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RESEARCH PAPER

A conserved AINTEGUMENTA–REVOLUTA module is a candidate to regulate carpel development in lychee

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Abstract

The lychee industry is vital to agricultural economies, boosting the livelihood of farmers and regional growth. However, instability of flowering causes yield fluctuations, severely limiting industry sustainability. Stable pistil development in female flowers is essential for yield improvement, yet its molecular regulation remains poorly understood. Although APETALA2 (AP2) transcription factors regulate floral organ differentiation and pistil development, their functional role in woody perennials such as lychee is uncharacterized. In this study, two *AP2* genes (*LITCHI007109* and *LITCHI010784*) were found to exhibit high and specific expression in carpels. *LITCHI007109*, designated as *LcANT1*, is an ortholog of Arabidopsis *AINTEGUMENTA* (*ANT*). We next systematically identified the direct downstream target genes of LcANT1, the set of which were significantly enriched in biological processes related to floral organ development and carpel morphology. Notably, the carpel development-related gene *LITCHI024703* (*LcREV*) exhibited a high level of co-expression with *LcANT1*. We found that the LcANT1 protein can directly bind to the promoter region of *LcREV*. Further evolutionary analysis indicates that the ANT–REV regulatory module is highly conserved in angiosperms, especially in Sapindaceae. Our findings establish a novel theoretical framework for understanding female flower development in lychee and offer critical gene resources and regulatory networks for molecular breeding strategies aimed at developing high-yield, stable cultivars.

Keywords: AP2 transcription factor, carpel development, DAP-seq, floral sex differentiation, lychee.

Abbreviations: AG, AGAMOUS; AIL, AINTEGUMENTA-like; ANT, AINTEGUMENTA; Ant, aintegumenta; AP2, APETALA2; DREB, dehydration-responsive element-binding proteis; ERF, Ethylene Response Factor; GO, Gene Ontology; RT–qPCR, quantitative reverse transcription–PCR; TSS, transcription start site; WGCNA, weighted gene co-expression network analysis.

Introduction

Lychee (*Litchi chinensis* Sonn.), a precious fruit tree originating from Yunnan, China, holds a significant position among global fruit crops due to its unique flavor and nutritional value (Zhao *et al.*, 2020; Hu *et al.*, 2022). However, the lychee industry has long faced significant challenges, including low overall yields and unstable production. Notably, the 'full blooms, half fruits' paradox in lychee production is primarily attributed to unstable flowering and severe physiological fruit drop during fruit development (Abbas *et al.*, 2022). The process of flower development in lychee is highly complex, with female flowers, male flowers, and functional male flowers co-existing within the same inflorescence of the same plant. While the pistils of female flowers eventually develop into fruits, the pistils of male flowers degenerate completely in later stages, and those of functional male flowers remain underdeveloped (Robbertse *et al.*, 1995; Guan *et al.*, 2021). Therefore, identifying the genes that regulate stable pistil development in female flowers is of great theoretical and practical significance for improving lychee yield and breeding new varieties with high and stable production.

The *APETALA2* (*AP2*) gene, a member of the APETALA2/Ethylene Response Factor (AP2/ERF) superfamily (Wessler, 2005), plays a vital role in plant growth and development, including floral development, somatic embryogenesis, meristem activity, and leaf growth, while also regulating hormone signaling and stress responses (Licausi *et al.*, 2013; Xie *et al.*, 2019; Wang *et al.*, 2022). Based on the number of AP2 domains and other DNA-binding domains, the AP2/ERF superfamily is divided into five subfamilies: AP2; dehydration-responsive element-binding proteins (DREB); ethylene-responsive element-binding proteins (ERFs); Related to ABI3/VP (RAV); and Soloist (Feng *et al.*, 2020). Among them, the AP2 subfamily is distinguished by its two highly similar and tandemly repeated AP2 domains. The AP2 subfamily can be further classified into three subgroups: euAP2 (characterized by the miR172-binding motif), euANT, and basalANT, based on differences in the amino acid sequences of the AP2 domains and nuclear localization signals (Men *et al.*, 2021). In contrast, other subfamilies such as ERF and DREB contain only a single AP2 domain, with their primary distinction lying in the differences at the 14th and 19th amino acid residues (Men *et al.*, 2021). The RAV subfamily, on the other hand, includes one AP2 domain and one B3 domain (J. Li *et al.*, 2023). As the smallest group within the AP2/ERF superfamily, the Soloist subfamily exhibits significant differences in protein sequence and gene structure compared with the other subfamilies (X. Li *et al.*, 2023).

AP2 genes play a pivotal role in floral development in plants. In Arabidopsis, *AP2* homologs are critical for the establishment of floral meristems, the development of floral organs, and the regulation of flowering time. During the initial stages of floral development, *AP2* collaborates with meristem identity genes such as *APETALA1* (*AP1*), *CAULIFLOWER* (*CAL*), and *LEAFY* (*LFY*) to collectively determine the identity of the floral meristem, thereby initiating floral differentiation (Huala and Sussex, 1992; Okamuro *et al.*, 1997; Pineiro and Coupland, 1998). In the ABCDE model of floral organ development, *AP2* functions as an A-class gene that not only participates in the developmental regulation of sepals but also co-regulates petal formation with the B-class genes *AP3* and *PISTILLATA* (*PI*) (Weigel and Meyerowitz, 1994). Additionally, it inhibits the development of stamens and carpels by antagonizing the action of the C-class gene *AGAMOUS* (*AG*) (Shannon and Meeks-Wagner, 1993; Krogan *et al.*, 2012; Huang *et al.*, 2017). In Arabidopsis, mutations in the *AP2* gene lead to the transformation of floral organs into reproductive organs, manifested as the homeotic transformation of sepals and petals into carpels and stamens, respectively (Kunst *et al.*, 1989). In the sixth stage of flower development, AG terminates the activity of *WUSCHEL* (*WUS*) by acting directly on the *WUS* locus or indirectly on its target gene *KNUCKLES* (*KNU*), thereby promoting floral determinacy and determining the number of carpels (Bollier *et al.*, 2018). Furthermore, the miR172–AP2 module regulates flowering time by inhibiting the expression of key flowering genes *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) and *AG* (Yant *et al.*, 2010).

AINTEGUMENTA (*ANT*), as a member of the AP2 subfamily, plays a crucial role in various developmental processes in Arabidopsis, including ovule development, floral organ formation, carpel development, and the determination of floral organ size (Krizek *et al.*, 2021). A female-sterile mutant, *aintegumenta* (*ant*), exhibits a phenotype characterized by carpel separation, impaired ovule development, a lack of integument development, and a blockage of megasporogenesis at the tetrad stage (Klucher *et al.*, 1996; Krizek, 1999; Liu *et al.*, 2000; Mizukami and Fischer, 2000). Moreover, *ANT* and *REV* in Arabidopsis collaborate to maintain morphogenetic balance during the development of the carpel marginal meristem (CMM), although the precise mechanisms remain unclear (Nole-Wilson *et al.*, 2010). The *ant* mutant in Arabidopsis also displayed reduced floral organ and leaf size, while overexpression of *ANT* resulted in enlarged floral organs, siliques, and leaves (Mizukami and Fischer, 2000). It was found that the changes in the expression levels of *ANT* in apple are consistent with the changes in the expression of cell cycle genes A- and B-type *CYCLIN* and B-type *CYCLIN-DEPENDENT KINASE* (*CDKB*) and *MdDEL1* (Dash and Malladi, 2012). Further it was proved that *ANT* participates in the auxin-dependent regulatory process of *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*) in Arabidopsis, regulating the final size of organs through modulation of cell growth, proliferation, and meristematic capacity

(Hu *et al.*, 2003). Through quantitative reverse transcription–PCR (RT–qPCR) analysis, seven *AINTEGUMENTA-like* (*AIL*) genes were identified in Arabidopsis, among which *AIL1*, *AIL5*, *AIL6*, and *AIL7* are expressed in the inflorescence, with *AIL5* showing a similar expression pattern to *ANT* (Nole-Wilson *et al.*, 2005). This suggests that *AIL* genes play regulatory roles in the development of the meristem and are involved in the regulatory pathways of ovule and floral organ development.

