Transcriptomic and Targeted Metabolomic Analysis Identifies Genes Controlling for Early Bolting and Flowering in Angelica Sinensis

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Abstract

Background: The root of the perennial herb *Angelica sinensis* is a widely used source for traditional Chinese medicines. While the plant thrives in cool-moist regions of western China, early bolting and flowering (EBF) for young plants, significantly reduces root quality and yield. Approaches to inhibit EBF by changes in physiology during the vernalization process have been investigated, however the mechanism for activating EBF has not been identified. Here, transcript profiles for bolted and unbolted plants (BP and UBP, respectively) are compared.

Results: A total of over 72,000 unigenes were detected with *ca.* 2,600 differentially expressed genes (DEGs) observed in the BP compared with UBP. While various signaling pathways participate in flower induction, it is genes associated with floral development and the sucrose pathway that are observed to be coordinated in EBF plants, to coherently up and down regulate flowering genes that activate and inhibit flowering, respectively. Down-stream signal accumulation including gibberellic acids and sucrose metabolites were also monitored by HPLC-MS/MS for EBF plants.

Conclusions: The signature transcripts pattern for the developmental pathways that drive flowering provides insight into the molecular signals that activate plant EBF.

Background

Angelica sinensis is a perennial herb that is distributed mainly in cool-moist regions of western China at elevations ranging from 2,200 to 3,000 m above sea level [1–3]. Roots (Danggui) are prepared as a traditional Chinese tonic reported to nourish the blood and harmonize vital energy. Over 140 root metabolites have been identified, including polysaccharides, organic acids, phthalides, and essential oils [4, 5]. These compounds confer pharmacological activities including: anti-inflammatory, antioxidant, anticancer and cardio-cerebrovascular effects [6–10].

Due to an increasing demand for traditional Chinese medicines, *A. sinensis* is farmed to meet commercial demand [3]. For industrialized planting, seeds are sown in early summer, plants are collected in Fall and overwintering indoors; the following spring, seedlings are planted for vegetative growth and either harvested in Fall of the second year to obtain non-lignified roots (Fig. S1A) or kept in the field till mid-summer of the third year for seed collection (Fig. S1B). Early bolting and flowering (EBF) occur in the second year for up to 40% of the plants, substantially reducing root yield and quality due to lignification and reduced concentrations of bioactive compounds [11, 12].

In order to reduce *A. sinensis* EBF, several internal (*e.g.* seed maturity, seedling age and weight) as well as external factors (*e.g.* altitude, light and nutrients) have been identified [1, 13–15]. For example, the purple stemmed (Mingui No.1) and green-stemmed phenotype (Mingui No.2), albeit quite rare, lower EBF rates compared to the more common purple-stemmed phenotype [3]. And for EBF to occur, the plant must experience vernalization [i.e. an extended period of cool weather (0 to 5^{o}C)] and long-day (LD) conditions (> 12 hr/day)]; thus, avoiding vernalization or LD conditions can reduce EBF [16–18]. Planting medium-

maturity seeds, shortening seedlings rearing time and reducing seedling size can also reduce EBF [15, 16, 19]. Cultivating at higher elevations, reducing nitrogen and phosphorus exogenous applications are both external factors that reduce EBF [13, 14, 16].

The transition from vegetative growth to flowering involves multiple signaling pathways that are transcriptionally regulated including: photoperiodic, autonomous/vernalization, sucrose, and gibberellin (GA) pathways. All pathways converge by increasing the expression of the two meristem identity genes: *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) that is also known as *AGAMOUSLIKE 20* (*AGL20*) and *LEAFY* (*LFY*). *SOC1* and *LFY*, in turn, regulate the floral homeotic genes to produce the floral organs [20, 21]. The photoperiodic pathway is initiated by phytochromes and cryptochromes. The interaction of photoreceptors with a circadian clock activates the expression of the gene *CONSTANS* (*CO*) that encodes a zinc-finger transcription factor that promotes flowering. In the dual autonomous/vernalization pathway, flowering occurs either in response to internal signals, the production of a fixed number of leaves, or to low temperatures that reduces the expression of the flowering repressor gene *FLOWERING LOCUS C* (*FLC*). The sucrose pathway reflects the metabolic state of the plant and sucrose stimulates flowering by increasing *LFY* expression. Lastly, the GA pathway can participate in early flowering and for flowering under noninductive short days.

Besides the previously mentioned developmental pathways that promote flowering, *Arabidopsis* mutants that exhibit early flowering have revealed the involvement of genes that repress flowering. For example, *EMBRYONIC FLOWER1* (*EMF1*) and *EMF2* act as strong flowering repressors [22]; *CURLY LEAF* (*CLF*) by preventing the expression of the floral meristem identity gene *AGAMOUS* (*AG*) during vegetative growth [23]; *EARLY BOLTING IN SHORTDAYS* (*EBS*) participates in the regulation of flowering time by specifically repressing the expression of *FLOWERING LOCUS T* (*FT*) [24]; *Cyclic dof factor 2* (*CDF2*) delays flowering by repressing *CO* transcription [25]; and *MicroProtein 1A* (*MIP1A*) and *MIP1B* repress flowering by forming heterodimeric complexes [26].

During *A. sinensis* seedling vernalization with winter storage, levels of soluble sugars, amino acids and organic acids increase as well as nitrate reductase activity [27]. In contrast, during the photoperiodic stage of plant growth, soluble sugars and protein levels are reduced. The amounts of amino acids, GA_3, zeatin riboside and polyamines as well as the activities of peroxidase and polyphenoloxidase increase in bolting plants (BP) compared with unbolted plants (UBP) [28]. Although *ca.* 5,100 genes have been differentially expressed in the apical meristem of vegetative growth compared to flower buds of early flowering and 13 DEGs were involved in photoperiodic, vernalization, sucrose and GA pathway [29], early bolting-dependent changes that impact genes expression and GAs metabolism have not been mapped and identified. In this study, functional leaves, and lateral roots of BP and UBP were measured by transcriptomic analysis and 40 DEGs associated with EBF were mapped on pathways associated with flower control. Gene expression levels were validated by qRT-PCR and down-stream GA metabolites were profiled by HPLC-MS/MS.

Results

Global gene analysis

A robust data set was collected (Fig. S2) and after data filtering, 60.7 and 52.4 million high-quality reads were obtained for the BP and UBP, respectively; 44.7 and 37.4 million unique reads as well as 7.8 and 6.4 million multiple reads were mapped. From the 72,502 compiled genes and annotated against the databases including NR, SwissProt, KEGG, KOG, and GO (Table 1 and Table 2, Fig. S3-S7), 2,645 DEGs were obtained (Fig. S8). Of these 2,645 DEGs, 369 were unidentified by SwissProt, KOG, GO and KEGG databases. Of the 2,276 identified DEGs, 738 and 846 were up- and down-regulated, respectively. Based on biological function and physiological characteristics, genes were divided into 11 categories: photosynthesis/energy (79), primary metabolism (285), secondary metabolism (80), hormone biosynthesis (34), bio-signaling (201), cell morphogenesis (197), polynucleotide biosynthesis (87), transcription factor (167), translation (119), transport (233), and stress response (102) (Fig. 1). Based on flower driving genes characterized in higher plants [21], 40 DEGs (29 UR and 11 DR) were identified as potential regulatory genes for EBF (Fig. 1).

