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# Transcription Profiles Reveal Sugar and Hormone Signaling Pathways Mediating Flower Induction in Apple (*Malus domestica* Borkh.)

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Regular Paper

**Flower induction in apple (*Malus domestica* Borkh.) is regulated by complex gene networks that involve multiple signal pathways to ensure flower bud formation in the next year, but the molecular determinants of apple flower induction are still unknown. In this research, transcriptomic profiles from differentiating buds allowed us to identify genes potentially involved in signaling pathways that mediate the regulatory mechanisms of flower induction. A hypothetical model for this regulatory mechanism was obtained by analysis of the available transcriptomic data, suggesting that sugar-, hormone- and flowering-related genes, as well as those involved in cell-cycle induction, participated in the apple flower induction process. Sugar levels and metabolism-related gene expression profiles revealed that sucrose is the initiation signal in flower induction. Complex hormone regulatory networks involved in cytokinin (CK), abscisic acid (ABA) and gibberellic acid pathways also induce apple flower formation. CK plays a key role in the regulation of cell formation and differentiation, and in affecting flowering-related gene expression levels during these processes. Meanwhile, ABA levels and ABA-related gene expression levels gradually increased, as did those of sugar metabolism-related genes, in developing buds, indicating that ABA signals regulate apple flower induction by participating in the sugar-mediated flowering pathway. Furthermore, changes in sugar and starch deposition levels in buds can be affected by ABA content and the expression of the genes involved in the ABA signaling pathway. Thus, multiple pathways, which are mainly mediated by crosstalk between sugar and hormone signals, regulate the molecular network involved in bud growth and flower induction in apple trees.**

**Keywords:** Cell cycle • Hormone signaling • *Malus domestica* • Sugar signaling • Flower induction.

**Abbreviations:** ABA, abscisic acid; AI, acid invertase; AUX, auxin; DAFB, days after full flower bloom; CK, cytokinin; DEGs, differentially expressed genes; ES, early stage of flower bud differentiation; GA, gibberellic acid; LS, late stage of flower bud differentiation; MS, middle stage of flower bud differentiation; NI, neutral invertase; SAM, shoot apical meristem; SDH, sorbitol dehydrogenase; SOX, sorbitol oxidase; SPS, sucrose phosphate synthase; SS, sucrose synthase.

## Introduction

Flower induction in plants is regulated by complex networks that involve endogenous and multiple environmental signals to ensure that flowering occurs at the appropriate time (Kurokura et al. 2013). In *Arabidopsis*, the flowering process involves multiple pathways, such as the photoperiod, vernalization, autonomous, gibberellic acid (GA), thermosensory and aging pathways (Turnbull 2011). A recent study showed that flowering was the primary trait affected by ambient temperature changes involving a key regulator within the thermosensory pathway (Lee and Lee 2010). Flowering is an important aspect of the life history of plants, especially in the woody fruit trees because it directly determines fruit production (Shabala et al. 2014). However, molecular mechanisms regulating flower induction and related pathways that promote flowering in perennial woody plants are very different when compared with the model plant *Arabidopsis thaliana*. For example, in *Arabidopsis*, GA promotes the transition from vegetative development to the first inflorescence phase of reproductive development (Yamaguchi et al. 2014); however, in woody trees, such as mango (Nakagawa et al. 2012) and apple (Wilkie et al. 2008), GA plays a negative role in flower bud formation. Consequently, research on the molecular mechanisms regulating floral induction in woody plants is particularly important.

Sugars play important roles in plant flowering (Turnbull 2011). Studies have shown that sugar, as the energy substance, regulates the growth and flowering transition in grape (Caspari et al. 1998) and citrus (Shalom et al. 2014). Recently, research showed that sugars, such as sucrose, glucose and T6P, as signal molecules, mediated the regulation of flowering induction (Wahl et al. 2013). In addition, sugar signals produced from 'source' leaves were transported to the shoot apical meristem (SAM), which determined flower development and induction (Bernier and Périlleux 2005). A study also showed that sugar-related signals were integrated into a flowering pathway, in which the transcription factor AtIDD8 regulates photoperiodic flowering

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by modulating sugar transport and metabolism in *A. thaliana* (Seo et al. 2011). The expression level of the key starch synthase key gene *GBSSI*, which affects starch deposition, can regulate the increased expression of the *CONSTANS* (*CO*) gene to promote flowering in plants (Serrano et al. 2009). Sugars may participate in the regulation of the flowering process in plants in multiple ways that involve different flowering pathways.

Hormones play a key role in the regulation of flowering (Amasino 2010), and the constitutive expression of *MADS-box* genes leads to precocious flowering in *A. thaliana*, which involves an intermediary event in a cytokinin (CK)- and/or GA-triggered signal transduction pathway (Bonhomme et al. 2000). The closest homolog of *FLOWERING LOCUS T* (*FT*), *TWIN SISTER OF FT* (*TSF*) is necessary for the flowering response to $N^6$-benzylaminopurine, suggesting that CKs play an important role in flowering by activating the expression of *FT* and *TSF* genes (D'Aloia et al. 2011). It has been reported that GA acts positively, then negatively, to control the onset of flower formation in *Arabidopsis*, indicating that GA promotes the termination of vegetative development and also inhibits flower formation (Yamaguchi et al. 2014). *PPC1*, as a regulator of defense against the stress-activated transition to flowering, can be regulated by ABA signaling (Mir et al. 2013). *FUS3* genes, which regulate phase transitions and flowering processes, are involved in the complex crosstalk between GA and ABA signaling (Tsai and Gazzarrini 2012). In addition, the ABAs' mediating of the plant flowering process is involved in sugar signaling (Gazzarrini and Tsai, 2014) and also in the regulation of the photoperiodic flowering pathway (Seung et al. 2012).

Apple (*Malus domestica* Borkh.) is a dominant temperate fruit crop worldwide, and 'Fuji' accounts for 65% of the apple planting area in China. However, a long juvenile phase before flowering and fruiting, and the poor quality of 'Fuji' flower buds, has seriously restricted the development of the apple industry in China. In addition, flower initiation and flowering occur in separate growing seasons for many woody fruits, including apple, peach and pear trees (Kurokura et al. 2013). Thus, the study of the molecular regulatory mechanisms of flower induction in apple is particularly important for understanding and solving the problems associated with flowering. In the present study, to determine the network of regulatory processes involved in complex signaling pathways, we used a high-throughput sequencing analysis to comprehensively provide the first global monitoring of gene expression level changes that occur during flowering, a physiological differentiation stage in apple.

## Results

### Shoot growth after full flower bloom

Shoot growth kinetics after full flower bloom were studied in 'Fuji' trees (**Fig. 1**). The shoot lengths increased gradually with an 'S' model curve, which showed large changes from 0 to 28 d after full flower bloom (DAFB) but little change between 28 and 42 DAFB (**Fig. 1**). The data indicated that shoots stopped growing after 28 DAFB, when flower bud induction begins (Lauri and Lespinasse 2001).

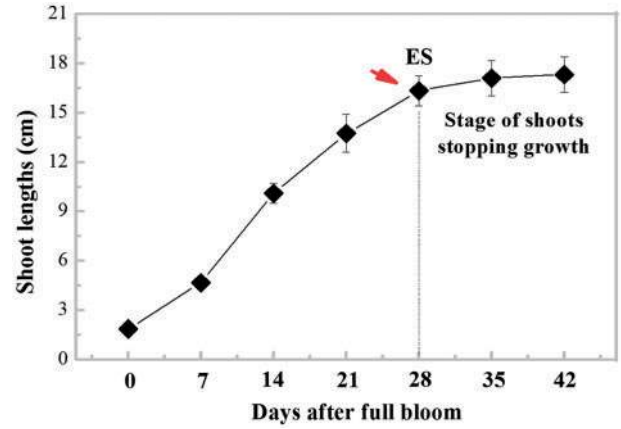


**Fig. 1** Shoot length changes days after full bloom (DAFB) in 'Fuji' apple.

### Bud elongation during flower induction

Growth kinetics during the flower induction period were studied in 'Fuji' buds (**Fig. 2**). In 'Fuji' buds, the bud length increased >85% between the early and middle and stages of the flower induction process (ES and MS, respectively) but only ~10% between MS and the late stage of the flower induction process (LS) (**Fig. 2A, D**). Additionally, bud width increased ~85% between ES and MS (**Fig. 2B, E**). The fresh weight of buds increased ~72% between ES and MS, but little change was observed between MS and LS (**Fig. 2B, E**). These data demonstrate that 'Fuji' buds mainly changed size in the early period of flower bud induction (from ES to MS).

### Sugar content, nitrogen (N) content and carbon (C)/N ratios in buds and bud-adjacent leaves during flower induction

The levels of sugar and N, the C/N ratio,and their catabolites were analyzed in 'Fuji' buds at three time points during flower induction (**Fig. 3**). In 'Fuji' buds, the contents of sucrose and N decreased 69% and 30%, respectively, from ES to LS (**Fig. 3A, G**). Glucose and fructose contents underwent a similar change, decreasing by 72% and 80%, respectively, between ES and MS with little change occurring between MS and LS (**Fig. 3B, C**). The total sugar content and the C/N ratio increased by 38% and 51%, respectively, from ES to MS, but decreased by 16% and 10%, respectively, between MS and LS (**Fig. 3F, H**). The starch content increased by ~80% at MS and ~90% at LS (**Fig. 3E**). The bud sorbitol content increased 47% between ES and MS and 48% between MS and LS (**Fig. 3D**).