Despite the extensive research conducted on the role of AP2 in the development of floral organs in plants, studies focusing on its function in perennial fruit trees such as lychee remain largely unexplored. In this study, we successfully identified two *AP2* genes, *LITCHI007109* and *LITCHI010784*, that are specifically and highly expressed in the carpels of female flowers, through weighted gene co-expression network analysis (WGCNA) of transcriptome data. Phylogenetic analysis indicated that *LITCHI007109* (*LcANT1*) is an ortholog of *ANT* in Arabidopsis. Moreover, DNA affinity purification sequencing (DAP-seq) analysis further revealed that LcANT1 can directly target genes related to floral organ development and carpel development, with LcANT–LcREV representing a potential conserved pathway for regulating carpel development in lychee. These findings not only provide a solid foundation for further investigation into the regulatory role of lychee *AP2* genes in the development of female flowers, but also offer important theoretical support and candidate gene resources for the molecular breeding of varieties with high and stable yields.

Materials and methods

Identification of *LcAP2* subfamily genes in lychee

To identify the *AP2* gene family members in lychee, GffRead was used to translate the coding sequences (CDSs) of all transcripts into protein sequences (Pertea and Pertea, 2020). TBtools V2.069 was utilized to extract representative transcript numbers from the genome annotation file (Chen *et al.*, 2023), while SeqKit was used to retrieve the corresponding transcript CDSs from all protein sequences (Shen *et al.*, 2016). The protein sequences of 17 *AP2* gene family members from *Arabidopsis thaliana* were downloaded from PlantTFDB (https://planttfdb.gao-lab.Org/family.php?sp=Ath&fam=AP2) as reference sequences (Jin *et al.*, 2017). Subsequently, BLASTP was used to search for potential *AP2* genes within the lychee genome (Altschul *et al.*, 1990), with results further validated by querying the UniProtKB/Swiss-Prot database to mitigate false positives. Then, the CDD (https://www.ncbi.nlm.nih.gov/cdd) (Marchler-Bauer and Bryant, 2004) and Pfam (http://pfam-legacy.xfam.org/) (Mistry *et al.*, 2021) databases were utilized to identify the conserved structural domains, ensuring a focus on members possessing two AP2 domains of AP2 members in lychee.

Intron–exon organization, gene structure, protein structure, and motif analysis of *LcAP2* genes

MUSCLE (Edgar, 2004) was utilized to perform multiple sequence alignment of protein sequences from 16 *AP2* genes in lychee, followed by TrimAL (Capella-Gutiérrez *et al.*, 2009) to eliminate poorly aligned regions. IQtree2 was employed to construct a phylogenetic tree using the maximum likelihood method with bootstrap testing, assigning a bootstrap value of 1000. TBtools V2.069 (Chen *et al.*, 2023) was employed to analyze the exons and introns of each *LcAP2* member. Subsequently, the NCBI Batch CD-search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was utilized to detect conserved structural domains. The MEME-Suite 5.5.5 (http://meme-suite.org/meme/tools/meme) (Bailey *et al.*, 2015) was employed to analyze sequence motifs, thereby elucidating the differences among *LcAP2* gene family members. The structure, conserved structural domains, and motif information of *LcAP2* genes were visualized using TBtools V2.069 (Chen *et al.*, 2023). The highly accurate protein structures of AtANT and LcANT domains were obtained from the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/).

Chromosomal distribution and gene duplication events of *LcAP2* genes

The positional information of *LcAP2* family members was retrieved from the lychee genome annotation file. The gene duplication events were analyzed using MCScanX (Wang *et al.*, 2012) with default parameters. The collinear relationships among *LcAP2*, *AtAP2*, and *OsAP2* gene family members were analyzed by using MCScanX. TBtools V2.069 was used to visualize all the results (Chen *et al.*, 2023).

RNA-seq data analysis, weighted gene co-expression network analysis, and Gene Ontology analysis

Two sets of transcriptome data were archived in NCBI under the accession number GSE182447 (Guan *et al.*, 2021). Fastp (Chen *et al.*, 2018) was employed for quality control and the removal of adapters from the raw RNA-seq data. Subsequently, STAR (Dobin *et al.*, 2013) was utilized to map the clean reads to the lychee genome, while StringTie (Pertea *et al.*, 2015) was applied to normalize transcript expression levels to transcripts per million (TPM) values. The construction of the network and detection of modules were performed by using WGCNA (Langfelder and Horvath, 2008). Gene Ontology (GO) enrichment analysis was performed with the 'TopGO' and 'GO.db' packages. Plots were drawn using the 'ggplot2' package of R (Villanueva and Chen, 2019).

DNA affinity purification sequencing and data analysis

The DAP-seq experiment was performed according to the previously published protocol (Zhang *et al.*, 2024). Initially, 5 μg of genomic DNA was extracted from the young leaves of lychee and fragmented into 200 bp segments using ultrasonication. These fragments were ligated with Illumina-based sequencing adaptors to create a DNA library. The CDS of *LcANT1* was cloned into the pIX-Halo vector and translated *in vitro* utilizing the TNT® SP6 High-Yield Wheat Germ Protein Expression System (L3260) from Promega Corporation. Primers are listed in Supplementary Table S1. Following the incubation of the lychee genomic DNA library with HALO-tagged LcANT1, the DNA–protein complex was eluted to be amplified with indexed primers, followed by sequencing at Novogene (Beijing, China).

Bowtie (Langmead, 2010) was used to align the clean reads to the lychee genome, while MACS (Zhang *et al.*, 2008) was utilized to identify the binding sites of AP2, using the BAMPE mode to discern peak values. TBtools on Linux was utilized to extract the sequences located 50 bp upstream and downstream of the binding sites, and MEME-Chip (Bailey *et al.*, 2015) was applied to enrich the combined sequences. FIMO (Grant *et al.*, 2011) was used to identify binding regions that contain conserved motifs. Genes exhibiting peaks within 2 kb upstream of the transcription start site (TSS) or downstream of the transcription termination site (TTS) were designated as target genes of AP2.

The logical implementation of the Python package ReCallpeaks

In response to the analytical requirements of this project, we have meticulously developed the DAP-seq data analysis tool, ReCallpeaks, from the ground up using Python. ReCallpeaks was specifically optimized for data pertaining to transcription factors with low DNA binding capacity in DAP-seq experiments. Initially, genome coverage files in bedgraph format were generated for each sample via the bamCoverage function of Bamtools, based on the BAM file for each dataset (Barnett *et al.*, 2011). Subsequently, the coverage of genes underwent repeated processing to obtain an average according to a designated sliding window length. The 'scipy.signal.find_peaks' method was used to identify potential binding signals, thereby eliminating regions devoid of read enrichment based on a pre-determined threshold. Upon isolating the regions with binding signals, the samtools depth method (Li *et al.*, 2009) was utilized to calculate the sequencing depth for each base within these regions. The 'scipy.signal.find_peaks' method was then applied once more to pinpoint the positions where the binding signal peaks manifest. Extending 50 bp on either side of these positions delineates the candidate binding sites for transcription factors. TBtools was utilized to extract the sequences of these target regions under Linux (Chen *et al.*, 2023). The MEME-ChIP tool was employed to enrich the potential binding sequences for transcription factors (Bailey *et al.*, 2015), while FIMO was utilized to detect binding regions containing conserved motifs (Grant *et al.*, 2011). The source code was hosted on GitHub (https://github.com/FanXuRong/ReCallPeaks.git).

Phylogenetic analysis and collinear relationships analysis

Protein sequences of 26 representative angiosperm species of ANT were downloaded from PlantTFDB (https://planttfdb.gao-lab.org/). The genomic and annotation files for Sapindaceae species were downloaded from SapBase (http://www.sapindaceae.com/Download.html) and other eudicots species were collected from the NCBI database. Gffread (Pertea and Pertea, 2020) was utilized to extract all the protein sequences, and Diamond (Buchfink *et al.*, 2015) was utilized to perform homology comparison in ultra-sensitive mode with an e-value set at 100. Orthogroup, single-copy ortholog sequences, and species trees were inferred using Orthofinder2 (v2.5.4) (Emms and Kelly, 2019) with parameters '-M msa -S diamond'. MUSCLE was utilized to perform multiple sequence alignment, followed by TrimAL to eliminate poorly aligned regions (Edgar, 2004; Capella-Gutiérrez *et al.*, 2009). Phylogenetic analysis was conducted using IQtree2, employing the maximum likelihood method along with bootstrap testing, with a bootstrap value of 1000. Homologous gene pairs were identified by JCVI (v1.2.7) (Tang *et al.*, 2008), and the collinear relationships between species were visualized with its graphics module. The conserved motif logos were generated with the WebLogo (https://weblogo.threeplusone.com/).

