

Integration of mRNA-miRNA revealed the possible role of PyCYCD3 may increasing branches through bud-notching in pear (*Pyrus bretschneideri* Rehd)

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Abstract

Background: The initiation of branching from the main stem of a pear tree is hindered in the absence of external stimuli, resulting in poor quality branching. Endogenous hormones and associated genes are pivotal in regulating the branching process in pear trees, while the patterns of branching in fruit trees and the underlying chemical mechanisms remain enigmatic.

Methods: The impact of notching on 'Huang guan' pear buds was investigated through the assessment of germination rate, branching rate, and hormone levels. Transcriptome sequencing was employed to examine mRNA and miRNA expression in notched and control buds, leading to the identification of a mRNA-miRNA network, which was subsequently validated using qRT-PCR. Furthermore, the *CYCD3* gene was genetically manipulated and analyzed in notched and control buds of pears.

Results: Based on KEGG analysis, a notable proportion of the differentially expressed genes (DEGs) are linked to the biosynthesis pathway of plant compounds involved in signal transduction within the organism. Within this subset of DEGs, *PyCYCD3* was identified as an up-regulated gene, potentially indicating its involvement in the regulation of branching processes. Furthermore, a comprehensive investigation identified fifteen miRNAs that target three genes associated with plant hormones. The differentially expressed genes (DEGs) were validated through quantitative real-time polymerase chain reaction (qRT-PCR). *PyCYCD3* was chosen for transformation into wild Arabidopsis plants, resulting in the generation of transgenic Arabidopsis plants. Subsequent analysis of branching patterns, plant hormone levels, and relative gene expression suggests that *PyCYCD3* may play a significant role in promoting lateral branches in pear trees through bud-notching.

Conclusions: This study suggests that the transport of brassinosteroids from lateral branches to the stem in pear trees may be crucial for the development of lateral buds. *PyCYCD3* likely plays a significant regulatory role in both bud germination and branch growth.

Background

The pear (*Pyrus bretschneideri* Rehd) is recognized as one of the most significant fruit crops globally due to its abundant nutrition, high output, and substantial financial value [1]. In order to enhance yield and optimize profitability, the utilization of well-branched fruit trees in production is crucial [2, 3]. However, the natural branching from the main stem of fruit trees such as apple and pear trees is often challenging or of subpar quality in the absence of external conditions. Consequently, nursery trees are frequently delivered with fewer branches than desired or, in some cases, with no branches at all [4–8]. The majority of the lateral branches of the fruit tree are currently in a dormant state, rendering them incapable of germination, potentially due to the dominant nature of the visible top of the fruit tree [9]. To achieve objectives such as crown suppression, fruit production, and increased yield, the technique of bud-notching plays a crucial role in the development of lateral branches on fruit trees [10].

Researchers have discovered that bud-notching disrupts the movement of auxin from the stem tip, leading to the stimulation of lateral branch germination [11]. Furthermore, the levels of other endogenous hormones within the bud, such as GA3, ABA, ZR, and BR, have been found to significantly change following notching [12]. Exogenous hormones are utilized to enhance crown formation and branch proliferation in orchard seedlings. Specifically, the application of cytokinin at a specific concentration in the spring prior to apple tree sprouting can effectively trigger the growth of dormant buds [13]. Several studies and reports have been conducted on the molecular mechanisms by which plant endogenous hormones impact the genes related to fruit tree branches. The auxin signaling pathway plays a significant role in influencing cell growth, with *Aux/IAA* and the transcription factor *ARF* responding to auxin to regulate stem development through the control of cell division [14]. The *ARF* factors, which transcribe DNA segments into RNA, are crucial components of plant hormone signaling that govern the expression of downstream target proteins involved in various growth and developmental processes, including tip control and shoot elongation [15–17]. Research has demonstrated that treatment with indole-3-acetic acid (IAA) can increase the levels of *ARF8*, *ARF9*, *AUX/IAA1*, and *GH3.12* gene expressions. Conversely, N-1-naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, has been shown to decrease the levels of *PIN1* and *GH3.12* gene expressions while increasing the expression of *AUX/IAA1* [18]. Previous studies compared the axillary bud outgrowth patterns of *Malus spectabilis* in a more-branching mutant (MB) and its wild-type (WT) using external chemical treatments. The up-regulation of the plant hormone response gene *MsGH3.11* was observed at stage "1" in MB compared to the transcript levels of WT at stage '0' [19]. Additionally, at stage "1" in MB, the up-regulation of cell proliferation genes such as *MsCYCD3* and *MsKAC2* was observed, while *MsPCNA2* was down-regulated compared to stage "0". The increased levels of *MsAHP2* and *MsARR3/5* detected in the present study during the bud development phases MB1 and MB2 could potentially be attributed to the essential role of type-A *ARRs* in cytokinin-mediated bud elongation [19, 20]. *ARF5* can inhibit the expression of chemical assembly instructions in living things by binding to *ARR15*, allowing for CTK signaling. Plant growth is typically regulated by the BR signal transduction pathway mediated by receptor kinases. The BR signal transduction pathway comprises numerous components, such as *BRI1*, *BAK1*, *BKI1*, *BSK1*, *CDG1*, *BSU1*, *BIN2*, and *BZR1/2*. Following treatment with BR, the expression levels of cell-growth related proteins *MdCYCD1;1*, *MdCYCD3;2*, and *MdCYCB3;1* were significantly elevated in BR-treated apple trees compared to control trees [21–24]. The suppression of abscisic acid (ABA) and subsequent activation of *DREB/CBFs* and *GA2OX* family members, in addition to the upregulation of *GIDs*, GA biosynthesis genes, and induction of genes positively regulated by GA, indicate that GA-mediated signaling likely contributes to the release of paradormancy and/or induction of growth [25]. *Arabidopsis CYCD3* was shown to be essential for cytokinin-mediated functions, in particular the regeneration of shoots from callus [26].

The utilization of whole-genome sequencing technology facilitated the analysis of mRNA and miRNA expression in both notched and control buds of pear trees, providing valuable insights into the regulatory mechanisms underlying pear tree branching through bud-notching. An extensive number of differentially expressed genes (DEGs) have been identified in plant hormone-related pathways through RNA sequencing analysis. This study also highlights several miRNAs and potential genes with altered

expression levels. Through integrative mRNA-miRNA interaction analysis, the molecular mechanism underlying the promotion of branching by bud-notching in pears through hormone signaling is elucidated. The *pCAMBIA-PyCYCD3-GFP* construct was introduced into wild *Arabidopsis* plants, resulting in the generation of transgenic *Arabidopsis* plants. Through analysis of branching patterns, plant hormone levels, and relative expression of *PyCYCD3* in the transgenic *Arabidopsis* plants, it was hypothesized that *PyCYCD3* may be involved in the promotion of lateral branches in pear trees through bud-notching. The findings of the study suggest that the transport of brassinosteroids from lateral branches to the stem may be crucial for the development of lateral buds in pear trees, with *PyCYCD3* potentially serving as a key regulator in bud germination and branch growth.

Materials and methods

Plant materials and sample collection

Experimental treatment materials were obtained from 2-year-old grafted sapling pear variety 'Huang guan', and the plant were planted at the National Modern Agricultural Pear Industrial Technology Experimental Station of Tiaoshan Group (Jingtai, Gansu Province, China).

All plants were cultivated in the same orchard under uniform management practices and with identical genetic backgrounds. Seedlings of similar growth and height were carefully chosen prior to budding in late March. Notching was performed by making epidermal incisions 30 cm below the treetop, positioned 1 cm above each bud. Control trees remained unaltered. The incision extended on both sides of the bud and encompassed approximately one-third of the stem's circumference. Tissue samples were obtained from the site of branch emergence 15 days post-notching. The first bud below 30 cm from the treetop in the control tree was designated as the initial sample (S0), showing impaired branching. The second bud nearest to the treetop in the control tree was designated as the subsequent sample (S1), exhibiting normal branching. The third sample (S2) was collected from the first notching bud located below 30 cm from the treetop in the notching tree, which exhibited branching potential. A minimum of ten individuals were sampled for each position, with samples pooled and repeated three times. The sample collection process is illustrated in Fig. 1. Samples were collected from a minimum of ten individuals for each position and pooled together, with this process repeated three times.