The contents of sucrose, sorbitol and total sugar in 'Fuji' leaves increased by ~62%, 72% and 58%, respectively, between ES and MS but little change was observed between MS and LS (**Fig. S1**). In 'Fuji' leaves, glucose, fructose and N contents displayed high levels between ES and MS, but then decreased sharply by ~79%, 72% and 63%, respectively, from MS to LS (**Fig. S1**). The starch content and C/N ratio showed low levels

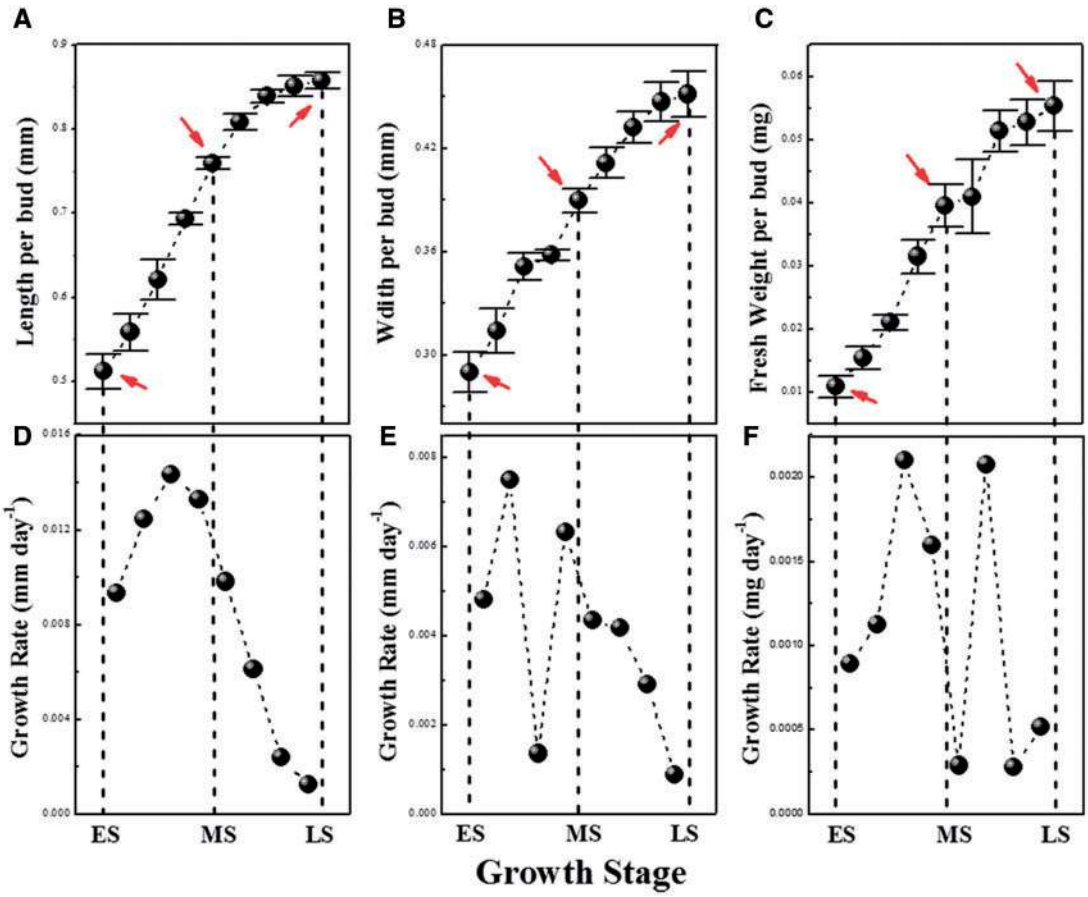


**Fig. 2** Bud growth and growth rates during the flower bud physiological differentiation stage in 'Fuji' apple. (A) Length, (B) width, (C) fresh weight, (D) growth rate of bud length, (E) width and (F) fresh weight.

between ES and LS, and then sharply increased by ~68% and 70%, respectively (**Fig. S1**).

## Sugar metabolism-related enzyme activities in buds and bud-adjacent leaves during flower induction

The activities of key enzymes in sugar metabolism were analyzed in 'Fuji' buds at three time points during flower induction (**Fig. S2**). The activities of sucrose phosphate synthase (SPS), neutral invertase (NI), and sorbitol oxidase (SOX) decreased by ~95%, 91% and 67%, respectively, from ES to MS but almost no changes were observed between MS and LS (**Fig. S2**). The acid invertase (AI) activity decreased by ~17% from ES to MS and by 27% from MS to LS (**Fig. S2C**). The sucrose synthase (SS) activity was high between ES and MS, but decreased sharply by ~87% from MS to LS (**Fig. S2B**). The sorbitol dehydrogenase (SDH) activity increased by ~17% between ES and MS, then decreased by ~8% between MS and LS (**Fig. S2E**).

In 'Fuji' leaves, the SPS activity increased by ~53% between ES and LS, with little change observed between MS and LS (**Fig. S3**). The SS and SDH activities increased by ~34% and 53%, respectively, from ES to MS, and ~17% and 31%, respectively, from MS to LS (**Fig. S3**). Interestingly, a completely opposite trend was observed for AI and SOX activities, which decreased by ~66% and 17%, respectively, from ES to MS, and then by ~49% and 35%, respectively, from MS to LS (**Fig. S3**). The activity of NI increased by ~19% between ES and MS, but decreased by ~74% from MS to LS (**Fig. S3**). The correlation coefficients of sugars, sugar metabolism-related enzyme activities, N levels and C/N ratios between buds and bud-adjacent leaves during the flower induction process can be seen in **Table S1**.

## Hormone contents in buds and bud-adjacent leaves during flower induction

The levels of hormones and their catabolites were analyzed in 'Fuji' buds at three time points during flower induction (**Fig. 4**). Auxin (Aux) and GA levels were high between ES and MS, and then decreased by 61% and 17%, respectively, from MS to LS (**Fig. 4A** and **C**). The CK content decreased sharply by ~55% between ES and MS, and then by ~28% between MS and LS (**Fig. 4B**). Interestingly, the ABA content increased by ~34% between ES and MS, and then by ~67% between MS and LS (**Fig. 4D**).

In 'Fuji' leaves, the Aux content increased by ~14% between ES and MS, and then decreased by ~40% between MS and LS (**Fig. S4**). The GA content showed low levels between ES and MS, but then increased by ~70% from MS to LS (**Fig. S4**). The CK content displayed a high level in ES, the beginning of flower induction, and then decreased sharply by ~79% from ES

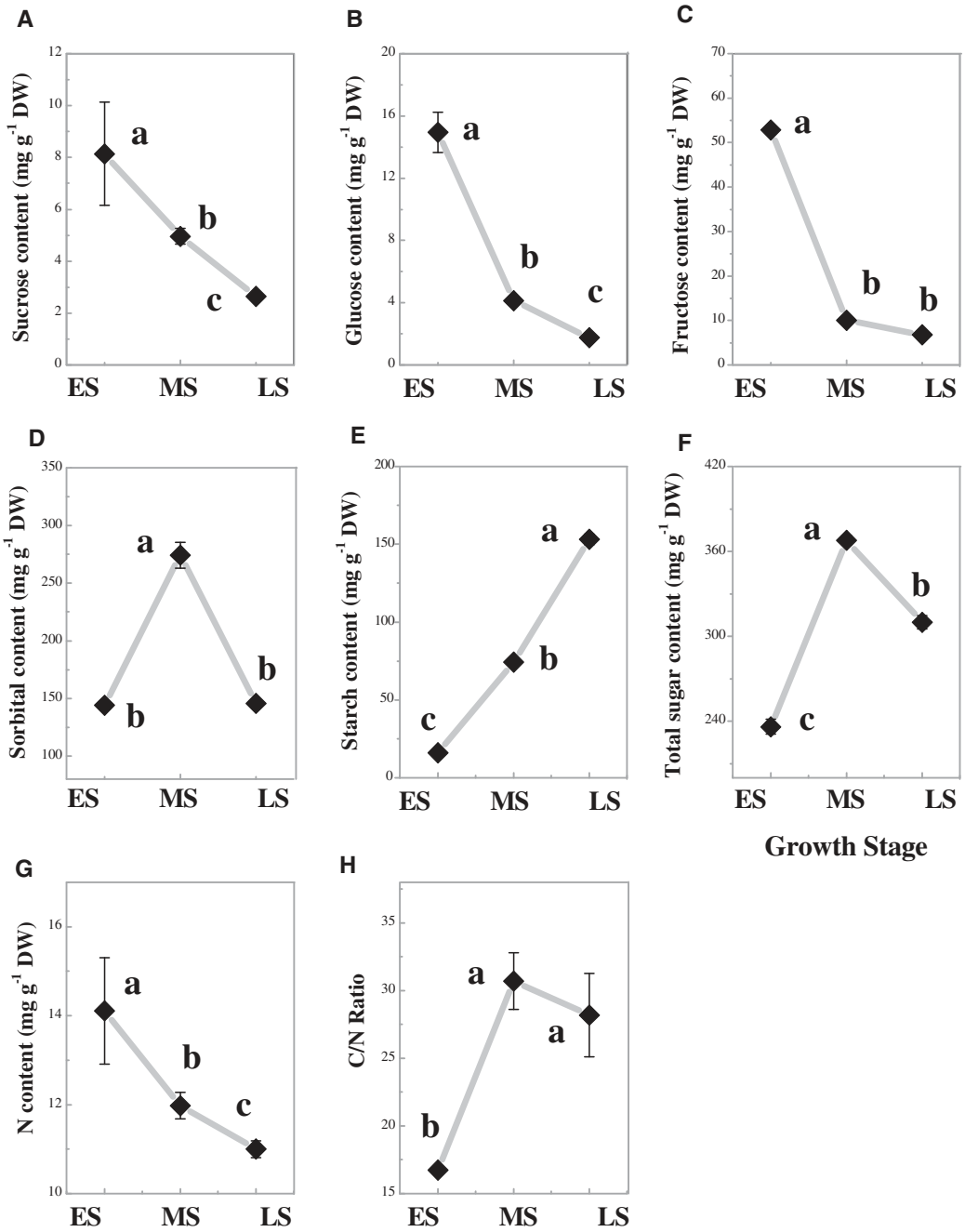


**Fig. 3** Bud sugar content, nitrogen (N) content and carbon/nitrogen (C/N) ratio during the flower bud physiological differentiation stage in 'Fuji' apple. (A) Sucrose, (B) glucose, (C) fructose, (D) sorbitol, (E) starch, (F) total sugar content, (G) N content and (H) C/N ratio. Values are means of three replicates ± SE.

to MS, and by ~56% from MS to LS (**Fig. S4**). However, the ABA content decreased by ~53% between ES and MS, and then by ~30% from MS to LS (**Fig. S4**).