Quantitative reverse transcription–PCR

RT–qPCR was conducted with Promega GoTaq® qPCR Master Mix (A6001) in a BioRad CFX384 Real-Time PCR Detection System, with each assay being replicated three times both biologically and technically. Actin was used as the reference gene according to the study published by Zhong *et al.* (2011). *LcANT1* and *LcREV* primers were designed by using primer3 (https://www.primer3plus.com) and are listed in Supplementary Table S2. The specificity of the primers was tested with melting curves and resequencing of PCR products. The relative expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

Electrophoretic mobility shift assay

The coding sequence of *LcANT1* was cloned into the pGEX-4T-1 vector using the primers listed in Supplementary Table S3 and then was expressed in *Escherichia coli* Rosseta (DE3). Expression and purification of the recombinant protein were performed according to the manufacturer's instructions for the GST-tag Protein Purification Kit (Beyotime). The electrophoretic mobility shift assay (EMSA) was conducted using the LightShift® Chemiluminescent EMSA Kit (Thermo Fisher Scientific) (Zhang *et al.*, 2024). The double-stranded probes with 3' biotin labeling were made by annealing separately synthesized strands. The probes used for EMSA are listed in Supplementary Table S3.

Results

LITCHI007109 and *LITCHI010784* are expressed specifically in the carpel of lychee female flowers

Lychee is a monoecious species that produces both male and female flowers on the same plant, including male flowers with entirely degenerated pistils, female flowers with aborted stamens, and functional male flowers exhibiting incomplete pistil development (Fig. 1A). In male and functionally male flowers, the ovaries are small and eventually degenerate, whereas in female flowers, the ovaries are larger and ultimately develop into the lychee fruit. Therefore, the stable development of female flower ovaries is one of the key factors determining lychee yield. Thus, understanding the molecular mechanisms of ovary development has important theoretical significance and practical value. Although *AP2* genes are known to play essential roles in floral organ development, their involvement in regulating ovary development in lychee remains unclear.

To screen for key *AP2* genes involved in the ovary development of lychee female flowers, we identified 16 *LcAP2* genes (Supplementary Table S4) from the lychee genome using BLASTP and Hidden Markov Model (HMM) search based on the AP2 domain (Pfam ID: PF00847). A phylogenetic analysis of the AP2 gene family was subsequently conducted (Fig. 1B; Supplementary S1A, B). Following the established classification systems for *AP2* genes in Arabidopsis and rice (Kim *et al.*, 2006; Zhao *et al.*, 2019), the lychee *AP2* genes were classified into three analogous groups: euAP2, euANT, and basalANT. Subsequently, to explore the potential roles of *LcAP2* genes in flower development, we performed an expression pattern analysis of these genes utilizing published RNA-seq data (Guan *et al.*, 2021). The results indicated that among the 16 *AP2* genes, *LITCHI027031*, *LITCHI024773*, *LITCHI027926*, *LITCHI018533*, *LITCHI006915*, *LITCHI031776*, and *LITCHI016016* were expressed in the stamen and carpel of female, male, and functional male flowers, while *LITCHI002341*, *LITCHI024007*, *LITCHI017889*, and *LITCHI005122* showed low or no expression in all tissues. Notably, five *AP2* genes (*LITCHI016388*, *LITCHI007217*, *LITCHI030251*, *LITCHI010784*, and *LITCHI007109*) exhibited high expression levels in female flowers. Strikingly, *LITCHI007109* and *LITCHI010784*, namely *LcANT1* and *LcANT2*, displayed exclusive, tissue-specific up-regulation in carpels (Fig. 1B), implicating their potential functional significance in carpel development during lychee female flower formation.

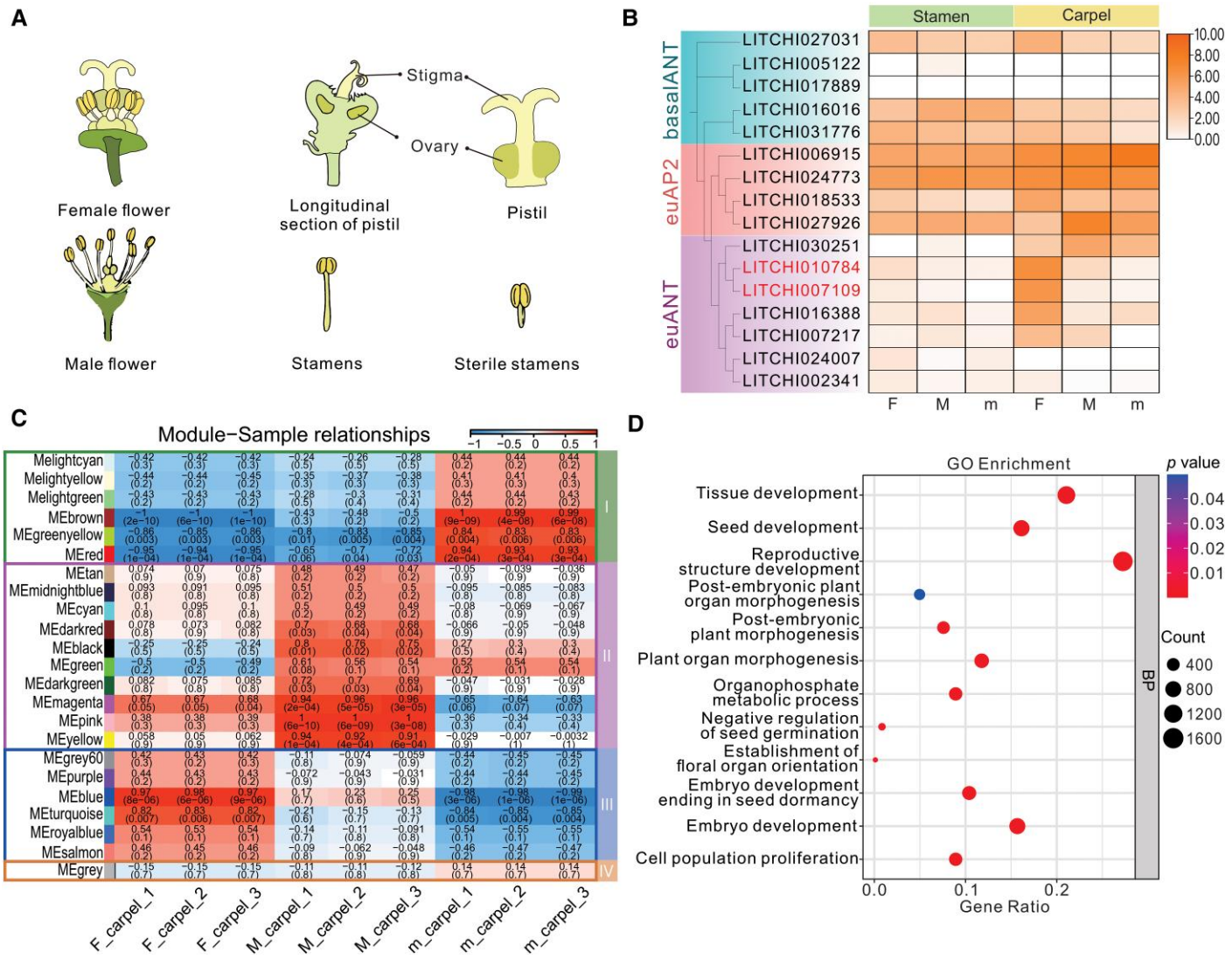

Fig. 1. *LITCHI007109* and *LITCHI010784* exhibit expression specificity in the carpel of lychee female flowers. (A) Schematic diagram of different tissues of lychee at full-bloom stage. (B) Expression profiles of *LcAP2* at different stages of various lychee flower tissues. F, female flower; M, male flower; m, functional male flower. *LITCHI007109* (*LcANT1*) showed the highest expression in the carpel of female flowers. (C) Module–trait relationship of WGCNA. Twenty-three different modules were grouped for all expressed genes. Colors and numbers indicate the correlation coefficient between gene expression and compound contents. Numbers in parentheses show the *P*-values of the significance. (D) Gene Ontology (GO) enrichment analysis for genes in the MEturquoise module associated with carpel development. Fig.1A is sourced from https://www.sapindaceae.com.