Measurement of emergence rate, branching rate and hormone levels of lateral buds

Sprouting and branching rate were recorded for S0, S1, and S2 pear trees. High-performance liquid chromatography was used to quantify the contents of indole acetic acid (IAA), zeatin riboside (ZR), abscisic acid (ABA), and gibberellic acid (GA3), 0.2 g of freshly collected lateral buds at 15 d accordance with previously described methods [27], quantify the contents of brassinosteroid (BR) need 1 g of freshly collected lateral buds at 15 d. Samples consisted of three biological replicates each comprising three technical replicates.

RNA preparation and transcriptome sequencing

Total RNA was isolated utilizing the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's guidelines. The NanoDrop 2000 Spectrophotometer was used to measure RNA concentration and purity (Thermo Fisher Scientific, Wilmington, DE). The RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 System was implemented to assess RNA integrity (Agilent Technologies, CA, USA). Following a manufacturer's recommendations, sequencing libraries were generated using NEBNextR Ultra™ Directional RNA Library Prep Kit for IlluminaR (NEB, USA) and the small RNA Sample Library Prep Kit for IlluminaR (NEB, USA), and index codes were added to attribute sequences to each sample. Biomarker Technologies Corporation addressed library preparation and sequencing (Beijing, China). The Agilent Bioanalyzer 2100 and qPCR were employed to analyze library quality. The RNA-seq data was submitted to the NCBI SAR database under the accession number PRJNA933274 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA933274>).

Differential expressed genes (DEGs) analysis

Differential expression analysis was performed using the DESeq (2010) R package. The resulting FDR (false discovery rate) were adjusted using the PPDE (posterior probability of being DE). The $FDR < 0.05$ and $|\log_2(\text{FoldChange})| \geq 1$ was set as the threshold for significantly differential expression. Gene function was annotated based on KEGG database (Kyoto Encyclopedia of Genes and Genomes). KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathway [28].

Quantitative real-time PCR (qRT-PCR) validation

A total of twelve DEGs with significant expression patterns of KEGG pathway were selected for qRT-PCR verification. Purification and reverse transcription of RNA were used PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA). Primers were designed by online software Primer3 input (version 4.0, <http://primer3.ut.ee/>), and the sequences were exhibited in Table S1. The qRT-PCR was conducted on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) by using SYBR®Premix ExTaq™ II. The data were analyzed by $2^{-\Delta\Delta Ct}$.

Overexpression PyCYCD3 in Arabidopsis and subcellular localization analysis

Genomic sequences of the CDS of the *PyCYCD3* gene was amplified from pears genomic DNA by the use of primers (*F*:AGAACACGGGGGACGAGCTCATGGGTGGTTGGGCAATTGCAG; *R*:ACCATGGTGTGCGACTCTAGACTCCCAAATCCCAACCTCCATG), after which the sequences were cloned into *pCAMBIA2300-GFP* vectors, double digestion using *SacI* and *XbaI*, resulting in *pCAMBIA2300-PyCYCD3-GFP* constructs. The inverted trifoliate and inverted quadrifoliate leaves of Ben's tobacco were selected for transient transformation of *PyCYCD3* to determine the subcellular localization of the gene. Subsequently, the *PyCYCD3* gene was transfected into *Arabidopsis* Columbia using the floc dipping method, and the positive plants were screened on MS plates containing 50 mg/L Kan and 20 mg/L tauramycin, after which the transgenes obtained from the screening were identified by PCR assay, and the T3 generation of pure transgenic *Arabidopsis* plants were obtained after two screenings. Finally, the

number of transgenic *Arabidopsis* plants branching, the relative expression of *PyCYCD3* gene (*PyCYCD3-F*: CGCCGTTTCGGGATTAATA; *PyCYCD3-R*: GAGGTAGGAGTGAGGTGATTG. *Actin-F*: GCCGACAGAATGAGCAAAGAG; *Actin-R*: AGGTACTGAGGGAGGCCAAGA) and its endogenous hormone content were determined.

Data analysis

A total of thirty plants of both transgenic and wild-type *Arabidopsis thaliana* were selected for observation of lateral shoot growth following a period of shoot extraction. The data obtained was subjected to statistical analysis using the Independent Samples T-test feature in SPSS 22.0 software for comparison purposes. Two-tailed t-tests were employed to identify significant differences in the sample results. Additionally, Microsoft Excel 2019 was utilized for the statistical analysis of the raw data and graphing was performed using Origin 9.0.

Results

Analyzing germination rate, branching rate and endogenous hormone levels in lateral buds

The germination rate of pear tree seedlings following bud-notching treatment was subjected to statistical analysis. The sprouts of pear tree seedlings treated with bud-notching predominantly emerged within a 60 cm radius from the top bud. The germination rate of pear tree seedlings treated with bud-notching reached 90.01%, while the control group had a germination rate of 89.53%. There was no significant difference in germination rates between the two groups (Fig. 2a, Table S2-1). The application of bud-notching treatment significantly enhanced the sprout rate of pear seedlings, as evidenced by the results presented in Fig. 2b and Table S2-2. Specifically, the average growth promotion rate of pear trees in the bud-notching group (91.21%) was significantly higher than that of the control group (33.63%) ($P < 0.01$). Additionally, there was no significant difference in the average promotion rate of medium branches between the control group (4.16%) and the bud-notching group (4.18%) ($P < 0.05$). The mean rate of short shoot promotion in the control group was 77.60%, compared to 20.01% in the bud-notching group. The short shoot promotion rate in the control group was found to be significantly higher than that in the treatment group ($P < 0.01$) (Fig. 2b). The comparison between the bud-notching treatment and control groups indicated that bud-notching significantly enhanced the growth of pear seedlings on the initiating side.

The peak area values of five hormones were quantified using liquid chromatography, and the concentrations of the hormones were subsequently determined (Fig. 2c, Table S3). The findings indicated that the levels of ZT, GA3, IAA, and BR in S1 and S2 were significantly elevated compared to those in S0, while the concentration of ABA did not exhibit a significant difference among S0, S1, and S2. The ratio of BR/ABA in S1 was found to be statistically less significant than that in S0 ($P < 0.05$), while the BR/ABA ratio in S2 was significantly higher than that in both S1 and S0 ($P < 0.05$) (Table S3). It is

hypothesized that the upregulation of growth-promoting hormones such as ZT, GA3, IAA, and BR, along with the maintenance of ABA at a stable level, may positively regulate the initiation of lateral branches following bud-notching.

Analysis of sequencing data and DEGs

To better understand the molecular mechanism of shoot branching by bud-notching in 'Huang guan' pears, we conducted a comparative transcriptomic analysis on new young shoots branching between S0, S1 and S2 groups. After quality control, 65.15 Gb Clean Data were obtained, with average quality Q30 scores of > 93.96% for each library, respectively, and an average GC content of 44.64%. For the three sample libraries, 68,728,487, 73,564,496, and 751,55,150 reads were mapped to the pear genome, representing 64.24%-66.49% of the clean reads from the three samples.

A total of 2154 DEGs were detected between S0 and S1, of which 1131 were up regulated and 1023 were down regulated. We also detect 3036 DEGs between S0 and S2, 1339 were up regulated, and 1697 were down regulated. Of these, 492 genes were found to be differentially expressed at both two confrontations (C1: S0_vs_S1, C2: S0_vs_S2), whole expression profile of 492 DEGs was presented (Fig. 3a), 238 were up regulated, 254 were down regulated (Fig. 3b). Clean reads with lengths from 18 to 30 bp were obtained from S0, S1, S2. The percentage of bases Q30 reached at least 98.52% of all the samples. The analysis identified 505 miRNAs, including 141 known and 364 new miRNAs (Table S3). Examination of miRNA expression in 3 samples identified 149 and 39 differentially expressed miRNAs (DEGs) at two confrontations C1 and C2, respectively. Out of the total, 130 genes were found to be up-regulated and 19 genes were down-regulated in C1. Additionally, 27 genes were up-regulated and 12 genes were down-regulated in C2. At all confrontations C1 and C2, 9 differentially expressed genes (DEGs) exhibited upregulation, while 2 DEGs displayed downregulation (Fig. 3d). In the same way, the expression profile of often differentially regulated miRNAs was presented (Fig. 3c).