Table 1
Summary of sequencing data for *A. sinensis* transcriptome

	Bolted	Unbolted
Unfiltered data		
Data of reads number (million)	60.73	52.48
Reads length	150	150
GC (%)	44.69	45.12
Data of reads number × read length (million)	9110	7872
Q20(%)	98.50	98.47
Q30(%)	95.25	95.18
Filtered data[1]		
Data of reads number (million)	60.66	52.41
Data of reads number × read length (million)	9098	7862
Q20(%)	98.56	98.53
Q30(%)	95.34	95.26
Mapped data[2]		
Data of unique mapped reads (million)	44.70	37.40
Data of multiple mapped reads (million)	7.80	6.40
Mapping ratio (%)	86.56	83.57
Compiled data		
Total number of unigenes	72,502	
Total Length (bp) (million)	64.14	
N50 (bp)	1,534	
Max length (bp)	15,601	
Min length (bp)	201	
Average Length (bp)	884	
GC content (%)	41.17	

[1] Reads with a quality score < 30 and length < 60 bp were excluded;

[2] Mapping ratio = (Unique mapped reads + Multiple mapped reads) / Filtered reads.

Table 2
Database searches for collected *A. sinensis* nucleotide sequences

BLASTx searching against specific platforms	Values	Percentage (%)
NR	44,708	61.66
SwissProt	30,471	42.03
KOG	22,959	31.67
KEGG	18,056	24.90
GO	12,473	17.20

DEGs Linked with Bolting and Flowering

Eight DEGs directly participate in floral development including: *SOC1*, *MADS8*, *AGL8*, *AGL12*, *DEFA*, *AP1*, *AP2* and *ANT* (Table 3). The RELs of these genes were consistent with RPKM values, with up regulation of 1.1-, 2.4-, 6.8-, 1.1-, 1.3- and 1.3-fold for *SOC1*, *MADS8*, *AGL8*, *AGL12*, *DEFA* and *AP1*, respectively, in bolted compared to unbolted plants; down-regulation of 0.6- and 0.9-fold was observed for *AP2* and *ANT* (Fig. 2A).

Table 3
Bolting/flowering genes differentially expressed in bolted and unbolted *A. sinensis*.

Gene name	Protein name	log^2 Ratio (B_{RPKM}/UB_{RPKM})
Floral development (8)		
Genes favor flowering		
SOC1	MADS-box protein SOC1	1.06
MADS8	MADS-box transcription factor 8	7.21
AGL8	Agamous-like MADS-box protein AGL8	4.16
AGL12	Agamous-like MADS-box protein AGL12	3.42
DEFA	Floral homeotic protein DEFICIENS	1.11
AP1	Floral homeotic protein APETALA 1	4.29
Genes disfavor flowering		
AP2	Floral homeotic protein APETALA 2	-6.14
ANT	AP2-like ethylene-responsive transcription factor ANT	-3.27
Sucrose pathway (11)		
Genes favor flowering		
SUS1	Sucrose synthase isoform 1	1.31
SUS3	Sucrose synthase 3	1.40
INVA	Alkaline/neutral invertase A, mitochondrial	1.41
INVB	Probable alkaline/neutral invertase B	1.22
INVE	Alkaline/neutral invertase E, chloroplastic	1.09
SUS7	Sucrose synthase 7	-2.70
AMY1.1	Alpha-amylase	1.03
BAM1	Beta-amylase 1, chloroplastic	1.62
BAM3	Beta-amylase 3, chloroplastic	1.05
BAM9	Inactive beta-amylase 9	1.30
Genes disfavor flowering		
INV Inh	Invertase inhibitor	-1.83
GA pathway (7)		

Gene name	Protein name	log^2 Ratio (B_{RPKM}/UB_{RPKM})
Genes favor flowering		
KO	Ent-kaurene oxidase, chloroplastic	2.04
GA20OX1	Gibberellin 20 oxidase 1	1.77
Genes disfavor flowering		
GA2OX1	Gibberellin 2-beta-dioxygenase 1	-1.41
GA2OX6	Gibberellin 2-beta-dioxygenase 6	2.53
GA2OX8	Gibberellin 2-beta-dioxygenase 8	1.65
GAI	DELLA protein GAI	-3.49
GAIP	DELLA protein GAIP	2.15
Photoperiodic induction (14)		
Genes favor flowering		
CO3	Zinc finger protein CO3	2.58
COL2	Zinc finger protein CONSTANS-LIKE 2	3.5
HD3A	Protein HEADING DATE 3A	13.41
FTIP1	FT-interacting protein 1	2.13
FD	Protein FD	3.26
SVP	MADS-box protein SVP	-1.25
Genes disfavor flowering		
COL3	Zinc finger protein CONSTANS-LIKE 3	-2.79
COL16	Zinc finger protein CONSTANS-LIKE 16	-1.33
AS1	Transcription factor AS1	-2.23
CDF2	Cyclic dof factor 2	3.03
MIP1A	B-box domain protein 30	2.58
MIP1B	B-box domain protein 31	3.50
EFM	EARLY FLOWERING MYB PROTEIN	1.12

Eleven DEGs associated with sucrose pathway including: Suc metabolism (*SUS1*, *SUS3*, *SUS7*, *INVA*, *INVB*, *INVE* and *INV Inh*) and starch metabolism (*AMY1.1*, *BAM1*, *BAM3* and *BAM9*) (Table 4) were

transcriptionally regulated so as to favor flowering in BPs. The RELs were consistent with RPKM values, with down-regulated 0.3-fold for the *INV Inh* gene, and up-regulated 1.3- to 6.1-fold for the other 10 genes in the BP compared to the UBP (Fig. 2B).

