of the *LcKNAT1* ORF) was cloned into pGEX4T-1 and expressed in *E. coli* BL21 (DE3) cells. Purified recombinant proteins were used for an electrophoretic mobility shift assay (EMSA) along with biotin-labeled fragments of *LcACS/ACO* promoters. EMSA was performed using the LightShift™ Chemiluminescent EMSA Kit (Thermo Fisher Scientific), as reported previously (Tan *et al.*, 2018). The primers used here are listed in Supplementary Table S1.

LcKNAT1 polyclonal antibody production

The coding sequence of *LcKNAT1* was inserted into pET32a(+) generating a His– LcKNAT1 construct and transformed into *E. coli* strain BL21 (DE3). The recombinant His–LcKNAT1 proteins were induced and affinity purified using His60 Ni Superflow Resin (Clontech). The fusion proteins were separated by SDS-PAGE. The bands of interest were excised and used as antigens for antibody production. Antibody was produced in rabbit by HuaAn Biotechnology Company (Hangzhou, China).

Chromatin immunoprecipitation–qPCR analysis

Chromatin immunoprecipitation (ChIP)–qPCR assays were performed, as previously described (Gendrel *et al.*, 2005). Chromatin was extracted from the AZ tissues (0.3 g), after fixation with formaldehyde, and the chromatin was extracted and then sheared to an average length of 500 bp by sonication. The chromatin was immunoprecipitated with a specific antibody, LcKANT1. The reaction with pre-immune serum IgG was used as the negative control. Equal amounts of the sonicated chromatin solution was set aside as the input sample. After cross-linking was reversed, the amount of precipitated DNA fragments and input DNA was detected by quantitative real-time PCR using specific primers, as listed in Supplementary Table S1. The percentage of the input was calculated by determining $2^{-\Delta Ct}$ ($=2^{-[Ct(ChIP)-Ct(Input)]}$).

Ethylene production measurements

The amount of ethylene production was determined using a gas chromatograph (GC2014, Shimadzu, https://www.shimadzu.com/), equipped with a flame ionization detector, as described previously (Yan *et al.*, 2011). For litchi, five fruits from each treatment on each tree were collected and enclosed in a glass jar for 2 h at 25 °C. The ethylene production rate was expressed as microliters of C_2H_4 kg^{-1} h^{-1}.

Agrobacterium-mediated transient expression in N. benthamiana leaves

For transcriptional activity assays, the coding sequence of *LcKNAT1* was subcloned as the effector into the GAL4-DBD vector. The double reporter vector includes a GAL4 DNA consensus binding site derived from the yeast *GAL4* gene cloned upstream of the firefly luciferase gene (*GAL4-LUC*) and an internal control *Renilla* (*REN*) luciferase gene driven by the *35S* promoter. *GAL4-LUC* contains five copies of the GAL4-binding element and the minimal TATA region of the CaMV *35S* cloned upstream of the genes encoding LUC and REN, respectively. Both the effector and reporter plasmids were co-transfected into *N. benthamiana* leaves in *A. tumefaciens* strain GV3101, as reported

previously (Cheng *et al.*, 2017). LUC and REN luciferase activities were assayed using Dual-Luciferase® Reporter Assay System kits (Promega, Madison, WI, USA). At least six assays were performed for each effector and reporter pair.

To assay the binding activity of LcKNAT1 to the *LcACS1*, *LcACS7*, *LcACO2*, and *LcACO3* promoters, the promoters were cloned into the pGreenII 0800 double-reporter vector (Hellens *et al.*, 2005), while *LcKNAT1* was cloned into the pGreenII 62-SK vector (Sainsbury *et al.*, 2009) as effectors. The effector and reporter plasmids were transiently co-expressed in tobacco leaves. LUC and REN luciferase activities were measured as described above. The results were calculated as the ratio of LUC to REN. The primers used here are listed in Supplementary Table S1.

Determination of abscission rate

The cumulative litchi fruitlet abscission rate was calculated according to our previous method (Kuang *et al.*, 2012). In brief, cumulative fruit abscission was calculated by dividing the number of dropped fruitlets by the initial number of fruitlets, and multiplying by 100. To analyse tomato pedicel abscission, flowers were removed with a sharp razor blade, and then the bases of the flower explants were placed in 1.5 ml Eppendorf tubes containing 1 ml water, and pedicel abscission was monitored at 0, 13, 20, 26, 34, 47, 57, and 66 h after flower removal. A total of 100 flowers were used for each treatment.

RNA-Seq experiments

RNAs from pedicel AZ tissues of Micro-Tom (0, 4, 8, and 14 h after flower removal) and the *35S:LcKNAT1-1* transgenic tomato line (0, 4, 8, and 14 h after flower removal) were separately extracted from each of the three randomly selected individual plants as one biological replicate and pooled after measuring the individual total RNA quantities and concentrations with the RNA Nano 6000 Assay Kit on an Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Size-selected, adaptor-ligated cDNAs were incubated with 3 µl USER™ Enzyme (NEB, USA) at 37 °C for 15 min, followed by 5 min at 95 °C prior to PCR. The amplified fragments were then sequenced using the Illumina HiSeq X™ Ten platform to obtain 150 bp paired-end reads.

For the assembly library, raw reads were filtered to remove the sequences containing adaptors and reads with >5% unknown nucleotides. Low-quality reads greater than 20% low Q-value (≤10) bases were also removed. The clean reads were then subjected to the Basic Local Alignment Search Tool (BLAST) analysis against *S. lycopersicum* mRNA and protein sequences from the ITAG3.0 annotations (ftp://ftp.solgenomics.net/tomato_genome/annotation/ITAG3.0_release/). Differential expression analysis was performed using DEGSeq, with a *q*-value (or false discovery rate) of <0.01 and $|\log_2(\text{fold-change})|$ of >1 set as the threshold for significant differential expression (Wang *et al.*, 2010).

Statistical analysis

Data are reported as means ±SD. One-way analysis of variance (ANOVA) followed by Duncan's multiple range test was performed to determine whether there were significant differences among all the treatments (Duncan, 1955) using SPSS Statistics (version 21.0, IBM Corp., Armonk, NY, USA). Significant differences between pairs of groups were then identified using Student's *t*-test (Student, 1908). Means were considered significantly different at $P<0.05$ or $P<0.01$.

Accession numbers

Accession numbers are as follows: *LcKNAT1* (MK413639), *LcACO1* (MK413640), *LcACO2* (MK413641), *LcACO3* (MK413642), *LcACS1* (MK413643), *LcACS2* (MK413644), *LcACS3* (MK413645), *LcACS4* (MK413646), *LcACS5* (MK413647), *LcACS6* (MK413648), *LcACS7* (MK413649), and *LcACS8* (MK413650).

Results

Activation of LcACS/ACO *genes in fruit abscission zone cells coincides with fruitlet abscission in litchi*

GPD treatment is an effective method for inducing carbohydrate stress in litchi (Li *et al.*, 2015*a*). After GPD treatment, fruitlet abscission was significantly induced 3 d after treatment (DAT), and the cumulative abscission rate reached almost 100% at 5 DAT. In contrast, only about 10% of the fruitlets had dropped at 5 DAT in the control (Fig. 1A). Ethylene emission in GPD-treated fruit was significantly higher than in the control from the first day, and reached a high level at 2 DAT prior to fruitlet abscission (Fig. 1B).