## RNA deep sequencing and the quantitative reverse-transcription polymerase chain reaction (qRT-PCR) identification of sugar-, hormone- and flowering-related genes

A total of 30,365,960, 27,041,176 and 33,396,838 raw reads were generated by the high-throughput Illumina HiSeq 2000 Sequencing System for ES, MS and LS, respectively (**Table S2**). After processing primary reads, 15,722,113 (51.78%), 13,794,918 (51.01%) and 17,353,029 (51.96%) total clean reads were selected from ES, MS, and LS, respectively (**Table S3**). Venn diagrams of the differentially expressed genes (DEGs) in buds with ES vs MS, MS vs LS and ES vs LS can be seen in **Fig. S5A**, and the corresponding number of DEGs can be seen in **Fig. S5B**. The eight expression model profiles of DEGs in buds were developed using a strategy for clustering a time series (ES, MS and LS) (**Fig. S6**). The GO analysis also revealed that the DEGs belonged to seven major GO terms, including hormone/signaling,

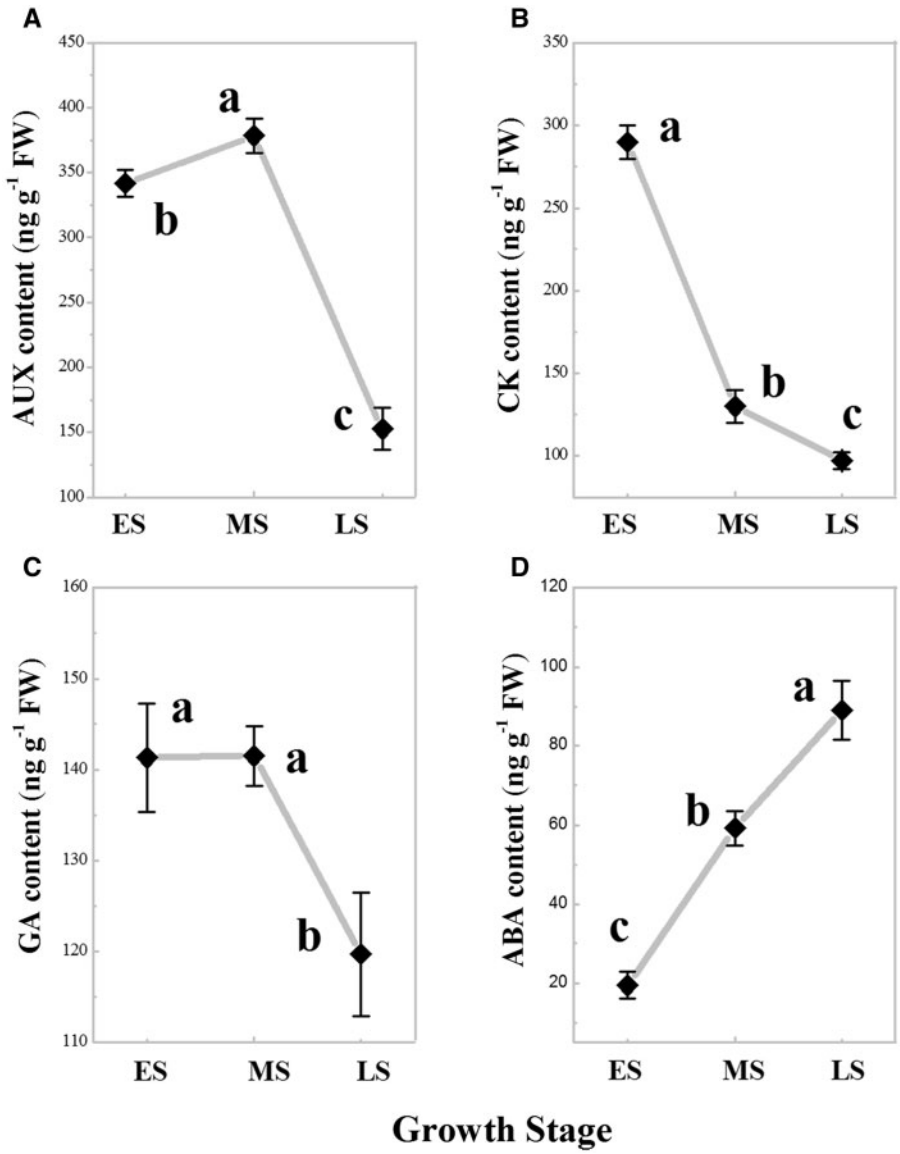


**Fig. 4** Hormone content of buds during the flower bud physiological differentiation stage in 'Fuji' apple. (A) AUX, Auxin; (B) CK, cytokinin; (C) GA, gibberellin; (D) ABA, abscisic acid.

carbohydrates, and cell and flowering (**Fig. S7**), as well as belonging to cellular component, molecular function and biological process (**Fig. S8**). A total of 40 KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were identified for DEGs from the ES, MS and LS profiles (**Table S4**). In addition, the identification by qRT-PCR of sugar-, hormone- and flowering-related genes expressed in buds and leaves during the flower bud physiological differentiation stage in 'Fuji' apple can been seen in **Figs. 5**, **S12**, **6**, **S13**, **7** and **S14**. The linear relationship between qRT-PCR data and RNA-seq data can be seen in **Fig. S15**.

## Expression profiles of sugar-related genes in buds during flower induction

Genes representing carbohydrate biosynthetic and metabolic, sugar transport, as well as sugar signaling-related, transcriptional signatures in buds during physiological differentiation are clustered in **Fig. 5A** and **B**, and listed in **Table S5** and **Supplementary file 2**. A hierarchical cluster analysis grouped these genes into six major clusters (**Fig. 5A**). Genes in cluster 1 (27 genes) typically showed high expression levels in the ES stage and then decreased gradually until the LS stage, when the physiological differentiation phase was completed (**Fig. 5B**). Among them, the tubulin beta 8 and small ubiquitin-like modifier 2 genes are involved in sugar gluconeogenesis, and the subtilisin-like serine protease 2 and alpha-glucan phosphorylase 2 genes are involved in the starch biosynthetic process (**Table S5**). The ADP-glucose pyrophosphorylase small subunit 2 and respiratory burst oxidase homolog D/F, which are involved in the starch biosynthetic process and carbohydrate homeostasis, respectively, are members of the second cluster (seven genes) (**Fig. 5B**). Cluster 3 included 135 genes that had increased in expression levels from ES to LS and were mainly related to the metabolism and biosynthesis of starch,

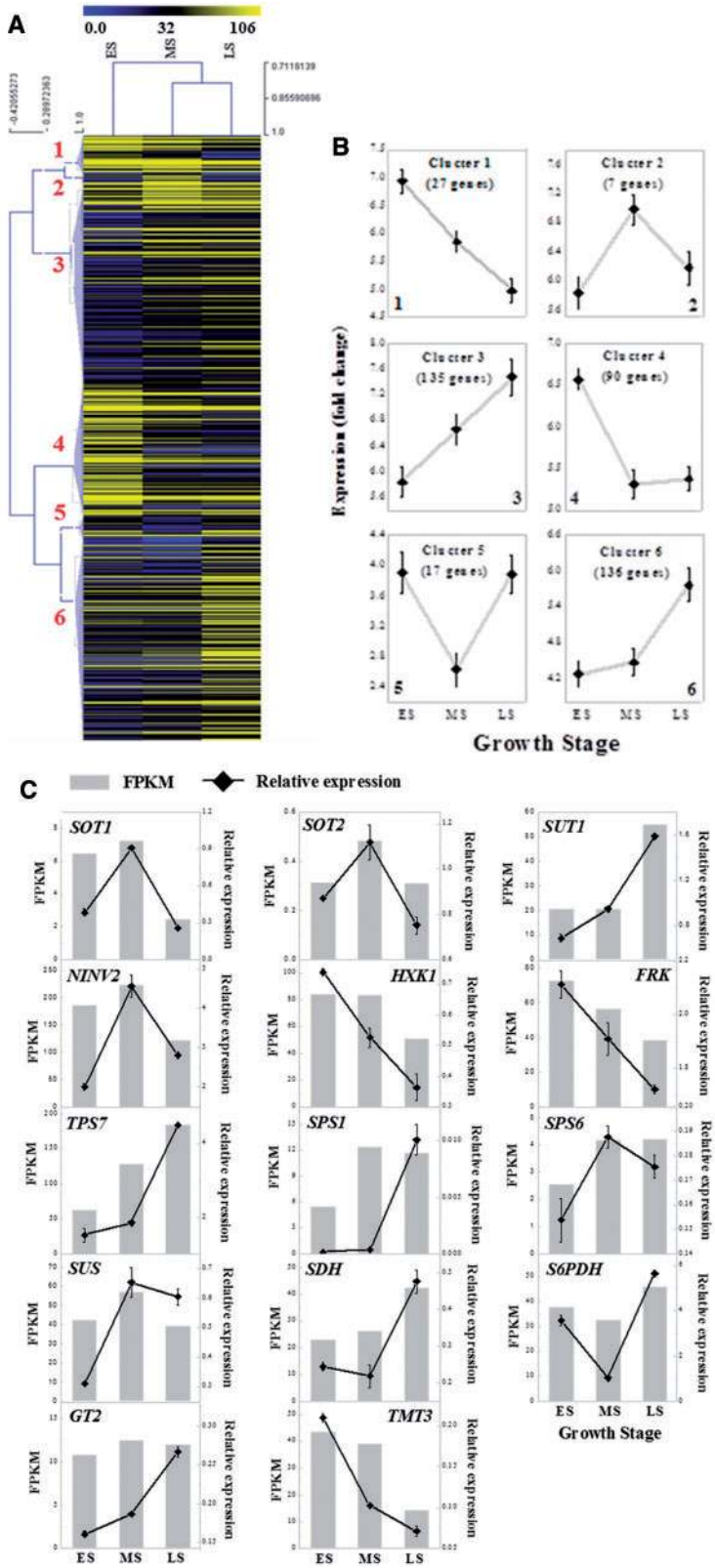


**Fig. 5** Expression profiles of sugar-related genes, as well as identification by qRT-PCR of sugar-related genes expression levels in buds during the flower bud physiological differentiation stage in 'Fuji' apple. (A) Hierarchical cluster analysis was performed on sugar-related genes with similar expression patterns. FPKM values were used for the cluster analysis. Expression data for a given gene are shown relative to its expression at early (ES), middle (MS) and late (LS) stages of flower bud differentiation. Numbers assigned to major clusters are indicated on the dendrogram. (B) Fold change in expression of representative genes from each of the major clusters in 'A' is presented (clusters 1–6). (C) Identification by qRT-PCR of sugar-related genes expression levels in buds. The bar graph and line graph are derived from RNA-seq and qRT-PCR data, respectively. Values are means of three replicates ± SE.