Table 4
Sequences of primer used in the qRT-PCR analysis

Gene name	Sequences (5' to 3')	Amplicon size (bp)
ACT	Forward: TGGTATTGTGCTGGATTCTGGT	109
	Reverse: TGAGATCACCACCAGCAAGG	
Floral development		
SOC1	Forward: CGAAACGGCGAAATGGACTG	200
	Reverse: CTGAATGTCTTGCCCAGCAG	
MADS8	Forward: GAGATGAGCGGAGGCCAAAG	101
	Reverse: AGCTTCCCCATGTTCTGTTCC	
AGL8	Forward: CGGAGCAACAAAACCAAGAGAG	102
	Reverse: CTCCGATGTTGCGTATGGCTT	
AGL12	Forward: ATGGATGAACTGCATGTGCTTG	191
	Reverse: ACCGGAACATCAATGTTACTAAACC	
DEFA	Forward: ACTCCGGAATGAGATCGGC	116
	Reverse: GCTCGCGTATGATGGCAAC	
AP1	Forward: TCGAAGTCCTGCAACGGAAC	200
	Reverse: CAGTTGGTTGTTCTGCCCC	
AP2	Forward: CTCTTCGACAGCCACCTTCA	163
	Reverse: AGGCTCCAACTGACTCGAAC	
ANT	Forward: GCGAGGCGTAACAAAGACA	153
	Reverse: CTGCTGTGGGAACTGATCTAGC	
Sucrose pathway		
SUS1	Forward: ATGAAGTCCACACAGGAAGCC	112
	Reverse: CGACGACAAGGTGATGAGTG	
SUS3	Forward: GGAGAGAAGACTAACGTGCCT	169
	Reverse: CGCGTAGCACTCTACTAGCC	
SUS7	Forward: TGACTTTATCGTCATCGGTTGG	166

Gene name	Sequences (5' to 3')	Amplicon size (bp)
	Reverse: AGAGCCGTGTTGAGTGTCT	
INVA	Forward: TAAGTTGTGCGGTTTGCGAG	174
	Reverse: ACTACACTCCTACCGCCAGT	
INVB	Forward: TTGCCGTCCTTTTCACCTCC	132
	Reverse: CTCATGCCCAAACTGTGACCT	
INVE	Forward: CAATTCGGGAAATCGAGTCCT	145
	Reverse: CTGCCGCACTAACACTTTCT	
AMY1.1	Forward: AAATCCAGACACCGGCACTC	128
	Reverse: ATCTCCAGCCAACGAATCCA	
BAM1	Forward: GGGAAGCTCAGGTACCACTT	127
	Reverse: GCACACATTTCCTCTGCTCC	
BAM3	Forward: AGTCTCCGAGCAAGAAATTCG	127
	Reverse: TGCTATGTGGCCGAGATGAC	
BAM9	Forward: TTCCCGCAGAAAATCTGTTGA	118
	Reverse: TGCGGAAATTGCTTTTGCGT	
INV Inh	Forward: TCGAGCTTTATGATGATGCCCTC	180
	Reverse: TAGCATTGTTCCTGGCGGTT	
GA pathway		
KO	Forward: ACTTGCTGTGAGTGGCCCG	104
	Reverse: AAGGAGGGAGATTCGAGGGTG	
GA20OX1	Forward: AACCGTTCTACCAAGCGAAT	175
	Reverse: CCGGAGAGAAAGCCTCCTATG	
GA2OX1	Forward: ACCAACACATTGATTCCCCTC	171
	Reverse: GGACATAGGTGAAGAGAAAAACTTG	
GAI	Forward: GTATTCGGCTGGTCCACGTT	144
	Reverse: AGCCAGCCACCTTACCAAAG	

Gene name	Sequences (5' to 3')	Amplicon size (bp)
GA2OX6	Forward: GCCTTGACTTGCTAAGGGTG	152
	Reverse: AGCCTCAGACCAAGAGAGTT	
GA2OX8	Forward: AGTTGGCAGAAATCTTAGCGG	119
	Reverse: CTCCGGTATTGGACATGGTGG	
GAIP	Forward: TGTATCATCCCCCTCCTGCT	132
	Reverse: GCATTCACTATCTCCTTTGCTTCC	
Photoperiodic induction		
CO3	Forward: CTCAGCTTATGGGACCACC	101
	Reverse: CGAGCACGTTGGCGAC	
COL2	Forward: TTCATCCCCAGCAAGAGCAG	131
	Reverse: GGATACAGAAACGCTGTGGC	
HD3A	Forward: GAAAGTCCGAGGCCATCAGC	121
	Reverse: CGGCAAAGTCTCTAGTATTGAATTG	
FTIP1	Forward: AACGCTGACAAAATCACAAGCA	193
	Reverse: GATTCCACTCGGGGTTCACT	
FD	Forward: TACCACCCCCACCACTAAAC	160
	Reverse: AGAGCTGCTGCTAGGGTTG	
SVP	Forward: GCCGGATTAAGTCGCGTGAT	150
	Reverse: TGGGATTAGCTTTCCACCTTTAGA	
COL3	Forward: AAAGGTACAGAGCAAAGCGTG	127
	Reverse: CCTTGAGCTTCATCATTGCGT	
COL16	Forward: GATGGGTCAGCAGAGACGAT	116
	Reverse: TCGCTGTTTGTCATTTCCGT	
AS1	Forward: ATCCCGACCAACCTGCTTC	138
	Reverse: AAGTTGGGAGTGGAGCTGTT	
CDF2	Forward: TCGATCCCCAACAACGAAGAG	196

Gene name	Sequences (5' to 3')	Amplicon size (bp)
	Reverse: ATGTTGTTGGACCACCCCTC	
MIP1A	Forward: GCGATACAAGGGTTCATGCTG	104
	Reverse: GAGTCCGATGAGATAGCGGTG	
MIP1B	Forward: TGATTGCCACGCTCTAACCC	156
	Reverse: GGCCTAATGAGTTGGTTGGTG	
HDR1	Forward: GCAAGAGATCAGGACAGGGAG	146
	Reverse: TGGTTTTCCAGCATTGTGTTTTG	
EFM	Forward: TGACTTGCACCGGAGATTTGT	200
	Reverse: TTTGAACTCGGGCCAACCA	

Flower-regulating DEGs inarticulately expressed with EBF

Since GA accumulation can promote flowering, transcripts that encode for GA biosynthesis were screened for up regulation in EBF plants. The 7 DEGs that are associated with GA signals include: GA biosynthesis (*KO*, *GA2OX1*, *GA2OX6*, *GA2OX8* and *GA20OX1*) and GA mediated signaling pathway (*GAI* and *GAIP*) (Table 3). The RELs of the 7 genes were consistent with RPKM values, with up-regulated 1.1-, 1.02-, 2.3-, 5.2- and 1.3-fold for the genes *KO*, *GA20OX1*, *GA2OX6*, *GA2OX8* and *GAIP*, respectively, in the BP compared to the UBP, and with down-regulated 0.9- and 0.7-fold for the genes *GA2OX1* and *GAI* in the BP (Fig. 3A).

The 14 DEGs that are associated with photoperiodic induction include: *CO3*, *COL2*, *COL3*, *COL16*, *FTIP1*, *FD*, *HDR1*, *HD3A*, *MIP1A*, *MIP1B*, *CDF2*, *SVP*, *EFM* and *AS1* (Table 3). RPKM based expression values of the 14 genes were validated by qRT-PCR, and their RELs were observed to be consistent with RPKM values, with up-regulated 1.3-, 2.0-, 3.3-, 1.2-, 4.4-, 1.2-, 2.2-, 1.7-, 3.7- and 1.8-fold for the genes *CO3*, *COL2*, *FTIP1*, *FD*, *HDR1*, *HD3A*, *MIP1A*, *MIP1B*, *CDF2* and *EFM*, respectively, in the BP compared to the UBP, and with down-regulated 0.7-, 0.98-, 0.9- and 0.8-fold for the genes *COL3*, *COL16*, *SVP* and *AS1* in the BP (Fig. 3B).