To examine the effect of GPD treatment on the expression of ethylene biosynthetic genes, eight *LcACS* genes and three *LcACO* genes were identified in the litchi genome. *LcACO2* accumulated gradually and reached ~50-fold expression of the control at 4 DAT (Fig. 2). The expression of another five genes (*LcACO3*, *LcACS4*, *LcACS6*, *LcACS7*, and *LcACS8*) was also significantly higher at 2 DAT. The expression of *LcACS3* was higher at 4 DAT, whereas the expression of *LcACS1* was elevated at 1 DAT and 2 DAT. The expression of three other genes (*LcACO1*, *LcACS2*, and *LcACS5*) was not significantly affected by GPD treatment (Fig. 2).

To further confirm which *LcACS/ACO* genes are the key components in ethylene biosynthesis involved in fruitlet

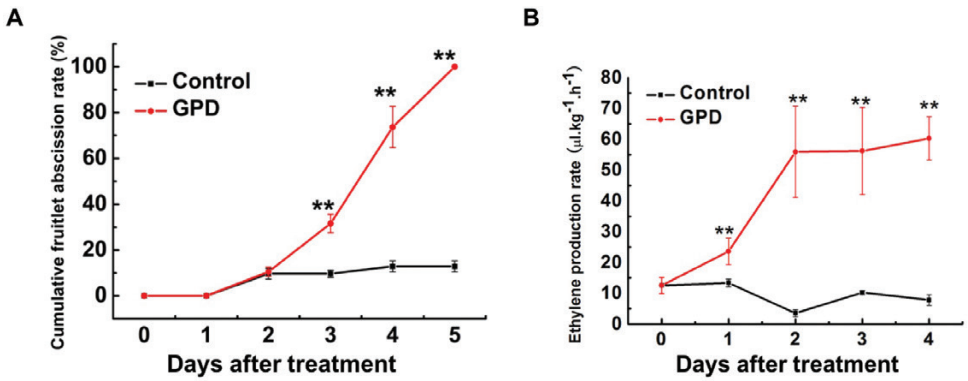

Fig. 1. Effects of GPD treatment on ethylene emission and fruitlet abscission in litchi. (A) GPD-induced fruitlet abscission in litchi. (B) The effect of GPD on ethylene production during fruitlet abscission in litchi. Results are the means of three biological replicates; error bars represent ±SD. Asterisks indicate a significant difference (Student's *t*-test: $^{**}P<0.01$). (This figure is available in color at *JXB* online.)

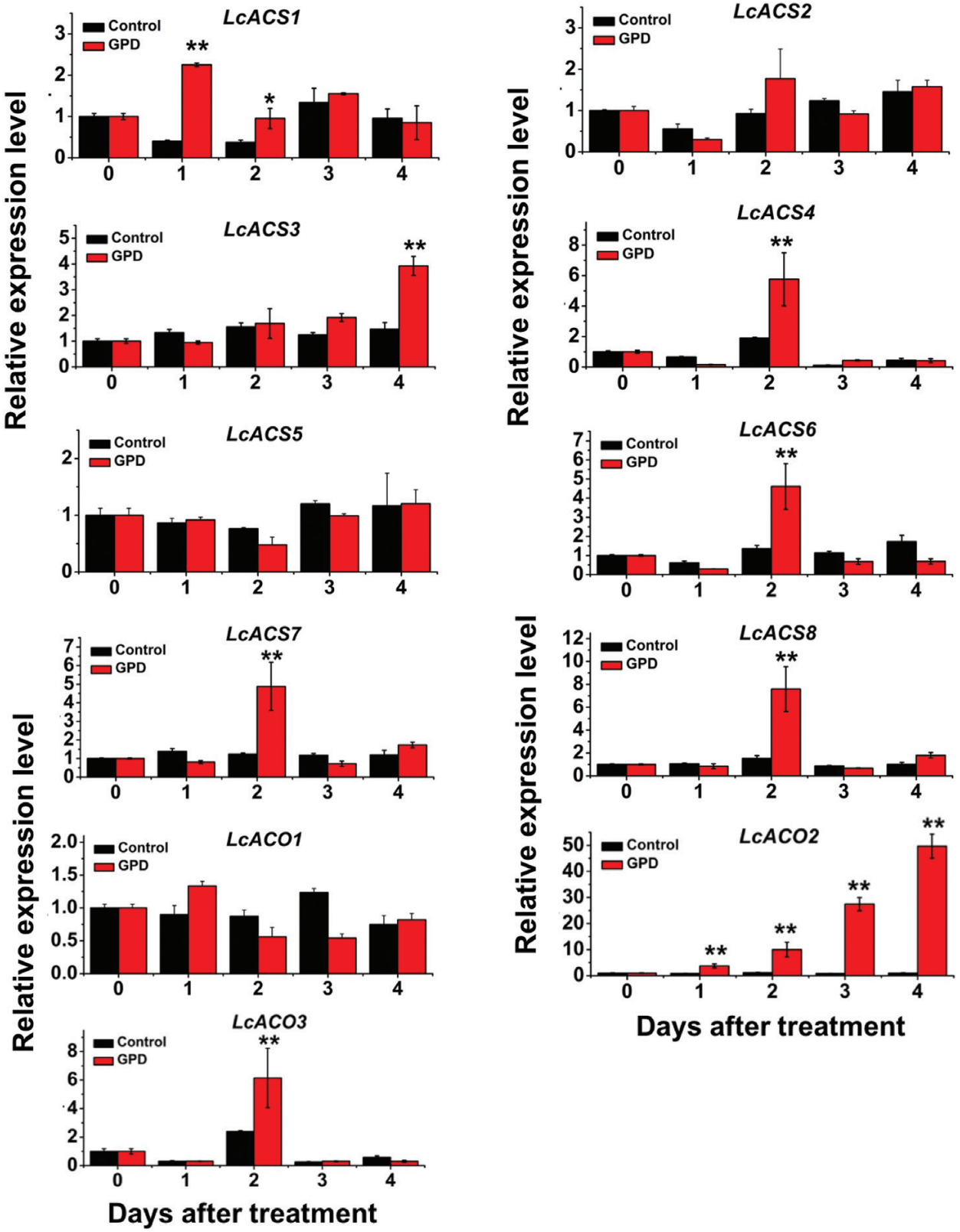

Fig. 2. Effects of GPD treatment on expression of *LcACS/ACO* genes in the fruit abscission zone (FAZ) during litchi fruitlet abscission. Expression of the *LcEF-1a* gene was used as an internal control. Data represent the mean of three biological replicates with three technical replicates each. Error bars represent ±SD. Asterisks indicate a significant difference (Student's *t*-test: **P*<0.05, ***P*<0.01). (This figure is available in color at *JXB* online.)

abscission in litchi, we compared the expression patterns of *LcACS/ACO* genes in the present study with the results of our two previous studies. One study was conducted in 2015 using RNA-Seq (see Supplementary Fig. S1A–C) and another study was performed in 2017 using qRT-PCR (Supplementary Fig. S1D–F). We found that *LcACS1*, *LcACS7*, and *LcACO2*

exhibited elevated expression in all the studies and that the expression of these genes was induced before the onset of ethylene emission and fruitlet abscission (Fig. 2; Supplementary Fig. S1).

