

Cross-talk between transcriptome, phytohormone and HD-ZIP gene family analysis illuminates the molecular mechanism underlying fruitlet abscission in sweet cherry (*Prunus avium* L)

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Abstract

Background

The shedding of premature sweet cherry (*Prunus avium* L) fruit has significantly impacted production, which in turn has a consequential effect on economic benefits.

Result

To better understand the molecular mechanism of sweet cherry fruitlet abscission, pollens viability and structure had observed from the pollination trees. Subsequently, the morphological characters of shedding fruitlet, the plant hormone titers of dropping carpodium, transcriptome of the abscising carpodium, as well as the HD-ZIP gene family were investigated. These findings showed that the pollens giving rise to heavy fruitlet abscission were malformed in structure, and their viability was lower than the average level. The abscising fruitlet and carpodium characterized in red color, and embryos of abscising fruitlets were aborted, which was highly ascribed to the low pollen viability and malformation. Transcriptome analysis showed 6,462 were significantly differentially expressed, of which 2,456 genes were up-regulated and 4,006 down-regulated. Among these genes, the auxin biosynthesis and signal transduction genes (*α-Trp*, *AUX1*), was down-regulated, while 1-aminocyclopropane-1-carboxylate oxidase gene (*ACO*) affected in ethylene biosynthesis, was up-regulated in abscising carpodium. About genes related to cell wall remodeling (*CEL*, *PAL*, *PG EXP*, *XTH*), were up-regulated in carpodium abscission, which reflecting the key roles in regulating the abscission process. The results of transcriptome analysis considerably conformed with those of proteome analysis as documented previously. In comparison with those of the retention fruitlet, the auxin contents in abscising carpodium were significantly low, which presumably increased the ethylene sensitivity of the abscission zone, conversely, the abscisic acid (ABA) accumulation was considerably higher in abscising carpodium. Furthermore, the ratio of (TZ + IAA + GA3) / ABA also obviously lower in abscising carpodium. Besides, the HD-ZIP gene family analysis showed that *PavHB16* and *PavHB18* were up-regulated in abscising organs.

Conclusion

Our findings combine morphology, cytology and transcriptional regulation to reveal the molecular mechanism of sweet cherry fruitlet abscission. It provides a new perspective for further study of plant organ shedding.

Background

Abscission is a highly programmed mechanism in which plants remove senescent, injured, infected, or dispensable organs, such as leaves, flowers, petals, sepals, and fruits [1]. The process involves cell separation which occurs in specialized cells in the petiole and pedicel, which is known as the abscission

zone (AZ) [2]. The AZ is consist of several layers of microscopic cells are distinct from the surrounding cells, and these cells originally developed before the organ separation [3]. Earlier studies have shown that a few MADS-box genes have been related to the differentiation of AZ, including *JOINTLESS*, *MACROCAYLYX*, and *SVP* [4–5]. The AZ cells are trend to respond to shedding signals [2]. Under fields conditions, the AZ firmly attaches the organ to the plant body [6]. Once AZ receives the abscission signals, such as development, environmental stresses, senescence, as well as abnormal fertilization, the abscission process will be triggered [7].

Advancement in physiological, genetic, molecular, and biochemical approaches has significantly enhanced the understanding of abscission over the last few decades [8–9]. In particular, in-depth research of model plants such as *Arabidopsis* and tomato makes it easier to understand the mechanism of plant organ shedding mechanism [10–11]. Numerous studies have shown that plant hormones play a vital role in the shedding of plant organs [12–13]. In typical ordinary situations, phytohormones that exist in the plant are in equilibrium. However, Some internal or external factors, such as drought, carbohydrate starvation, pests, diseases, and abnormal pollination, have broken the balance, and this event has become a signal of plant organ shedding [7]. Among these phytohormones, ethylene (ETH) and abscisic acid (ABA) play a positive role in shedding [14]. Nevertheless, auxin (IAA), cytokinin (CTK) gibberellin (GA), and Polyamines (PAs) play a negative role in the abscission process [7]. Usually, the balance between ethylene and auxin plays a crucial role in the shedding process [1]. In addition, the lack of carbohydrates can also contribute to the abscission of plant organs [7, 15]. Genes that synthesize these phytohormones and signal transduction pathways, therefore, also play an essential role in the abscission process of plant organs [16, 17].

Additionally, the plant cell wall is consist of cellulose, hemicellulose, pectin, lignin, and structural proteins [18]. Upon induction, these cells secrete cell wall modifying and hydrolyzing enzymes, which loosen the cell wall and degrade the middle lamella between adjacent cells [2]. Studies have shown that some cell wall hydrolases and helper proteins can damage the structure of plant cell walls and eventually lead to the shedding of plant organs [19–21]. These hydrolases include cellulases (CELs), polygalacturonases (PGs), pectin methylesterases (PEMs) and pectate lyases (PLs). Moreover, some studies have shown that hydrolysis seems essential for cell expansion coordinated by other enzymes like expansins (EXPs), as well as xyloglucan endotransglucosylase/hydrolases (XTHs) [22]. After the cell wall degradation and the plant organs were abscission, the development of a protective layer can limit water loss and cast a physical barrier against opportunistic pathogen attacks [23]. In order to support anatomical findings for the formation of the protective layer in the AZ, it has been shown that stress-related peroxidase activity within AZ is increased, which is assumed to play a role in the lignification process of AZ [24].

A working model for abscission was developed based on several years of plant organ abscission research [25]. Abscission is an organized, regulated developmental process consisting of four discernible steps: (1) differentiation of the AZ; (2) acquisition of competence to respond to abscission signals, (3) activation of plant organs abscission, and (4) post-abscission differentiation of a protective layer. During the whole shedding process of plant organs, transcription regulation also plays a vital role [18]. Therefore,

unveiling the mechanisms of transcriptional regulation will enable a more clear understanding of the onset of abscission [6]. In the stage of AZ differentiation, *JOINTLESS* interacts with the MADS-box gene *MACROCALYX* to regulate the development of the abscission zone in the flower pedicel [4]. During plant hormone signal transduction, the auxin response factor (ARF) and ethylene response factor (ERF) was regulated by auxin and ethylene in the plant hormone signal transduction [1, 26]. Furthermore, cell wall modification enzymes degrade the primary cell wall or middle lamella of AZ tissues to allow abscission organs to easily detach from the parent plant. [6]. For example, the *AtDOF4.7*, which is a transcription factor, which has the functions as a shedding inhibitor to regulate the expression levels of *ADPG2* by binds its promoter. Then the *ADPG2* encodes a cell wall-hydrolyzing enzyme, to degrade the cell wall, and initiate cell separation, induce organ abscission [10]. Additionally, in lychee, when fruits feel a shedding signals ethylene stimulation or carbohydrate deficiency, the expression levels of *LcHB2* are up-regulated. The *LcHB2* binds the promoters of *LcCEL2* and *LcCEL8* induced their expressed in the flower abscission zone, and the cellulase activity increases. As a result, the cellulose contents are reduced, and eventually the fruitlet shedding due to the cell wall degradation and cell separation in the flower abscission zone [27].

Sweet cherry (*Prunus avium* L.) is one of the most popular fruits containing various sugar (glucose, fructose, sucrose, and sorbitol), dietary fiber [27], and melatonin [28], Additionally, it also is rich in melatonin and dietary fiber, simple sugars, sweet cherry also contains various vitamins, minerals primarily potassium, phenolic compounds (flavonoids and anthocyanins). Moreover, eating sweet cherries can reduce the risk of cancer and joint pain, as well as prevent neurodegenerative diseases[29]. Owing to productive nutritional value of sweet cherry, it is extensively cultivated in countries such as China, America, and Japan, Chile. However, fruitlet shedding dramatically reduces yield and economic efficiency. Therefore, efforts on the unveiling molecular mechanism of fruitlet abscission in sweet cherry play a vital role in high-yield breeding varieties.

In the present study, the pollen vitality of sweet cherry pollination tree has been determined, the transcriptome and phytohormone content of abscising carpodium have been analyzed and the transcriptome was verified by qRT-PCR, the HD-ZIP gene family was be analyzed. (1) This study found that the pollen vigor of the pollination tree is low, and the structure of the pollen electron microscope is deformed; (2) The transduction of plant hormone signals and genes linked to cell wall modifications is significantly up-regulated in carpodium abscission; (3) the *PavHB16*, cell wall remodeling protein, and plant hormone biosynthesis and signal transduction were regulated significantly between transcriptome and proteome. (4) the *PavHB16* and *PavHB18* were up-regulated in abscising organs (flower, fruit, carpodium, leaf, and petiole). These findings contribute to our understanding of the sweet cherry fruitlet abscission transcriptional regulatory mechanisms.

Results

Pollen vitality of the main pollination tree

The pollen vigor showed that the pollen germination rate of the pollination tree, was 15.56% (Figure.1 b). According to pollen's morphological characteristics, it can be divided into two types, in which the pollen is oval, the plump pollen was called type I (Figure.1 c), and the pollen is irregular and withered, we called type II (Figure.1 d). The statistical data showed that the type I pollen accounted for 11.19% in the pollination tree (Figure.1 b). This result suggests that pollen vigor can be correlated with pollen morphology. And this lower pollen vigor of pollination trees will contribute to embryo abortion, which is a cause for abscission from sweet cherry fruitlet.