Functional classification of mRNA and miRNA was conducted using the KEGG database (Fig. 3e and 3f). In this study, 22 important KEGG pathways were selected to analysis. In mRNA KEGG pathway analysis we found there were 25 and 32 DEGs in C1 and C2 from 'Cellular Processes', there were 45 and 35 DEGs in C1 and C2 from 'Environmental Information Processing', there were 53 and 86 DEGs in C1 and C2 from 'Genetic Information Processing', there were 110 and 155 DEGs in C1 and C2 from 'Metabolism' and there were 21 and 24 DEGs in C1 and C2 from 'Organismal Systems' (Fig. 3e, Table S4). In miRNA KEGG pathway analysis we found there were 7 and 10 DEGs in C1 and C2 from 'Cellular Processes', there were 13 and 11 DEGs in C1 and C2 from 'Environmental Information Processing', there were 66 and 55 DEGs in C1 and C2 from 'Genetic Information Processing', there were 60 and 35 DEGs in C1 and C2 from 'Metabolism' and there were 13 and 8 DEGs in C1 and C2 from 'Organismal Systems' (Fig. 3f, Table S4). 'Plant hormone signal transduction', 'Protein processing in endoplasmic reticulum' and 'Phenylpropanoid biosynthesis' were top three pathways with largest number of associated DEGs in C1 and C2 (Fig. 3e). A

significant number of DEGs in C1 and C2 were related to 'plant hormone signal transduction', with 43 and 31 genes in mRNA KEGG pathway and 9 and 6 genes in miRNA KEGG pathway, respectively.

Plant hormone signaling pathway analysis of mRNA DEGs

Based on the KEGG pathway analysis we found 'plant hormone signaling' pathway was essential for this research, therefore, mRNA DEGs about 'plant hormone signaling' pathway was used to deeply analysis. Within auxin signaling transduction pathway genes, *AUX1*, *AUX/IAA*, *SUAR*, *ARF*, *GH3*, *PP2C*, *ABF*, *BRI1*, and *CYCD3*, were found to be enriched (Fig. 4a, Table S5). And most of the predicted auxin-induced protein genes (gene38584, 38585, 38587, 17817, 27027, newgene 29561) belong to *SUAR* family were identified to be down-regulated in both S1 and S2. *ARF* (Pyrus_newGene_32024) was identified to be up-regulated in both S1 and S2. *GH3* (gene11049) was identified to be down-regulated in both S1 and S2, *GH3* (gene1810, gene34115, gene34117) were identified to be up-regulated in both S1 and S2. Within gibberellin signaling transduction pathway genes, *GID1* and *TF*, were found to be enriched, and predicted gibberellin genes *GID1* (gene22380) and *TF* (gene28794) were identified to be down-regulated in both S1 and S2. Within abscisic acid signaling transduction pathway genes, *PYR/PYL*, *PP2C* and *ABF*, were found to be enriched, and predicted abscisic acid genes *PYR/PYL* (gene24413) was identified to be down-regulated in S1 and up-regulated in S2. *PP2C* (gene12403, gene24075, gene26342) was identified to be up-regulated in S1 and down-regulated in S2. *ABF* (gene23064) was identified to be up-regulated in both S1 and S2. Within cytokinin signaling transduction pathway genes, *AHP* and *B-ARR*, were found to be enriched, and predicted abscisic acid genes *AHP* (gene22385) and *B-ARR* (gene9903) were identified to be up-regulated in S1 and down-regulated in S2. Within brassinosteroid signaling transduction pathway genes, *BRI1*, *BSK* and *CYCD3*, were found to be enriched, and predicted brassinosteroid genes *BRI1* (gene6361) and *CYCD3* (gene1164) were identified to be down-regulated in S1 and up-regulated in S2, *BSK* (Pyrus_newGene_23435) was identified to be up-regulated in S1 and down-regulated in S2.

Analysis of the interaction network of miRNAs and target mRNAs

The identification of key genes associated with shoot branching following bud-notching in pears was achieved through KEGG pathway and miRNA-mRNA analysis. Through an analysis of the regulatory mechanisms of miRNAs and their target genes, a significant number of miRNAs were identified as targeting seven genes associated with shoot branching in pear trees (Table S6). After analysis, only one miRNA was identified to target at least two pear shoot branching-related genes, which was cpa-miR166e (*GH3* and *ABF*). Ten miRNA was identified to target only one pear shoot branching-related gene, which was aly-miR157d-3p (*ABF*), cem-miR166g (*ABF*), atr-miR166b (*ABF*), gma-miR166m (*ABF*), bdi-miR166e-3p (*ABF*), hbr-miR166a (*ABF*), cpa-miR166e (*ABF*), aly-miR166a-3p (*ABF*), mes-miR166i (*ABF*), cis-miR166a (*ABF*). One miRNA was identified to target one pear shoot branching-related genes, which were cpa-miR166e (*GH3*), gma-miR390e (*SAUR*), gma-miR162a (*SAUR*), hbr-miR156 (*PP2C*), aly-miR390a-5p (*BRI1*) and cme-miR396e (*CYCD3*). The detail information of these fifteen miRNAs and seven mRNAs

were presented in Table S4. In addition, a regulatory network of miRNA-mRNA related to pear shoot branching-related genes was constructed (Fig. 4b, Table S7).

Validation of differential gene expression by qRT-PCR

The reliability of transcriptomic data, 12 DEGs were selected for qRT-PCR validation. 12 DEGs accession number were obtained by *Pyrus bretschneideri* (taxid:225117) alignment in NCBI (Table S7). *PySAUR21-like1* (gene38584), *PyAUX15A* (gene38585), *PySAUR21-like2* (gene38587), *PyAUX15A-like1* (gene27027), *PyAUX15A-like2* (newgene29561), *PyBRI1* (gene6361), *PyPP2C24* (gene24075), *PyPYR/PYL4* (gene24413), *PyGID1C-like* (gene22380), *PyGH3.9* (gene34117), *PyAUX/IAA6* (gene31506), *PyAUX/TLP2* (gene25464). 12 DEGs from the S0, S1, and S2 were randomly chosen, and the relative expression levels of these DEGs were examined using qRT-PCR. In general, the outcomes demonstrated that the expression patterns of RNAs discovered using qRT-PCR are comparable to those discovered using RNA-seq data, strengthening the validity of the DEGs discovered in this work (Fig. 5, Table S8).

Overexpression *PyCYCD3* increased lateral branch formation of transgenic *Arabidopsis*

To explore the role of *PyCYCD3* with respect to the induction of lateral branch formation in pear trees, the CDS of the *PyCYCD3* was amplified, and the fusion protein of *pCAMBIA2300-PyCYCD3-GFP* was stably transformed into *Arabidopsis* (Fig. 6a and Figure S1). Transgenic *Arabidopsis* were obtained by the *Agrobacterium*-mediated leaf disc method and PCR identification (Figure S1). *CYCD3-1*, *CYCD3-2*, *CYCD3-3* were selected to explore whether *PyCYCD3* promotes branching in transgenic *Arabidopsis* (Fig. 6b). Through the photographic observation and rate of branches of wild type and *PyCYCD3* gene transfer *Arabidopsis* plants growing for 1 month (Fig. 6b and Fig. 6c Table S9), it was found that the growth and number of branches of transgenic *Arabidopsis* plants was significantly faster than that of wild type *Arabidopsis* plants during the same period. Six transgenic *Arabidopsis* plant lines (*CYCD3-1*, *CYCD3-2*, *CYCD3-3*, *CYCD3-4*, *CYCD3-5*, *CYCD3-6*) were selected to observe how *PyCYCD3* would be expressed by methods of the qRT-PCR (Figure.5d). The qRT-PCR results revealed that the expression level of *PyCYCD3* was significantly upregulated compared with WT (Fig. 6d Table S10).