Sucrose and GA Accumulation

Flowering can be initiated by the accumulation of active GAs including GA_1, GA_3, GA_4 and GA_7. Interestingly, GA_4 and GA_1 as well as the up-stream precursors GA_9 and GA_{20} had a 3.0-, 1.3-, 5.4- and 4.2-fold increase in BP while the down-stream inactive forms of GA_4 and GA_1, GA_8 had an 1.5-fold increase in UBP (Fig. 4A). Since GA_1 and GA_4 exhibit higher floral induction activity than other GAs that are produced in plants [21], an elevated level of GA_1 and GA_4 may promote EBF. In contrast, an almost 2-fold decrease

in soluble sugars in the BP was unexpected as elevated sugar is usually a driver of flowering (Fig. 4B) [28].

Discussion

The *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) can integrate signals from the photoperiodism, vernalization, sucrose and GA pathways and regulate the expression of *LFY*, which links floral induction and floral development, when associated with other MADS box genes [30]. MADS box proteins regulate different developmental processes including flowering time, floral meristem identity and floral organ development [31]. MADS8 that is structurally related to the *AGL2* family is involved in controlling flowering time [32]. *AGL8* promotes early floral meristem identity in synergy with *AP1* and *CAULIFLOWER* [33]. *AGL12* acts as promoter of the flowering transition through up-regulation of *SOC*, *FT* and *LFY* [34]. *DEFICIENS* (*DEFA*) is involved in the genetic control of floral development [35]. *APETALA1* (*AP1*) and *AP2* are required for the transition of an inflorescence meristem into a floral meristem and promote early floral meristem identity, with *AP1* regulating positively *AG* in cooperation with *LFY*, while *AP2* repressing *AG* by recruiting the transcriptional corepressor *TPL* and *HDA19* [36, 37]. *AINTEGUMENTA* (*ANT*), a member of the *AP2*-like family, is involved in flower organs initiation and development and mediates *AG* down-regulation [38, 39]. Positive regulators of flowering in the floral development pathway were observed to be up-regulated in EBF plants are while genes that disfavor flowering (*AP2* and *ANT*) were down-regulated, suggesting that transcription regulation of these genes may well be a driver for *A. sinensis* EBF.

Suc and its cleavage products glucose (Glc) and fructose (Fru) are central molecules for cellular biosynthesis and signal transduction throughout a plant's life cycle [40]. In this study, Suc synthases (SUSs) that are encoded by three *SUS1*, *SUS3* and *SUS7* genes catalyze a reversible conversion of Suc and UDP to UDP-Glc and Fru [41, 42]; Alkaline/neutral invertases (INVs) that are encoded by three *INVA*, *INVB* and *INVE* genes catalyze an irreversible hydrolysis of Suc to Glc and Fru [43–45]; and the invertase inhibitor (*INV Inh*) inhibits the INV activity by forming a complex with INV [46]. Two kinds of amylase enzymes including α-amylase (AMY) and β-amylase (BAM) could respectively produce α-maltose and β-maltose through the hydrolysis of amylopectin and amylose [47]. In this study, four DEGs encoding amylase enzymes include: AMY1.1 that can increase enzyme activity via accessory binding sites on the protein surface, BAM1 and BAM3 that play important roles in starch degradation and maltose metabolism and BAM9 is inactive due to lack the conserved Glu active site [47–49]. Since the genes (*SUS1*, *SUS3*, *SUS7*, *INVA*, *INVB*, *INVE*, *AMY1.1*, *BAM1*, *BAM3* and *BAM9*) that flavor flowering were up-regulated and the *INV Inh* gene that disflavors flowering was down-regulated, transcriptional regulation of sucrose pathway is consistent with EBF.

While genes associated with GA biosynthesis and GA mediated signaling were differentially regulated in BP versus UBP, the genes did not exhibit coherent transcriptional regulation with EBF, suggesting that transcriptional regulation of GA mediated genes is not a driver of early bolting. For example, with GA mediated signaling, DELLA proteins GA-INSENSITIVE (GAI) and GAIP function as inhibitors by interacting

in large multiprotein complexes that repress transcription of GA-inducible genes [50–52]. Inconsistent with promoting flowering, these genes are transcriptionally up-regulated in BP versus UBP. Inconsistency is also observed in genes that encode GA biosynthesis with a subset of genes up regulated such as *KO*, that catalyzes the conversion of ent-kaurene to kaurenoic acid early in the biosynthetic pathway [53] as well as *GA20OX1* that converts GA12/GA53 to GA9/GA20 [54] later in the pathway (Fig. S13), while *GA2OX8* that catalyzes 2-beta-hydroxylation of GA precursors, rendering them unable to be converted to active GAs is up-regulated under the same condition that promotes flowering (BP). This incoherent transcriptional regulation of GA biosynthesis and signaling for EBF suggests that early bolting may be regulated by events downstream of flowering signaling such as GA and/or sugar accumulation.

While *CONSTANS-LIKE* (*COL*) genes are regulators in the photoperiod pathway and flowering, transcripts in this pathway were also inconsistently induced providing an inarticulate signal for plant flowering. For example, while both *CO3* and *COL3* function as floral activators, the two genes were transcriptionally up- and down-regulated, respectively when comparing BP with UBP. Specifically, *CO3* up regulates the expression of *Heading date 3a* (*HD3A*) and *FLOWERING LOCUS T-LIKE* (*FTL*) under LD conditions [55, 56]. FT-interacting protein 1 (FTIP1) is an essential regulator required for the export of FT protein from the phloem companion cells to sieve elements through the plasmodesmata under LD conditions [57] and was observed to be up regulated in BP. The FT protein acts as a long-distance signal to induce flowering [58] and *FLOWERING LOCUS D* (*FD*) interacts with FT protein to activate the downstream floral meristem identity genes *AP1* to initiate floral development [59, 60]. While this is consistent with flower induction that is observed with BP, there are several transcriptional responses that are not down-regulated at expected. For example, *CDF2* is a transcriptional repressor that delays flowering by repressing *CO* transcription under LD conditions [25] was found to be up-regulated almost 4-fold in BP compared with UBP. *MIP1A* and *MIP1B* that repress flowering by forming heterodimeric complexes that sequester CO and COL proteins into non-functional complexes [26] were also found to be up-regulated in BP. Another inconsistent transcriptional response for flowering is up regulation of *HEADING DATE REPRESSOR 1* (*HDR1*), a flowering suppressor that up-regulates *HD1* in LD conditions [61]. Again, inconsistent regulation of photoperiod pathway transcripts associated with flowering in BP suggests down-stream signaling involvement in early bolting.