Morphology and anatomical structure of abscission fruit

According to fruitlet morphological character observations, the morphological structure of shedding fruits and carpodiums was red and the embryos were dried out. In contrast, the retention of fruits and carpodiums was green and the embryos were full (Figure.2a). Additionally, the weight of abscising embryos was also significantly lower than that of non-abscising embryos (Figure.2b). These results suggest that the embryo of the shedding fruit has aborted, which can trigger hormone imbalance and lead to the fruitlet being prematurely shedding. Such findings indicate that there be an excellent correlation between the shedding of sweet cherry fruitlet and embryo abortion.

Endogenous hormone analysis of abscising carpodium

To further study the hormonal regulation of physiological fruitlet abscission in sweet cherry, endogenous levels of Auxin: Indole-3-Acetic Acid (IAA) and Indole-3-butyric acid (IBA), gibberellins (GAs): GA3, GA4, and GA7, cytokinins: trans-zeatin (TZ), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylate were (ACC), jasmonic acid (JA), and Methyl jasmonic acid (MeJA) analyzed between the abscising carpodium and non-abscising ones in the fruitlet development stage, which precede the physiological abscission. The auxins (IAA, IBA) levels in the abscising carpodium were reported to be low than the non-abscising carpodium. Out of the three GAs analyzed, GA4 was detected at similar levels in both abscising carpodium and non-abscising carpodium, while the GA3 and GA7 levels significantly decreased in abscising carpodium. Cytokinins (TZ) levels were found to reduce significantly in abscising carpodium. However, the abscisic acid level was markedly increased in abscising carpodium. Incredibly, the levels of 1-aminocyclopropane-1-carboxylate were (ACC), jasmonic acid (JA), and Methyl jasmonic acid (MeJA) decreased significantly in abscising carpodium. To observe the balance of plant hormones comprehensively, the ratio of $(TZ + IAA + GA3) / ABA$ was calculated, and the results showed that the rate in abscising carpodium was significantly lower than that of non-abscising carpodium (Figure. 3). These results suggest that the reduced auxin may increase the sensitivity of abscission zone response ethylene, and the increased ABA content will also accelerate the abscission of sweet cherry fruitlet. However, in the abscising carpodium, the content of GA3 and CTK related to inhibition of sweet cherry fruitlet abscission in lower. All these hormone changes may be the reasons for sweet cherry fruit shedding, while the auxin decline decrease may be the main key reason.

Transcriptome profiling and identifying differentially expressed genes (DEGs)

To obtain comprehensive and efficient transcriptome information for the sweet cherry fruitlet abscission, the transcriptomes of abscising carpodium and non-abscising carpodium were analyzed using the RNA-seq. Before RNA-seq analysis, six cDNA libraries were constructed and generated paired-end sequence reads using the Illumina HiSeq 4000 platform. The raw data have been deposited in NCBI Sequence Read Archive (SRA) through Gene Expression Omnibus (GEO) (access number: PRJNA636209). A sum of 3.07 billion raw reads being generated and each sample provided an average production of 51.33 million. A mean of 50.54 million clean reads was obtained from each library with an adequate read ratio of 98.48 percent after eliminating adaptor sequences, low-quality, and N-containing reads. (Additional file 1: Table S1). The reference genome exactly matched the average mapping ratio of 89.87 percent to roughly 45.41 million clean reads across each library. The Pearson correlation coefficient with all gene expression levels between every three samples was determined to investigate the gene expression correlation between samples. (Additional file 2: Figure. S1).

A total of 43,673 genes were mapped from all the samples. Abscising and non-abscising carpodium gene expression levels were analyzed, and 6,462 differentially expressed genes (DEGs) were recognized. (Additional file 3: Table S2). Among such DEGs, the abscising carpodium had 2,456 DEGs up-regulated and 4,006 DEGs down-regulated. (Figure.4 a). The number of related down-regulated DEGs was higher than that of up-regulated ones; It is worth pointing out that the plant hormone signal transduction pathway and the galactose metabolic pathway were significantly enriched, and these two metabolic pathways may play a regulatory role in plant organ shedding(Figure.4 b).

Enrichment analysis of DEGs during carpodium abscission

Study of the KEGG pathways was also performed; according to the enrichment results, the top 20 pathways between abscising and non-abscising carpodium were shown in Figure. 4 b and Additional file 4 Table S3. In these pathways, the plant hormone signal transduction and galactose metabolism were involved, which may regulate sweet cherry fruitlet abscission. Besides, the auxin and ethylene biosynthesis relate pathways were also enriched (Additional file 4 Table S3). Additionally, some pathways associated with cell wall modification were also augmented. These results suggested that plant hormone biosynthesis, plant hormone signal transduction, and cell wall modification play crucial roles in fruitlet abscission regulation. There were other pathways including biosynthesis of amino acids, carbon metabolism, and phenylpropanoid biosynthesis was found to be enriched.

Gene Ontology (GO) classification showed that 2,596 DEGs between the abscising and non-abscising carpodium were graded into three categories: biological process, cellular component, and molecular function (Additional file 5 Table S4). In the biological process category, the metabolic process and the single-organism process were the most abundant terms. For the cellular component category, membrane, cell, and cell parts were the main terms. The top three terms of molecular function were bind, catalytic, and transporter activity (Figure.5).

Plant hormone biosynthesis and signal transduction

The KEGG enrichment analyses showed “plant hormone signal transduction” was a significant pathway. In the auxin signal transduction pathway, the down-regulated significantly genes include auxin influx carrier (AUX1), Auxin/Indole-3-Acetic Acid (AUX/IAA), auxin response factor (ARF), and small auxin up RNA (SAUR). In the cytokinin signal transduction pathway, the cytokinin receptor CRE1 was down-regulated. However, in the ABA signal transduction pathway, the PP2C and SnRK2 were up-regulated in abscising carpodium. Simultaneously, in the ethylene signal transduction pathway, the ethylene insensitive 3 (EIN3) and ethylene response factor (ERF) were up-regulated which may improve the fruit abscission. Also, some essential genes are differentially expressed in the plant hormone synthesis pathways. These genes include tryptophan synthase alpha chain (α -Trp), which is related to auxin biosynthesis. Additionally, the 1-aminocyclopropane-1-carboxylate synthase and 1-aminocyclopropane-1-carboxylate oxidase were up-regulated in abscising carpodium.

Cell wall remodeling related genes

Cell wall remodeling was one of the reasons regulated abscission. Our RNA-Seq analyses of abscising carpodium and non-abscising ones showed bidirectional changes in the expression of the cell wall remodeling genes. This phenomenon may be associated not only with the ongoing process of abscission but also with the progressive development of the organs that are not dropped (Additional file 6 Table S5). Among these cell wall remodeling-related DEGs, there were 4 cellulases (CELs), 7 polygalacturonases (PGs), 5 pectinases (PEs), 7 peroxidases (PODs), 2 beta-galactosidase (BGALs), 5 expansins (EXPs), and 5 xyloglucan endotransglucosylase/hydrolase (XTHs) was up-regulated, which may be regulated the cell wall remodeling. These results indicate that cell wall remodeling-related genes play a vital role during the fruitlet abscission. Due to the differential expression of these cell wall remodeling enzyme genes, the cell wall was remodeled, leading to the degradation of the cell wall or middle lamella, which leads to cell separation and fruitlet shedding.

Transcription Factor

Transcriptional regulation plays a pivotal role in the complex series of events leading to plant organ abscission. Therefore, transcription factors also enact an imperative role in the process. According to our data, there were 8 types of transcription factors that deserve attention, namely NAC, ERF, MYB, bZIP, WRKY, bHLH, MADS, HD-ZIP, which may regulate the shedding of sweet cherry fruitlet. Among these transcription factors, the most significant number of differential expressions was MYB, followed by WRKY and HD-ZIP (Figure.6). It is noteworthy that the genes of these three gene families are likely to regulate the shedding of plant organ abscission. Moreover, the HD-ZIP family has been shown to regulate shedding in litchi by regulating plant hormone biosynthesis genes and cell wall modification linked enzyme genes.

Cross-talk between carpodium transcriptome and proteome

To find out more precisely the primary genes of sweet cherry fruit shedding, this study conducted a joint proteomics and transcriptomics analysis. The differentially accumulated proteins (DAPs) with 1.5-fold and differentially expressed genes with 2 folds are being used for cooperative investigation. There were 337 genes/proteins common differential expression between the transcription level and the protein level. Among these genes, there were 166 genes frequent up-regulation and 133 genes common down-regulation (Fig. 7 a). It is evident that in the plant hormone biosynthesis and signal transduction pathways, three genes a linked related to ethylene biosynthesis, 1-aminocyclopropane-1-carboxylate oxidase (ACO), were identified to be up-regulated in the abscising carpodium; while one auxin efflux carrier (AUX1) gene was down-regulated which might involved in auxin transport. More significantly, enzymes relevant to the plant cell walls remodeling, such as cellulose, pectin acetylerase, and polygalacturonase have been up-regulated. Likewise, peroxidase associated with lignin biosynthesis in plant cell walls is also being up-regulated. However, some tubulins related to cell wall synthesis including tubulin alpha chain (α -TUB), tubulin beta chain (β -TUB), and microtubule-associated proteins (MAPs) showed a downward trend. Excitingly, a homeobox leucine zipper transcription factor (*PavHB16*) was up-regulated at both transcription and protein levels (Figure.7 b).