Subcellular localization of *pCAMBIA1300-PyCYCD3-GFP* transformed tobacco was studied. The results showed that *PyCYCD3* gene was mainly located in the cytoplasm, while unloaded *pCAMBIA1300-GFP* was expressed in both cytoplasm and membrane (Fig. 6e). This indicates that *PyCYCD3* gene plays different biological characteristics and functions in different cell physiological processes, *PyCYCD3* gene may be related to cell division, mitosis and other processes, so it is mainly located in the cytoplasm. The ZT content in three transgenic *Arabidopsis* plant lines *CYCD3-1*, *CYCD3-2* and *CYCD3-3* were 21.57 ng/g, 36.69 ng/g and 33.99 ng/g, respectively, and the GA3 content were 52.91 ng/g, 69.87 ng/g and 77.12 ng/g, respectively. The contents of IAA were 47.82 ng/g, 54.89 ng/g and 68.11 ng/g, respectively, which were significantly higher than those of ZT (10.02 ng/g), GA3 (87.68 ng/g) and IAA (17.12 ng/g) in wild

Arabidopsis ($P < 0.01$) (Fig. 6f, Table S11) and BR/ABA values (0.31, 0.26, 0.29) were significantly higher than those of wild *Arabidopsis* (0.17) ($P < 0.05$) (Fig. 6g, Table S11). These results indicated that the heterologous expression of *CYCD3* may promote the synthesis of growth-promoting hormones ZT, GA, IAA and BR, and regulate the stability of growth-inhibiting endogenous hormone ABA, thereby regulating the growth of branches of plants.

Discussion

The process of increased branching is a crucial aspect of pear tree growth and development. In the cultivation of fruit trees, bud-notching is commonly employed to stimulate the growth of branching and enhance yield. Research findings indicate that bud-notching treatment disrupts the initial hormonal balance within buds, resulting in a significant increase in growth-promoting hormones such as IAA, GA3, and ZR, while levels of the growth-inhibiting hormone ABA are reduced in apples [29]. Hence, the research focused on two-year-old 'Huang guan' pear young seeds as the subject of investigation. Bud-notching was performed at a 1cm distance from the upper part of the bud to induce new branch growth. The study compared the branching capabilities of S0, a reference object unable to branch normally, with S1 and S2, which exhibited normal branching and enhanced branching after bud-notching, respectively. This study examined the expression levels of mRNA and miRNA, and constructed regulatory pathways between miRNA and mRNA to enhance understanding of the molecular mechanisms underlying increased branching in pear trees. Although the importance of bud-notching in fruit trees to push branching has been recognized, the underlying mechanism of bud differentiation regulation square measure still for the most part not understood. Various studies have additionally shown that plant hormones play necessary roles within the method of branching formation [30]. Classical theory believes that apical dominance regulates the moving of auxin along the main stem through competing auxin sources and controls the activation of lateral buds [31, 32]. In apples, the method of auxin transport from axillary buds to stems is concerned within the growth of axillary buds, which can be thought about essential for the expansion of buds.

Most differentially expressed genes related to hormone signal transduction in this study are auxin-related, including *AUX1*, *AUX/IAA*, *ARF*, *GH3*, and *SUAR*. Transcriptome sequencing and KEGG enrichment analysis identified these genes. Genes activated by auxin were up-regulated in S2 (such as *AUX1*, *AUX/IAA*, *GH3*), and *ARF* was up-regulated in both S1 and S2 [33, 34]. Cytokinin (CTK) may regulate the growth of apple branches by activating certain factors that indirectly suppress the expression of genes linked to branching [35, 36]. The article showed that a type-B *ARR* can act as a transcription factor for a type-A *ARR*, as seen with *ARR1/ARR2* activating *ARR6* in plants [37]. *ARR1/ARR2/ARR10* induce *ARR4/5/6/7* transcription in response to cytokinin in protoplasts via *AHK4* and/or *CKI1* [38]. The *Arabidopsis* His-Asp phosphorelay network includes 11 type-B *ARR* family members, which are crucial transcriptional regulators [39]. The study found that *AHP* and *B-ARR* genes were enriched in the cytokinin signaling pathway. Gene22385 (*AHP*) and gene9903 (*B-ARR*) were up-

regulated in S1 and down-regulated in S2, indicating that phosphorylated *AHPs* move into the nucleus [40, 41]. Previous studies have shown that exogenous GA spraying benefits lateral bud initiation and branch extraction [5, 9, 35]. This study confirmed this conclusion and identified down-regulated gibberellin genes *GID1* and *TF* in both S1 and S2. Research has demonstrated that *PYR/PYL/RCAR* proteins inhibit *PP2C* phosphatase activity, which is a known inhibitor of the ABA signaling pathway. This suggests that *PYR/PYL/RCAR* and *PP2C* work together to negatively regulate the ABA signaling pathway [42, 43]. *PYR/PYL*, *PP2C*, and *ABF*, genes in the abscisic acid signaling pathway, were identified in this study. *PYR/PYL* (gene24413) was down-regulated in S1 and up-regulated in S2, while *PP2C* (gene12403, gene24075, gene26342) was up-regulated in S1 and down-regulated in S2. *ABF* (gene23064) was up-regulated in both S1 and S2. Previous research suggests that ABA indirectly promotes bud differentiation rather than playing a direct role [44, 45]. Several studies have indicated that ABA increases vacuole sugar content, leading to enhanced leaf dehydration [46, 47]. It has also been shown that root pruning and ring cutting may enhance the differentiation process of buds [48]. Decreased IAA content and increased ABA and CTK contents in xylem SAP during flower bud differentiation may lead to increased shoot branching in pear trees through the up-regulation of genes involved in ABA signaling. *BRI1*, *BSK*, and *CYCD3* genes were found to be enriched in the brassinosteroid signaling pathway. *BRI1* and *CYCD3* were down-regulated in S1 and up-regulated in S2, while *BSK* was up-regulated in S1 and down-regulated in S2. In the course of this investigation, the up-regulation of *CYCD3* (gene1164) was observed in S2, a finding consistent with the results reported by Tan et al. Additionally, the expression of cell proliferation genes, such as *CYCLIN D3* (*MsCYCD3*), was found to be up-regulated at stage "1" in MB.

MiRNAs are short noncoding RNAs, around 19–24 nucleotides long, that can interact with longer target RNAs [49]. In ‘Huang guan’ pears, 505 miRNAs were identified, including 143 known and 362 new ones. The interactions between miRNAs and their target genes can be one-to-many or many-to-one, rather than strictly one-to-one, leading to significant potential regulatory effects. One miRNA can target multiple genes related to branching, and vice versa, one gene can be targeted by multiple miRNAs, indicating a complex relationship in controlling branching in young pear plants. Such as ten miRNAs were identified to target only one pear shoot branching-related gene, which was aly-miR157d-3p (*ABF*), cem-miR166g (*ABF*), atr-miR166b (*ABF*), gma-miR166m (*ABF*), bdi-miR166e-3p (*ABF*), hbr-miR166a (*ABF*), cpa-miR166e (*ABF*), aly-miR166a-3p (*ABF*), mes-miR166i (*ABF*), cis-miR166a (*ABF*). only one miRNA was identified to target at least two pear shoot branching-related genes, which was cpa-miR166e (*GH3* and *ABF*). One miRNA was identified to target one pear shoot branching-related genes, which were cpa-miR166e (*GH3*), gma-miR390e (*SAUR*), gma-miR162a (*SAUR*), hbr-miR156 (*PP2C*), aly-miR390a-5p (*BIR1*) and cme-miR396e (*CYCD3*). *CYCD3;1* expression in *Arabidopsis* is linked to growing tissues like meristems and developing leaves, not differentiated tissues. Studies indicate that *CYCD3* plays a crucial role in transitioning from cell proliferation to differentiation during plant development [50]. Research has shown that *BIR1* can prevent cell death and inactivating it in yeast leads to various cell division defects [51]. Our study found that the upregulation of *BIR1* and *CYCD3* is crucial for shoot branching in pears. In summary, cap-miR166e was upregulated and *GH3* (gene11049) was downregulated in S2 compared to S0. gma-

miR162a, hbr-miR156, aly-miR390a, and cem-miR396e were downregulated, while SAUR (gene27024), *PP2C* (gene12403), *BIR1* (gene6361), and *CYCD3* (gene1164) were upregulated in S2 compared to S0.