Flowering is a process in which plants transition from vegetative to reproductive growth via a complex pathway of signaling networks. The DEGs observed comparing BP and UBP suggests transcription-based regulation of EBF. Specifically, genes associated with floral development and sucrose signaling are transcriptionally correlated with bolting (Fig. 5). For the floral development, *SOC1* can integrate signals from the photoperiodic, GA and sucrose pathways to initiate early floral meristem identity by regulating the over-expression of *LFY*; meanwhile, *AP1* in synergy with *MADS*, *AGL8* and *AGL12* that are repressed by *AP2* and *ANT*, promotes early floral meristem identity. Lastly, the early floral meristem identity induces early bolting and flowering of *A. sinensis* plants. For sugar signaling, over-expression of genes *AMY1.1*, *BAM1* and *BAM3* enhances starch degradation while differential expression *SUS*s, *INV*s and *INV* Inh cleavage Suc to Glc and Fru that can also promote *SOC1* expression.

Conclusions

The DEGs observed comparing BP and UBP suggests transcription-based regulation of EBF. This transcriptomic and analysis focuses on four pathways that can mediate a transition from vegetative to reproductive growth: photoperiodic, GA signaling, autonomous and floral development. While genes associated with EBF have been identified and mapped here, a causative role of these genes in activating and/or regulating EBF will require the knocking out of specific genes via a CRISPR-Cas 9 system.

Methods

Plant material

Mature seeds of 3-year-old *A. sinensis* (Mingui No. 1) (family Apiaceae, alt. Umbelliferae) were permitted to collect from the county-owned garden located in Minxian county (2,520 m asl; 34^{o}28'33"N, 104^{o}05'51"E) of Gansu province, P. R. China in July 2017. The species was identified by professor ling Jin (Gansu University of Chinese Medicine, Lanzhou, Gansu, China). A voucher specimen (No. 20200182) was deposited in the herbarium of College of Life Science and Technology, Gansu Agricultural University, Lanzhou, Gansu, China. Seeds were pre-treated in water (30°C) for 24 hrs. and sown at a soil depth of 0.5 cm located in Minxian county (2,730 m asl; 34^{o}28'8"N, 104^{o}36'22"E) in June 2018. Seedlings were dug up in October 2018, aired in the shade for approximately 15 days and then stored in a natural-rain-proof environment for the winter.

On April 3, 2019, the stored seedlings (root tip diameter 4.5-5.0 mm) were transplanted into pots (diameter 17 cm, depth 20 cm; one seedling per pot) with nutrition matrix and seedlings were greenhouse grown with controlling matrix volumetric moisture content of 60%-70%, light condition of 10-12 hrs. per day and air temperature 15-22°C. No additional fertilizer was applied after the transplant. On July 3, 2019, samples including the second-tip leaves and lateral roots (1:1, g/g fresh weight) from BP and UBP (Fig.S10) were collected (n = 20 plants) and then flash frozen in liquid nitrogen for transcriptomic analysis and GA metabolite analysis.

Total RNA isolation and Illumina sequencing

Total RNA samples were extracted using a Trizol reagent, enriched using Oligo (dT) beads (Invitrogen, CA, USA), fragmented into short mRNA segments (200-700 nt) using a fragmentation buffer and reverse transcribed into cDNA with random primers. Second-strand cDNA was synthesized via DNA polymerase I, RNase H, dNTP and buffer and cDNA fragments were purified using a QiAquick PCR extraction kit, successively repaired-end, added poly (A), and ligated to Illumina sequencing adapters. Finally, the ligation products were sequenced using an Illumina HiSeq^{TM} 4000 platform by Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China).

Sequence filtration, assembly and unigene expression analysis

Raw reads obtained from the Illumina sequencing were further filtered to get high quality clean reads by removing reads containing adapters, more than 10% unknown nucleotides as well as more than 40% low quality (Q-value≤10) bases. *De novo* assembly of clean reads was carried out using Trinity software [62] that combined three components: *Inchworm*, *Chrysalis* and *Butterfly*, respectively for assembling a collection of linear contigs, building graphs for each cluster of related contigs and outputting one linear sequence for each alternatively spliced isoform and transcripts. The expression level of each transcript was calculated and normalized to reads per kb per million reads (RPKM) [63]. In this study, the level of differential expression for each transcript with a criterion of $|\log_2$ (fold-change) ≥ 1 and p value ≤ 0.05 to identify DEGs between BP and UBP.

Basic annotation of DEGs and gene cluster analysis

Unigenes were annotated against the databases including: NCBI non-redundant protein (NR), Swiss-Prot protein, Kyoto Encyclopedia of Genes and Genomes (KEGG), euKaryotic orthologous groups of proteins (KOG), and gene ontology (GO) by using a BLASTx procedure with an e-value $\leq 10^{-5}$ [64]. Molecular Evolutionary Genetics Analysis (MEGA) 7.0 was used for the gene cluster analysis (Fig. S11).

qRT-PCR validation

Total RNA samples from samples of the BP and UBP plants were extracted using a plant RNA kit. Primer sequences of the 40 DEGs (Table 4) were designed with the tools for primer-blast in NCBI. First-strand cDNA was synthesized using a FastKing RT kit with one cycle at 42°C for 15 min and then 95°C for 3 min. PCR amplification was carried out using a SuperReal PreMix with one cycle at 95°C for 15 min, followed by 40 cycles at 95°C for 10 s, 60°C for 20 s and 72°C for 30 s. Melting curves were analyzed after an incubation at 72^{o}C for 34 s. *Actin* was used as an internal standard, the relative expression level (REL) of gene was calculated based on a $2^{-\Delta\Delta Ct}$ method [65].

GA quantification and identification

Five GAs including GA_1, GA_4, GA_8, GA_9 and GA_{20} were quantified and identified using a HPLC (Agilent1290, USA)-MS/MS (QTRAP 6500, AB SCIEX, USA) by Shanghai Biotree biotech Co., Ltd. (Shanghai, China). Representative chromatograms of reference standard of the 5 GAs are shown in Fig. S12, and representative chromatograms of the BP and UBP are shown in Fig. S13. The content of the 5 GAs was calculated based on calibration curves (Table S1).

Soluble sugar measurement

Soluble sugar was measured using a sulfuric acid-phenol protocol [66]. A dried powder (1.0 g) was soaked in 10% EtOH (25 mL) for 72 hrs. at 22^{o}C and then centrifuged (4°C, 8000 r/min, 10 min). Extracts (30 μL) were added into 9% phenol reagent (1 mL), sulfuric acid (3 mL) was added after oscillation and then reacted at 22^{o}C for 30 min. Absorbance was measured at 485 nm, soluble sugar content was evaluated based on mg of Suc.

Statistical analysis

All the measurements were performed using three replicates. A t-test for independent samples was performed and SPSS 22.0 was used, with p 0.05 as the basis for significant differences.