Verification of differential expression gene by quantitative real-time PCR

To confirm findings of gene expression obtained from transcriptome data, 15 DEGs concerned to plant organ abscission were chosen for qRT-PCR. These DEGs are mainly involved in phytohormone biosynthesis, plant hormone signal transduction, plant cell wall remodeling. As shown in, the 15 DEGs had very similar expression patterns based on the transcriptome data and qRT-PCR results, which indicates the trustworthiness of the transcriptomic analysis (Additional file 7 Table S6 Figure. 8).

Identification and expression profiling of HD-ZIP gene family

According to previous studies, the HD-ZIP gene family plays an influential, role in the shedding of plant organs. To explore the relationship between HD-ZIP and sweet cherry fruitlet shedding, this study identified the HD-ZIP gene family of sweet cherry. The phylogenetic tree was constructed with 27 HD-ZIP TFs in sweet cherry plants and 55 ZmHD-ZIP TFs in *Zea mays* (Figure.9 a). The 27 PavHBs proteins were divided into 4 classes, namely HD-Zip I, HD-Zip II, HD-Zip III, as well as HD-Zip IV, and the proportion of each subgroup of HD-ZIP was calculated in the sweet cherry plant. HD-Zip I (ten members), which accounted for 37%, was the largest group of PavHB TFs, followed by HD-ZIP IV and HD-ZIP II, with 26% (seven members) and 22%(six members), respectively; the smallest was HD-Zip III (four members), with just 15%. These sweet cherry HD-Zip TFs were divided into four subgroups based on the *Zea mays* classification (I, II, III, and IV). (Mao et al., 2016). According to the gene structure analysis, HD-Zip III and HD-Zip IV had more motifs than the other two groups, and most proteins in HD-Zip III had 15 or 16 motifs. Additionally, the same subfamily the similar motifs (Figure. 10). Among these HD-ZIPs, it is noticeable that *PavHB16*, which was up-regulated in the transcriptome and the proteome, belongs to the HD-ZIP I subgroup.

Discussion

In the present study, we combined the morphology, transcriptome, proteome, phytohormone and HD-ZIP gene family profiling to reveal the molecular mechanism of sweet cherry fruitlet abscission. Our research reveals that the abscission of sweet cherry fruitlet might be associated with the plant hormone biosynthesis, cell wall remodeling, cytoskeleton, and transcription factor. Interestingly, we found a transcription factor that may be related to sweet cherry flowers, leaves, and fruits. Understanding the shedding of plant organs at the molecular level is of great significance for sparse flowers and fruits and mechanical picking.

Pollen abortion leads to fruitlet shedding

As known, the healthy development of the embryo makes the hormone of fruit in a relatively stable state, subsequently promotes the further growth of the fruit. On the contrary, the embryo abortion could substantially lead to the shedding of the fruit [33]. However, the natural development of the embryo highly depends on successful fertilization [34]. When the fertilization has failed, this will lead to embryo abortion, which will trigger the hormone imbalance and fruit shedding. Numerous studies have found that the regulation of endogenous hormones is closely related to the embryonic development of plants [35]. In this study, the sweet cherry 'Santina' is a cross-pollinated variety. Therefore, the pollen vigor of its pollination tree plays a vital role in embryo development. This analysis found that Santina's pollination tree's pollen germination rate was just 15.56%.

Moreover, the pollen morphology showed that most of the pollen was deformed. This trend indicates that sweet cherry embryos abortion may be affected by irregular pollen from pollinating trees. Previous research has shown premature fruit drop can be caused by several factors, like an example lack of pollination or embryonic degeneration [36]. Therefore, for cross-pollinated fruit trees, the perfect development pollen of pollination trees plays a fatal role in the regular fruit setting.

Phytohormones involve in the fruitlet abscission of sweet cherry

In the whole process of abscission, the regulatory effects of phytohormones are of significant relevance since they mediate responses of plant organs to stress [7]. Phytohormones perhaps play a role in promoting or inhibiting shedding signals depending on the different tissues, the concentrations, the homeostasis and the affinities of their receptors, their transport or their interactions with each other, and responses are complex [37]. Several phytohormones, including ethylene (ETH), abscisic acid (ABA), jasmonic acid (JA), and methyl jasmonate (MeJA), act as abscission-accelerating signals [7, 38], while auxin, gibberellins (GA), cytokinin (CTK) and polyamines are considered as abscission inhibitors [7, 39]. Since phytohormones are involved in the entire plant development cycle, several genes that control abscission also form part of the biosynthetic phytohormone and signal transduction pathways, or influence their metabolism [12, 40]. Though the roles of the many phytohormones remains unclear,

auxin/ethylene balance and ABA have been proved to touch off shedding [41]. At present, the concentrations of auxin in abscising carpodium was substantially lower than that of retention carpodium.

Moreover, increased abscisic acid may also be responsible for regulating the shedding of carpodium. However, the decreased ACC does not represent the ethylene concentrations lower. Although the ACC concentrations lower in the abscising carpodium, If the 1-aminocyclopropane-1-Carboxylate Oxidase (ACO) content increases, it would lead to an increase in ethylene concentration [16]. Therefore, the ACO plays a vital role in the ethylene biosynthesis, and the transcriptome result showed that the ACO was up-regulated. Additionally, the reduction of auxin content can increase the sensitivity of the abscission zone to ethylene [42]. Hence, once the auxin content in the abscission zone decreases, the sensitivity of the abscission zone to ethylene will increase, and even a little of ethylene will trigger shedding [43]. Therefore, auxin content in the abscission zone plays a vital role in regulating shedding.

Transcription factors regulate fruitlet abscission of sweet cherry

Multiple genes regulate the shedding of plant organs, and transcription factors also play a critical role [18, 25, 27]. In the shedding of the tomato flower organ, the *jointless*, which is the MADS-box gene family plays an important role in the differentiation of the abscission zone [44]. Also, during the process of litchi fruitlet abscission, when fruitlet abscission begins or is induced by girdling plus defoliation (GPD) or ethylene (ETH) treatment, *LcHB2/3* was induced, which then accelerate the biosynthesis of ethylene and abscisic acid by directly binding to the promoters of *LcACO2/3*, *LcACS1/4/7*, and *LcNCED3* genes [41]. In recent research, some transcription factors of the MADS family have also been significantly up-regulated, which may also play a necessary role in the differentiation of the abscission zone. The most noteworthy is the genes of the homeobox family, especially *PavHB16* and *PavHB18*, which belongs to the HD-ZIP I subfamily and has a close relationship with *LcHB2* in litchi [41]. Moreover, according to sequence alignment analysis, the promoter of the sweet cherry gene *PavCEL* gene of sweet cherry and the promoter of the *LcCEL2* of litchi have the same sequence (AAATTAAA) that can be combined with the *LcHB2* (Additional file 9). Therefore, it can be speculated that the genes of the HD-ZIP gene family play an extensive role in regulating the shedding of sweet cherry.

Cell wall remodeling implicates in fruitlet abscission of sweet cherry

The remodeling of cell walls that accompanies the shedding of plant organs, so genes related to cell wall remodeling are particularly important in promoting the shedding of plant organs. The genes of cellulase, pectinase, polygalacturonase, expansin, xyloglucan endotransglucosylase / hydrolase, and peroxidase were found to be significantly up-regulated during the abscission cycle of citrus, litchi and tomatoes. [2, 45, 46]. Additionally, in litchi, it was proved that *LcCEL2* and *LcCEL8* could be regulated by *LcHB2*, thereby promoting the litchi shedding [20]. Through this research, we also found that several genes linked to cell wall remodeling were significantly up-regulated in the abscising carpodium that was about to

shedding. The reason why these cell wall modifying enzyme genes can be associated with the shedding of plant organs is that each protein they encode has a specific role. The primary function of cellulase is to hydrolyze cellulose which is the main component of the cell wall [47]; both pectinase and polygalacturonase are involved in the hydrolysis of pectin [48]; Xyloglucan endotransglucosylase/hydrolase mainly hydrolyzes the hemicellulose in the cell wall, and the expansin plays a fatal role in the ripening and softening of the fruit [22]; Besides, peroxidase plays a crucial role in the synthesis of lignin; in the study of plant organ shedding, although the relationship between the accumulation of lignin and the shedding of plant organs has not been found, it was found that lignin is produced in other plants in the abscission region [22]. This evidence showed that peroxidase plays an important role in promoting lignin synthesis. Therefore, the up-regulated expression of the peroxidase gene may play a positive role in the shedding of plant organs. In recent research, the genes of cellulase, pectinase, polygalacturonase, expansin, xyloglucan endotransglucosylase/hydrolase, and peroxidase were found to be significantly up-regulated in the abscising carpodium. In addition, previous proteomic studies have also shown the same results [49]. These results suggested that the remodeling of cell walls might promote the sweet cherry fruitlet shedding.

The cytoskeleton regulate fruitlet abscission of sweet cherry

The cytoskeleton is the fundamental component of a cell and plays a crucial role in the entire cell development process. Furthermore, the pectin transport and its modifying proteins occur principally through the actin cytoskeleton. Therefore, it is unsurprising that defects in actin filament organization affect cell adhesion. The Actin-related protein2/3 complex (Arp2/3) is highly conserved and is the prime component in regulating branching and nucleation of actin filaments [50]. Additionally, the microtubule skeleton is the basic skeleton that constitutes a cell [51]. And there have been reports that the arrangement of microtubules has also been related to plant organ shedding [44]. However, we must correlate the cytoskeleton with plant organ shedding. In this study, some cytoskeleton-related genes were down-regulated. This result implies that the formation of plant cells is hindered, which in turn leads to programmed cell death and the shedding of plant organs. In addition, the proteomics at the early stage of this research group also showed that a close relationship existed between the cytoskeleton and the shedding of sweet cherry fruit[49].