Conclusions

In this study, we sequenced the transcriptome of notched and control buds in pear tree, focusing on plant hormone signal transduction pathways and analyzing mRNA-miRNA interactions. A total of 15 miRNA-mRNA pairs were investigated, revealing both positive and negative correlations. Subsequent analysis identified upregulation of genes *SAUR* (gene 27024), *PP2C* (gene 12403), *BIR1* (gene 6361), and *CYCD3* (gene 1164), while *GH3* (gene 11049) showed downregulation. These genes are suggested to have a substantial regulatory role in the shoot branching process of pear trees. Ultimately, *PyCYCD3* was chosen for transformation into wild *Arabidopsis* plants, resulting in the generation of transgenic *Arabidopsis* plants. Analysis of branching patterns, plant hormone levels, and the relative expression of *PyCYCD3* in these transgenic *Arabidopsis* plants suggests that *PyCYCD3* may potentially play a significant role in promoting lateral branches in pear trees through bud-notching techniques. The findings presented in the summary suggest that brassinosteroid transport from the lateral branches to the stem in pear trees may be crucial for the development of lateral buds, with *PyCYCD3* potentially serving as a significant regulatory factor in bud germination and branch growth. The primary research framework of this study is illustrated in Fig. 7.

Declarations

Supplementary Information

The online version contains supplementary material available at

Acknowledgements

Not applicable.

Author contributions

B H and J M designed the experiments. Z H, W F, and Z S performed the experiments. C W, Z H, and Z S performed data analysis. Z S wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Availability of data and materials

The data that support the finding of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

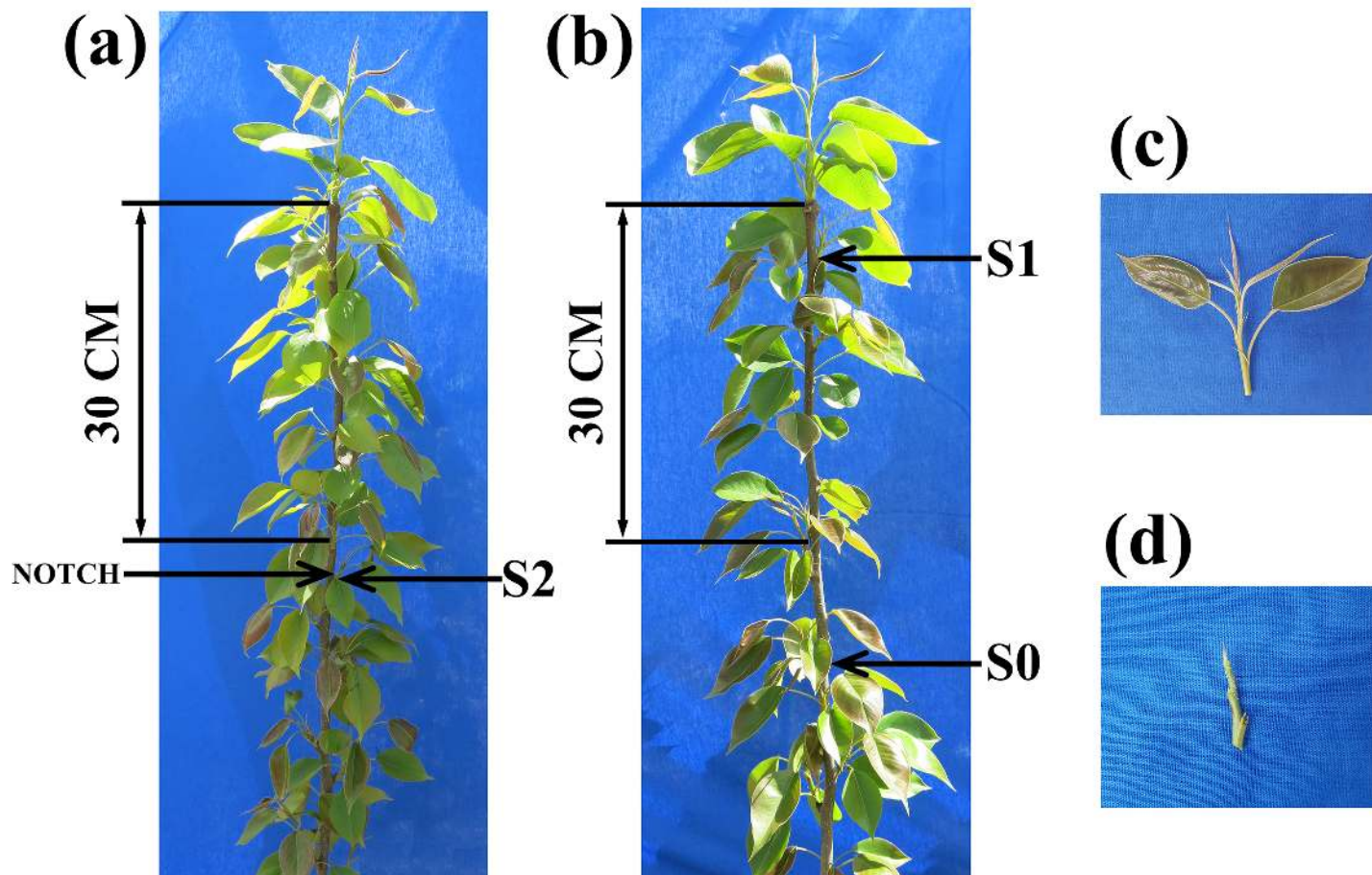


Figure 1

Schematic diagram of sample collection.