To further uncover *AP2* genes related to carpel development in lychee flowers, we conducted WGCNA on RNA-seq data from carpels of different flower sex types, classifying the input genes into 23 modules (Fig. 1C). Through correlation analysis of the expression patterns of module EngineGene with the samples, we found that six modules (Group 1) were relatively highly expressed in the carpel of functional male flowers, 10 modules (Group 2) were relatively highly expressed in the carpels of male flowers, and six modules (Group 3) were primarily highly expressed in the carpels of female flowers (Fig. 1C). Additionally, GO enrichment analysis based on the genes in Group 3 showed that these genes were significantly enriched in pathways such as seed development, reproductive structure development, plant organ morphogenesis, floral organ orientation, and cell population proliferation (Fig. 1D). Notably, the *AP2* genes *LcANT1* and *LcANT2* were identified within the MEturquoise module, exhibiting specific high expression in the carpel of female flowers, while its expression was nearly undetectable in the ovary of male and functional male flowers. Therefore, it is hypothesized that *LcANT1* and *LcANT2* may be involved in regulating the carpel development of lychee female flowers. Moreover, most of the genes in the MEturquoise module were highly expressed in female flowers, further supporting this inference.

LITCHI007109 (*LcANT1*) is an orthologous gene of Arabidopsis ANT

To explore the origin and function of *LcANT1* and *LcANT2* in lychee, we selected 21 angiosperm species characterized by hierarchical evolutionary relationships, extending from the basal angiosperm *Amborella trichopoda* to various species within the Sapindaceae family (Fig. 2A). Subsequently, we identified the orthologs of *LcANT1* and *LcANT2* utilizing OrthoFinder. As depicted in Fig. 2B, the ANT homologous gene has only one copy in basal angiosperms such as *A. trichopoda* and *Nymphaea tetragona*, and retains a single-copy form in *A. thaliana*. However, it has stably differentiated into two copies in Rosaceae species (Fig. 2A). Among 21 angiosperms, *LcANT1* (*LITCHI007109*) belongs to the same orthogroup as *AtANT*, which contains 65 genes (Fig. 2B), most of which are annotated as *ANT* homologous genes in the NCBI database, indicating that they share common ancestral genes. Further analysis revealed that both LcANT1 and AtANT contain two highly conserved DNA-binding AP2 domains (Fig. 2C; Supplementary Fig. S2), belonging to euANT. Additionally, the three-dimensional structure of the domains in all LcAP2 proteins comprises an antiparallel three-stranded β-sheet (β1, β2, and β3), accompanied by an α-helix that aligns approximately parallel to the β-sheet (Fig. 2D).

Previous studies have elucidated that the *AtANT* gene plays a crucial role in regulating carpel development in *A. thaliana*; single *ant-1*, *ant-3*, *ant-4*, and *ant-9* mutants exhibit severe defects in ovule morphogenesis, including the absence of integuments and a block in embryo sac development (Elliott *et al.*, 1996). Additionally, the number of ovules produced by these mutant plants is markedly reduced compared with that of wild-type plants (Klucher *et al.*, 1996). By integrating these results with the expression profile of lychee floral organs and WGCNA, we hypothesized that *LcANT1* and *LcANT2* may influence the development of female flower carpels in lychee by controlling the expression of prospective downstream genes. Moreover, phylogenetic analysis reveals that LcANT1 is a ortholog of AtANT, whereas LcANT2 is categorized as a paralog (Fig. 2B). To better understand the regulatory network of LcANTs in lychee carpel development, we chose LcANT1 for our next analysis due to its closer relationship to AtANT and potentially more conserved functions.

DAP-seq showed that LcANT1 targets multiple plant growth and development pathways, including carpel development

To identify the biological pathways potentially regulated by LcANT1, we screened for genes that LcANT1 may target within the lychee genome by using DAP-seq. Due to the relatively weak binding peaks, many potential binding sites for LcANT1 were overlooked during the initial peak calling using the MACS2 software. To accurately identify the binding motifs of LcANT1, we developed a Python package called ReCallpeaks (Fig. 3A, see the Materials and methods for details). We specified a distance of 10 kb between binding sites, set the peak height to be no less than 20 and no greater than 350, and defined that the peak value of the experimental group must be more than twice that of the control group. These parameters were established to avoid false positives caused by repetitive sequences and erroneous read mappings. After processing the data with the ReCallpeaks package, we identified 16 099 potential binding sites for LcANT1, among which 7497 were located in intergenic regions, 5494 in the exonic regions of genes, 1689 in intronic regions, 734 in the upstream 2000 bp region of genes (promoter), and 685 in the downstream 2000 bp region (Fig. 3B).

To determine the binding motif of LcANT1, MEME-ChIP analysis was performed on the base sequences of all binding regions (extended by 50 bp on each side of the binding site), and a highly enriched binding motif was identified (Fig. 3C). This binding motif is similar to ZmANT1 in maize (Liu *et al.*, 2020) and also resembles the AtANT-binding motif in Arabidopsis found in JASPAR (https://jaspar.elixir.no/). Then, we utilized FIMO to scan all binding regions within the DAP-seq data, revealing that 998 genes, along with their 2000 bp upstream and downstream regions, contain target sites for this binding motif. Moreover, GO enrichment analysis was conducted on 998 genes, and it was found that these genes were enriched in reproductive system development, regulation of flowering, flower development, flower organ development, and carpel development pathways (Fig. 3D), indicating that LcANT1 may regulate the expression of these genes and affect the ovary development of lychee female flowers. In addition, by classifying these GO entries, it was found that these genes are also involved in pathways such as cell wall, lipid metabolism, secondary metabolism, development, transport, and response to hormone (Fig. 3E). Most of the entries related to development are enriched in pathways involved in flower development, also including fruit, seed, shoot, leaf, organ, and root development (Fig. 3E), suggesting that LcANT1 can also participate in regulating these tissue and development processes, which is consistent with previously reported ANT-regulated pathways.

LcANT1 may regulate carpel development in lychee flowers by influencing the expression of *LcREV*

To further identify downstream target genes of LcANT1 in the ovary of female lychee flowers, we integrated the DAP-seq-identified binding genes within Module 3 based on previous WGCNA (Fig. 1C), which was specific to the carpel of female flowers, resulting in the identification of 400 overlapping genes (Fig. 4A). Next, to investigate the consequential role of these 400 target genes, we performed a GO enrichment analysis (Fig. 4B). Our results demonstrated that these shared target genes play significant roles across a broad spectrum of lychee flowers and reproductive development.