Conclusion

A variety of reasons causes the shedding of plant organs. Because the sweet cherry can abscission when they are over-mature, it is essential to distinguish the abscission zone during the development phase. However, owing to the pollination tree's malformed pollen, pollen tube germination was restricted, and normal pollination and fertilization could not be completed, which led to the abortion of the embryo. The embryo abortion causes imbalances in plant hormones, such as increased ethylene production and decreased auxin production. Reduced auxin content will increase the sensitivity of the abscission zone to ethylene, thereby activating the expression of transcription factors related to plant organ shedding in

cells. The transcription factors were translated into proteins and then bound to the promoters of genes, which can degradation cell wall. After that, the cell wall degradation-relate genes, such as *CEL* and *PG*, were initiated, thereby increasing the content of these cell wall degrading enzymes. Under the suitable pH and temperature, these enzymatic activity rises, consequently degrading the middle lamella and cell wall, which persuade sweet cherry fruit dropping. It is worth noting that the transcription factors of the HD-ZIP gene family may play an indispensable role in regulating the shedding of plant organs. In the future, the *PavHB16*, *PavHB18* and other transcription factors will be studied in detail, to further reveal the molecular regulation mechanism of sweet cherry fruitlet shedding (Figure. 13).

Methods

Plant materials

The sweet cherry 'Santina', grown in Weining County, Guizhou Province, China (E:104.12, N: 27.25) was used as the material, and the abscising carpodium and the non-abscising carpodium were taken. Three plants with similar growth vigor were selected, and abscising carpodium and non-abscising carpodium were taken at 20 days after flowering (20DAF). This carpodium were quickly frozen in liquid nitrogen and brought back to the laboratory for storage in of -80 °C refrigerator. The pollen of 'Brooks', the pollination tree of sweet cherry 'Santina', was used to detect the pollen viability and morphology characters. Different tissues of sweet cherry 'Santina' were used for tissue-specific expression pattern analysis.

Determination of pollen germination ratios and morphology characters.

In vitro pollen germination was performed according to previously published methods, which be modified slightly [31], the pollen germination rate was determined by *in vitro* culture. The medium formula was: MS medium + 10% sucrose + 0.5% agarose + 0.1 g/L of boric acid. Briefly, dropping the medium in the groove on the glass slide, after it was solidified, the pollen was sprinkled on the medium with a dissecting needle, placed in a petri dish with moist filter paper and covered, and placed in a 25 °C incubator. Four hours later, the pollen germination rate was counted after microscopic observation under the microscope. The pollen tube length was greater than the pollen grain diameter, which was recorded as germination. Otherwise, it was recorded as no germination, and three replicates per treatment. Each replicate was randomly selected for 3 fields of view, and the number of pollens per field was counted. Percentage pollen germination was calculated using the formula:

$$\text{germination rate (\%)} = \frac{\text{Number of germinated pollen}}{\text{Total number of observed pollen}} \times 100\%.$$

Scanning of electron microscopy sample preparation, spreading pollen evenly on a metal stage with double-sided tape, spraying gold by conventional vacuum spraying for 3~4 min and placing it under the

scanning electron microscope (HITACHI, S-3400N) (working voltage 30kV).

Extraction and determination of hormone levels

The contents of, IAA, IBA, GA3, GA4, GA7, ABA, ACC, JA, and MeJA were determined by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Approximately 0.5 g carpopodium were ground in a pre-cooled mortar that contained a 5 mL extraction buffer composed of isopropanol/hydrochloric acid. The extract was shaken at 4 °C for 30 min. Then, 10 mL of dichloromethane was added, the sample was stirred at 4 °C for 30 min, and centrifuged at 12,000 g for 5 min at the same temperature. Later, extracted the lower organic phase. The organic phase was dried under N₂, then dissolved in 200 µL of methanol (0.1% methane acid), and filtered through a 0.22 µm filter membrane. The purified product was then analyzed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). ZORBAX SB-C18 (Agilent Technologies) chromatographic column (2.1mm 150 mm;3.5mm) HPLC analysis was performed. The solvent of mobile phase A consists of methanol /0.1% methanoic acid, and the solvent of mobile phase B consists of ultrapure water /0.1% methanoic acid. The injection volume is 2 L. The MS conditions were as follows: the spray voltage was 4500 V; the pressure of the air curtain, atomizer, and auxiliary gas was 15, 65, and 70 psi respectively. The atomization temperature is 400 °C.

Total RNA Extraction, Illumina library construction and sequencing

Total RNA was extracted using the RNA prep Pure Polysaccharide Polyphenol Plant Total RNA Extraction Kit (Tiangen, Beijing, China), following the manufacturer's protocol. Three biological replicates were performed. RNA quality and concentration were measured using a Nanodrop 2000 micro-spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and agarose gel electrophoresis. The integrity of RNA was assessed using an Agilent 2100 bioanalyzer (Agilent Technologies). The mRNA was enriched using magnetic beads with Oligo (dT) and cleaved into short fragments (~200 nt) in fragmentation buffer. The reverse transcription was conducted with random hexamer primer and then the second-strand cDNA was synthesized. After end repair, the 5' tails were phosphorylated, the 3' tails were added with an adenine. Sequencing adaptors were ligated to the double-stranded DNA fragments. Afterward, the fragments were amplified by PCR to construct the cDNA library. The library was sequenced using the Illumina cluster station and Illumina HiSeq 4000 sequencing platform.

Bioinformatics analysis of RNA-Seq data

The genome (Prunus_avium_v1.0.a1) of sweet cherry from the Genome Database for Rosaceae (GDR) (https://www.rosaceae.org/species/prunus_avium/genome_v1.0.a1) was used as the reference and for transcript annotations. The gene expression level was calculated with normalized to FPKM (Fragments Per Kb per Million reads). log₂ ratio counted the relative gene expression levels between the two samples.

Differentially expressed genes (DEGs) were identified using the DEseq2 method and screened with the criteria of fold change ≥ 2 and Q-value ≤ 0.001 . The gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses of the DEGs were implemented using omicshare (<https://www.omicshare.com/tools/>), which is a bioinformatics platform.

Quantitative real-time PCR (qRT-PCR) analysis

The expression levels of samples under the various plant tissues, including abscising and non-abscising carpopodium, were validated by qRT-PCR using PowerUp SYBR Green Master Mix (ThermoFisher, Chongqing, China) in a volume of 10 μL , containing 5 μL of SYBR Green Master Mix, 100 ng of cDNA template, and 0.5 μM of each of the forward and reverse primers. The qRT-PCR amplification was performed as follows: 95 $^{\circ}\text{C}$ for 30 s, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 5 s and 60 $^{\circ}\text{C}$ for 30 s. The *PavEF-1a* and *PavRSP3* were used as internal control, and the primer sequences are listed in Additional file 10: Table S7. The relative gene expression level was executed using the $2^{-\Delta\text{Ct}}$ method [32] with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA).

HD-ZIP gene family bioinformatics and expression levels analysis

A Hidden Markov Model (HMM) profile of HD-ZIP domain (protein family ID: PF00046) was downloaded from Pfam (<http://pfam.xfam.org/>), and used as the query to search against a previous assembly sweet cherry genome database (https://www.rosaceae.org/species/prunus_avium/genome_v1.0.a1) using the HMMER 3.0 software (<http://hmmer.org/>). Additionally, the obtained sequences were submitted to the SMART and Pfam websites for the prediction of conserved domains. Proteins containing both the homeobox domain (HD, PF00046) and the homeobox-associated leucine Zipper domain (HALZ, PF02183) are classified as candidate proteins. A phylogenetic tree was constructed with MEGA (version 7.0) using the neighbor-joining method. Motif location was analyzed using MEME (version 4.12.0) (<http://meme-suite.org/tools/meme>). The expression levels were analyzed using semi-quantitative and quantitative methods, The *PavEF-1a* and *PavRSP3* were used as internal control, and the primer sequences are listed in Additional file 11: Table S8. The functional interacting networks of *PavHB16* and *PavHB18* integrated into STRING (version 11.0).

Abbreviations

ABA: Abscisic acid; ACC:1-aminocyclopropane-1-carboxylate were; ACO:1-aminocyclopropane-1-carboxylate oxidase; AE:abscising fruit embryo; AF:abscising fruit; ARF:auxin response factor; AUX/IAA:Auxin/Indole-3-Acetic; AUX1:auxin efflux carrier; AZ:abscising carpopodium abscission zone; CA:Abscising carpopodium; CN:Non-abscission carpopodium in the first stage; CTK:Cytokinin; DAF:days after flowering; EG:endoglucanase; EIN3:ethylene insensitive 3; ERF:ethylene response factor; EXP:expansin; FA:Abscising fruit in the first stage; Fb:Flower bud; FL:Flower; FN:Non-abscising fruit in the first stage; FPKM:Fragments Per Kb per Million reads; GA:Gibberellin; HPLC-MS/MS:high-performance

liquid chromatography-tandem mass spectrometry; IBA:indole-3-butyric acid; JA:Jasmonic acid; KEGG:Kyoto Encyclopedia of Genes and Genomes; MeJA:Methyl jasmonate; NAF:non-abscising fruit; NAZ:non-abscising carpopodium abscission zone; NE:non-abscising embryo IAA:indole-3-acetic acid; NPR5:Non-expressor of PR5; PAL:pectin acetylerase; PAO:polyamine oxidase; PEM:pectin methylesterase; PG:polygalacturonase; PL:pectate lyase; POD:peroxidase; SAUR small auxin up RNA; St:Stem; TZ:Trans-zeatin; XTH:xyloglucan endotransglucosylase/hydrolases; α -Trp:tryptophan synthase alpha chain; β -gal:beta-galactosidase; β -glc1:beta-glucanase.