such as *SS4*, *SSI1*, *RP1* and *BAM7*, as well as sugar transport, such as *PMT5*, *SUC2*, *TMT2*,and *MSSI* (**Fig. 5B**). In addition, trehalose-phosphatase/synthase 7 (*TPS7*) and phosphoglucan water dikinase genes also belong to this cluster (**Fig. 5B**). The expression profiles of 90 genes in Cluster 4 typically displayed a sharp decrease between ES and MS stages, and then remained constant or slightly declined between the MS and LS stages (**Fig. 5B**). These genes are involved in glycolysis, such as *TPI*, *LOS2*, *CCR2*, *MABI* and *PFD6*; gluconeogenesis, such as *RNR1*, *RPN10*, *ACT11* and *RPT2a*; and starch biosynthesis, such as *THI1*, *HCEF1*, *CLPP5*, *NDF6* and *CP31B* (**Table S5** and **Supplementary file 2**). Interestingly, the genes in cluster 5 (17 genes) displayed a completely opposite expression profile compared with genes in cluster 2 (**Fig. 5B**), such as succinate dehydrogenase 2-3 and beta glucosidase 13 (**Table S5** and **Supplementary file 2**). Genes within the sixth cluster (136 genes) were characterized by enhanced expression levels during the later physiological differentiation period (**Fig. 5B**). Many of these genes belong to biological processes. Among them, beta-amylase 3 (*BMY3*), alpha-amylase-like 2 (*AMY2*) and sedoheptulose-bisphosphatase (*SBPASE*) are involved in starch metabolic and biosynthetic processes, while polyol/monosaccharide transporter 1 and sucrose transporter 4 (*SUT4*) are related to sugar transport. SNF1-related, protein kinase, regulatory subunit gamma 1 is involved in carbohydrate metabolic process (**Table S5** and **Supplementary file 2**). In addition, a model of the sugar biosynthetic, metabolic, and transport pathways in apple during the flower induction processes (**Fig. S16**) suggests that sugars play important roles in bud growth and flower bud formation.

## Expression profiles in buds of hormone regulatory genes during flower induction

Genes encoding hormone signaling pathways were analyzed and showed a significant change in expression levels, by at least 2-fold, among the different stages of bud development (ES vs MS, MS vs LS and ES vs LS). Six main gene clusters were determined by hierarchical clustering based on their expression profiles during bud development (**Fig. 6A, B** and **Supplementary file 2**). Cluster 1 included 75 genes that had decreased expression levels from the ES to MS stages, and then remained constant thereafter (**Fig. 6B**). The genes that respond to Aux, such as *RUBI*, *GH3.9*, *RPN12a*, *TT5* and *FQRI*, and Aux polar transport-related genes, such as *ACL5* and *MT3*, as well as Aux-activated signaling-related genes, such as *SHY2*, *IAA9* and *CNX7*, belong to this cluster (**Table S6**). The brassinosteroid signaling positive regulator family protein (*BZR1*) and general regulatory factor 8, which are the key genes of brassinosteroid-mediated signaling, had the same expression profiles (**Table S6**). While jasmonic acid-mediated signaling pathway genes, such as *CRB*, *DMR6*, *EFE*, *MYB113* and *JAZ8*, were grouped within this cluster (**Table S6**). Genes within cluster 2 (59 genes) typically displayed high expression levels during the early stages of bud development, and then gradually decreased until the LS stage (**Fig. 6B**). Genes associated with karrikin-responses, such as *ERD9*, *ZIFL1* and *AHP3*, and GA-mediated signaling-related genes, including *GASA1*, *GASA4* and *AGL20*, displayed this expression profile. In addition, the Aux efflux carrier family protein and phytochrome-associated protein 2 also grouped within this cluster (**Table S6** and **Supplementary file 2**). Several ABA-activated, signaling pathway genes, such as *MYB15*, *S6K2*, *PBP1*, *PYL4* and *CYP707A4*, and Aux-related genes *PIN3* and *TIR1* belong to the third cluster (55 genes). These genes decreased in expression from ES to MS and increased in expression between MS and LS (**Fig. 6B**). The fourth cluster contained 128 genes, which exhibited almost constant expression levels from ES to MS, and then sharply increased from MS to LS (**Fig. 6B**). Among them, GA-mediated signaling genes, such as *RVE1*, *GIDIB* and *GP ALPHA1*, the GA biosynthetic gene, as well as ethylene-activated signaling and response genes, such as *ERF7*, *EIN3*, *TEM1*, *RAP2.7*, *ERF1* and *RYP2.4* (**Table S6** and **Supplementary file 2**). In addition, CK oxidase 5 (*CKX5*), the cytochrome P450, family 714, subfamily A, polypeptide 1 gene and nine-cis-epoxycarotenoid dioxygenase 5 (*NCED5*), as the key genes related to ABA synthesis exhibited this expression pattern (**Table S6**). Indole-3-acetic acid inducible 29 and the flavin-containing monooxygenase family protein associated with Aux biosynthesis also belong to this cluster (**Table S6**). Twenty genes were included in cluster 5, which typically displayed a sharp increase in expression between ES and MS, followed by a sharp decrease in expression levels between MS and LS (**Fig. 6B**). ATP-binding cassette subfamily B19, BEL1-like homeodomain 1 and lipoxygenase 3 exhibited this pattern of expression (**Table S6**). Cluster 6 included 203 genes, some that increased in expression during flower bud physiological differentiation from ES to LS, and some that increased in expression from ES to MS. The expression levels of these genes then remained constant (**Fig. 6B**). Genes associated with ABA-activated signaling, such as *MYC2*, *ABF3* and *SPHK1*, and with ethylene-activated signaling genes, such as *ARF19*, *ETO1* and *RCD1*, as well as with CK response genes, such as *KNAT3*, *KNAT2* and *MNS1*, grouped within this cluster (**Table S6**). In addition, the tetratricopeptide repeat-like superfamily protein (*SPY*), which is involved in GA signaling, and jasmonic acid-mediated signaling-related genes, such as *COI1*, *SVP* and *WRKY40*, as well as two genes associated with brassinosteroid signaling exhibited this expression pattern (**Table S6**).

## Expression profiles of flowering genes in buds during flower induction

Expression profiles of flowering genes during the flower induction period were analyzed here by hierarchical clustering, which grouped these genes into five major clusters (**Fig. 7A, B** and **Supplementary file 2**). Sixty-nine genes within cluster 1 displayed high expression levels in ES, and then directly decreased until LS (**Fig. 7B**). Genes associated with the vegetative to reproductive phase transition of meristem, such as *ARPN*, *CCR2*, *IAA9*, *RAP2.7* and *TCP4*, as well as *SPL9*, *SPL4* and *SPL5* of the Squamosa-promoter binding protein-like gene family (SPL4/5 clade) involved in the regulation of the vegetative phase change, also displayed this expression profile (**Table S7**). MADS-box family member genes, such as *AGL19*, *AGL20*, *AGL42* and *SOC1*, vernalization process-related genes and