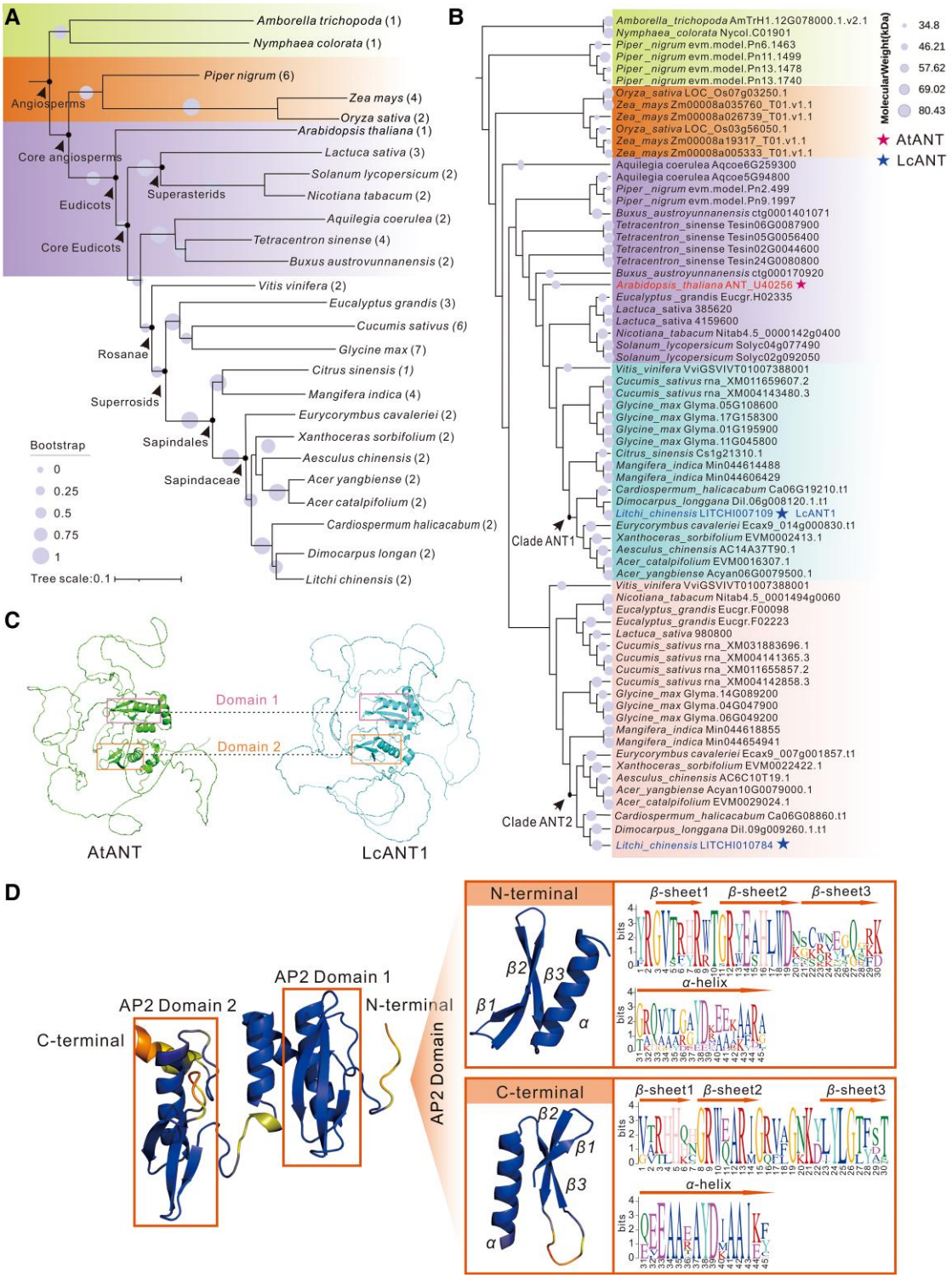

Fig. 2. Phylogenetic analysis of ANT homologs across the 26 angiosperms species. (A) Phylogenetic analysis of angiosperm species was conducted using single-copy genes extracted from the entire genome, with the numbers of *ANT* genes indicated in parentheses. (B) A phylogenetic tree was constructed based on the protein sequences of all *ANT* genes obtained from 26 representative species. The red star represents *ANT* genes from *Arabidopsis thaliana*, and blue stars indicate two ANT genes from lychee. (C) The prediction of protein folding for AtANT and LcANT1. The AP2 domains were visualized using different color schemes for alignment. (D) Three-dimensional structural models of AP2 domains complexed with the DNA double helix were generated using AlphaFold2, alongside the sequence logo of AP2 domains produced by MEME. The heights of the symbols within the stack signify the relative frequency of each amino acid at that particular position.

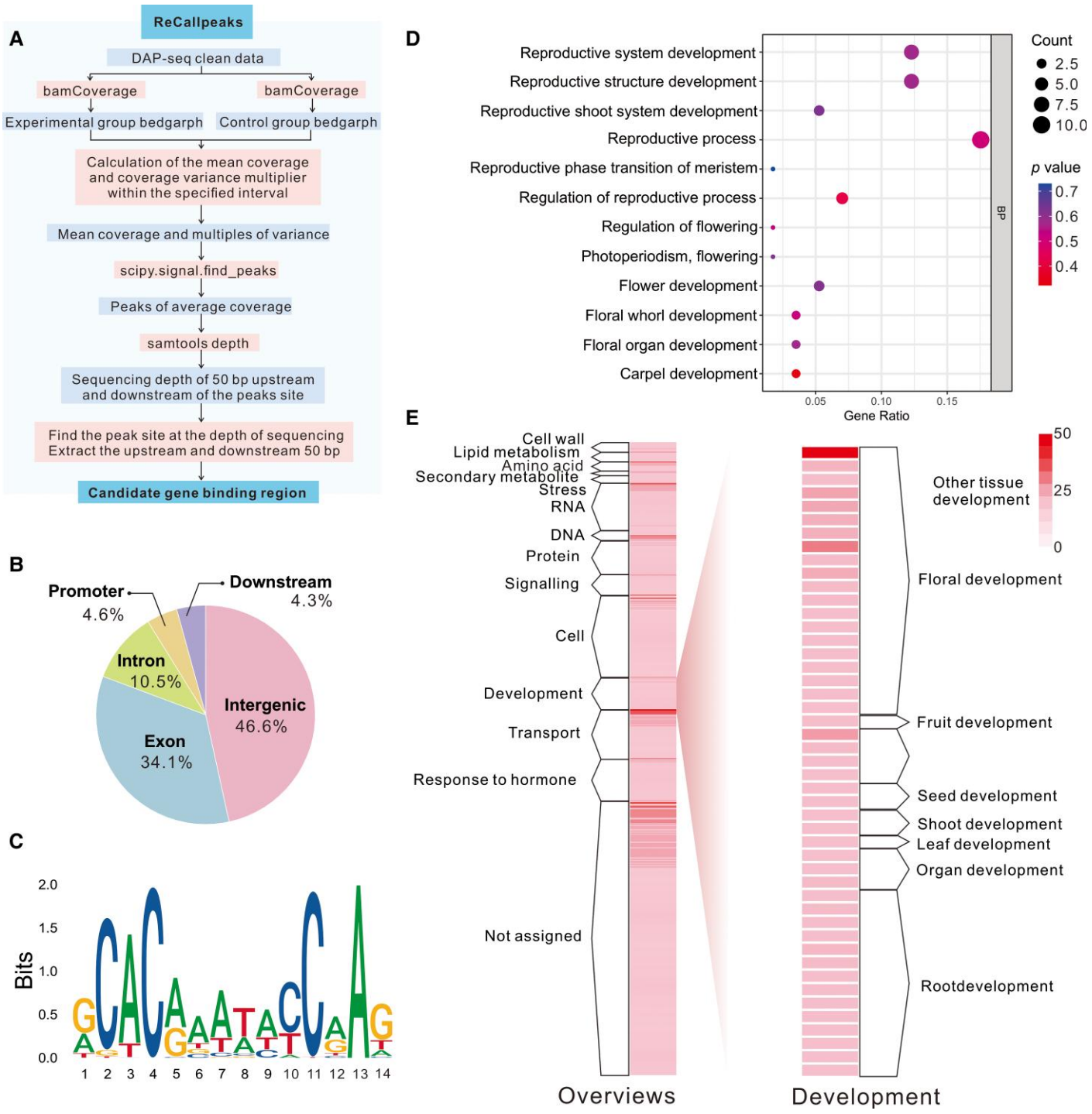

Fig. 3. DAP-seq showed that LcANT1 targets multiple plant growth and development pathways, including carpel development. (A) The flowchart of Recall peaks. (B) Analysis of LcANT1-enriched regions in the DAP-seq assay. The pie chart shows the percentage distribution of LcANT1-binding peaks in each category. (C) LcANT1-binding motif identified by DAP-seq analysis. (D) Gene Ontology (GO) enrichment analysis of LcANT1 downstream genes identified by DAP-seq analysis. (E) GO category enrichment analysis of putative LcANT1 downstream genes in each module, including the category general overview and tissue development-related pathways. The color scale shows the enrichment score.