Declarations

Ethics approval and consent to participate

The experiments did not utilize transgenic technology or involve protected species. Santana used in this study were obtained from an orchard in Weining country, which was a demonstration base of Guizhou University, China. It does not require specific permits.

Consent for publication

Not applicable.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The datasets used and analyzed during the current study are available from the authors on reasonable request (Zhilang Qiu, 18786621377@163.com).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XPW designed the research. ZLQ carried out the experiments with the help of ZW, QDH, GQ, KY and HY. ZLQ collected the experimental data and drafted the manuscript. XPW reviewed the manuscript. All authors read and approved the final manuscript.

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Supplementary Information

Additional file 1: Table S1. Summary of read numbers from abscising carpopodium and non-abscising carpopodium.

Additional file 2: Figure S1. The pearson correlation between samples.

Additional file 3: Table. S2 Differentially expressed genes between abscising carpopodium and non-abscising carpopodium.

Additional file 4: Table S3. The enriched KEGG pathways of DEGs.

Additional file 5: Table S4. GO classification of DEGs.

Additional file 6: Table S5. The plant cell wall remodeling related gene expression levels.

Additional file 7: Table S6. Validation of RNA-seq results via qRT-PCR.

Additional file 8: The original gel images of internal genes and HD-ZIP gene family.

Additional file 9: The promoter sequence of *PavCEL*.

Additional file 10: Table S7. Primers for transcriptome data verification by qRT-PCR analysis in carpodium.

Additional file 11: Table S8. A list of primer sequences of the 27 selected HD-ZIP genes for expression levels analysis.

Figures

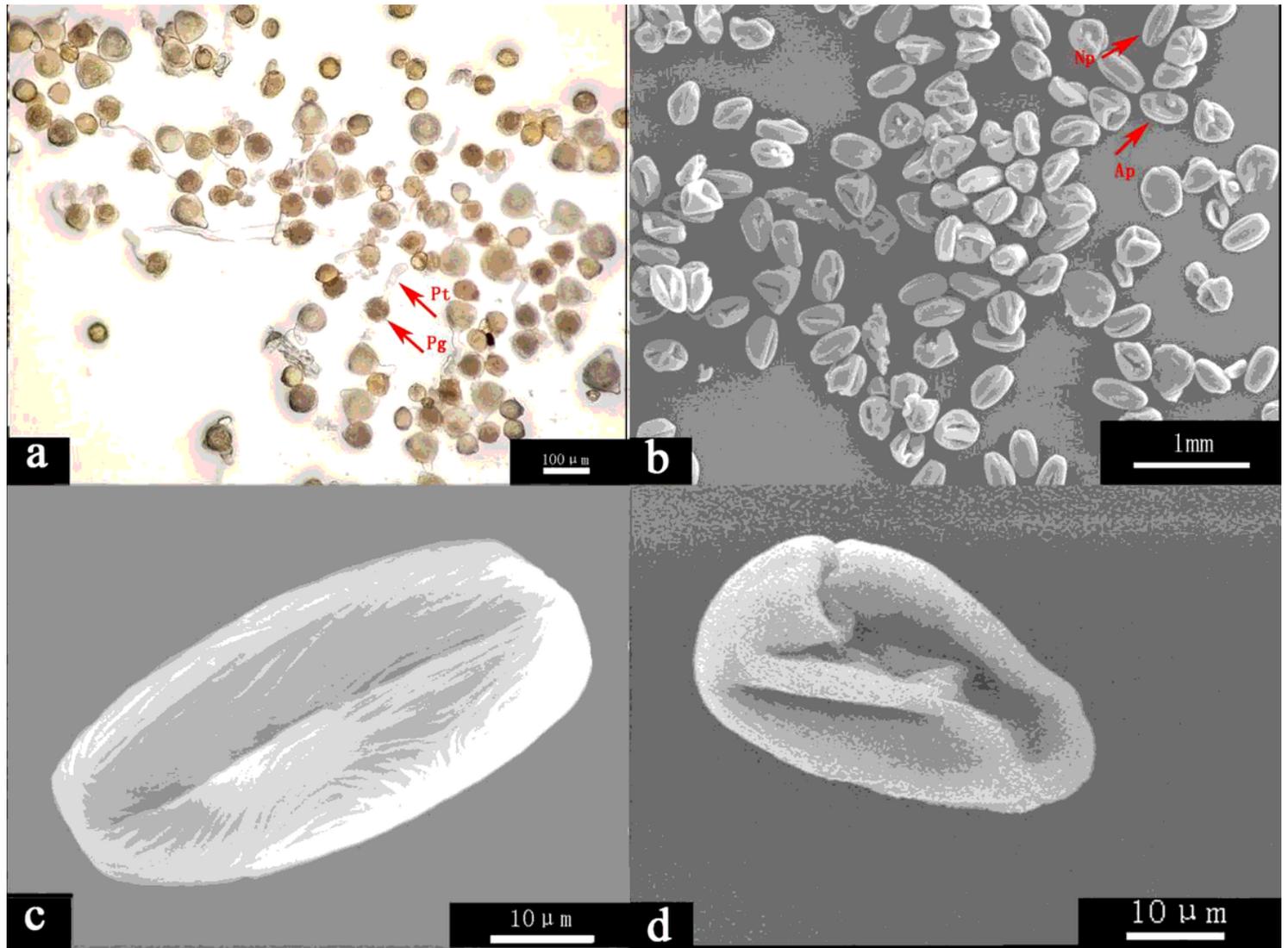


Figure 1

The pollen vigor and structure in the scanning electron microscope. Pg: pollen grain; Pt: pollen tube; Ap: abortion pollen; Np: normal pollen.

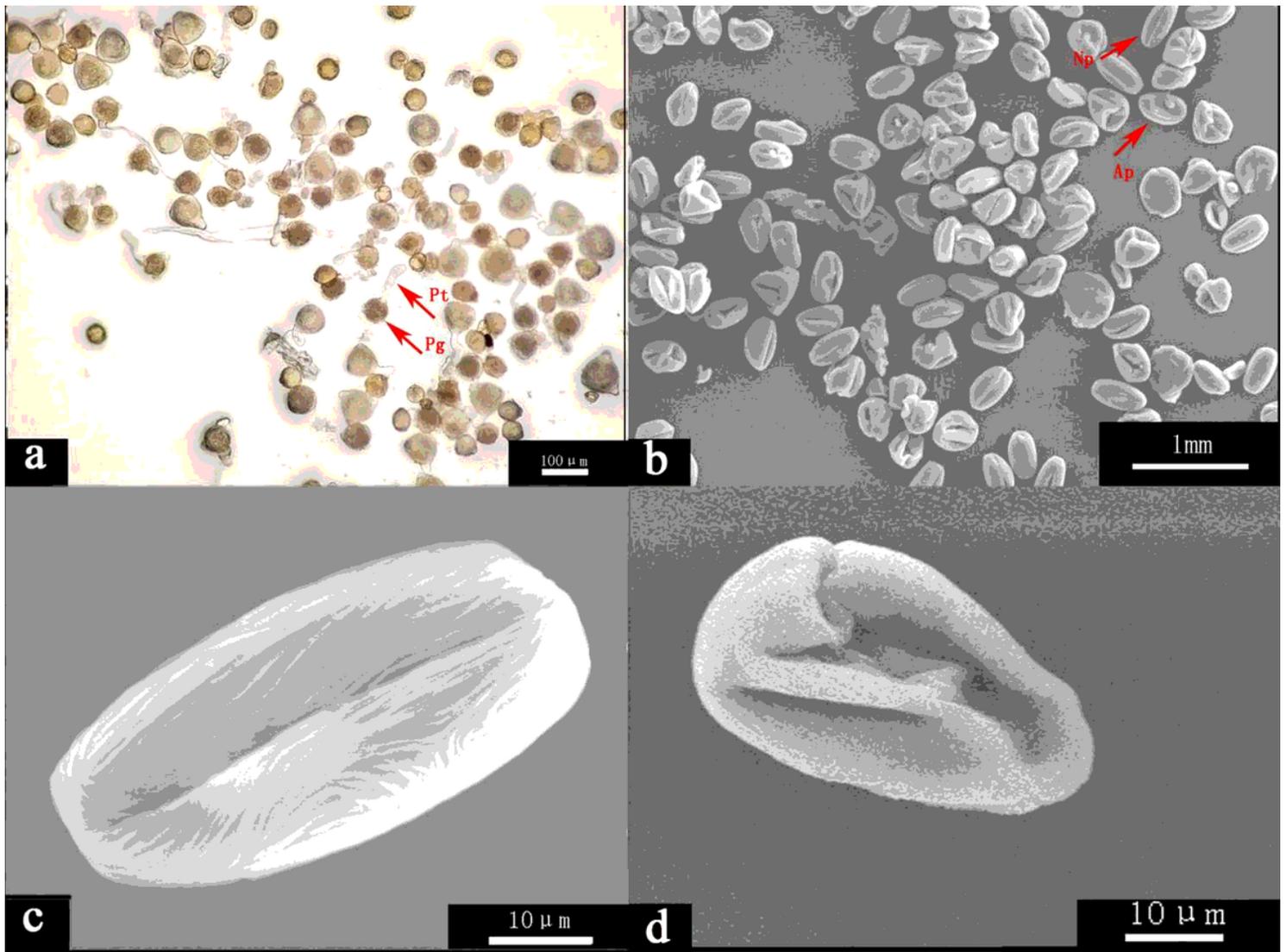
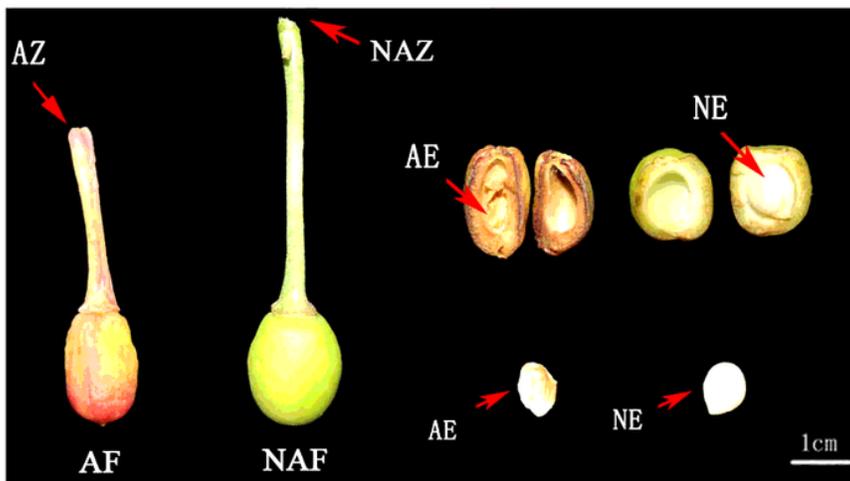
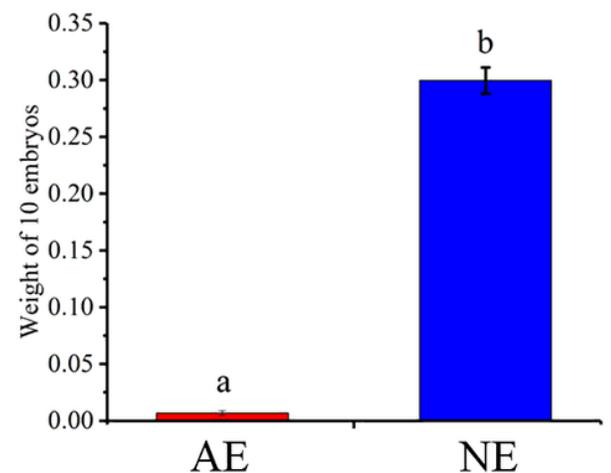