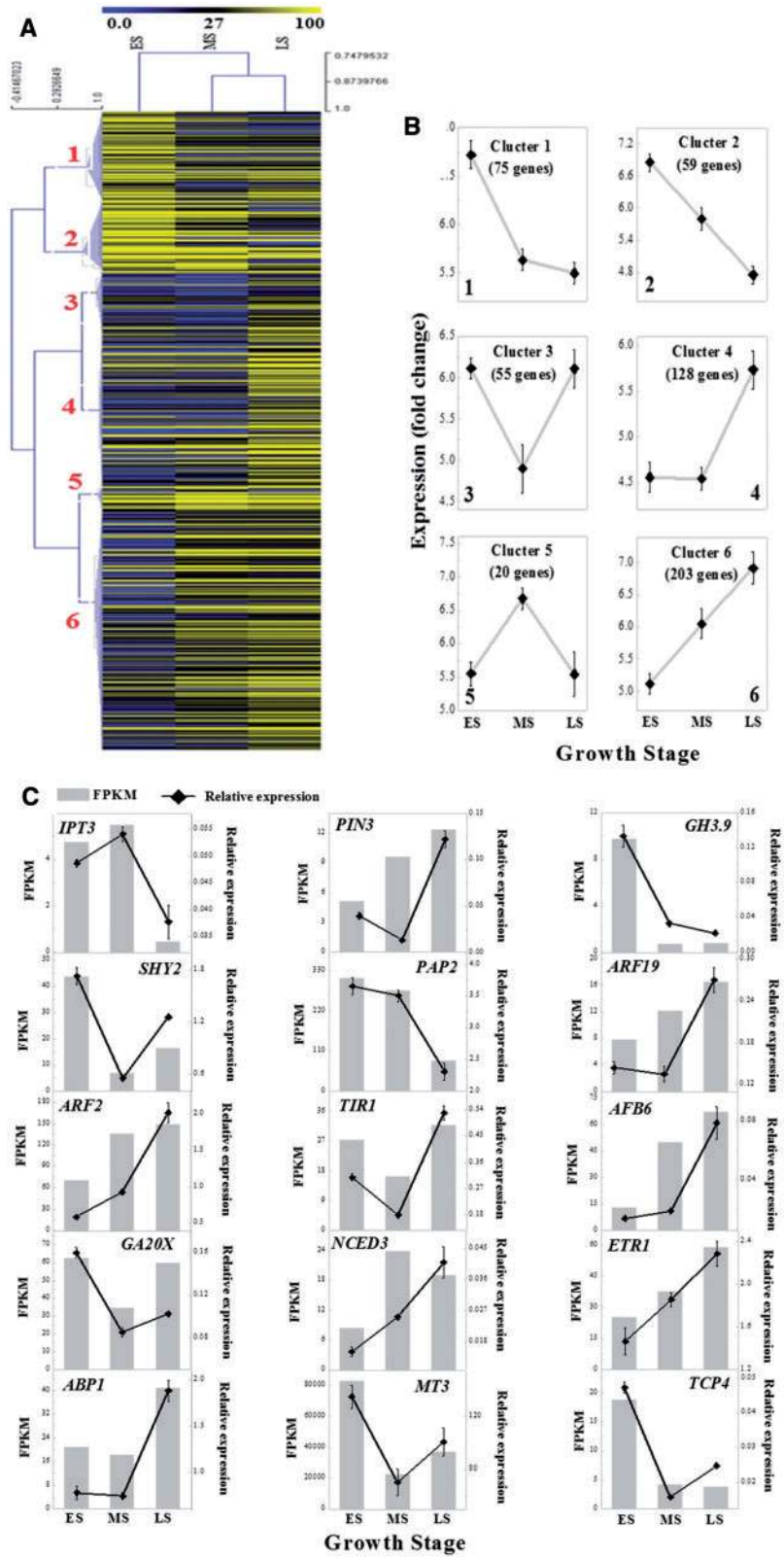


**Fig. 6** Expression profiles of hormone-related genes, as well as identification by qRT-PCR of hormone-related genes expression levels in buds during the flower bud physiological differentiation stage in 'Fuji' apple. (A) Hierarchical cluster analysis was performed on hormone-related genes with similar expression patterns. FPKM values were used for the cluster analysis. Expression data for a given gene are shown relative to its expression at early (ES), middle (MS) and late (LS) stages of flower bud differentiation. Numbers assigned to major clusters are indicated on the dendrogram. (B) Fold change in expression of representative genes from each of the major clusters in 'A' is presented (clusters 1–6). (C) Identification by qRT-PCR of hormone-related genes expression levels in buds. The bar graph and line graph are derived from RNA-seq and qRT-PCR data, respectively. Values are means of three replicates ± SE.

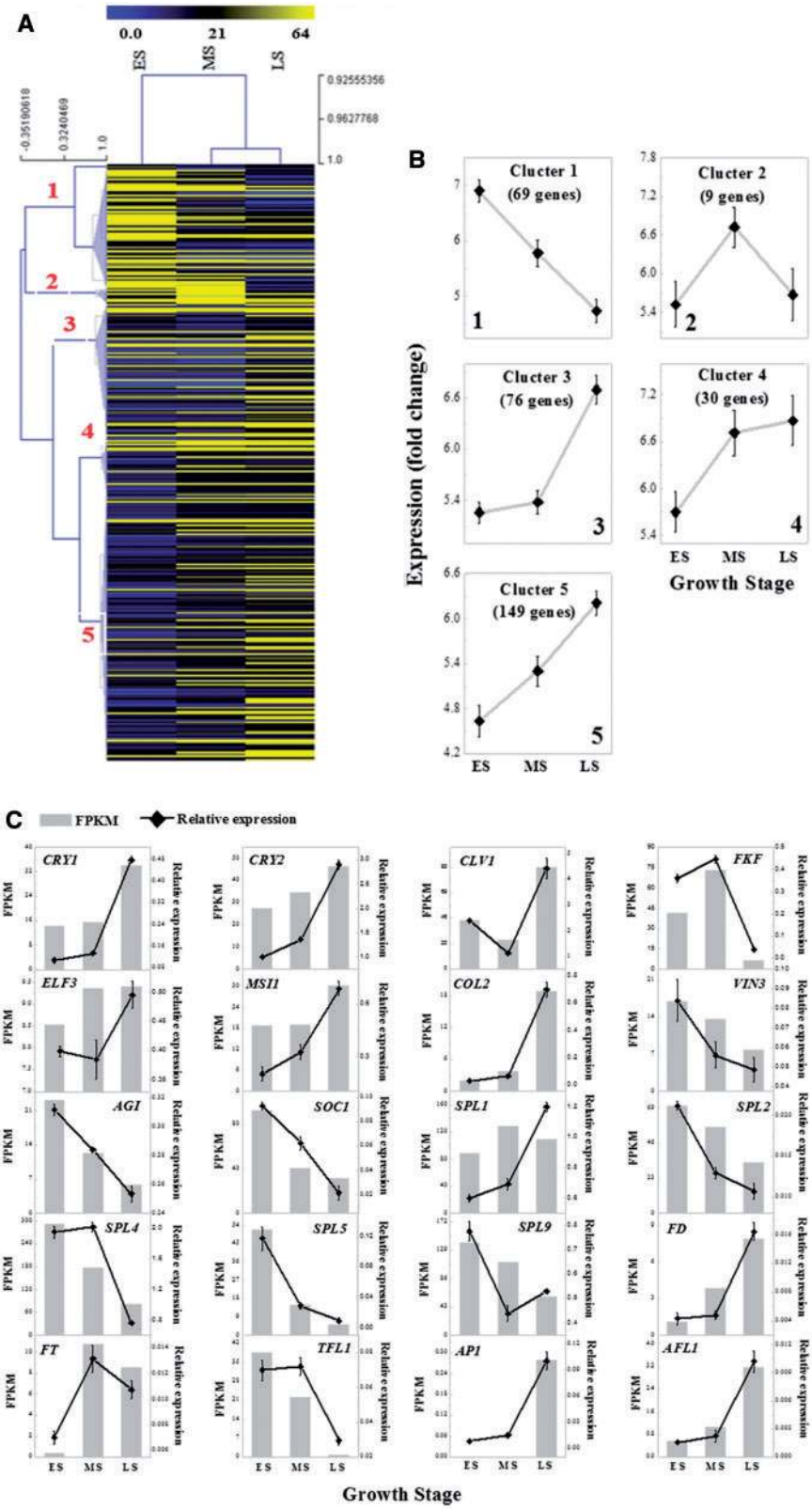


**Fig. 7** Expression profiles of flower-related genes, and identification by qRT-PCR of flower-related gene expression levels in buds during the flower bud physiological differentiation stage in 'Fuji' apple. (A) Hierarchical cluster analysis was performed on flower-related genes with similar expression patterns. FPKM values were used for the cluster analysis. Expression data for a given gene are shown relative to its expression at early (ES), middle (MS) and late (LS) stages of flower bud differentiation. Numbers assigned to major clusters are indicated on the dendrogram. (B) Fold change in expression of representative genes from each of the major clusters in 'A' is presented (clusters 1–6). (C) Identification by qRT-PCR of flower-related genes expression levels in buds. The bar graph and line graph are derived from RNA-seq and qRT-PCR data, respectively. Values are means of three replicates ± SE.

*TFL1*, a FT/TFL1 family member involved in flower development and the regulation of flower development processes, were grouped within this cluster (**Table S7**). In addition, expansin B1, which is associated with sexual reproduction, and *CP29*, *OHP* and *PCL1*, which are involved in circadian rhythms, displayed this cluster 1 expression profile (**Table S7**). Cluster 2 included nine genes that increased in expression from ES to MS, and then declined sharply from MS to LS (**Fig. 7B**). Among them, a flavin-binding Kelch repeat F-box (*FKF1*) protein regulating the transition to flowering, a gigantea (*GI*) protein involved in regulating circadian rhythms, as well as circadian rhythm-related genes exhibited this expression pattern (**Table S7**). In addition, the GRAS family transcription factor family protein (*GAI*) involved in repressing GA-induced vegetative growth and floral initiation also belongs to this cluster (**Table S7**). Genes within cluster 3 (76 genes) typically displayed high expression levels during LS (**Fig. 7B**). These genes are mainly involved in several biological processes, such as flower development and the regulation of flower development (*FER1*, *SWC2*, *CPD* and *COL2/5*), the vegetative to reproductive phase transition of meristem and its regulation (*MYB17*, *HB8*, *TOR*, *CDF2* and *UBC2*), as well as circadian rhythms and the regulation of circadian rhythms (*CRY1*, *CRY2* and *TIR1*) (**Table S7** and **Supplementary file 2**). In addition, several genes associated with the photoperiodism of the flowering process, such as *TEM1*, *EID1*, *CUTA* and *TIM17-2*, also exhibited this expression pattern (**Table S7**). Interestingly, genes within cluster 4 (30 genes) typically displayed low expression levels during ES, but exhibited high expression levels in the MS and LS stages (**Fig. 7B**). This cluster contains several floral organ formation genes, such as *AGO4*, *COBL1* and *ACA9*, as well as K-box region and MADS-box transcription factor family proteins associated with flower development and floral meristem determinacy (**Table S7**). Cluster 6 included 149 genes that increased constantly in expression from the ES to LS stages (**Fig. 7B**). Flower development and regulation genes, such as *DCAF1*, *VIP5*, *FD* and *FT* (FT/TFL family members), and several flowering genes, such as *LFY*, *FCA*, *VRN1* and *EFL3*, exhibited this expression pattern (**Table S7**). Pseudo-response regulator 5/7 (*PRR5/7*) and FKF1-like protein 2 (*ZTL*), which are involved in circadian rhythms also belong to this cluster (**Table S7**). In addition, several phytochrome family members, such as *PHYA*, *PHYB* and *PHYC*, which are involved in the regulation of photomorphogenesis and the photoperiodism of flowering-related genes also displayed the expression profile of cluster 5 (**Table S7**). In addition, we examined the expression levels of flowering-related genes in buds during flower induction (from ES to LS) using qRT-PCR (**Fig. 3C**). Expression levels of some