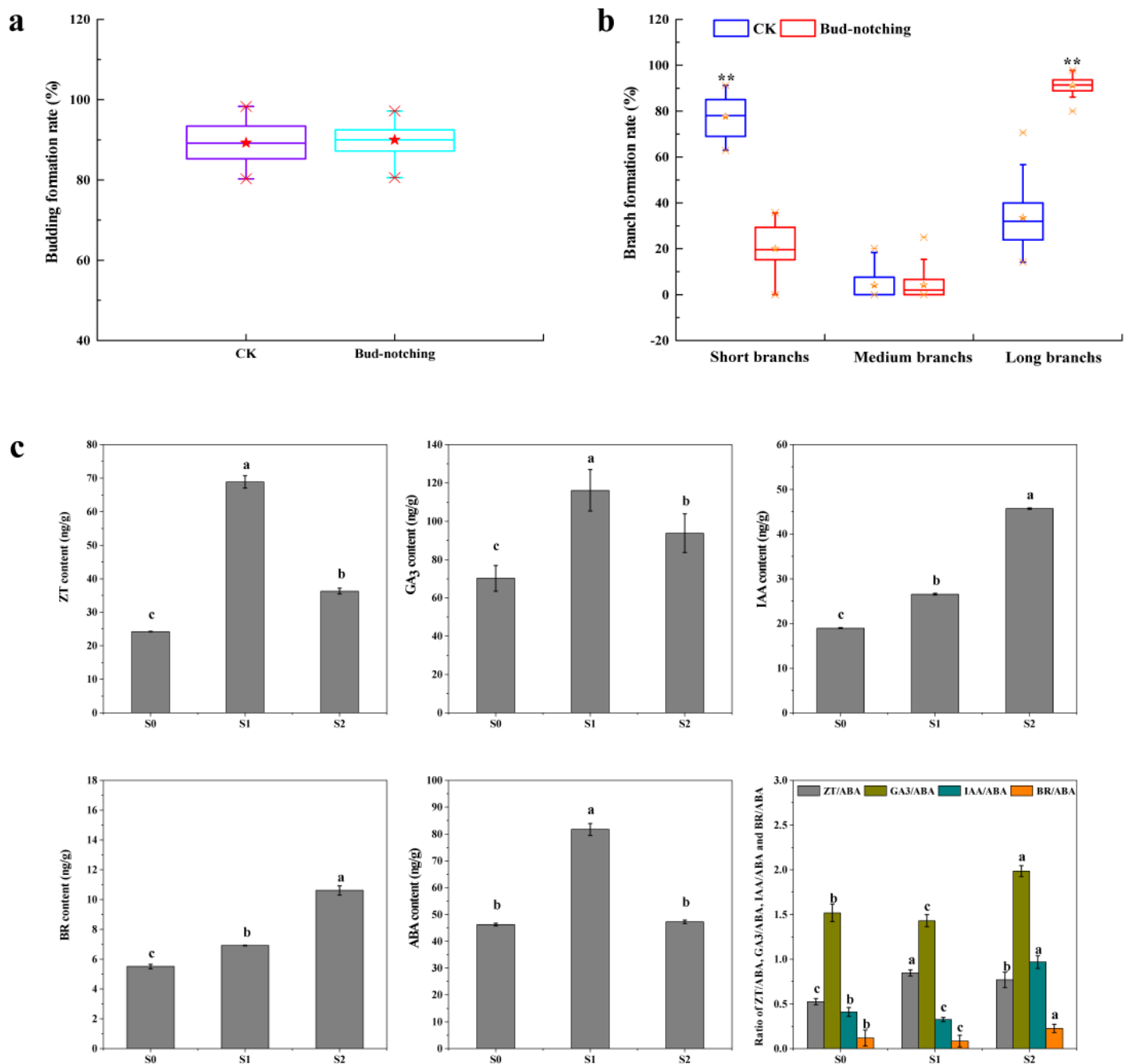


Figure 2

Germination rate, branching rate and endogenous hormone levels in lateral buds.

a, Sprouting rate of pear trees after bud-notching treatment. b, Comparison of branch formation rate between control and bud-notching treatment groups. Note: The test in figure.2a and figure.2b is the independent samples t-test; $P < 0.05$ indicates a significant difference (*) and $P < 0.01$ indicates a highly significant difference (**). c, The endogenous content and ratio of hormones. Note: The hormone contents and the ratios of the hormone contents and ratios are the means of three independent samples. a, b, and c indicate $P < 0.05$ level of significant difference.

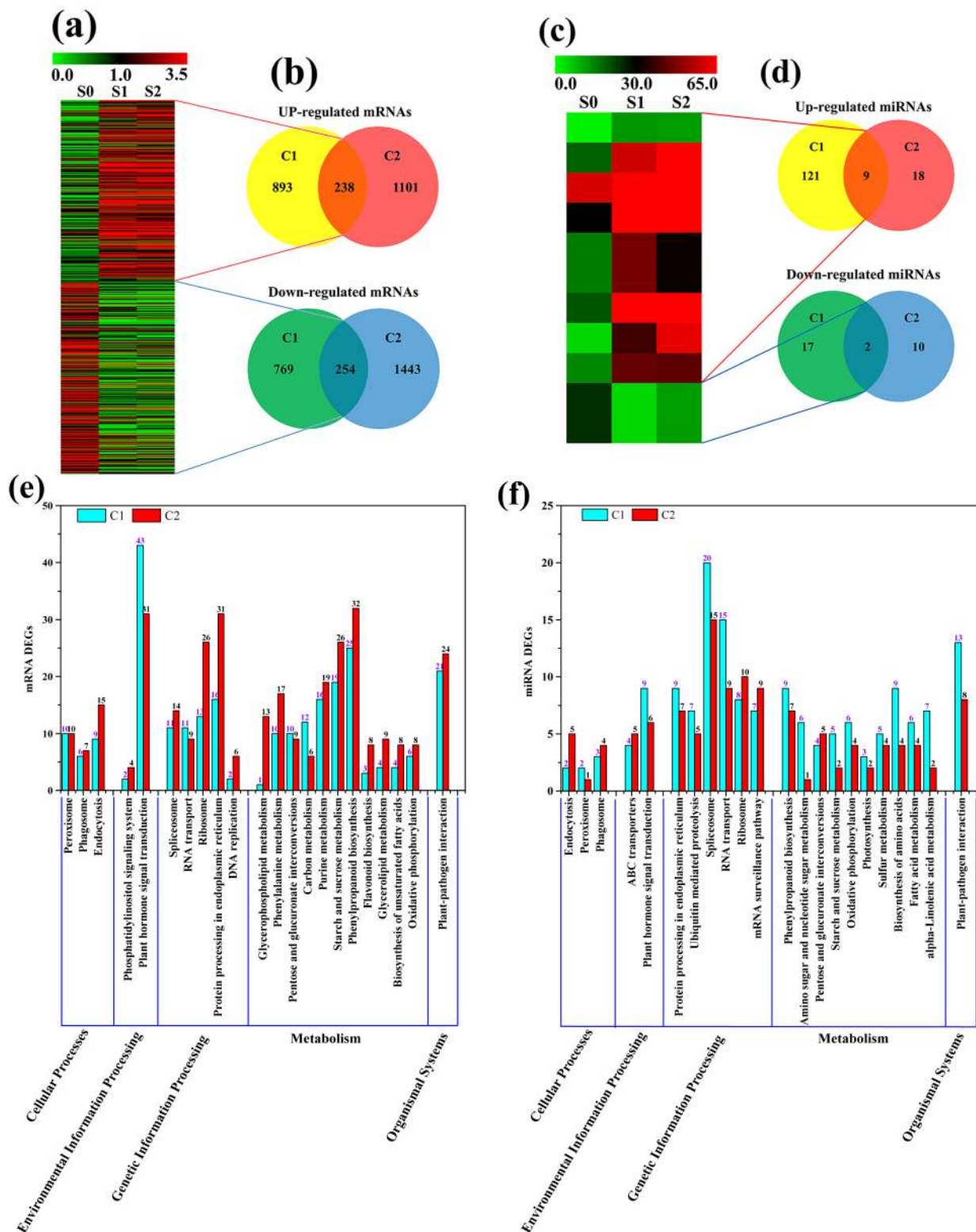


Figure 3

Differentially expressed mRNAs and miRNAs at both confrontation C1 and C2.

(a) The cluster heatmap of 492 DEGs at both confrontation C1 and C2. (b) The number of up (yellow, red) and down (green, blue) regulated DEGs detected between C1 and C2. (c) The cluster heatmap of 11 DEGs at both confrontation C1 and C2. (d) The number of up (yellow, red) and down (green, blue)

regulated miRNAs detected between C1 and C2. (e) Pathway analysis of mRNA DEGs based on the KEGG database C1 and C2. (f) Pathway analysis of miRNA DEMs based on the KEGG database at C1 and C2.