Moreover, to further determine whether those events occurring during the GPD-induced abscission process are similar to those accompanying the natural abscission process, the expression patterns of *LcACS1/7* and *LcACO2* during wave I of abscission occurring naturally in the 'Huaizhi' litchi were analysed. Fruitlets of 'Huaizhi' were first sampled at 0 dpa, then the fruitlets were sampled every week for 3 weeks. The relative fruit abscission rate and ethylene production rate were examined. As shown in Supplementary Fig. S2A, a peak in the relative abscission rate was observed at 7 dpa, indicating that wave I of fruitlet abscission occurred within 1 week after anthesis. In addition, we found that the fruitlets within 1 week after anthesis remained at a high ethylene production rate, and then decreased sharply (see Supplementary Fig. S2B). qRT-PCR assays showed that the expression level of *LcCEL8* at the AZ was induced greatly at 7 dpa and declined gradually at 14 and 21 dpa, indicating that a cell separation process occurred naturally within the AZ tissues, as *LcCEL8* has been proved to be closely associated with fruitlet abscission in litchi (Li *et al.*, 2019). As expected, *LcACS1/7* and *LcACO2* were induced at 7 dpa and declined thereafter (Supplementary Fig. S2C). Furthermore, those molecular events were also examined during the male flower abscission process. Generally, the male flowers will drop within 1 week after the stamens protrude. AZ tissues of the first day (stage I), third day (stage II) and seventh day (stage III) after the stamens protruded were harvested for analysis (Supplementary Fig. S2D). Similar to what was found during the natural fruitlet abscission process, *LcACS1/7* and *LcACO2* were induced at stage III (Supplementary Fig. S2E). Collectively, these results demonstrate that *LcACS1/7* and *LcACO2* are the key genes associated with litchi fruitlet abscission.

The expression of LcKNAT1 *is down-regulated during the abscission process*

A previous study showed that AtKNAT1/BP acts as a suppressor during floral organ abscission in Arabidopsis (Shi *et al.*, 2011). We identified seven typical KNOX gene family members (designated *LcKNAT1*–7) and one truncated *KNOX* (named *LcKNATM*) that lacks the homeodomain (HD) in the litchi genome. Phylogenetic analysis showed that these KNOX proteins grouped into three clades: Class I, Class II, and Class M. LcKNAT1, 2, 3, 5, and 6 clustered into Class I. Among these proteins, LcKNAT1 is most closely related to Arabidopsis AtKNAT1/BP. LcKNAT4/7 grouped into Class II, while LcKNATM clustered into Class M (Fig. 3A). All of these LcKNOX proteins contain a conserved MEINOX domain that is responsible for protein–protein interactions and a C-terminal HD domain (except for LcKNATM) for binding to DNA via a specific *cis*-element (see Supplementary Fig. S3A, B).

To determine whether the KNOX-like genes in litchi are involved in fruitlet abscission, transgenic Arabidopsis plants with the ectopic expression of *LcKNAT* genes driven by the Cauliflower Mosaic Virus (CaMV) *35S* promoter were generated for analysis. Among the transformants, only *35S:LcKNAT1* exhibited a completely inhibited floral organ abscission phenotype (see Supplementary Fig. S4), suggesting that LcKNAT1 is involved in organ abscission.

To gain a better understanding of the involvement of LcKNAT1 in fruitlet abscission in litchi, we examined the expression profile of *LcKNAT1* in the peduncle of litchi, which is divided into three regions: the fruit abscission zone (FAZ), the distal region (between the fruitlet and the FAZ), and the proximal region (Fig. 3B). The transcript of *LcKNAT1* was significantly more abundant in the FAZ than in the adjacent tissues (Fig. 3C). We also analysed the expression pattern of *LcKNAT1* using a histochemical GUS staining assay in tomato. A construct carrying the GUS reporter, driven by the *LcKNAT1* promoter, was transformed into tomato. Strong GUS signals were detected in the pedicel AZ of tomato. In addition, the GUS signals in the pedicel AZ were decreased when the pedicel began to abscise (Fig. 3D).

Moreover, we examined the expression of *LcKNAT1* in the FAZ during GPD and ethylene-induced fruitlet abscission in litchi. Expression of *LcKNAT1* was significantly lower than that of the control from 1 to 4 DAT after GPD/ethylene treatments (Fig. 4A). Additionally, as shown in Supplementary Figure S2C, E, *LcKNAT1* was down-regulated during natural fruitlet abscission and the male flower abscission process in litchi. Furthermore, when a *LcKNAT1pro:GUS* reporter was transiently expressed in tobacco leaves, the GUS signal was very low at 2 DAT, following ethylene treatment, while the control expressing *35S:GUS* showed no response to ethylene treatment (Fig. 4B). Together, these findings suggest that *LcKNAT1* is predominantly expressed in the AZ and its expression is decreased during the abscission process.

LcKNAT1 represses the LcACS1, LcACS7, LcACO2, *and* LcACO3 *genes by directly binding to their promoters*

As *LcKNAT1* was down-regulated and *LcACS/ACO* genes were up-regulated during the fruitlet abscission in litchi (Figs 2, 4A; Supplementary Fig. S2C, E), we propose that LcKNAT1 might regulate the expression of *LcACS/ACO* genes directly or indirectly. To test this hypothesis, we first analysed the promoter regions of *LcACS/ACO* genes and identified several potential *cis*-elements (two adjacent TGAC motifs) responsible for KNOX protein binding in the promoters of *LcACS1* (two potential binding sites), *LcACS7*, *LcACO2*, and *LcACO3* (two potential binding sites) (Fig. 4A). EMSA experiments showed that a recombinant LcKNAT1 protein was able to bind to the labeled probes containing two adjacent TGAC motifs derived from *LcACS1*, *LcACS7*, *LcACO2*, and *LcACO3* (Fig. 5A). Furthermore, the shifted bands could be effectively competed out by the addition of the unlabeled competitor of the same sequence, but not by mutated competitors (Fig. 5A). To further test whether LcKNAT1 could target *LcACS1/7* and *LcACO2/3 in vivo*, the binding of LcKNAT1 with these promoters was verified by ChIP-qPCR. As expected, the promoter regions containing the KNOX TF binding site of