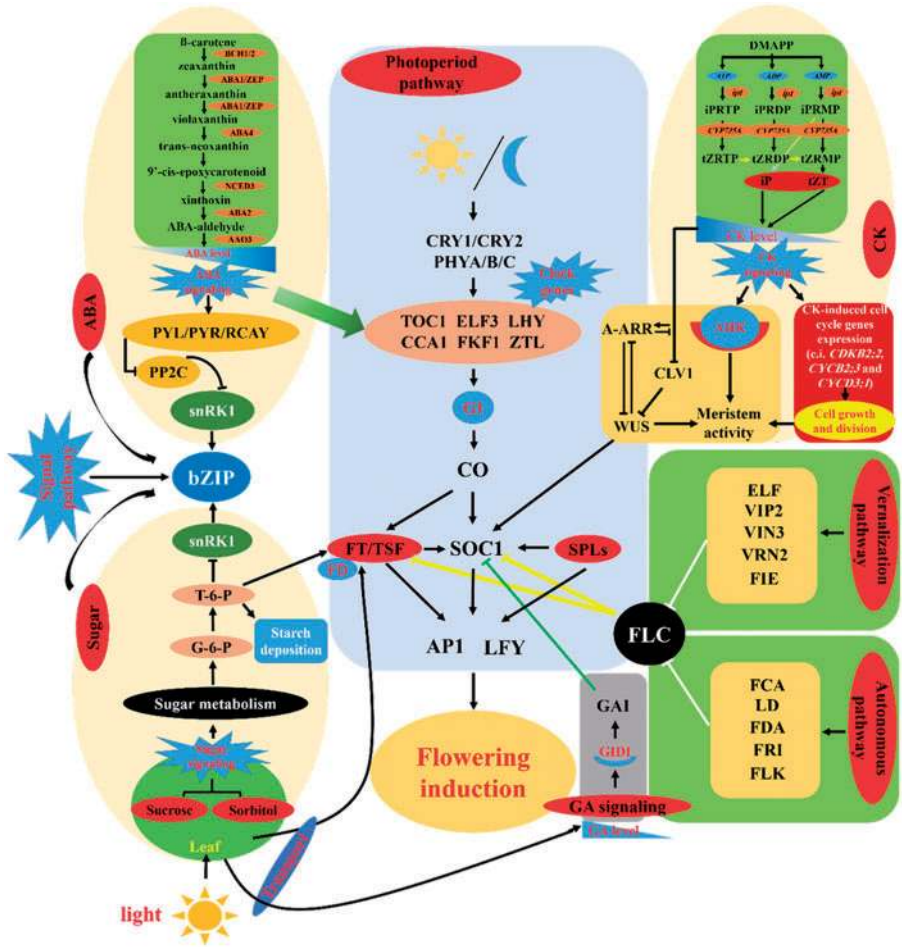


**Fig. 8** Hypothetical model for the regulation of flower induction in apple by sugar and hormone signaling crosstalk.

flowering genes such as *CRY1*, *CRY2*, *COL2*, *AP1*, *MSI1* and *AFL1* increased from ES to LS stages in buds (**Fig. 3C**), however, expression levels of other flowering genes (*FKF*, *TFL1*, *SPL4*, *AGI* and *VIN3*) showed an opposite results during flower induction process (**Fig. 3C**). Meanwhile, these qRT-PCR experiments also validated the RNA-seq results of expression levels for these flowering-related genes in buds during this process (**Fig. 3C**). The hypothetical model for the regulation of flower induction in apple by sugar- and hormone-signaling crosstalk can been seen in **Fig. 8**.

## Discussion

### Expression profile of cell cycle-related genes during flower induction

Cell cycle-related genes in apple buds during flower induction were analyzed, which suggested they have important roles in facilitating cell production, bud growth and flower induction in apple (**Fig. S9** and **Supplementary file 2**). However, a deep understanding of the molecular regulation in bud growth during flower induction in apple is still lacking. According to our results, several genes, such as *CDKB2;2*, *CYCB2;3*, *CYCD3;1* and *CYCB1;4*, associated with the cell cycle and the *MT3* genes showed high expression levels in the ES stage (**Fig. S9** and **Supplementary file 2**), similar to that observed in *Arabidopsis* (Boudolf et al. 2009). This suggests that these genes may play important roles in promoting cell division and in responding to flower initiation signals. The transcription factor family members, such as *NAC052*, *NAC082*, *NAC083* and *NAP*, gradually increased and displayed peak expression levels in LS (**Fig. S9** and **Supplementary file 2**), suggesting that these genes also have key roles in the regulation of cell wall thickening and cell size. Similarly, increases in cell size and the regulation of cell growth are observed as a result of the overexpression of *NAC* transcription factors in *Populus* species (Zhao et al. 2014). With flower induction, different expression profiles among these cell cycle-related genes revealed their importance in the molecular mechanisms regulating bud growth and flower induction.

### Sugar signaling mediates flower induction in apple

Previous studies have shown that sugars, as the main source of energy, participate in the plant flowering process (Gibson 2005). However, limited information is available about sugar's specific influence on flower induction in woody fruit trees, especially apple trees. Sugar levels in leaves and SAM (Smith et al. 2005), as well as the sugar flux between source and sink, are involved in determining whether an event, such as flower development and induction, occurs (Bernier and Périlleux 2005). Flower induction in plants, which involves sugar levels and responses, may actually be regulated by alterations in sugar flux or sugar signaling (Smeekens et al. 2010). Our results showed high sucrose levels in buds during the early stages, ES and MS, but lower levels in leaves at the same time. Then, they underwent changes, resulting in the opposite condition (**Fig. 3**). Several sugar transport genes, such as *PMT5*, *SUC2*, *TMT2* and *MSSI*, also increased gradually with flower induction (**Table S5**), suggesting that sucrose as a signal molecule is exported from leaves to buds by sugar transport-related genes in the early stage, potentially creating an instantaneous transitory signal to induce flower initiation. Similar results were reported in other species by Hisamatsu and King (2008).

The transcriptomic profiling studies performed here reinforced the view that flower induction during bud development involves sugar metabolism, biosynthesis and transport processes (**Fig. 5A** and **B** and **Table S5**). Sucrose efflux is increased by *SUT4* expression in the SAM and is involved in the sugar-mediated flower-induction process (Kühn and Grof 2010). Meanwhile, the levels of sucrose transporter-related genes *SUC2* and *SUT1* increased with bud development (**Table S5** and **Fig. 5A,B**), accompanied by an increase in the buds' sucrose content (**Fig. 3**), suggesting that *SUC2* and *SUT1* participate in flowering by responding to sugar regulation. More evidence supporting the close association of flowering with sugar-related genes has been reported (Seo et al. 2011). Indeed, *SOC1* and other homologous genes, such as *AGL19*, *AGL20* and *AGL42*, as the central factors of flower induction in the meristem, showed high expression levels in the early stages of flower induction, with a similar change and content in the buds' sucrose content (**Fig. 3**), suggesting that sucrose induced flowering by regulating the flowering-related gene expression levels, such as *SOC1* and *AGL24* (**Table S7**).

Currently, the signal molecule trehalose-6-phosphate (T6P) is thought to play a crucial role in the regulation of flowering and has been suggested to function as a proxy for carbohydrate status in plants (Wahl et al. 2013). Our results showed that the sucrose level was high in ES and gradually decreased (**Fig. 3**), while the expression of *TPS7* genes gradually increased during the flower induction process (**Table S5**), suggesting that both sucrose and T6P act as proxies for the carbohydrate status in plants. Similarly, T6P levels are closely related to sucrose, and an increase in the sucrose level in plants raises the T6P level, leading to sugar signaling and metabolic changes that initiate flower induction (Lastdrager et al. 2014). In addition, several SBP transcription factor gene family members, such as *SPL4*, *SPL5* and *SPL9*, which act as miR156 targets involved in the aging pathway of the flowering process, showed high expression levels in the ES, and decreased with bud growth during flower induction (**Table S7**). This is similar to what was observed in *Arabidopsis* (Wahl et al. 2013), suggesting that the sugars that induce flower initiation in apple trees may participate in the aging pathway of the flowering process. Plants with reduced *TPS1* expression levels had lower *FT* gene expression levels, which inhibited flower induction (Wahl et al. 2013). However, the expression of *FT* genes in buds increased from ES to LS, as did the expression levels of *TPS7* genes (**Tables S5** and **S7**), showing that sugars, as signals that promote flowering, may be involved in another flowering pathway (Tsai and Gazzarrini 2014) in which the *TPS* gene directly influences the expression levels of *FT* genes to promote flowering. Several lines of evidence indicate opposite roles for T6P and the SnRK1 kinases in the regulation of phase transitions and flower induction in response to carbohydrate levels (Zhang et al. 2009), and bZIP transcription factors

were found to mediate the effects of sugar signaling on the flower induction process (Abe et al. 2005), suggesting that the sugars participating in flower induction may be involved in multiple pathways.

Starch metabolism and biosynthetic processes are involved in flower induction, and the T6P signaling molecule is involved in the plant flowering pathway (Paul 2007). It is reported that the *CO* gene's expression level is regulated by *GBSSI*. Involved in starch synthesis, the overexpression of CO augmented *GBSS1* mRNA levels, affecting the capacity of cells to accumulate starch (Serrano et al. 2009). Indeed, our results showed that the starch content in both leaves and buds increased during the flower induction process (**Fig. 3** and **S1**), and the expression levels of several genes, *SS4*, *SSI1*, *GBSSI* and *SBPASE*, associated with the starch biosynthesis process in buds displayed similar changes (**Fig. 5A, B** and **Table S5**), but two genes, *AMY2* and *BMY3*, which are involved in the starch degradation process, showed low expression levels in ES and MS (**Fig. 5A, B** and **Table S5**). This suggests that starch plays an important role in the regulation of bud growth, cell division and carbohydrate levels that induce sugar signal and promote flowering (**Fig. S16**).

## Hormone signaling mediates flower induction in apple

Phytohormones have been reported to be involved in the complex regulation of floral transitions (Davis 2009); however, the complex regulatory mechanisms involved in floral induction and transition in woody plants are not very clear. In our study, to understand the roles of hormones in bud growth, flower induction and its regulation, we analyzed hormone levels and expression levels of genes association with hormone regulation and response during the physiological differentiation process (**Fig. 6A, B**; **Table S6** and **Supplementary file 2**).