Figure 1

The pollen vigor and structure in the scanning electron microscope. Pg: pollen grain; Pt: pollen tube; Ap: abortion pollen; Np: normal pollen.



(a)



(b)

Figure 2

The morphology structure and the weight of ten embryos of abscising sweet cherry fruitlet. AZ: abscising carpodium abscission zone; NAZ: non-abscising carpodium abscission zone; AF: abscising fruit; NAF: non-abscising fruit; AE: abscising fruit embryo; NE: non-abscising embryo.

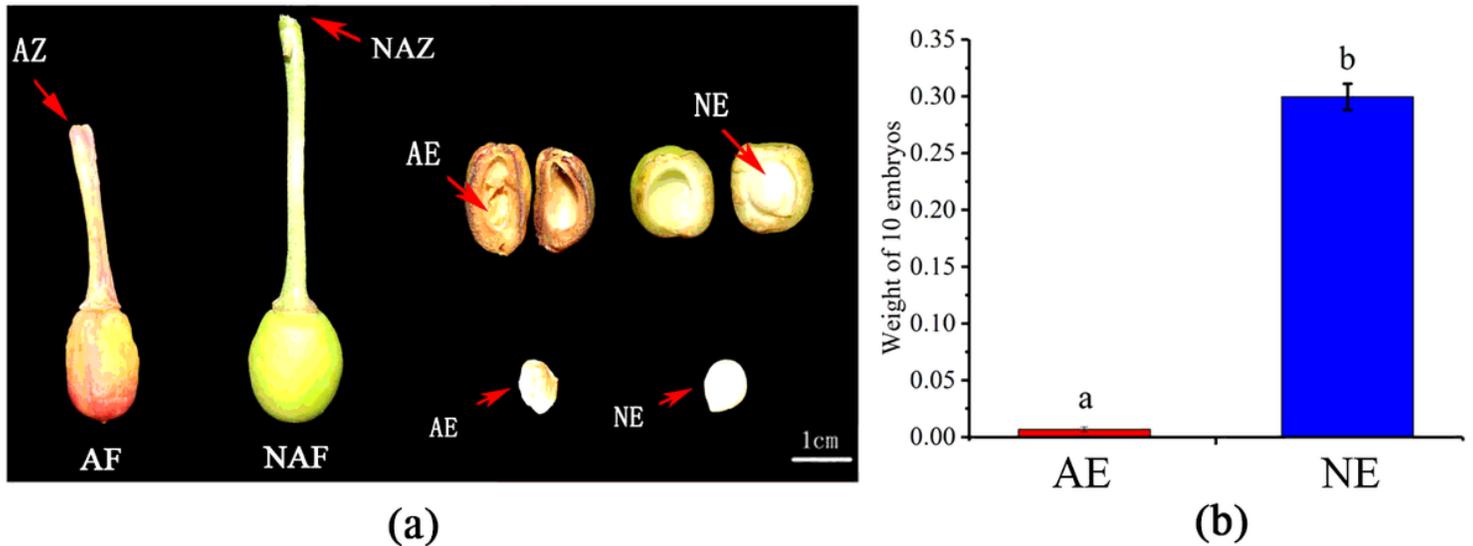


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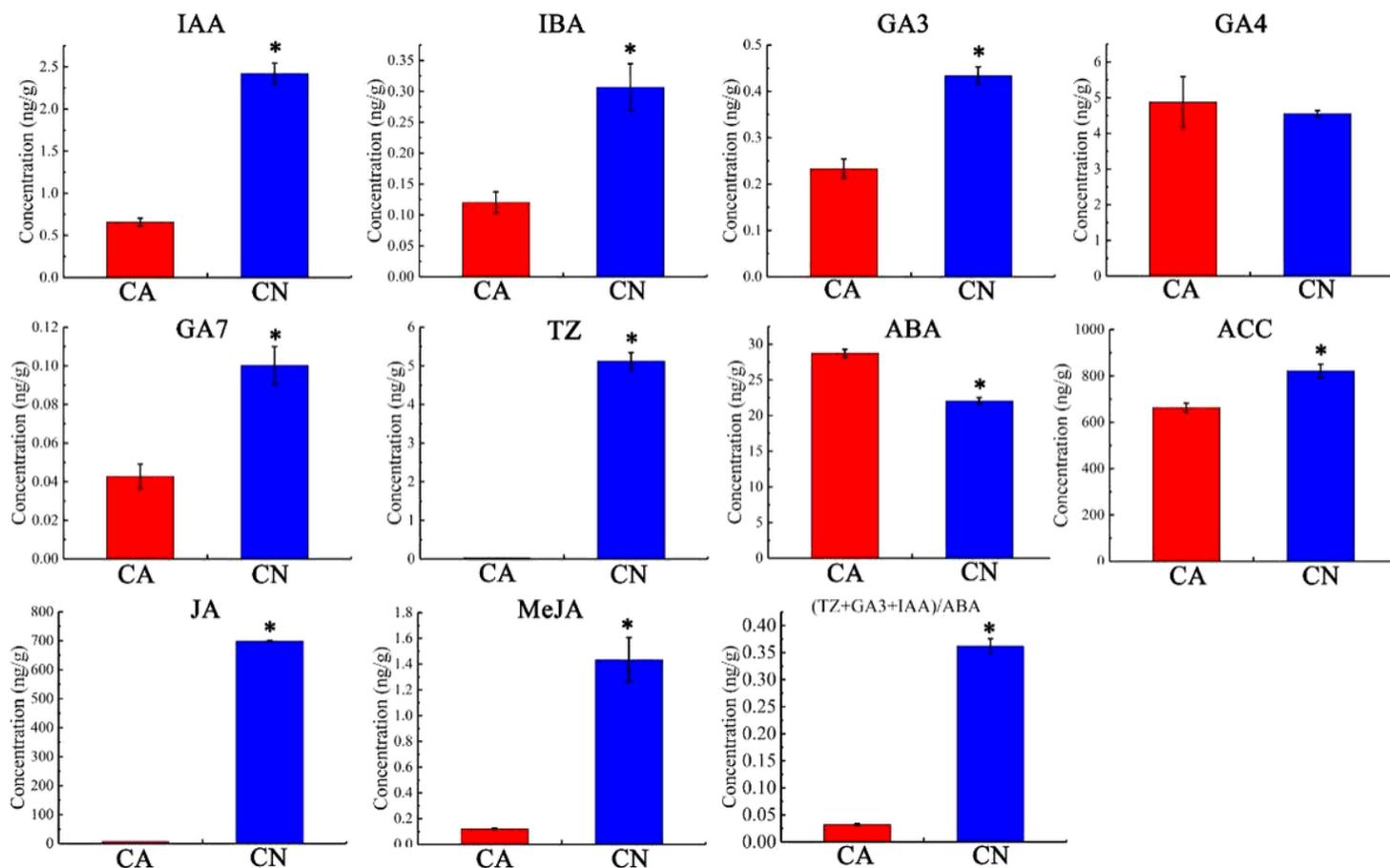