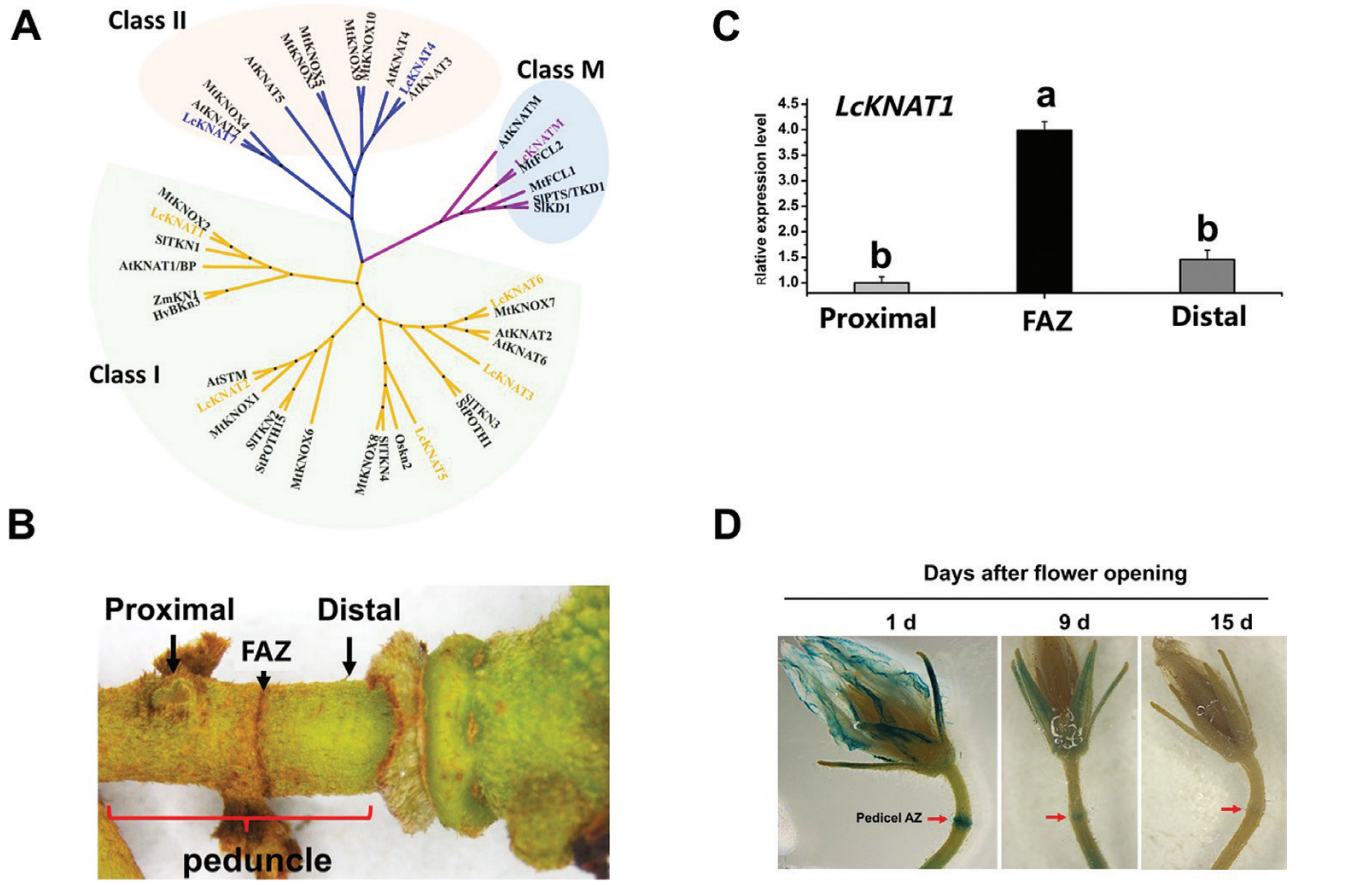

Fig. 3. *LcKNAT1* is expressed at AZ. (A) A maximum likelihood phylogenetic tree of KNOX proteins from Arabidopsis (*At*), tomato (*Sl*), *Medicago truncatula* (*Mt*), and *Litchi chinensis* (*Lc*). (B) Photograph showing the fruit abscission zone (FAZ), distal, and proximal regions of the litchi fruit peduncle. (C) *LcKNAT1* is predominantly expressed in the FAZ of litchi. (D) *LcKNAT1 is* expressed in the pedicel AZ of tomato. *GUS* reporter analysis of *LcKNAT1* expression in T_1 transgenic plants harboring the *GUS* gene driven by the *LcKNAT1* promoter. The process of tomato flower opening through abscission is shown over 15 d. Arrows indicate the abscission zones. (This figure is available in color at *JXB* online.)

LcACS1/7 and *LcACO2/3* were significantly enriched by the anti-LcKNAT1 antibody, but not by IgG. These data, along with the EMSA results, clearly reveal that LcKNAT1 directly targets *LcACS1/7* and *LcACO2/3 in vitro* and *in vivo* (Fig. 5B, C).

To evaluate the effects of LcKNAT1 on the transcriptional activity of *LcACS/ACO* genes, we tested whether LcKNAT1 is a transcriptional suppressor or activator in tobacco using a dual luciferase reporter system. Five copies of the GAL4 DNA-binding element and minimal CaMV *35S* were fused to the firefly luciferase (LUC) reporter, and a *Renilla* luciferase (REN) reporter under the control of the CaMV *35S* promoter was used as an internal control (Fig. 5D). We showed that pBD-LcKNAT1 significantly suppressed the expression of the *LUC* reporter compared with the pBD empty vector, suggesting that LcKNAT1 could act as a transcriptional suppressor. Subsequently, the dual-LUC system was used to test whether LcKNAT1 could repress the expression of *LcACS1*, *LcACS7*, *LcACO2*, and *LcACO3*. Upon transient co-expression of the *promoter:LUC* reporter and *35S:LcKNAT1* effector in tobacco leaves, a significantly lower LUC/REN ratio was observed (Fig. 5E), indicating that the promoter activities of *LcACS1*, *LcACS7*, *LcACO2*, and *LcACO3* were inhibited by LcKNAT1 in tobacco leaves. Collectively, these data suggest that LcKNAT1 acts as a transcriptional suppressor of ethylene biosynthetic genes by directly binding to their promoters.

Overexpression of LcKNAT1 *delayed the pedicel abscission in tomato*

As current genetic transformation strategies are not applicable for litchi, tomato plants were used for LcKNAT1 transformation and functional analysis of *LcKNAT1*. The role of LcKNAT1 in repressing abscission was confirmed by expressing *35S:LcKNAT1* in more than 30 independent transgenic tomato lines. Three representative homozygous lines, *35S:LcKNAT1-1*, *35S:LcKNAT1-2*, and *35S:LcKNAT1-3*, each differing in the *LcKNAT1* expression level, were chosen for further study (Fig. 6B). These *35S:LcKNAT1* transgenic lines exhibited a strong phenotype of lobed and serrated leaves (Fig. 6A). Flower removal (Fig. 6C), which is a good system for inducing tomato pedicel abscission (Meir *et al.*, 2010), was used to test the function of LcKNAT1 in regulating pedicel abscission. As shown in Fig. 6D, *35S:LcKNAT1* transgenic tomato lines displayed delayed pedicel abscission after flower removal, and the pedicel abscission rate was much lower (less than 40%) than that in control Micro-Tom (more than 80%) at 4 h after flower removal. At 70 h after flower removal, the abscission rate

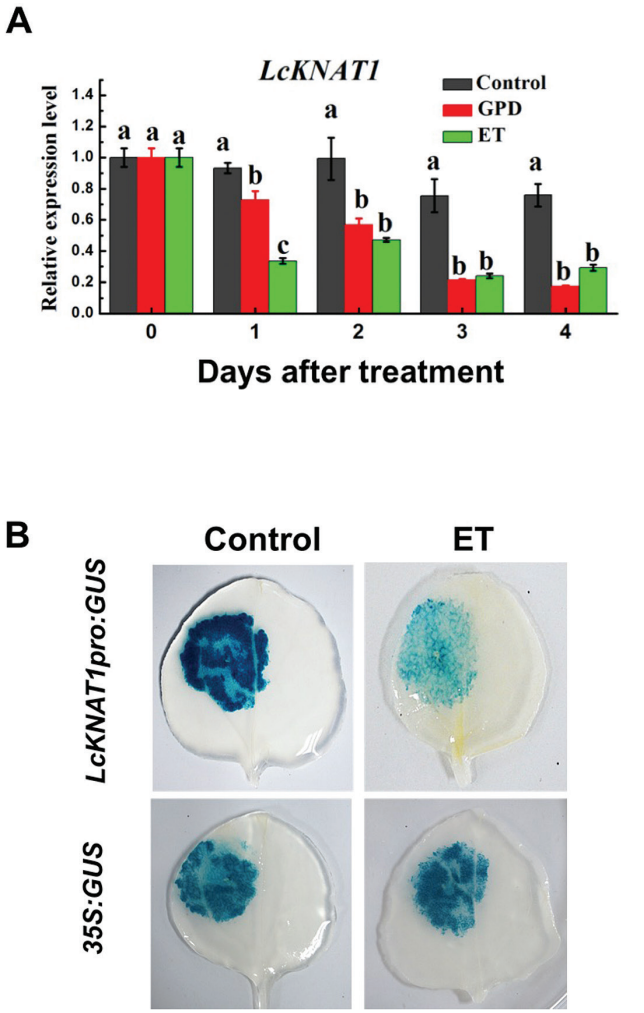

Fig. 4. *LcKNAT1* is repressed during litchi fruitlet abscission. (A) *LcKNAT1* expression is lower following fruitlet abscission in litchi. qRT-PCR was used to profile the expression pattern of *LcKNAT1* after GPD and ethylene (ET) treatments. Expression of the *LcEF-1a* gene was used as an internal control. The *y*-axis represents fold-change in expression relative to the control at 0 d, which was set to 1. Data represent the mean of three biological replicates with three technical replicates each. Error bars represent ±SD. Different letters indicate significant pairwise differences according to Duncan's test ($P<0.05$). (B) Transient expression assay of repression of *LcKNAT1* expression by ethylene. The *LcKNAT1pro:GUS* vector was introduced into tobacco leaves using *Agrobacterium* GV3101. The tobacco plants were incubated with (50 μl l^{-1}) or without ethylene for 48 h. The empty *35S:GUS* construct was used as a control. Similar results were obtained with six biological replicates, and images pictures are shown here. (This figure is available in color at *JXB* online.)