By integrating the results of co-expression and GO enrichment analysis, we preliminarily screened 15 genes potentially related to the development of female flower carpels in lychee from the 400 overlapping target genes (Fig. 4C). These genes shared the same co-expression module (MEturquoise) with *LcANT1* and exhibited equivalent or reverse expression

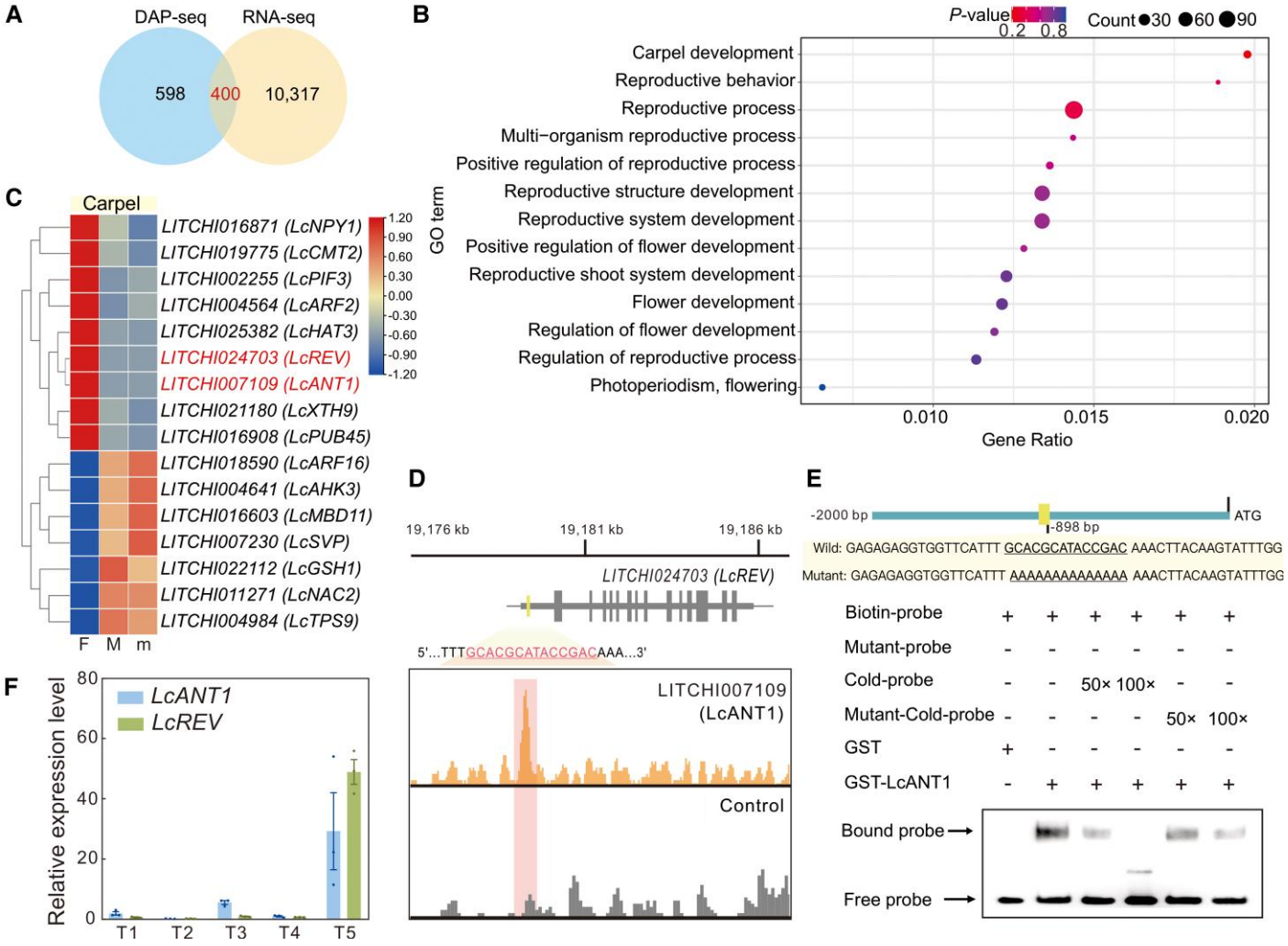

Fig. 4. LcANT1 regulates the transcription of *LcREV*. (A) Venn diagram assessing the count of key genes identified by DAP-seq and RNA-seq analysis. 400 represents the count of shared key genes. (B) Gene Ontology (GO) enrichment analysis on the overlapping genes between the downstream target genes identified by DAP-seq and the genes in pathways related to carpel development obtained from WGCNA. (C) Genes with high expression levels in carpel development among the genes potentially targeted by LcANT1. (D) The binding peaks of LcANT1 in the promoter of *LcREV*. (E) Electrophoretic mobility shift assay (EMSA) showing the binding ability of LcANT1 with the promoter of *LcREV in vitro*. The shifted bands indicated by arrows suggest the formation of DNA–protein complexes. '+' and '−' represent the presence and absence, respectively. GST protein and a biotin probe were used as the negative control. (F) Expression pattern analysis of *LcANT1* and *LcREV* genes using RT–qPCR across different lychee flower tissues. *P<0.05; Student's t-test. T1, 0.5–1.0 mm flower buds; T2, 1.0–1.5 mm flower buds; T3, 1.5–2.0 mm flower buds; T4, slight-bloom flowers; T5, full-bloom flowers.

patterns in the carpels of different flower varieties in lychee, suggesting that they may be regulated by LcANT1 during the development of carpels. Based on their expression patterns, the 15 genes were categorized into two groups: nine genes exhibited an expression pattern that was consistent with *LcANT1*, while the remaining six genes showed the opposite pattern. All of these genes have been reported to be involved in flower development. For instance, *AHK3*, a type of cytokinin receptor, was found to regulate plant organ size, flowering time, and plant longevity in Arabidopsis gain-of-function mutants (Bartrina *et al.*, 2017). Similarly, plants lacking *TPS9* consistently demonstrated delayed flowering times compared with the wild type (Tian *et al.*, 2021). Among these 15 genes, we identified *LcREV* as a homolog of the *AtREV* gene, which belongs to the Class III HD-ZIP family. In Arabidopsis, *rev* mutants produce flowers lacking full meristematic activity (Otsuga *et al.*, 2001; Prigge *et al.*, 2005). In addition, *ant rev* double mutants showed partial loss of the carpel marginal meristem (Nole-Wilson *et al.*, 2010), highlighting the critical role of REV in carpel development. Similarly, LcREV is likely to play an important role in lychee carpel development. LcANT1 may regulate lychee carpel development by controlling the expression of *LcREV*. The DAP-seq results identified a binding peak in the *LcREV* promoter region at −898 bp, characterized by the enriched motif sequence (GCACGCATACCGA). Next, EMSA validated that

LcANT1 could bind to this region (Fig. 4D, E). Moreover, the results of RT–qPCR showed that the expression trends of *LcANT1* and *LcREV* were similar (Fig. 4F). In summary, the above experiments indicated that LcANT1 probably influences lychee carpel development by regulating the expression of *LcREV*.

The evolutionary conservation of ANT–REV in land plants

To investigate possible evolutionary conservation of the ANT–REV pathway in plants, we selected 29 representative species spanning key evolutionary nodes (including two mosses, liverwort, two gymnosperms, and 24 angiosperms, 10 of which belong to the Sapindaceae family) for orthologous gene analysis of *ANT* and *REV*. This result indicated that *ANT* homologs are present in the green alga *Micromonas commoda*, whereas *REV* homologs first appear in the moss *Ceratodon purpureus*. The above findings suggested that the *ANT* gene originated significantly earlier than the *REV* gene, and the REV pathway probably formed a stable genetic module starting from bryophytes (Fig. 5A, B).

In order to further explore the molecular basis of the interaction between ANT and REV, this study further analyzed the conservation of the regulatory elements within the *REV* promoter region in 24 species of angiosperms. Using the high-affinity binding motif (GCACGCATACCGAC) as a reference, the fuzznuc tool (allowing for four-base mismatches) was employed to search for potential binding sites in the promoter regions (2000 bp upstream of ATG) of the *REV* homologous genes in each of the 24 species. The results showed that the promoter regions of all species contained at least one potential binding site. After conducting multiple sequence alignment and motif analysis of the potential binding sites, it was found that starting from the genus *Ceratodon*, the interaction motif between ANT and REV exhibited a high degree of conservation of the sequence (GCACGCATACCGAC). Especially within the Sapindaceae (excluding the 1 bp difference in *Aesculus* and *Nephelium*), the binding motif showed nearly complete sequence conservation (Fig. 5C; Supplementary Table S5). This result aligns with the known characteristic that the AP2 domains of the AtANT protein cooperatively bind to DNA fragments containing such conserved motifs, which serve as potential ANT-binding sites. These findings collectively indicate profound evolutionary conservation of the ANT–REV interaction mechanism in vascular plants.