Figure 3

Endogenous hormone analysis of the abscising carpodium (CA) and non-abscising carpodium (CN). Auxin: IAA (indole-3-acetic acid); IBA (indole-3-butyric acid); Gibberellin: GA3, GA4, GA7; Cytokinin (CTK): TZ (Trans-zeatin); Abscising acid: ABA; 1-aminocyclopropane-1-carboxylate were: ACC; Jasmonic acid: JA; Methyl jasmonate: MeJA

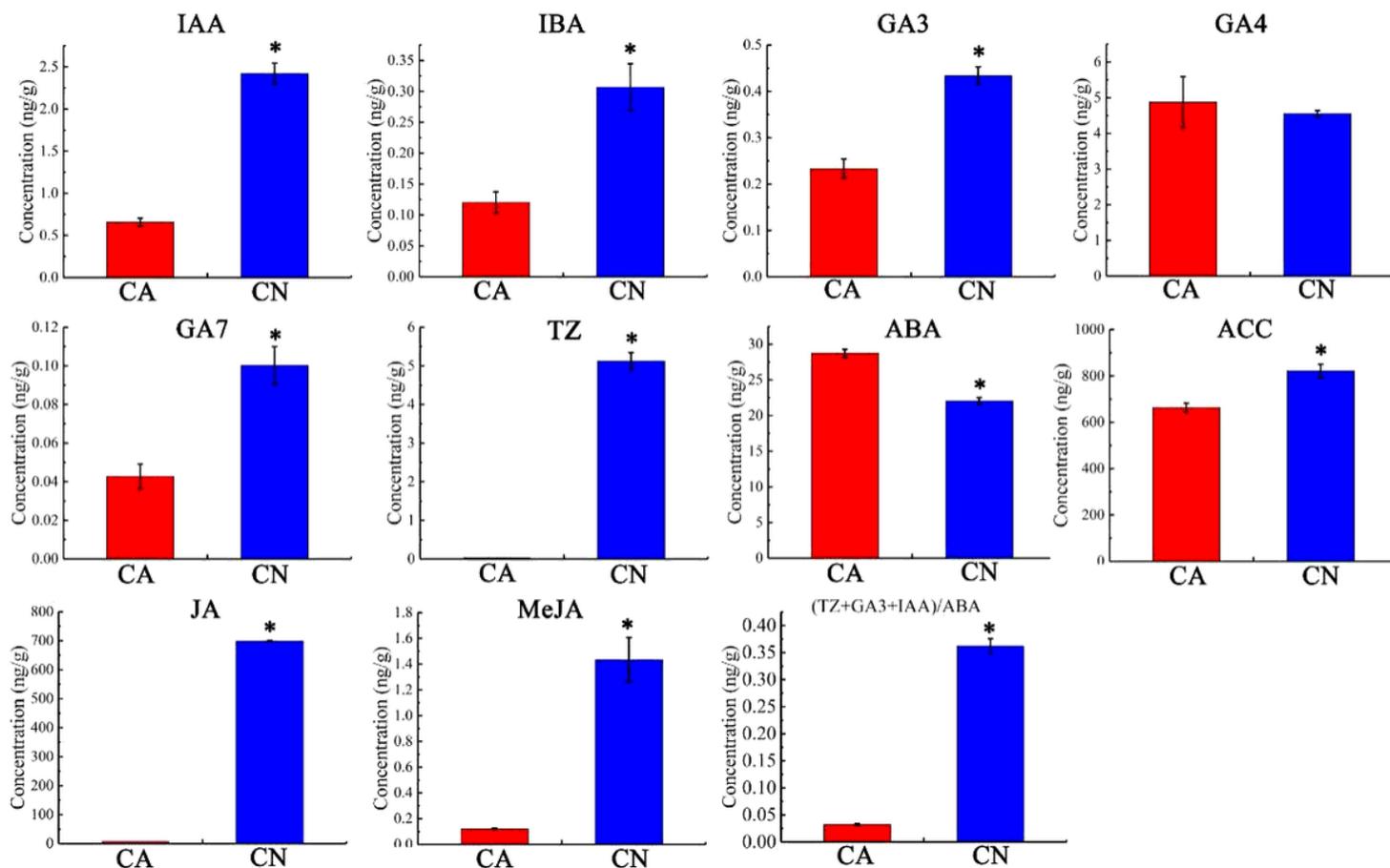


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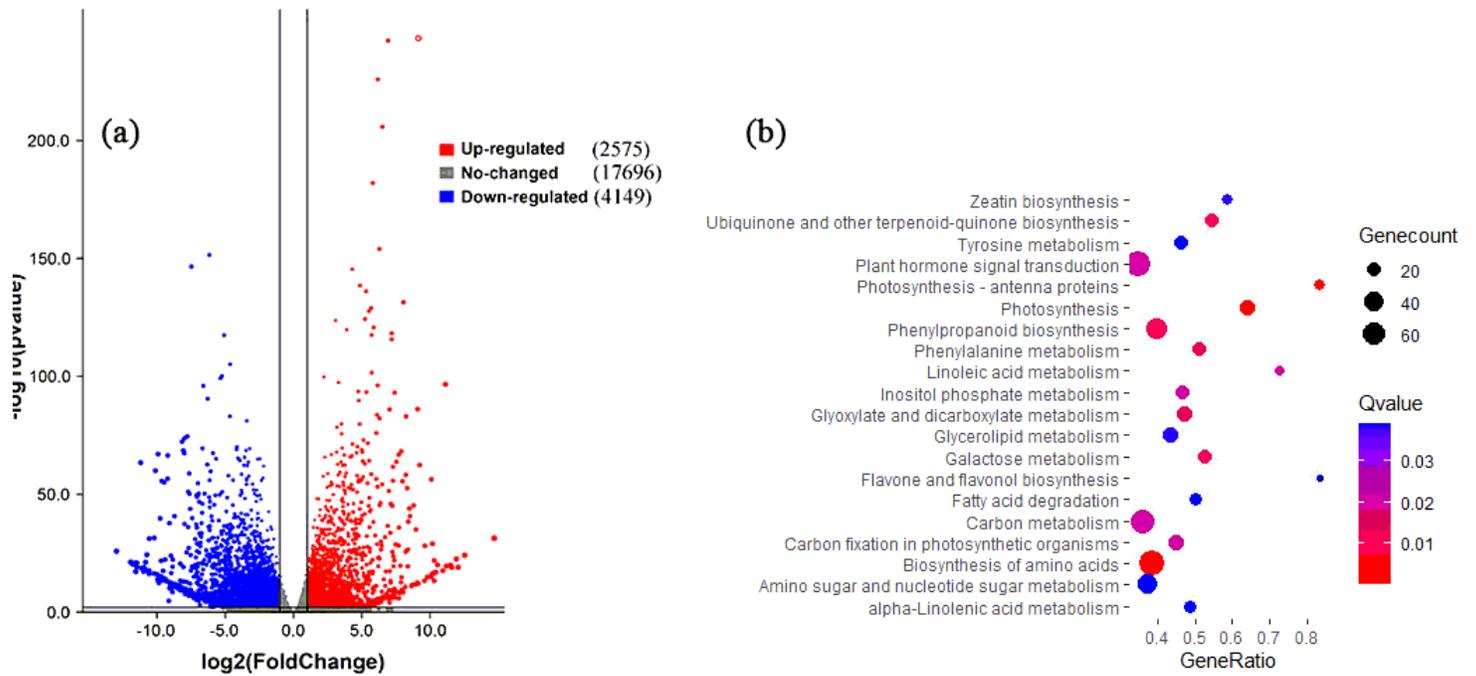


Figure 4

The differential expression gene (a) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (b).