reached 100% for Micro-Tom; however, less than 60% of pedicels abscised in the *35S:LcKNAT1* transgenic lines (Fig. 6D). A previous study showed that organ abscission is closely associated with an increase in cytosolic pH in AZ cells, which can be easily detected by BCECF-AM staining (Sundaresan *et al.*, 2015). Consistently, only weak BCECF signals were detected in the AZ of *35S:LcKNAT1-1* transgenic lines compared with those in Micro-Tom at 14 h after flower removal (Fig. 6E, F).

Overexpression of LcKNAT1 *leads to reduced expression of* ACS/ACO *genes and reduced ethylene sensitivity in tomato*

To gain further insights into the molecular mechanism of LcKNAT1 in delaying abscission in tomato, we compared the transcriptome of the pedicel AZ in the *35S:LcKNAT1-1* transgenic line with that of wild-type Micro-Tom using an RNA-Seq approach. After flower removal, pedicel AZ tissues from four time points (0, 4, 8, and 14 h) were collected for analysis. Our data indicated that 6629 genes were differentially regulated at 8 h, as compared with other time points, and 118 genes were co-regulated during pedicel abscission in the tomato AZ (see Supplementary Fig. S5A, B). We further carried out Gene Ontology (GO) analysis on all the identified differentially expressed genes (DEGs) and found that the GO terms 'protein binding', 'extracellular vesicular exosome', 'regulation of transcription', and 'DNA-template' were highly represented from the molecular function, cellular component, and biological process domains (Supplementary Fig. S5C). A full list of DEGs can be found in Supplementary Tables S2–5.

Previous work has demonstrated that genes related to cell wall remodeling accomplish cell separation in abscission zones (Estornell *et al.*, 2013). RNA-Seq data revealed that 35 cell wall remodeling genes were differentially expressed in the *35S:LcKNAT1-1* transgenic lines. Among these DEGs, 33 exhibited lower expression than in wild-type, including seven genes encoding polygalacturonases (PG), eight encoding cellulases (Cel), 12 encoding xyloglucan endotransglucosylases (XTH), and six encoding expansins (EXP). In addition, a gene encoding a T2/S-like RNase (LX), which is suppressed in tomato with a delay in leaf senescence and abscission (Lers *et al.*, 2006), was also down-regulated in *35S:LcKNAT1-1* plants (Fig. 7). Importantly, four *ACO* genes (*SlACO2*, *SlACO2*, *SlACO3*, and *SlACO6*) and *SlACS2*, which are involved in ethylene biosynthesis, were also down-regulated in *35S:LcKNAT1-1* transgenic plants (Fig. 7).

In addition, ethylene response can be monitored by ethylene-mediated triple response (Guzmán and Ecker, 1990). In the absence of ACC, both the root and hypocotyl length exhibited no significant difference between wild-type and *35S:LcKNAT1* transgenic lines (see Supplementary Fig. S6A, B). Upon 5 μM ACC treatment, however, *35S:LcKNAT1* transgenic lines showed less reduction in root elongation compared with wild type (Supplementary Fig. S6A, C). Taken together, these results indicate that LcKNAT1 can inhibit ethylene biosynthetic genes to reduce the expression of cell wall remodeling genes during abscission in tomato plants.

Discussion

Our previous work showed that ethylene production in fruitlets reaches a peak after GPD treatment at day 2 prior to fruitlet abscission in litchi (Li *et al.*, 2015*a*). This result is consistent with the view that ethylene is the inducer of fruit abscission (Davenport and Manners, 1982; Bonghi *et al.*, 2000; Katz *et al.*, 2004; Dal Cin *et al.*, 2005; Murayama *et al.*, 2006; Ollauri *et al.*, 2014; Eccher *et al.*, 2015). Similarly, we also observed that GPD-induced litchi fruitlet abscission is closely associated with ethylene emission in fruitlets in this study (Fig. 1A, B). Further, we inferred that *LcACS1*, *LcACS7*, and *LcACO2* may be the key genes involved in fruitlet abscission in litchi, since these genes were up-regulated under both natural fruitlet abscission and the GPD/ethylene-induced fruitlet abscission process (Fig. 2; Supplementary Figs S1, 2). Moreover, LcACO2 is