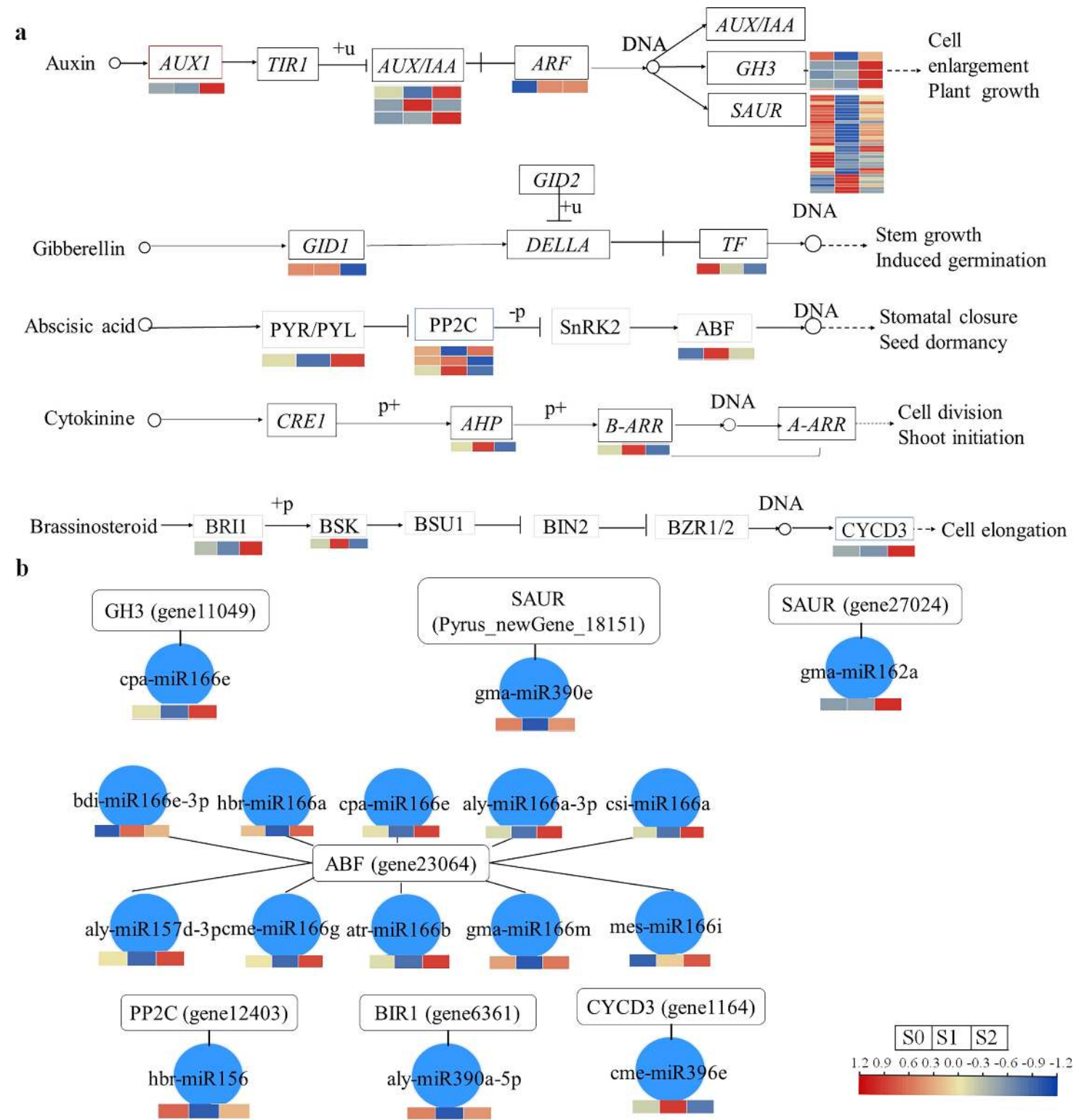


Figure 4

Pathway analysis of mRNA DEGs and miRNA DEGs based on the KEGG database. a. Pathway analysis of mRNA DEGs based on the KEGG database at Auxin, Gibberellin, Absciscic acid, Cytokinin and

Brassinosteroid. b. A regulatory network of miRNA-mRNA related to pear shoot branching-related genes.

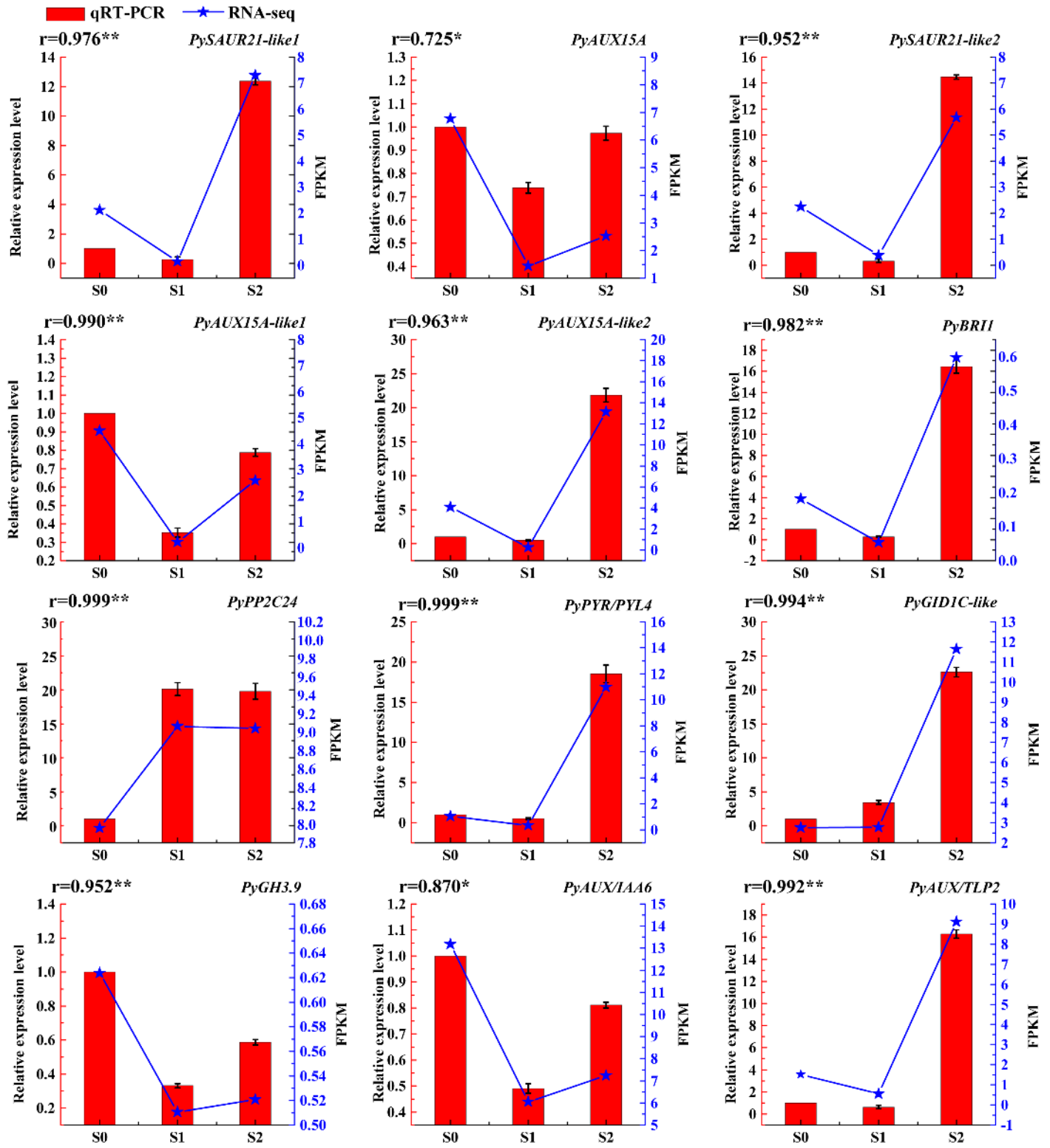


Figure 5

Real-time PCR validation of 12 DEGs in mRNA pairs. The x-axis represents RNA names, the left y-axis represents relative expression level, and right y-axis represents FPKM. Red bars represent data yielded by qRT-PCR, and blue star points represent data obtained by RNA sequencing, 'r' represents Pearson correlation coefficient. '*', correlation is significant at 0.05 level. '**', correlation is significant at 0.01 level.