CKs are important phytohormones involved in many aspects of plant growth and development, including the growth of SAM (Gordon et al. 2009), the regulation of the transition from vegetative growth to flowering (Amasino 2010) and the flower induction process (D'Aloia et al. 2011). Indeed, our results showed that leaves and buds displayed high CK levels during the ES and gradually decreased with the completion of flower induction (**Fig. 4** and **Fig. S4**). *IPT3* and *IPT5*, key genes of CK synthesis, showed high expression levels in buds and leaves during ES and MS (**Table S6** and **Fig. 6C**), suggesting that CK plays a role in the first regulatory step of flower induction. As a signal transmitted from leaves to SAM, a similar study reported that CKs of leaf origin triggered the flower induction (Jacqmard et al. 2002). It was reported that *SOC1* is an early flowering gene whose expression is up-regulated by increasing CK levels in the SAM during floral induction (Lee and Lee 2010). CK applications can induce plant flowering by promoting the expression of *TSF* genes, *FT* homolog genes (D'Aloia et al. 2011). According to our results, the *SOC1* expressions pattern, and those of several homologous genes, such as *AGL19*, *AGL20* and *AGL42*, displayed similar changes to the CK content in buds, showing high expression levels in the ES of flower induction, but then decreasing from ES to LS (**Table S7**). Additionally, there was a relatively lower expression level of *FT* (**Table S7**), suggesting that the role of CK in the initiation of flower bud induction in apple may be in activating the expression of *SOC1* and other homologous genes, as seen in previous research results (Lee and Lee 2010). CK, as part of the WUSCHEL (WUS)-CLAVATA (CLV) feedback circuit, is involved in SAM maintenance, and the growth and initiation of flowering (Gordon et al. 2009). Meanwhile, *CKX5* and *CKX6*, as CK degradation genes, and *CLV1*, which is involved in the regulation of SAM activity and growth with CK and *WUS* (Wang and Li 2008), displayed expression increases from ES to LS (**Table S6**). In addition, *CYCD3;1* genes associated with the cell cycle showed high expression levels in ES (**Fig. S9** and **Supplementary file 2**), suggesting that CK-related genes may also be involved in the process of flower induction regulation in apple.

ABA is an important hormone because of its critical regulatory roles in different phases of the plant life cycle associated with seed development, floral and phase transitions, and in plant responses to environmental stresses (Tsai and Gazzarrini 2014). Indeed, the ABA level gradually increased in buds and adjacent leaves during the flower induction process (**Fig. 4** and **Fig. S4**), suggesting that ABA may play a key role in the regulation of flower induction in fruit trees. In addition, expression profiles of ABA-related genes during the flower induction period were analyzed here by hierarchical clustering to determine the role of ABA in apple flower induction and molecular regulatory mechanisms (**Fig. 6A, B**). Several genes involved in ABA biosynthesis, including *ZEP*, *NCED3*, *NCED5*, *SDR*, *CYP707A4* and *AO4*, displayed similar changes in the ABA contents in buds and leaves (**Fig. 4**, **Fig. S4** and **Table S6**), suggesting that these changes may mediate the regulation of apple floral induction. In addition, ABA signaling is involved in the regulation of vegetative development and flower induction by T6P and SnRK1 pathways (Tsai and Gazzarrini 2014). *ABI3*, as a transcription factor, along with *AIP2*, belongs to the ABA signal pathway that promotes the transition from vegetative growth to reproductive initiation (i.e. flowering) (Zeng et al. 2013). Because the transcriptomic analyses revealed several ABA signaling and response-related genes, such as *PYL4*, *HY5*, *MYC2*, *ABF3*, *HAB1* (*ABI1*) and *KIN10* (*SnRK2.6*), expression levels in buds gradually increased from ES to LS and displayed high expression levels in LS (**Table S6** and **Fig. 4**), indicating that ABA may be involved in bud growth and flower induction during the physiological differentiation process. Indeed, *AKIN10*, as a homolog of *SnRK1* in *Arabidopsis*, and *FUS3*, the B3-domain transcription factor FUSCA3, both act as positive regulators of the floral transition response to ABA signaling in *Arabidopsis*, and *AKIN10* is also involved in the regulation of sugar signaling (Tsai and Gazzarrini 2012).

Hormonal signaling networks, especially the ABA signaling involved in the regulation of circadian rhythms, influence growth and development, as well as flower induction and transition in plants (de Montaigu et al. 2010). Indeed, our results showed that the expression profiles of these circadian rhythm-related genes, such as *TOC1*, *ZTL*, *FKF1*, *PRR5*, *PRR7* and *GI*,

showed similar changes in ABA levels to the ABA signaling-related genes in buds, which increased from ES to LS during the flower induction process (**Fig. 4** and **Table S7**). This may indicate that flower induction, and growth and development are regulated by circadian clock-dependent gating in the ABA signaling pathway. *LWD1/2* is a component of the clock, forming a positive regulatory feedback loop with *PRR9* that is involved in the photoperiodic regulation of flowering (Wang et al. 2011). *EID1*, which is involved in the regulation of circadian rhythms, plays an important role in ABA signaling and in the regulation of the floral transition in *Arabidopsis* (Koops et al. 2011). Meanwhile, our results also showed that the *EID1* and several *Constance-like* family members, including *COL2* and *COL5*, expression levels increased from ES to LS and have similar changes in ABA levels to the ABA signaling-related genes in buds (**Fig. 4** and **Table S7**). This is in agreement with the ABA-induced flowering involving a regulatory process in which *EDL3* over-expression results in an increased *CO* transcript level (Koops et al. 2011), suggesting that flower induction in apple trees through the photoperiod pathway may be related to the regulatory process of ABA signaling. In addition, exogenous ABA induces flower initiation by promoting *AP1* expression (Cui et al. 2013), and Pathogen and Circadian Controlled 1 (*PCC1*) is involved in ABA-mediated responses to the flower transition in plants (Mir et al. 2013). Our results indicated that ABA is involved in the regulation of flower bud induction in apple through multiple pathways.

Previous research showed that GA plays a positive role in flowering in *A. thaliana* through GA-dependent pathways (Mutasa-Göttgens and Hedden 2009). However, a decline in GA is beneficial for floral bud initiation in many woody species, such as citrus (Koshita et al. 1999) and apple (Wilkie et al. 2008). GA and CKs have opposite effects on the regulation of meristem activity and the initiation of flower induction, which is probably caused by the direct repression by CK of the expression of the GA biosynthetic gene GA20ox and by KNOX increasing the expression in SAM (Sakamoto et al. 2001). Our results showed that the GA content in buds was high between ES and MS, and decreased sharply from MS to LS, but in leaves increased gradually from ES to LS (**Fig. 4**). In addition, two key genes in GA synthesis, *GA20ox* and *GA30ox*, increased from ES to MS, but decreased from MS to LS (**Table S6**). In addition, *SPY*, as both a positive regulator of CK signaling and a repressor of GA signaling (Greenboim-Wainberg et al. 2005), increased from ES to LS during the flower induction process in buds (**Table S6**), suggesting that GA inhibits flower induction and development by repressing CK responses and signaling in apple trees. Tseng et al. (2004) also showed that *SPY* interacts with *GI*, which is involved with light signals that influence flowering. In this study, the expression of *FT* genes increased gradually from ES to LS, while there was a complete opposite change in the GA levels of buds (**Fig. 4** and **Table S7**). Additionally, GA3 inhibits flowering in woody fruits by repressing *FT* expression (Nakagawa et al. 2012), suggesting that the negative regulatory role of GAs in flowering is the inhibition of flowering genes and flowering signaling molecules levels in woody plants.

## Flowering pathway in apple during the physiological differentiation process

A complex network involved in photoperiod, vernalization, autonomous, GA-dependent and aging pathways, which responds to environmental and internal signals, and controls the flowering transition, has been reported in *Arabidopsis* (Kurokura et al. 2013). In this study, we analyzed expression profiles of flowering genes involved in flowering pathways using clustering methods during bud growth and development (**Table S7** and **Fig. 7A, B**). Our results showed that *TOC1*, *CCA1*, *ZTL*, *LHY*, *CRY1*, *PRR5/7*, *CRY2* and *ELF3* were involved in circadian rhythms, and several light photoreceptor genes were involved in the regulation of photoperiod, such as *PHYA*, *PHYB* and *PHYC*, as well as the COL-like genes *COL2* and *COL5*, which acted as the central regulators of the photoperiod pathway (Jang et al. 2008). They displayed increasing expression levels from ES to LS during the flower induction process in buds (**Table S7** and **Supplementary file 2**), and two other circadian rhythm genes, *FKF1* and *GI*, peaked at the MS stage in buds (**Table S7**), indicating that photoperiod-mediated floral induction is an important pathway in the flowering regulatory network during bud growth in apple trees. The up-regulation of the *CO* gene is the result of a combination of circadian rhythm controls expressed through *CRY*, *PHYA*, *PHYB* and *GI* on the CO protein (Valverde et al. 2004). Indeed, *CO* as a key gene controls the flowering transition in the photoperiod pathway by directly up-regulating the *FT* gene (Valverde et al. 2004). In our data, the *FT* gene and its homologous gene *TSF* showed changes similar to genes such as *CO*, *PHYA* and *CRY2* in the photoperiod pathway (**Fig. 7** and **Table S7**), but another homologous gene, *TFL1*, changed in the opposite manner (**Fig. 7** and **Table S7**). A high level of *MdTFL1* expression can be seen in the vegetative SAM, whereas it is down-regulated during the flower induction process in apple (Mimida et al. 2011). *FT* controls flowering through both PHYA-mediated photoperiodic regulation and photoperiod-independent regulation (Kong et al. 2010), suggesting that the *CO-FT* module controls photoperiodic flowering induction via the circadian clock genes' regulation. In addition, *FD*, a bZIP transcription factor interacting with *FT* gene to induce flowering, showed a similar change to *FT* genes in buds (**Fig. 7** and **Table S7**). A similar result can be seen in Jaeger and Wigge (2007).