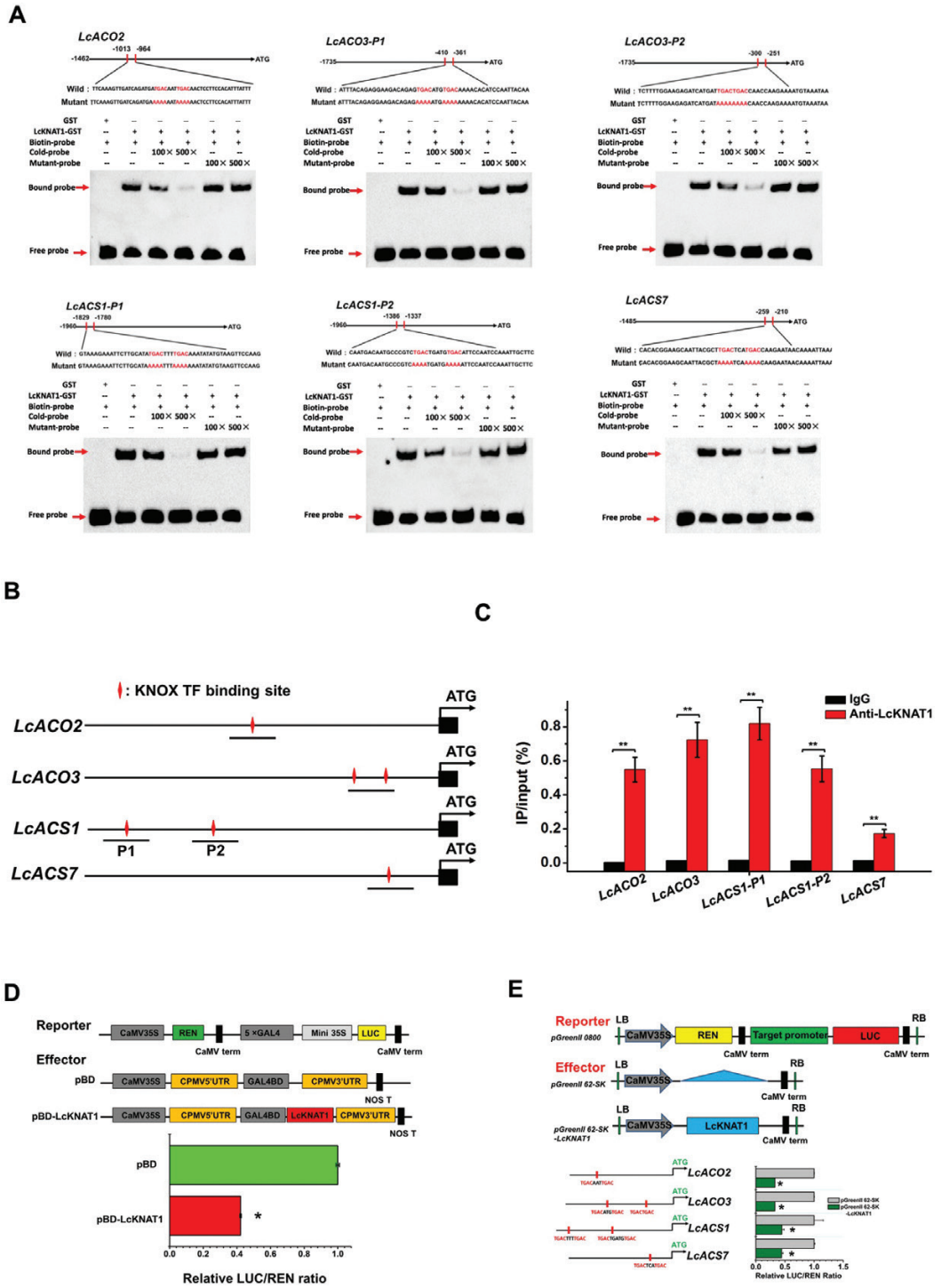

Fig. 5. Binding of LcKNAT1 to *LcACO2*, *LcACO3*, *LcACS1*, and *LcACS7* promoters to repress their activity. (A) Binding of LcKNAT1 to *LcACO2*, *LcACO3*, *LcACS1*, and *LcACS7* promoters *in vitro*. EMSA using purified LcKNAT1–GST fusion protein. Sequences of both the wild-type and mutant probes are shown at the top of the image (the core TGACTGAC motifs are indicated). Shifted bands, suggesting the formation of DNA–protein complexes, are indicated by arrows. '–' represents absence, '+' represents presence; 100× and 500× indicate increasing amounts of unlabeled or mutant probe used for competition and testing of binding specificity. Non-biotin-labeled probe was added as an unlabeled competitor. GST protein alone served as a negative control. (B, C) ChIP assay showing the binding of LcKNAT1 to the promoters of *LcACO2*, *LcACO3*, *LcACS1*, and *LcACS7*. DNA samples were immunoprecipitated with LcKNAT1 antibody or IgG control. $^{**}P<0.01$, difference from IgG, by Student's *t*-test. (D) Transcriptional repression by LcKNAT1 in tobacco leaves. Top panel shows diagrammatically the reporter and effector vectors. Transcriptional repression by LcKNAT1 is indicated as the ratio of LUC to REN fluorescence. The LUC/REN ratio from leaves expressing the empty pBD vector (negative control) was used to calibrate fluorescence (set as 1). Each value represents the mean ±SD of six biological replicates. Asterisk indicates a significant difference (Student's *t*-test: $P<0.05$). (E) Transient expression assays of repression of *LcACO2*, *LcACO3*, *LcACS1*, and *LcACS7* transcription by LcKNAT1. Top panel shows diagrammatically the double reporters and effector plasmids used in the assay. The reporter and effector vectors were co-introduced into tobacco leaves using *Agrobacterium* GV3101. The tobacco plants were incubated for 48 h, and the suppression of expression from the *LcACO2*, *LcACO3*, *LcACS1*, and *LcACS7* promoters by LcKNAT1 is indicated as the ratio of LUC to REN fluorescence. Each value represents the means of six biological replicates, and vertical bars represent the SD. Asterisks indicate a significant difference (Student's *t*-test: $P<0.05$). (This figure is available in color at *JXB* online.)

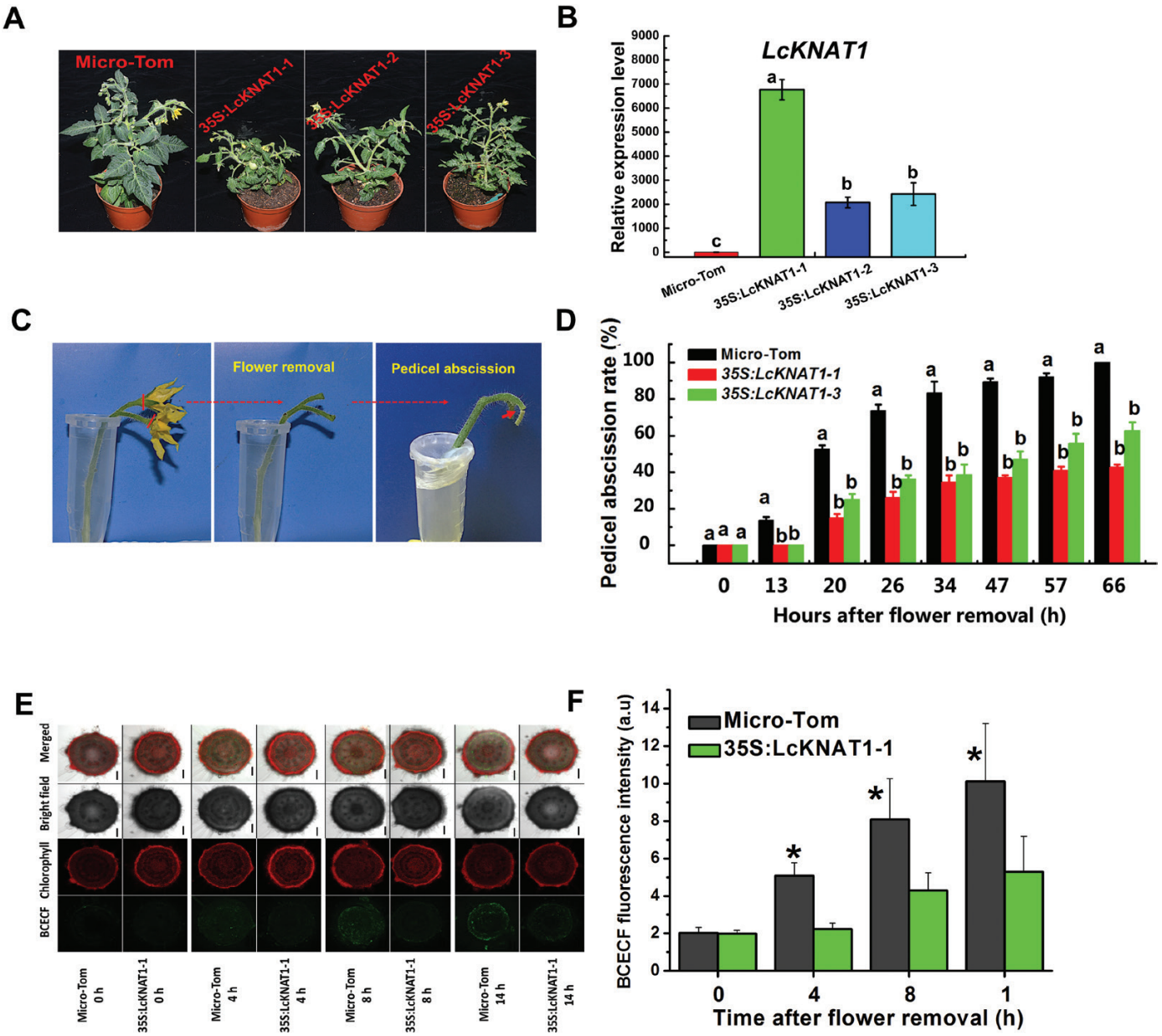

Fig. 6. Ectopic expression of *LcKNAT1* delayed pedicel abscission in tomato. (A) Whole plants of Micro-Tom and LcKNAT1 transgenic lines. (B) Expression levels of LcKNAT1 in transgenic tomato lines. Error bars represent ±SD. Different letters indicate significant pairwise differences according to Duncan's test ($P<0.05$). (C) The flower removal system to induce pedicel abscission. (D) The pedicel abscission rate of Micro-Tom control and LcKNAT1 transgenic lines after flower removal. The cumulative percentage of pedicel abscission was monitored at the indicated time intervals following flower removal. Error bars represent ±SD. Different letters indicate significant pairwise differences according to Duncan's test ($P<0.05$). (E) BCECF fluorescence micrographs of cross-sections of the AZ of tomato flower pedicels after flower removal. Scale bars: 10 μm. The experiment was repeated three times with three to four different biological samples from different flowering shoots. (F) Quantification of BCECF fluorescence intensity in the pedicel AZ at indicated time points after flower removal. Each quantification was calculated from 10 independent images. Asterisks indicate a significant difference (Student's *t*-test: $P<0.05$). (This figure is available in color at *JXB* online.)