Abbreviations

EBF: early bolting and flowering; BP: bolted plants; UBP: unbolted plants; DEGs: differentially expressed genes; asl: above sea level; GA: gibberellin; LD: long-day; RPKM: reads per kb per million reads; NR: NCBI non-redundant protein; KEGG: Kyoto Encyclopedia of Genes and Genomes; KOG: euKaryotic orthologous groups of proteins; GO: gene ontology; MEGA: Molecular Evolutionary Genetics Analysis; REL: relative expression level.

Declarations

Acknowledgments

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Authors' contributions

ML and JW conceived and designed the study. JL, HX and DY performed all the experiments and analyzed the transcriptome data. ML, PWP, RY and LJ wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during the current study are publicly available at National Center for Biotechnology Information (NCBI), with BioSample: SAMN13379532 and SRA: SRR10524260 to SRR10524265 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA591308?reviewer=pthe4ktocjjigtmm0dh136aa9t).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Figures

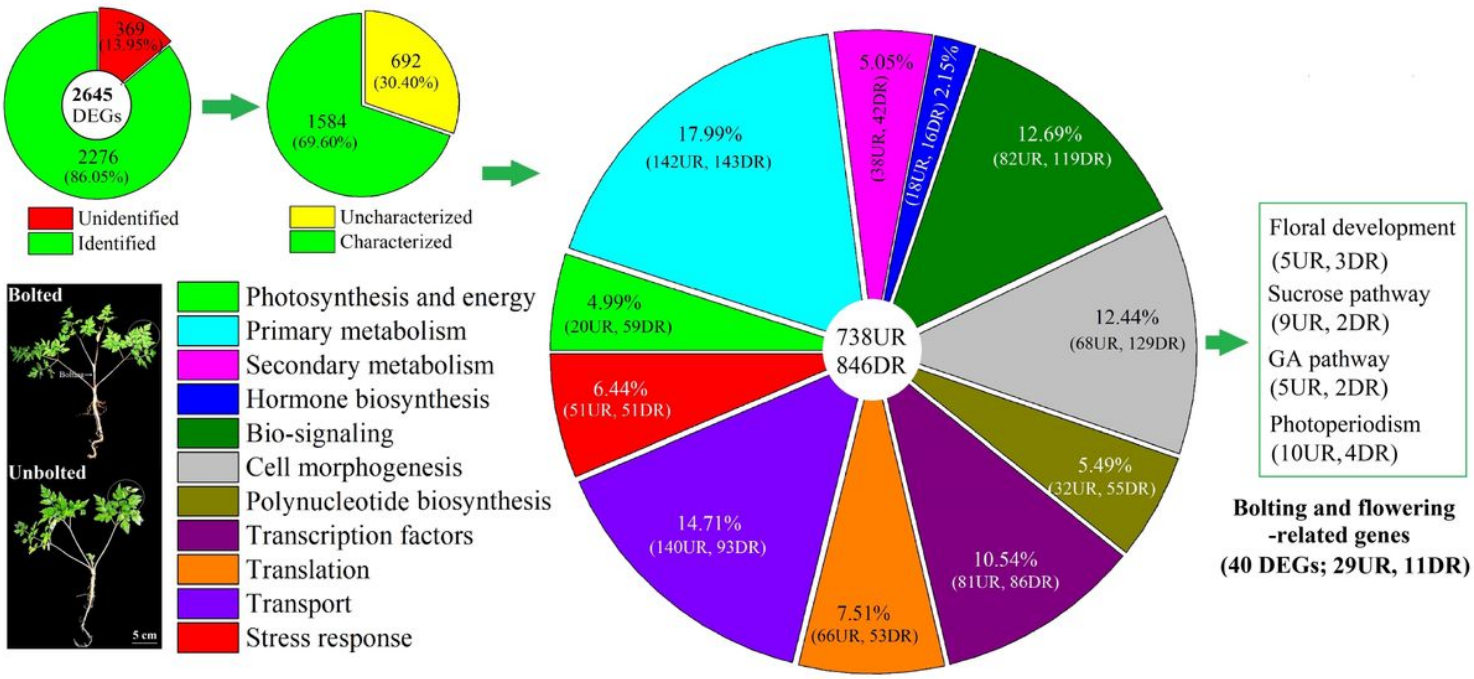

Figure 1

Distribution and classification of differentially expressed genes (DEGs) in bolted versus unbolted A. sinensis (UR, up-regulation; DR, down-regulation).