Discussion

The ANT–REV regulatory module potentially regulates floral organogenesis in lychee

As a core member of the AP2 subfamily within the AP2/ERF transcription factor family, the *ANT* gene in Arabidopsis exhibits a dual regulatory role in floral organ morphogenesis: it maintains the activity of the marginal meristem to influence carpel development and acts as a primary regulatory switch to coordinate cell proliferation and differentiation during organ growth. This study identified two direct *ANT* homologous genes (*LcANT1* and *LcANT2*) in the lychee genome (Fig. 2). These genes encode AP2/ERF proteins with two AP2 domains, each of 60–70 amino acids (Fig. 2; Supplementary Table S4). Furthermore, phylogenetic analysis reveals that they belong to the ANT evolutionary branch (Fig. 2). To reveal the potential mechanisms by which ANT regulates the development of lychee carpels, we selected LcANT1 for DAP-seq analysis based on its closer relatedness to AtANT1, which led to the identification of 15 potential target genes (Fig. 4). Based on their expression profiles, the target genes were divided into two categories: Class I genes that are co-expressed with *LcANT1* in female flower carpels (such as *LcREV/LITCHI024703*, *LcHAT3/LITCHI025382*, and *LcARF2/LITCHI004564*) and Class II genes that exhibit a negative correlation with *LcANT1* expression (such as *LcNAC2/LITCHI011271*, *LcARF16/LITCHI018590*, and *LcSVP/LITCHI007230*). Among the Class I genes, their Arabidopsis homolog *AtREV* influences gynoecia morphology partly by regulating auxin homeostasis (Nole-Wilson *et al.*, 2010), *AtHAT3* determines gynoecium symmetry through spatiotemporal regulation of auxin distribution (Carabelli *et al.*, 2021), and *AtARF2* promotes self-pollination by limiting gynoecium growth to maintain its proper position relative to the stamens, suggesting that these genes may mediate the positive regulatory function of LcANT1 (Schruff *et al.*, 2006). In contrast, the Arabidopsis homologs of Class II genes, such as *AtNAC2*, inhibit the expression of the flowering promoter *FT* by up-regulating *FLC* (Zhang *et al.*, 2018), *AtARF16* plays a promoting role in stamen development (Reeves *et al.*, 2012), and *AtSVP*, as a temperature-sensitive flowering inhibitor, can antagonize FT function (Hartmann *et al.*, 2000), suggesting that LcANT1 may maintain gynoecium development advantage by inhibiting such genes.

It is worth noting that *LITCHI024703* (*LcREV*), the homolog of *AtREV*, is a Class III HD-ZIP gene that has been reported to play a role in the development of the carpel marginal meristem in Arabidopsis. ANT and REV function in parallel and have overlapping roles, but the interaction between *ANT* and *REV* genes is still unclear (Nole-Wilson *et al.*, 2010). This study demonstrated through EMSA experiments that LcANT1 can directly bind to the *LcREV* promoter, suggesting a potential role in the regulation of *LcREV* expression (Fig. 4). Interestingly, the DNA-binding site sequence of LcANT1 within the promoter of *LcREV* is highly conserved (Fig. 5). Furthermore, the exclusive expression of this ANT–REV module in lychee female flower carpels mirrors the female-specific expression and function of the *CRC* gene in melon carpel determinacy and male organ suppression (Zhang *et al.*, 2022). This conserved expression pattern strongly

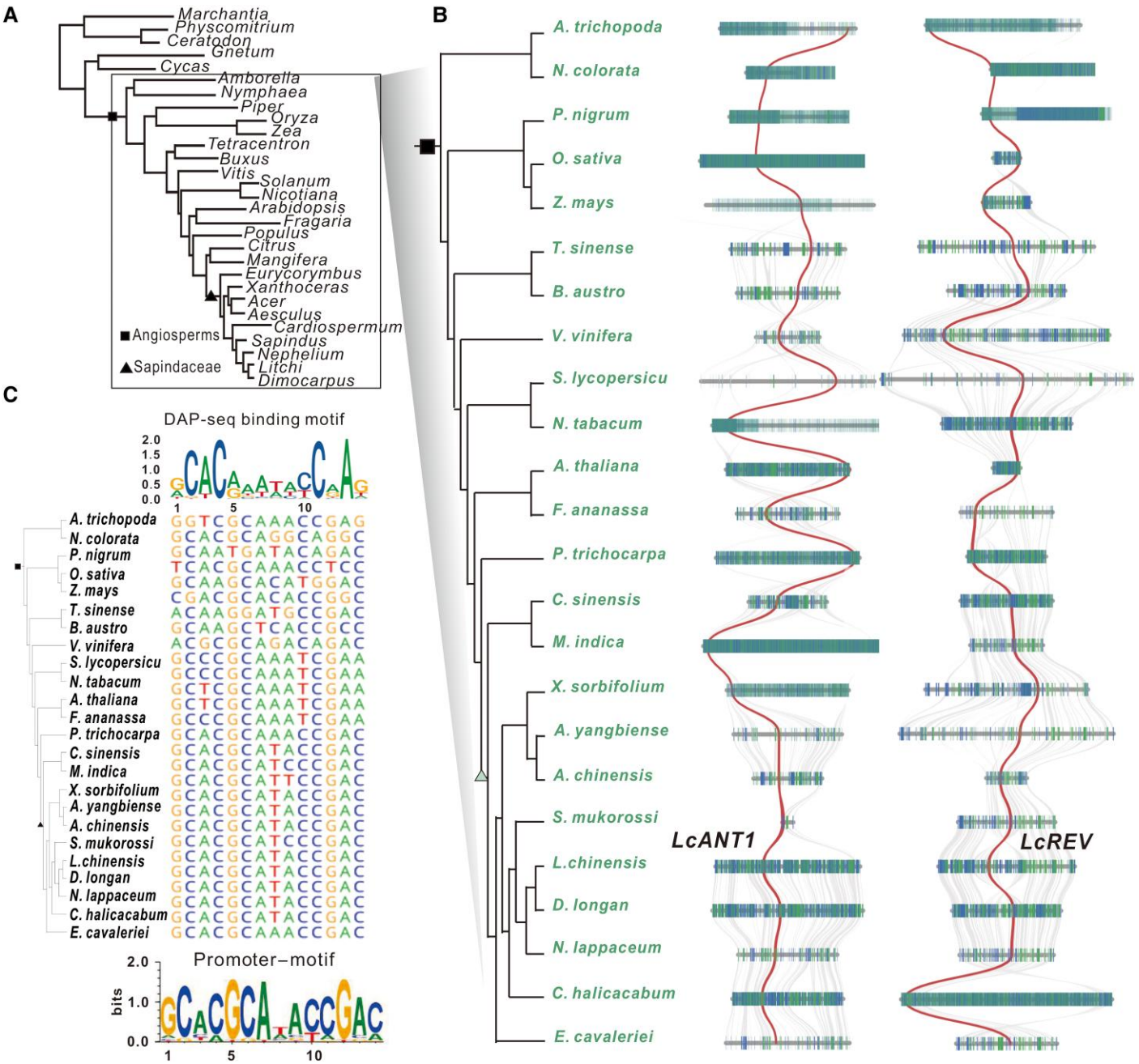

Fig. 5. The evolutionary conservation of the ANT–REV regulatory pathway in plants. (A) A phylogenetic tree of 29 species (comprising two moss, one Marchantiopsida, two gymnosperm, and 24 angiosperm species) constructed based on single-copy genes across the whole genome. (B) Collinearity analysis of *ANT* and *REV* genes in 24 angiosperm species. Syntenic blocks of *ANT* and *REV* genes are connected by red lines. (C) The conservation of the motif sequences of potential binding sites in the promoter region of *REV* in 24 angiosperm species.

indicates an analogous mechanism in lychee: promoting carpel formation/maintenance in female flowers, while its suppression in male flowers facilitates carpel degeneration and staminate development. Therefore, the ANT–REV pathway probably plays a central role in orchestrating floral organogenesis and contributing significantly to sex determination mechanisms in lychee (Fig. 6).