not closely clustered with any other ACO homologs, including MdACO1 (MDP0000195885) and CsACO1 (AJ297350.1), which have been reported to be involved in fruit abscission (Katz *et al.*, 2004; Botton *et al.*, 2011), indicating that LcACO2 could possess unique functions in litchi (Supplementary Fig. S7A). Interestingly, LcACS1 and LcACS7 are more closely related to other abscission-associated *ACS* genes, including *CsACS1* (AJ011095) from citrus, and *MdACS5A* (AB034992) and *MdACS5B* (AB034993) from apple (Katz *et al.*, 2004; Dal Cin *et al.*, 2005; Li and Yuan, 2008; Botton *et al.*, 2011) (Supplementary Fig. S6B). Notably, although abscission-related SlACS9 (Solyc12g056180.1.1) was also more closely related to LcACS1 and LcACS7 (Supplementary Fig. S6B; Sundaresan *et al.*, 2018), the transcripts of *SlACS9* did not changed significantly in the *35S:LcKNAT1* transgenic lines, indicating that *SlACS9* was not involved in LcKNAT1-mediated

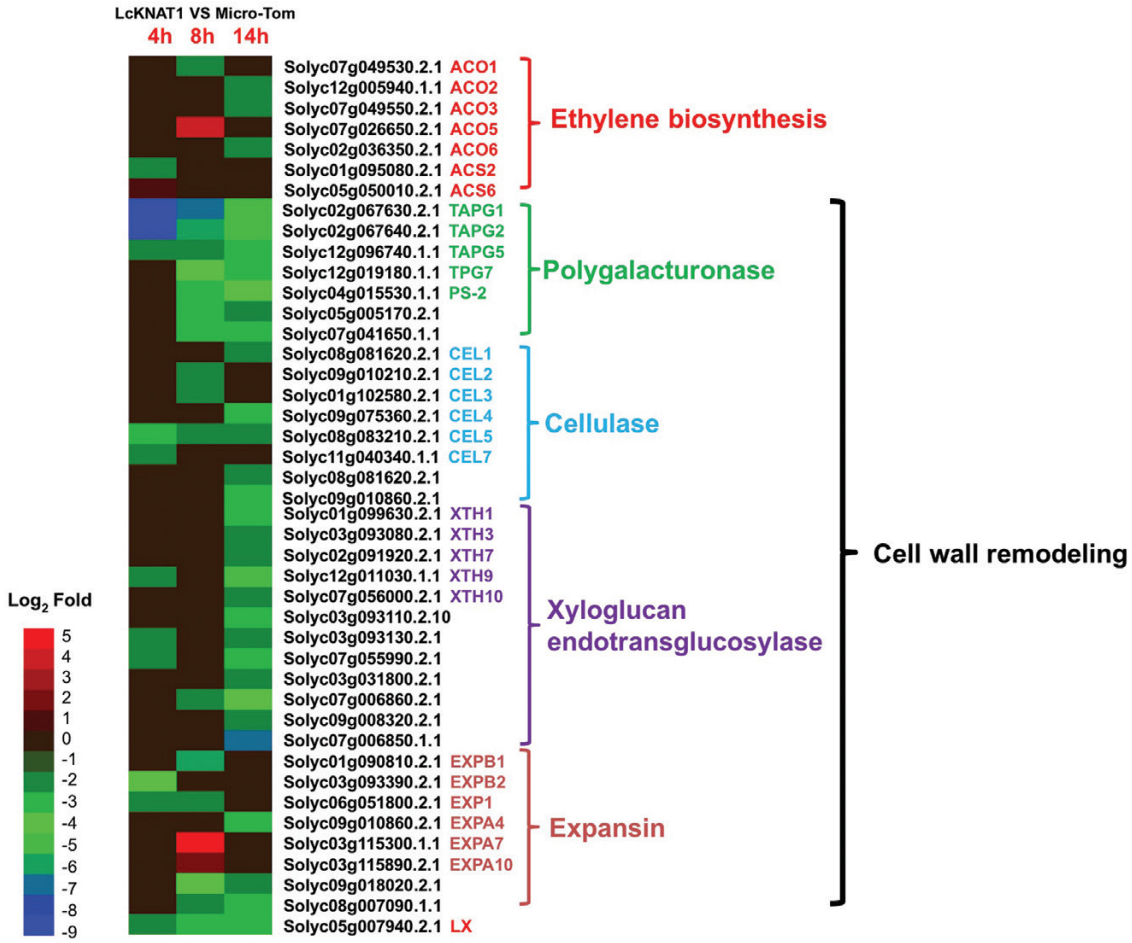

Fig. 7. Repression of *ACS/ACO* and cell wall remodeling genes in *LcKNAT1* transgenic tomato plants during flower abscission. Heatmap showing selected differentially expressed genes from the pedicels of abscising flowers identified in RNA-Seq experiments. The fold-change in mean expression (log_2 scale) of each gene is shown. (This figure is available in color at *JXB* online.)

pedicel abscission in tomato. Overall, our studies indicate that *LcACS1*, *LcACS7*, and *LcACO2* are the key genes involved in the ethylene biosynthesis associated with fruitlet abscission in litchi.

Transcriptome analyses in maize have shown that the auxin pathway is the most significantly regulated hormone pathway in *kn1* mutants. KNOTTED1 (KN1) directly targets auxin pathway genes at all levels, including auxin biosynthesis, perception, transport, and signaling, suggesting that auxin acts downstream of KN1 in regulating meristem identity in maize (Bolduc *et al.*, 2012). Our study identified a KNOX TF in litchi, LcKNAT1, which is highly expressed at the AZ, but was down-regulated during abscission (Fig. 4A; Supplementary Fig. S2C, E). LcKNAT1 is capable of binding to the promoters of four *LcACS/ACO* genes (*LcACS1*, *LcACS7*, *LcACO2*, and *LcACO3*) and repressing their transcriptional activity (Fig. 5). The importance of *LcACS1*, *LcACS7*, and *LcACO2* in litchi fruitlet abscission is particularly noteworthy. Therefore, we propose that LcKNAT1 represses litchi fruitlet abscission via the negative regulation of the ethylene biosynthetic genes *LcACS1*, *LcACS7*, and *LcACO2*. However, we cannot exclude the possibility that other LcACS/ACOs might also be involved in fruitlet abscission in a LcKNAT1-independent manner. Moreover, we provide strong evidence that LcKNAT1 acts as a repressor to control abscission in tomato (Fig. 6). Importantly, both *ACS/ACO* transcript abundance and ethylene sensitivity were suppressed in transgenic lines overexpressing *LcKNAT1* (Fig. 7; Supplementary Fig. S6), further supporting our hypothesis that the regulation of ethylene biosynthesis by KNAT1 is through the targeting of the *ACS/ACO* genes. Interestingly, a previous study in Arabidopsis has shown that KNAT2, another class-I KNOX gene, acts antagonistically with ethylene on leaf development, senescence, and shoot initiation (Hamant *et al.*, 2002).