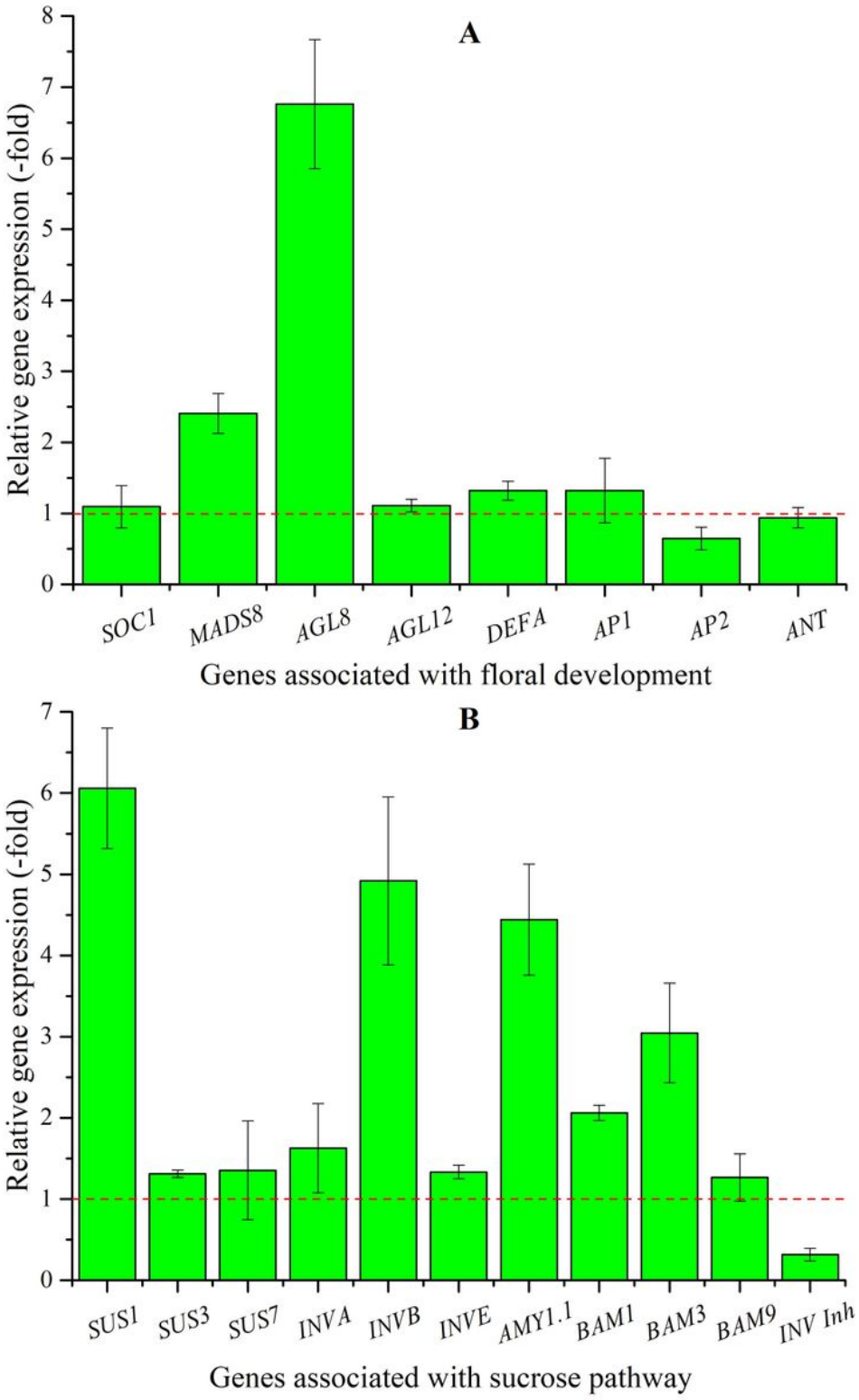

Figure 2

The relative expression level of genes associated with floral development (A) and sucrose pathway (B) in bolted compared with unbolted plants, as determined by qRT-PCR.

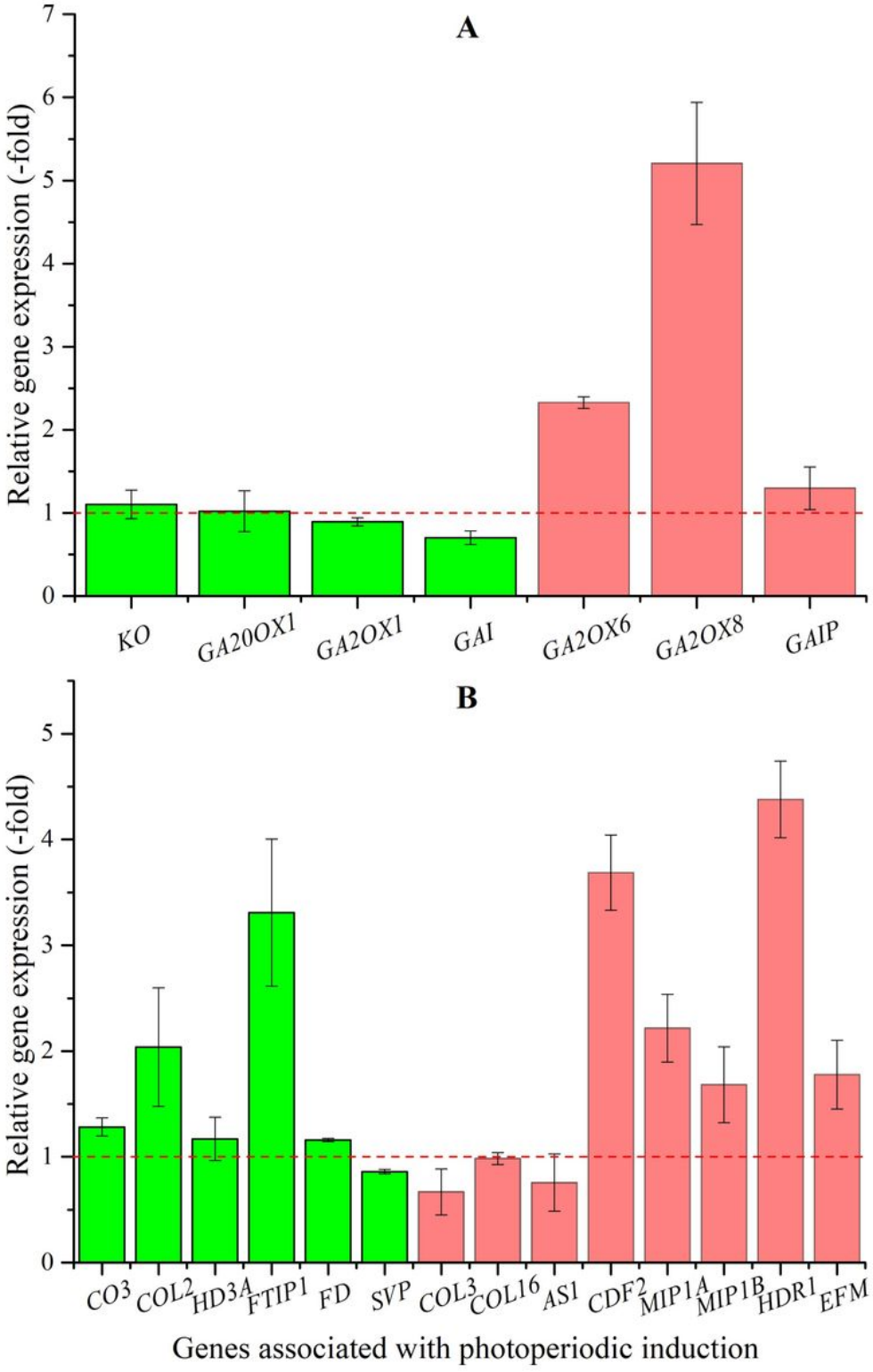

Figure 3

The relative expression level of genes associated with GA (A) and photoperiodic pathways (B) in bolted compared with unbolted plants, as determined by qRT-PCR. Column highlighted in green represents favor flowering and red represents disfavor flowering.