Evolutionary conservation of the ANT–REV regulatory module in land plants

Our phylogenetic analysis of ANT transcription factors across 29 representative species (including green algae, mosses, liverworts, and angiosperms) reveals their pivotal role in plant terrestrial evolution (Fig. 2). The presence of an *ANT* homolog

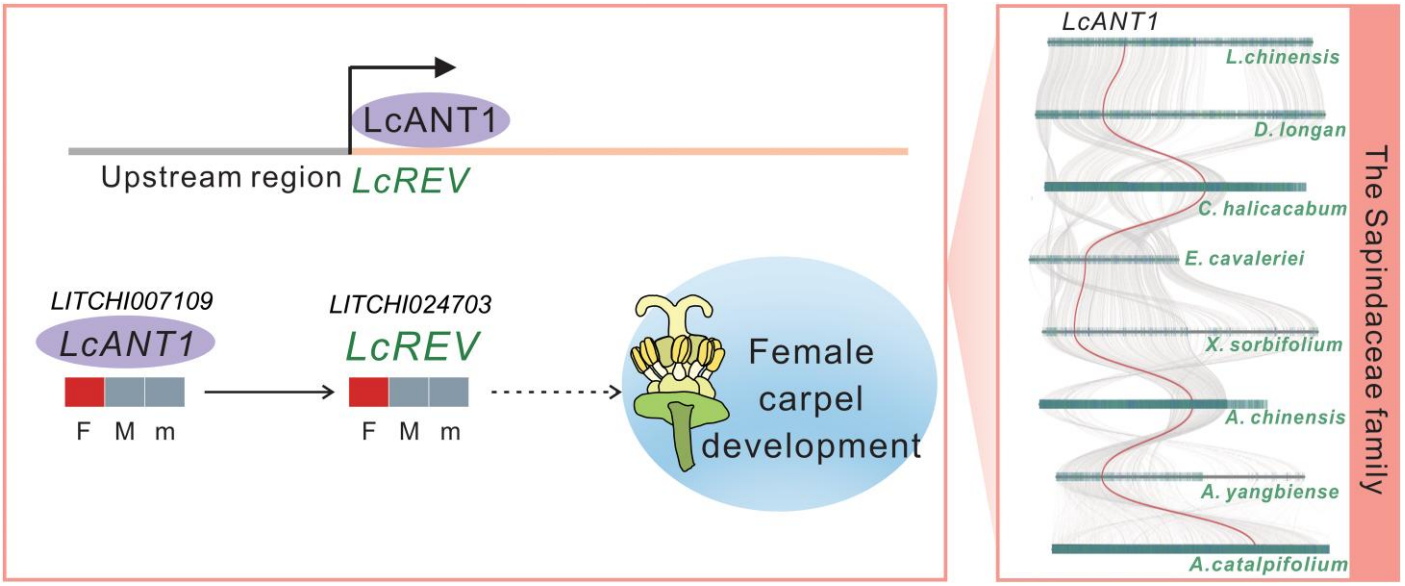

Fig. 6. A proposed model for the ANT–REV regulatory pathway within the *Sapindaceae* family. F, female flower; M, male flower; m, functional male flower.

in the green alga *M. commoda* indicates an ancient origin within the plant lineage (Supplementary Table S5). Supporting this, the moss *Physcomitrella patens* encodes four *euANT* genes, which function as molecular switches for diverse stem cell types and are auxin induced, mirroring the regulation of Arabidopsis AIL proteins (Aoyama *et al.*, 2012; Horstman *et al.*, 2014).

ANT homologs encode two AP2 domains, essential for DNA binding and function. Intriguingly, some researchers hypothesized that horizontal transfer of a bacterial HNH-AP2 endonuclease (bearing an AP2 domain) into plants may have initiated the origin of the AP2/ERF family (Magnani *et al.*, 2004). Duplication of the AP2 domain preceded the divergence of the two major AP2-like lineages: euAP2 and ANT (Kim *et al.*, 2006). The ANT clade itself subsequently diversified into basalANT and euANT subgroups. In Arabidopsis, the euANT subgroup includes eight members such as *ANT*, *AINTEGUMENTA-LIKE 1* (*AIL1*), *BABY BOOM* (*BBM*), and *PLETHORA* (*PLT*) genes, while the basalANT subgroup includes genes such as *WRINKLED1* (*WRI1*), *WRI3*, *WRI4*, and *ADAP* (Dipp-Álvarez and Cruz-Ramírez, 2019).

Our findings, integrated with existing evidence, underscore the pivotal evolutionary role of the ANT clade in facilitating plant terrestrialization. The conservation of this clade within the proposed ancestral toolkit for land plant life (Kim *et al.*, 2006; Shigyo *et al.*, 2006; Floyd and Bowman, 2007) and the lineage-specific absence of euANT and basalANT homologs in algae strongly support the hypothesis that the tandem duplication of pre-ANT and its subsequent divergence into distinct basalANT and euANT subgroups within the embryophyte ancestor constituted a critical molecular adaptation (Dipp-Álvarez and Cruz-Ramírez, 2019). We propose that this divergence was instrumental in enabling plants to colonize terrestrial habitats. This is mechanistically plausible given the crucial functions of the ANT clade: their involvement in desiccation tolerance directly addressed a paramount challenge of the aerial environment, while their roles in establishing, maintaining, and elaborating complex multicellular structures provided the necessary anatomical innovations absent or rudimentary in aquatic ancestors. The observed trend of functional specialization between the basalANT and euANT subgroups in Arabidopsis exemplifies this evolutionary role: members of the basalANT subgroup primarily regulate fundamental stress adaptation and metabolic processes such as drought response and fatty acid metabolism (Lee *et al.*, 2009; To *et al.*, 2012; Park *et al.*, 2016), while euANT members are chiefly responsible for governing intricate developmental programs, such as meristem maintenance and organogenesis (Galinha *et al.*, 2007; Yamaguchi *et al.*, 2016; Dipp-Álvarez and Cruz-Ramírez, 2019). It is important to note that some functional overlap exists; for instance, the euANT protein AtANT itself contributes to abscisic acid signaling and drought tolerance in seeds (Meng *et al.*, 2015). Nonetheless, the model for the overall pattern of subfunctionalization suggests that the duplication and subsequent specialization of pre-ANT allowed for the partitioning and refinement of essential ancestral function, thereby enhancing both stress resilience and developmental complexity.

Phylogenetic analysis of *REV* homologous genes indicates their first appearance in the moss *Ceratodon purpureus*, which was later than that of *ANT*, suggesting that they may have co-evolved as regulatory partners in bryophytes (Supplementary Table S5). We identified at least one potential *ANT–REV* binding site in the promoter regions of all 24 representative angiosperm species. Conservation of the ANT-binding motif

within the promoters of *REV* first emerged in the genus *Ceratodon* (Fig. 5C), reflecting refined regulatory requirements for meristem three-dimensional architecture during early land plant evolution. Notably, the DNA-binding motifs and target genes of cepurANT and AtANT exhibit high similarity, indicating that the core regulatory module of the ANT–REV pathway had begun to form during the moss stage (Supplementary Table S5). Moreover, it has been reported that both *ANT* and *REV* could be expressed in the integuments and participates in the positive regulation of integument development (Elliott *et al.*, 1996; Kelley *et al.*, 2009), supporting the likelihood of their co-regulation. Based on these findings, we propose that the conserved ANT–REV regulatory module originated in bryophytes and was later co-opted during angiosperm evolution. This module has potentially played a fundamental role in the development of complex reproductive structures such as flowers and fruits.

The evolutionary trajectory of the ANT–REV pathway reveals a hierarchical innovative mechanism for organ development in terrestrial plants: the ancient origin of ANT (possibly tracing back to green algae) provides a molecular basis for early land adaptation, while the later emergence of REV and the gradual conservation of its binding sites signify the refinement of meristem regulatory modules. The high conservation of pathway components in the Sapindaceae family aligns with the biological characteristics of their complex organ development, suggesting a general principle of this pathway in the regulation of key agronomic traits (Fig. 6).

Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. Phylogenetic analysis and conserved domain examination of the AP2 proteins in lychee.

Fig. S2. Conserved domain compositions of LcANT and AtANT proteins.

Table S1. Primers for DAP-seq.

Table S2. Primers for RT–qPCR.

Table S3. Primers and probes for EMSA.

Table S4. Physicochemical properties of and functional information on the lychee AP2 family proteins.

Table S5. The homologs of *ANT* and *REV* genes in 29 species.

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Author contributions

ZZ: designed and coordinated the research; HH, YX, and JZ: cloned crucial genes and carried out a validation experiment; XF, QW, CC, and FW: conducted the bioinformatics analysis; HH, YH, JX, XF, QW, and YL: drafted and wrote the paper; ZZ, YH, and RX: assisted in revising the article. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Data availability

The datasets presented in this study are publicly available. RNA-seq data are available via the NCBI with accession numbers GSE98698 and GSE182447.

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