Silencing *KD1* in tomato delayed pedicel and petiole abscission together with changes in the transcript abundance of auxin-related genes such as *PIN3-like 3*, *PIN9*, *SAUR*, and *ARF5* and altered the auxin content in the AZ (Ma *et al.*, 2015). Interestingly, transcripts of these genes were not detected in our *LcKNAT1* transgenic tomato line (Supplementary Tables S2–S5), suggesting that the function of LcKNAT1 in the control of abscission is independent of auxin. These results indicate that the molecular mechanisms underlying the control of abscission by LcKNAT1 and KD1 may be different. This distinction might be due to the differences in protein structure between LcKNAT1 and KD1 (Tsuda and Hake, 2015).

Recently, a strong correlation was demonstrated between pH changes in the AZ cells and organ abscission in three different plant species (Sundaresan *et al.*, 2015). In litchi, we also

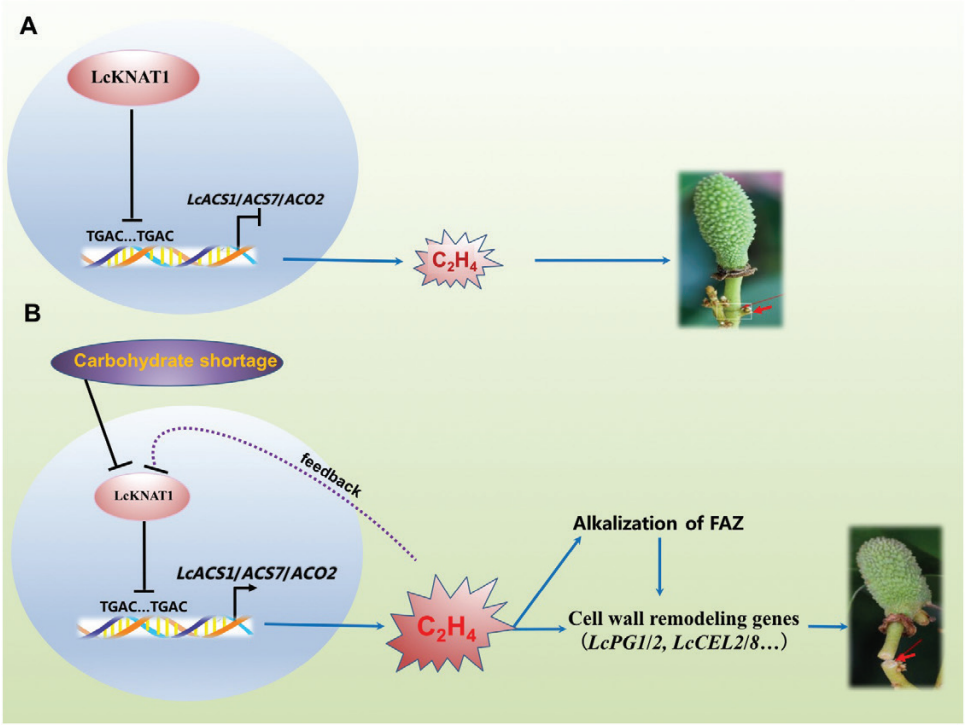

Fig. 8. Proposed model for regulation ethylene biosynthesis by LcKNAT1 during fruitlet abscission in litchi. (A) Under normal conditions, *LcKNAT1* is expressed at a relatively high level. LcKNAT1 directly binds to the core TGAC motif to repress the expression of *LcACS1/ACS7/ACO2*, leading to very low levels of ethylene production without fruitlet abscission. (B) Upon carbohydrate shortage, *LcKNAT1* expression becomes repressed, de-repressing *LcACS1/ACS7/ACO2* expression and resulting in ethylene production. Ethylene can also suppress the expression of *LcKNAT1* as a feedback mechanism to enhance ethylene's action, which leads to cytosolic alkalization and activation of cell wall remodeling genes (such as *LcPG1/2* and *LcCel2/8*) in FAZ cells, followed by fruitlet abscission. (This figure is available in color at *JXB* online.)

detected an increase in cytosolic pH in FAZ cells after GPD/ethylene treatment (unpublished data), suggesting that litchi fruitlet abscission is associated with the alkalization of the cytosol in FAZ cells. As pH can affect enzymatic activities or act as a signal for gene expression, it has been proposed that the increase in cytosolic pH in AZ cells might serve as a signal for abscission-related gene expression or optimize pH for the activity of cell wall remodeling proteins (Sundaresan *et al.*, 2015). In addition, our previous studies have demonstrated that two genes encoding cellulases (*LcCEL2/8*) and two genes encoding polygalacturonases (*LcPG1/2*) are closely associated with fruitlet abscission in litchi (Li *et al.*, 2019; Ma *et al.*, 2019). Thus, based on current findings and our previous studies, we propose a possible working model of LcKNAT1 function in the control of fruitlet abscission in litchi. Before the initiation of fruitlet abscission, *LcKNAT1* is expressed at a relatively high level and LcKNAT1 directly binds to the core TGAC motif to repress the expression of *LcACS1/ACS7/ACO2*, leading to very low levels of ethylene production (Fig. 8). However, when fruitlet abscission begins naturally or is induced by GPD/ethylene treatment, *LcKNAT1* expression is repressed, and then *LcACS1/ACS7/ACO2* expression is increased, resulting in ethylene production to a high level. Moreover, ethylene can also suppress the expression of *LcKNAT1* as a feedback mechanism to enhance ethylene action. Finally, it leads to cytosolic alkalization and the activation of cell wall remodeling genes (such as *LcPG1/2* and *LcCEL2/8*) in FAZ cells, followed by fruitlet abscission.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Ethylene production and *LcACS/ACO* genes expression during GPD-induced litchi fruitlet abscission.

Fig. S2. Ethylene production and LcACS/ACO/KNAT1 genes expression during natural fruitlet abscission and male flower abscission process in litchi.

Fig. S3. Multiple alignment of the litchi KNOX proteins with KNOX proteins from other plant species including Arabidopsis (*At*), *Solanum lycopersicum* (*Sl*), *Medicago truncatula* (*Mt*), *Zea mays* (*Zm*), *Hordeum vulgare* (*Hv*), *Solanum tuberosum* (*St*), and *Oryza sativa* (*Os*).

Fig. S4. Ectopic expression of *LcKNAT1* in Arabidopsis inhibited floral organ abscission.

Fig. S5. Overview of RNA-Seq analysis of gene expression in pedicel AZ of Micro-Tom control and *35S:LcKNAT1-1* transgenic line after flower removal.

Fig. S6. LcKNAT1 suppressed ethylene sensitivity in tomato.

Fig. S7. Phylogenetic analysis of litchi LcACS/ACO proteins with homologs from other plant species.

Table S1. Primers used in this study.

Table S2. DEGs from the LcKNAT1 compared with Micro-Tom at 0 h after flower removal.

Table S3. DEGs from the LcKNAT1 compared with Micro-Tom at 4 h after flower removal.

Table S4. DEGs from the LcKNAT1 compared with Micro-Tom at 8 h after flower removal.

Table S5. DEGs from the LcKNAT1 compared with Micro-Tom at 14 h after flower removal.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

J-GL, M-LZ, and C-LS conceived and designed the experiments; M-LZ performed most of the experiments; C-QL, X-SM, P-YY, M-JP, and JW provided assistance; M-LZ, C-LS, and J-GL wrote the paper; and RX, J-YC, and X-CL helped to analyse the data and revise the article.

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