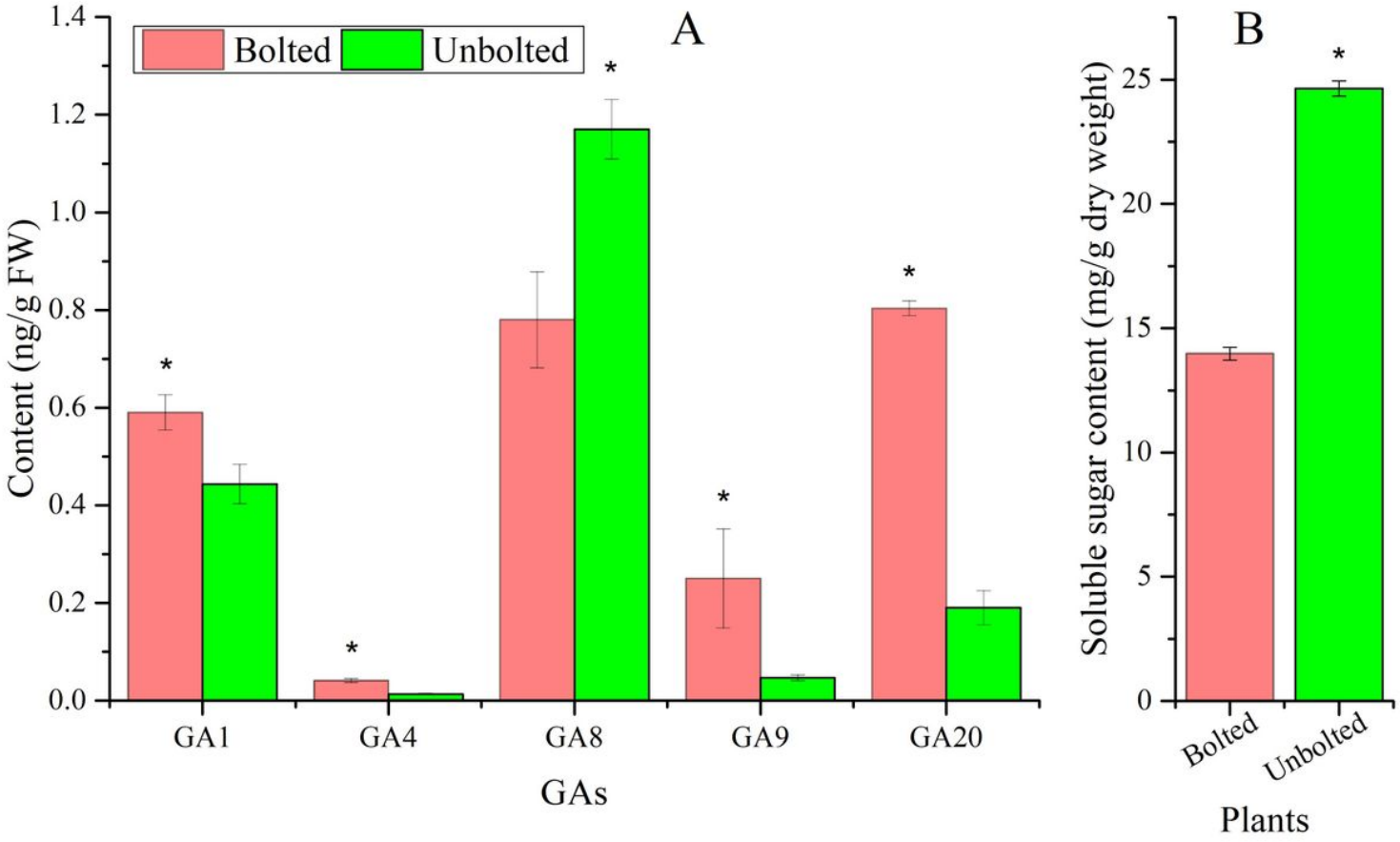

Figure 4

The contents of GAs (A) and soluble sugar (B) in bolted and unbolted plants, as determined by HPLC-MS/MS. An asterisk (*) represents a significant difference (P□0.05) between BP and UBP.

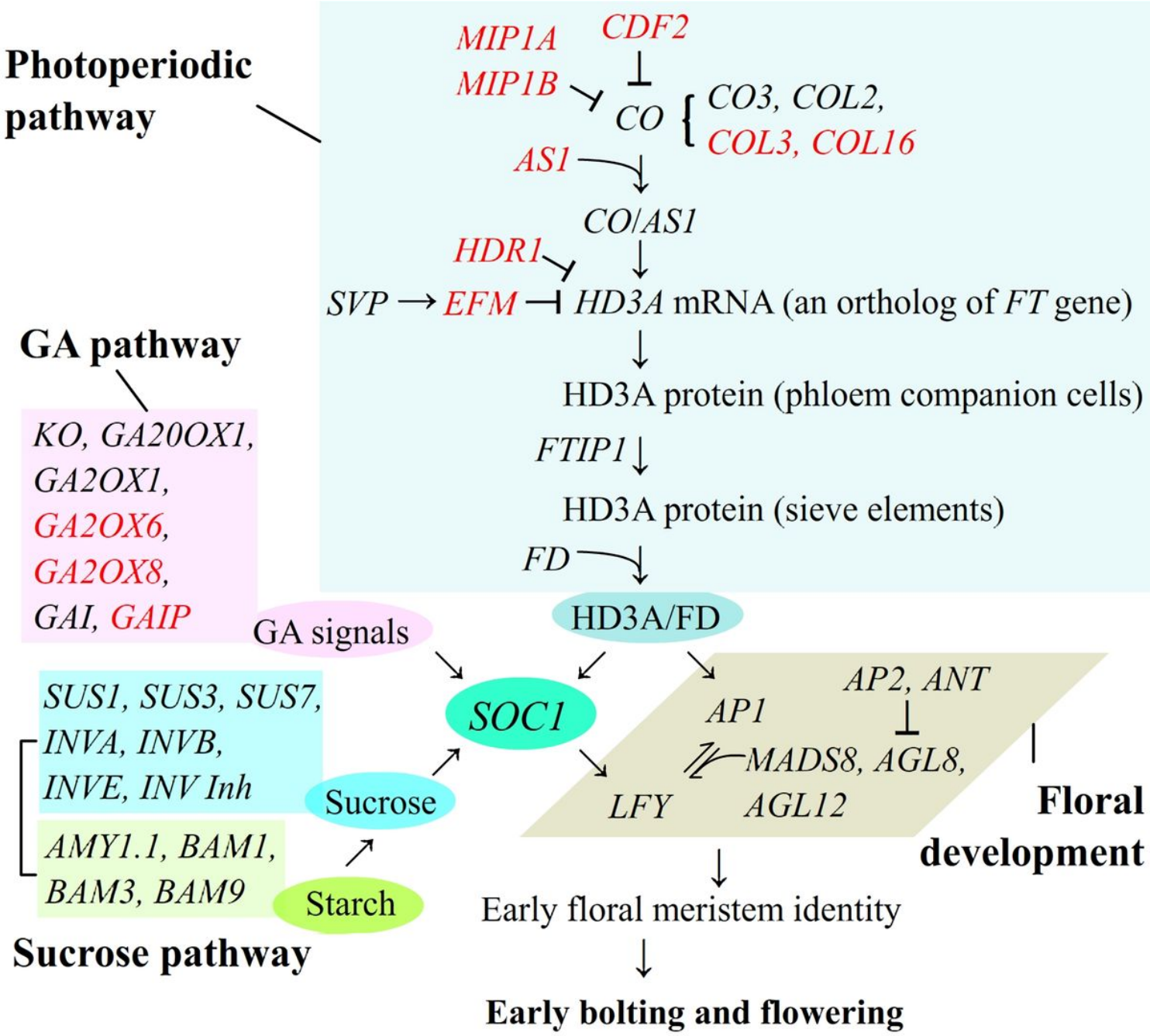

Figure 5

Schematic representation of speculated pathways of DEGs for regulating early bolting and flowering in A. sinensis. Genes highlighted in black represents favor flowering and red represents disfavor flowering.

Supplementary Files

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