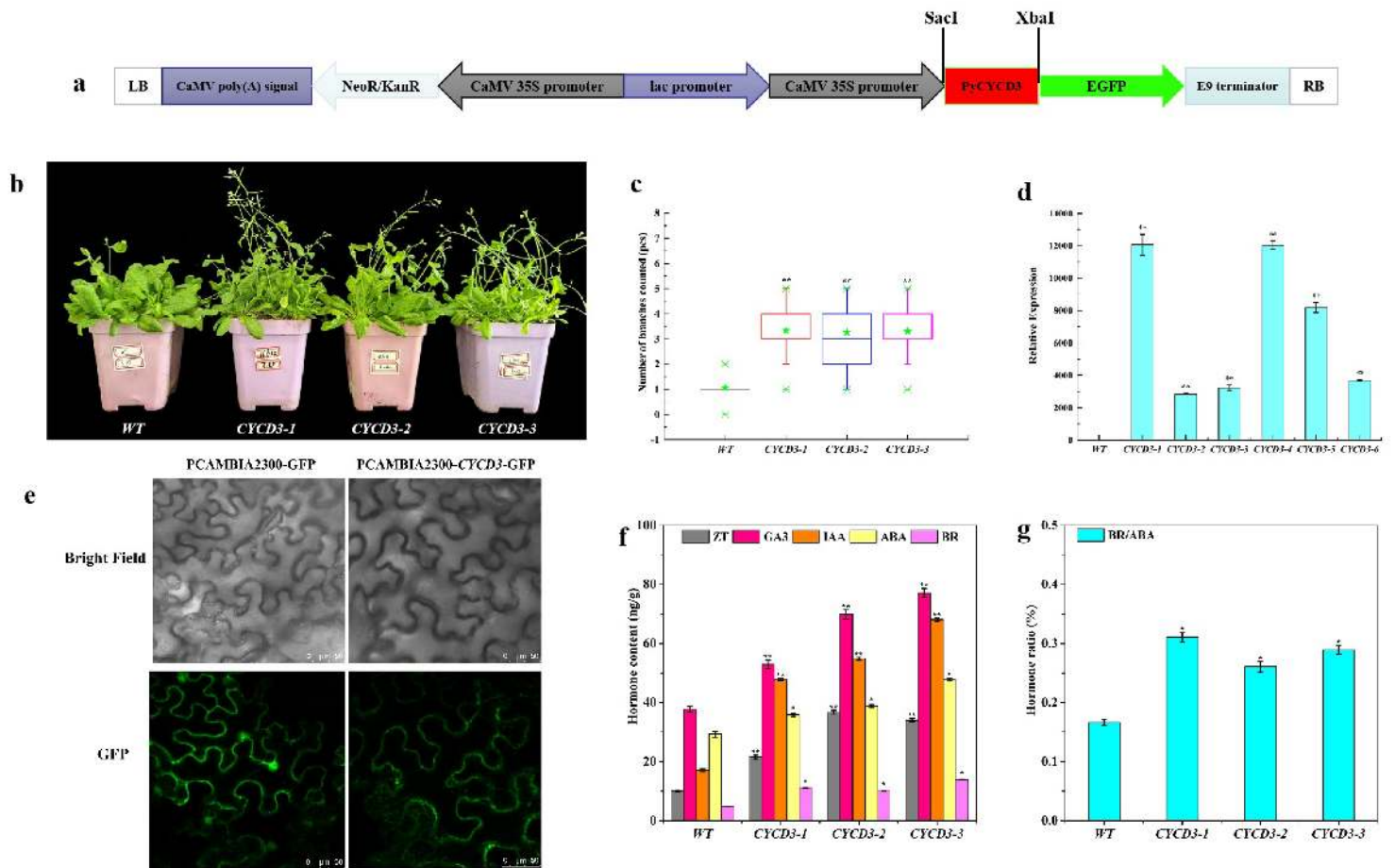


Figure 6

Overexpression *PyCYCD3* increased lateral branch formation of transgenic *Arabidopsis*. a, Schematic of the *pCAMBIA2300-PyCYCD3-GFP* fusion construct. b, The phenotypic difference of WT and transgenic *Arabidopsis thaliana*. c, Comparison of the number of outgrowths in WT and transgenic *Arabidopsis thaliana*. d, The relative expression levels of *PyCYCD3* in WT and transgenic lines. e, Subcellular localization of *PyCYCD3* gene in tobacco leaves. Bright Field: indicates bright field of view; GFP: indicates target protein GFP signal. Bar=50 μ m. f, The content of ZT, GA3, IAA, ABA, and BR in branches of WT and transgenic *Arabidopsis*. g, The BR/ABA of hormones in branches of WT and transgenic *Arabidopsis*. The test in figure is the independent samples t-test; $P < 0.05$ indicates a significant difference (*) and $P < 0.01$ indicates a highly significant difference (**).

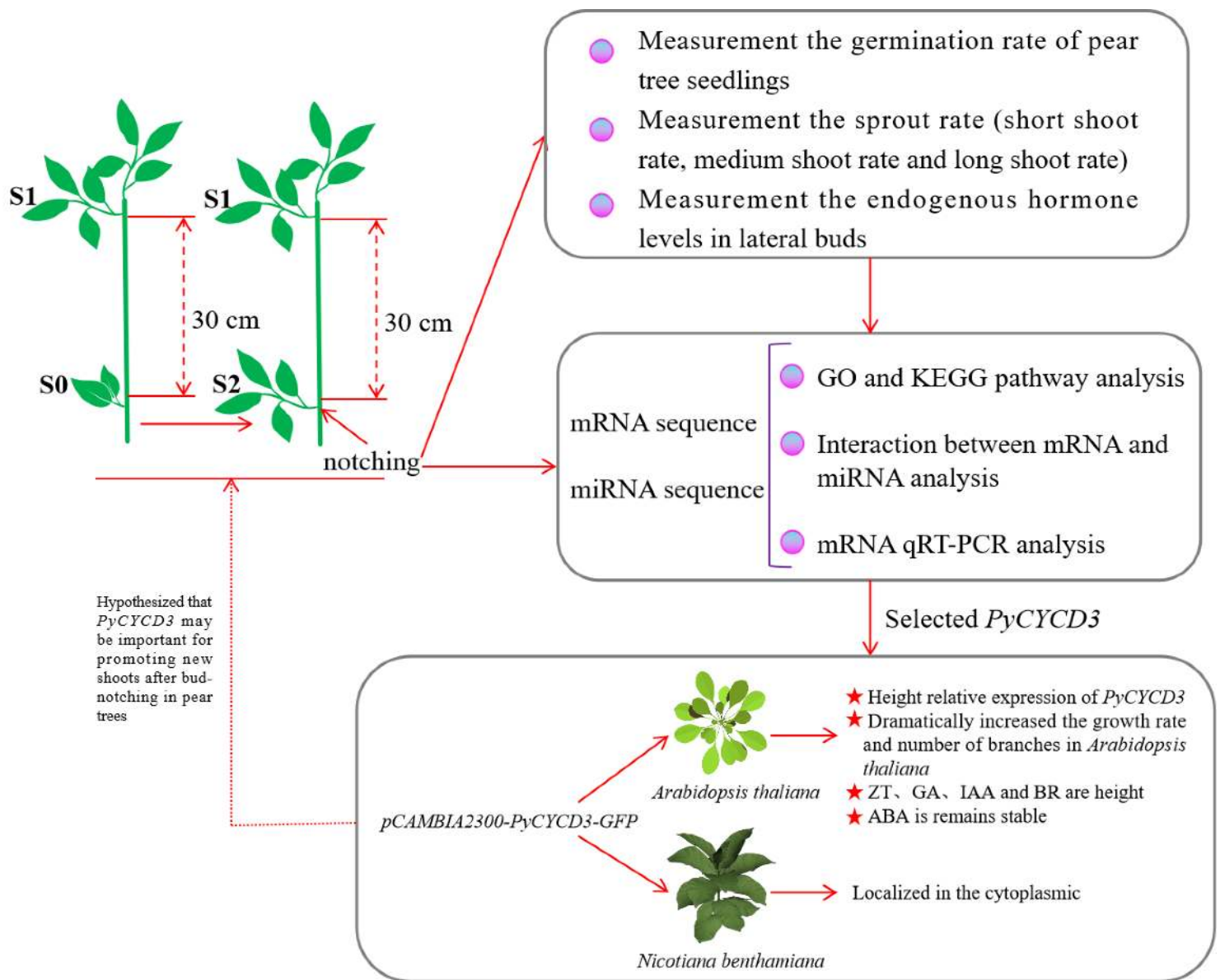


Figure 7

The main research simplified schemeatic diagram of this study

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