Our results showed that the expression levels of several *MADS* family members, such as *AGL19*, *ADL20*, *AGL42* and *SOC1*, were high in the early stages during flower induction, and then decreased gradually in the later stage (**Fig. 7** and **Table S7**). A similar study indicated that the expression of *SOC1*, a key early flowering gene, is up-regulated in early flower induction (Lee and Lee 2010). The *LYF* gene's expression displayed a change similar to those of *FT* and *FD* from ES to LS, suggesting that these flowering genes may be involved in the complex regulatory network of the apple flower induction process (**Fig. 7** and **Table S7**). A recent study suggested that the SPL family of transcription factors is involved in the age-related flowering transition pathway (Wang et al. 2009). The up-regulation of *SPL3*, *SPL4*, *SPL5* and *SPL9* is directly regulated by *SOC1*

expression in SAM (Porri et al. 2012). Similar expression profiles between *SPLs* (*SPL3*, *SPL4*, *SPL5* and *SPL9*) and the *MADS* family members (*AGL19*, *ADL20*, *AGL42* and *SOC1*) in buds and leaves, which displayed high expression levels in the early stage in our data (**Fig. 7** and **Table S7**), suggest that the *SPLs* mediate an age-related floral induction process in apple. In addition, this age-related pathway involved the miRNA156 family that is highly expressed in the juvenile phase (Wang et al. 2009).

Gocal et al. (2001) showed that GAs promote the expression of *LFY* by the GAMYB protein binding to its promoter. Meanwhile, *SOC1* regulates *LFY* expression by directly binding to its promoter, and GA interacts with *SOC1* to regulate *SPL* expression through *SOC1* binding directly to *SPL* genes (Porri et al. 2012). Indeed, our results showed that the expression levels of GAs, *MADS-box* family members, such as *AGL19*, *AGL20*, *AGL24*, *AGL42* and *SOC1*, and *SPLs*, such as *SPL3*, *SPL4*, *SPL5* and *SPL9*, displayed similar trends with high levels in the early stage during flower induction (**Table S7**). This suggests that the GA-signaling pathway of flower induction is also involved in the regulation of *MADS-box* family members, *SPLs* and *LFY* genes. Our data showed that *GAI*, as negative regulator of the response to GA signaling, decreased gradually in buds from ES to LS, and had relatively high expression levels in leaves during ES and MS but a high GA level in leaves during LS (**Fig. 7** and **Table S7**). Richter et al. (2010) showed that GAI, as a DELLA protein (which are degraded in response to GA), repressed GA signaling by inhibitory interactions with PHYTOCHROME-INTERACTING FACTOR (PIF) family transcription factors. In addition, GAs influenced the phase transition through the regulation of *SOC1* and *LFY* in SAM (Lee and Lee 2010), and GA signaling facilitated the activation of *FT* expression in leaves and the expression of the *SPL* genes in SAM, indicating that multiple GA-dependent pathways may be involved in flower induction in apple (**Fig. 7** and **Table S7**).

In our data, *FLOWERING LOCUS C* (*FLC*), as the key flowering repressor involved in vernalization and autonomous pathways (Kim et al. 2006), is barely expressed in buds during the flower bud differentiation phase (**Supplementary file 2** and **Table S7**). Meanwhile, in leaves its expression decreased (**Fig. S14**). In contrast, the expression of *FCA*, *FPA*, *FY*, *FLD*, *LD*, *FVE* and *FLK*, which are involved in the autonomous pathway, increased from ES to LS during flower induction (**Fig. 7** and **Table S7**) and inhibited *FLC* gene expression to promote flowering (Lim et al. 2004). *FLC* inhibited flowering by repressing the expression of *FT*, *FD* and *SOC1*, which bind to the promoters of *SOC1*, *FD* and the first intron of *FT* genes, respectively (Corbesier and Coupland 2006). In addition, the *MADS-box* transcription factor (*SVP*) repressed *FT* expression by directly binding to the vCArG III motif in the *FT* promoter (Li et al. 2008) and displayed a relatively lower expression level in buds during the early stage (**Table S7** and **Supplementary file 2**). Our results showed that *SVP* and two genes, *TEM1* and *TEM2*, displayed relatively lower expression levels in the early stage (**Table S7**), and (Sawa and Kay 2011) showed that *GI* promoted flowering by binding to *SVP*, *TEM1* and *TEM2* promoter regions, which were up-regulated by *FT* gene expression. Taken together, our results showed that *FLC* is a central flowering repressor and interacts with its partner *SVP* to regulate the expression of downstream flowering genes, such as *FT*, *FD* and *SOC1* that respond to environmental and endogenous signals.

## Materials and Methods

### Plant material and sample collection

Plant material was collected from 6-year-old 'Fuji' apple (*M. domestica* Borkh.) trees growing on M.26 rootstocks at the Apple Demonstration Nursery of Yangling Modern Agriculture Technology Park (Northwest Agriculture & Forestry University), Shaanxi Province of China (34°52′N, 108°7′E). Shoot elongation was measured on eight trees, with 15 shoots per tree being sampled at 0, 7, 14, 21, 28, 35 and 42 DAFB (**Fig. 1**). Buds on the top of spurs, as mixed samples from 48 6-year-old trees, were collected for size and weight nine times, and the sugar content, sugar metabolism-related enzyme activity and hormone levels were measured three times, from ES to MS, during the period of flower induction (**Fig. 2**). Leaves adjacent to the buds were collected at the same time and their levels of sugar, hormones and sugar metabolism-related enzyme activities were measured. All samples (buds and leaves) were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction and RNA-seq library construction.

### Measurement of sugar, sugar metabolism-related enzyme activity and hormone contents

At three developmental stages, ES, MS and LS, ~0.3 mg dry weight of buds and leaves were used for sugar and starch extractions, and the contents were determined as described in detail by Rosa et al. (2009). To measure sugar metabolism-related enzyme activities according to Li et al. (2012), ~0.5 g fresh weight of buds and leaves were used. Meanwhile, 0.5 g fresh weight of buds and leaves were used for phytohormone extractions (Ivanov Dobrev and Kamínek 2002), and hormonal analysis and quantification were performed using a high-performance liquid chromatograph (Waters 2498/UV, Visible Detector; Shaanxi, China) with a standard measure as described in detail by Dobrev and Vankova (2012) and Djilianov et al. (2013).

### RNA extraction, quantification and qPCR analyses

Total RNA was isolated from each sample by a modified method (Xia et al. 2012), and cDNA was synthesized as previously described (Shalom et al. 2012). The specific primers for real-time qPCR in this study were designed using Primer 3 software (**Table S8**). PCR was performed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) with 10 μl of 2X SYBR® Premix Ex Taq II (Takara, Beijing, China), and 0.8 μl of forward and reverse primers in a 20-μl final volume to determine the expression of the target genes, and then incubated in a Bio-Rad iCycler iQ5 (Bio-Rad, Plano, TX, USA) for 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 35 s at 60°C, followed by 81 cycles for the melt curve. The *actin* gene was used as the internal control for gene expression normalization. Each reaction was performed in three replicates. The correlation of target genes in expression profiles was measured by qRT-PCR and by RNA-seq.

### RNA deep sequencing and library construction

The quality and quantity of extracted RNA from buds at three time points, ES, MS and LS, during flower induction were determined using a NanoDrop ND1000 spectro-photometer (NanoDrop Technologies, Wilmington, DE, USA). A ~2-μg aliquot of total RNA from each sample was prepared and used for cDNA libraries and RNA-seq by the Biomarker Biotechnology Corporation (Beijing, China). The process of library construction was described in detail by Lou et al. (2014), and the three libraries were sequenced using an Illumina HiSeq™ 2000. The files of raw fastq were checked by FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), adaptor sequences were removed using fastqmcf (https://code.google.com/p/ea-utils/wiki/FastqMcf), and the reads were aligned to the apple (*Malus domestica* Borkh.) genome database (Velasco et al. 2010); http://www.nature.com/ng/journal/v42/n10/full/ng.654.html) using TopHat2 (Kim et al. 2013). All usable reads were then

normalized into Fragments Per Kilobase of transcript per million mapped reads (FPKM) values according to Mortazavi et al. (2008). The differences in these transcript abundance values among the samples were calculated using the ratio of FPKM values, and the significance of the differences was computed using the false discovery rate (FDR control method as the threshold of the *P* value in multiple tests) (Yekutieli and Benjamini 2001). The values with a log2 ratio $\geq 2$ and FDR significance score $<0.01$ can be used for later analyses. The methods of assessing the significance of DEGs have been used in our study according to Anders and Huber (2010). A Gene Ontology (GO) analysis was used to identify each gene in the various biological processes, molecular functions and cellular components (http://www.geneontology.org) (Du et al. 2010). Additionally, the KEGG database (fttp://fttp.genome.jp/pub/kegg/pathway) was used for KEGG pathway analyses. GO and KEGG terms with corrected *P* values $<0.05$ were considered significantly enriched.

## Time-series cluster analysis and hierarchical clustering

The time-series cluster method was used to analyze expression profiles of DEGs (Shalom et al. 2014). The raw expression values were converted to FPKM, and then, using a strategy for clustering times-series gene expression data (ES, MS and LS), we defined unique profiles. These expression model profiles were related to the actual or the expected number of genes assigned to each profile's model. Significant profiles have higher probabilities than expected using the Fisher's exact and multiple comparison tests. In addition, hierarchical clustering heat maps were generated by MultiExperiment Viewer software 4.2 (MEV4.2) (http://www.tm4.org/mev.html) using the FPKM values of each gene. The Pearson correlation with complete centroid linkage was adopted for all clustering analyses.

## Statistical analysis

For qPCR results, sugar and hormone levels, and sugar metabolism-related enzyme activities, a one-way analysis of variance with Tukey–Kramer multiple comparison tests was performed using DPS software, version 7.0 (DPS version 7.0; Zhejiang University, Hangzhou, China).

## Supplementary Data

**Supplementary data** are available at PCP online.

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