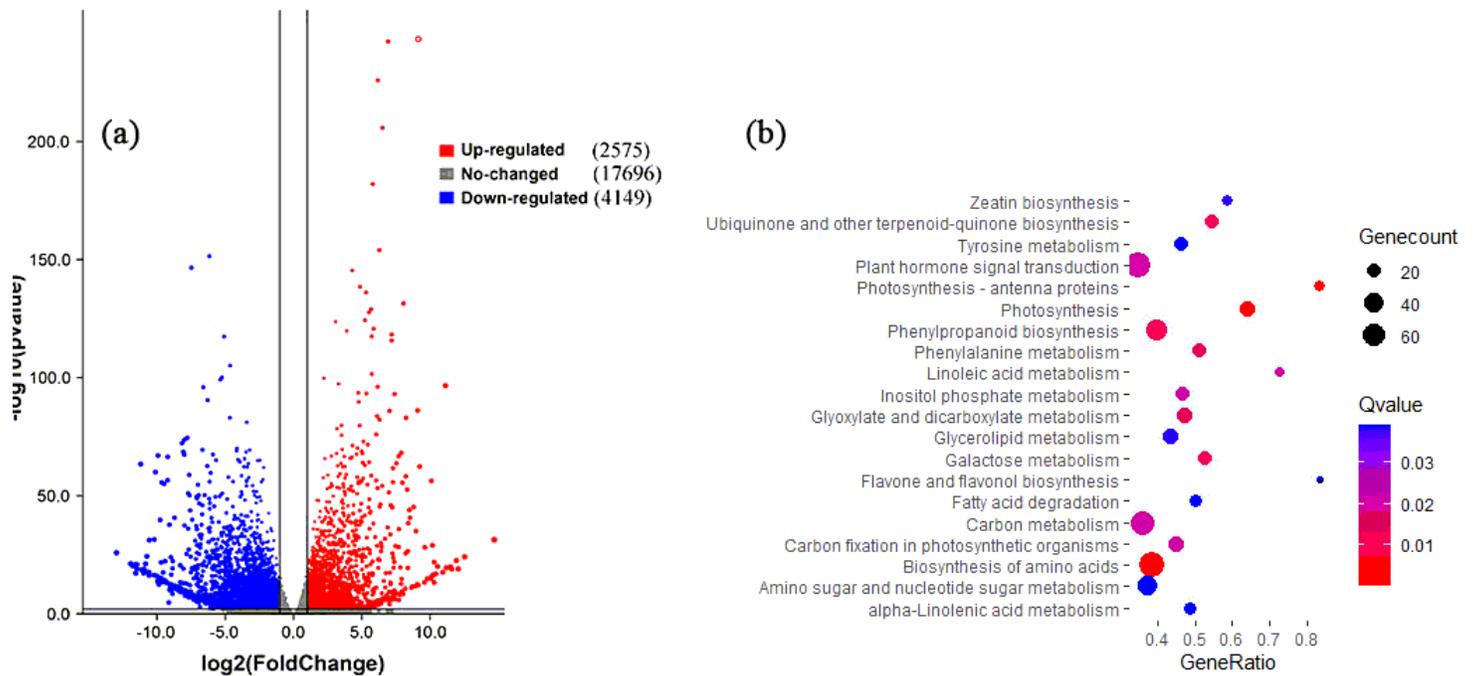


Figure 4

The differential expression gene (a) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment (b).

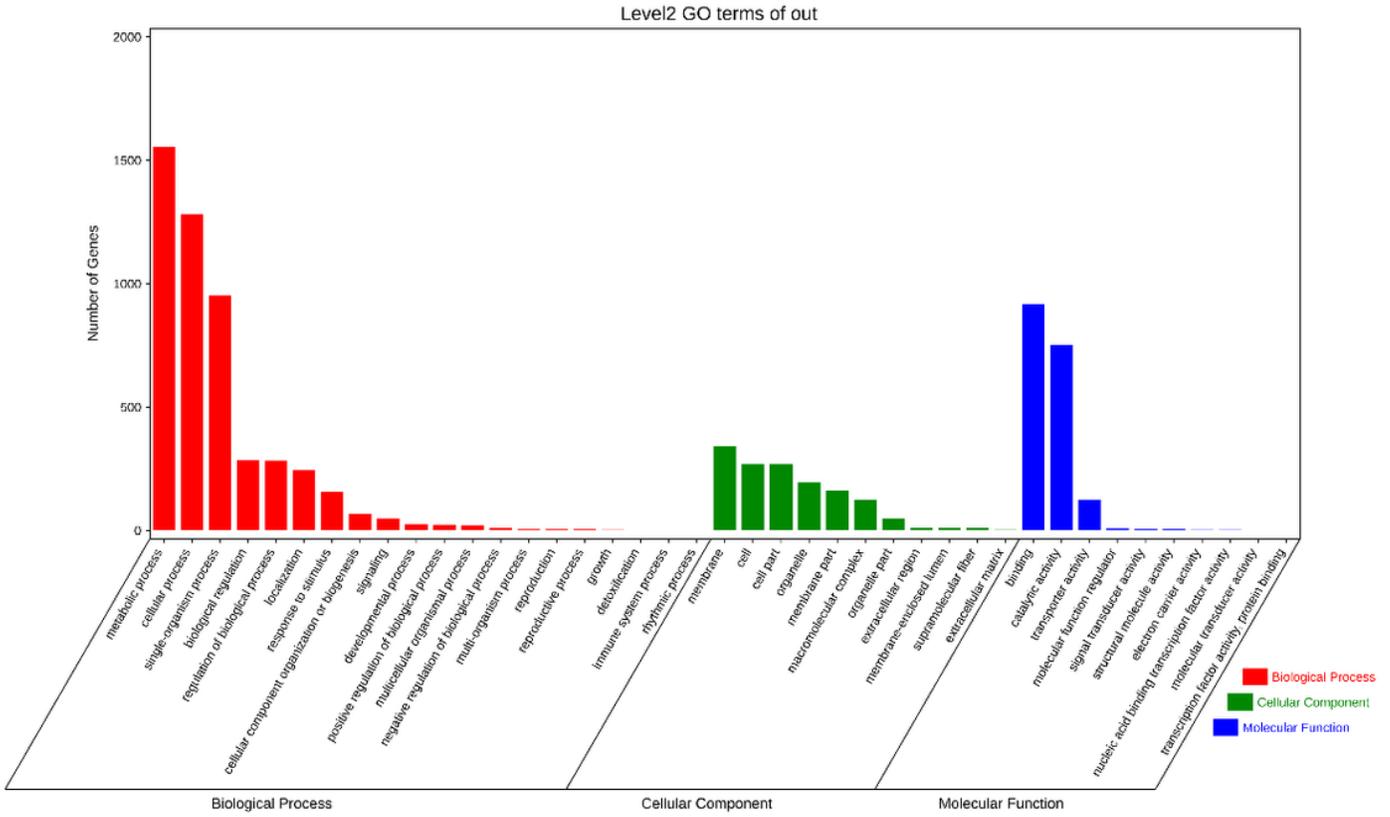


Figure 5

GO classification of DEGs. The X-axis represents the functional classification, and the Y-axis represents the number of genes annotated into the GO terms.

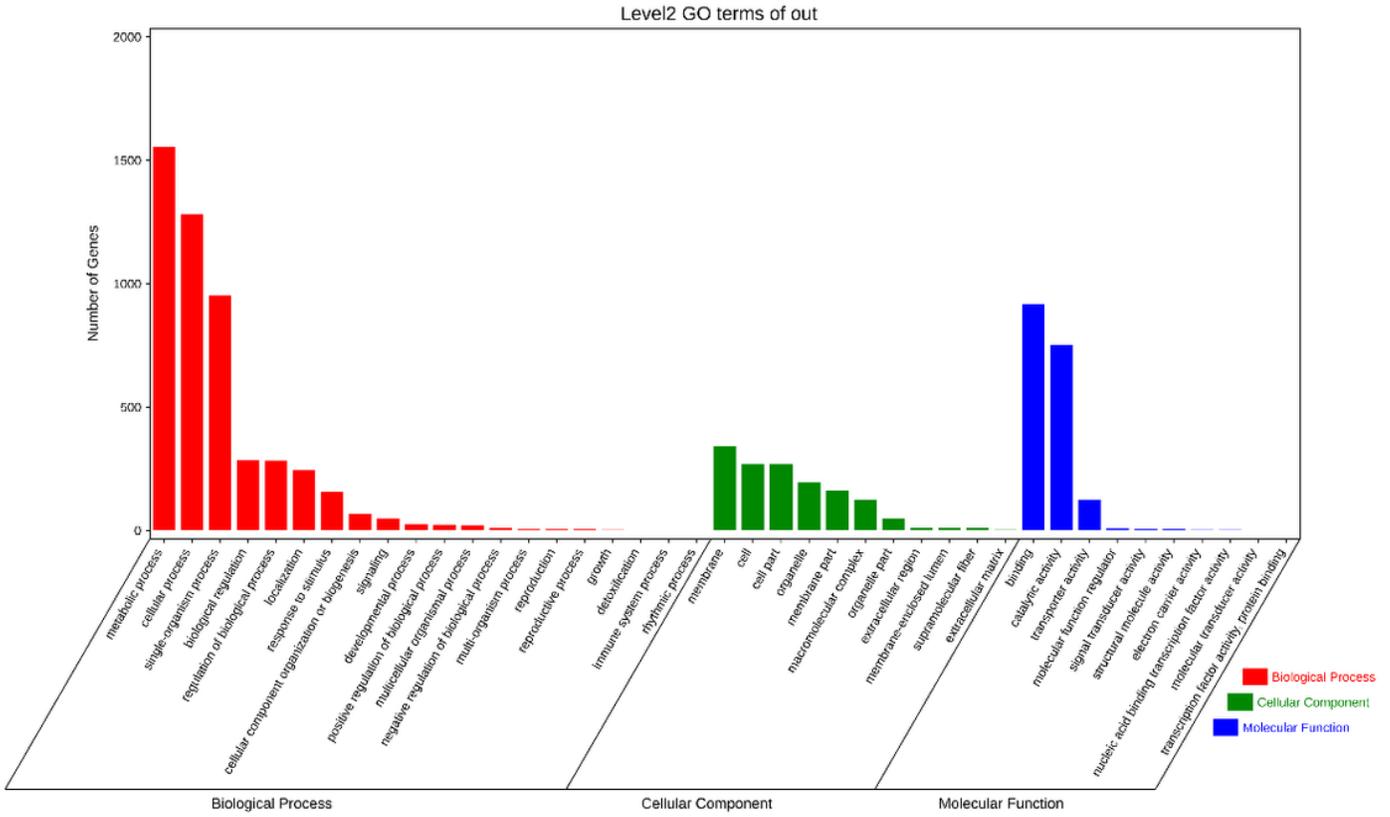


Figure 5

GO classification of DEGs. The X-axis represents the functional classification, and the Y-axis represents the number of genes annotated into the GO terms.

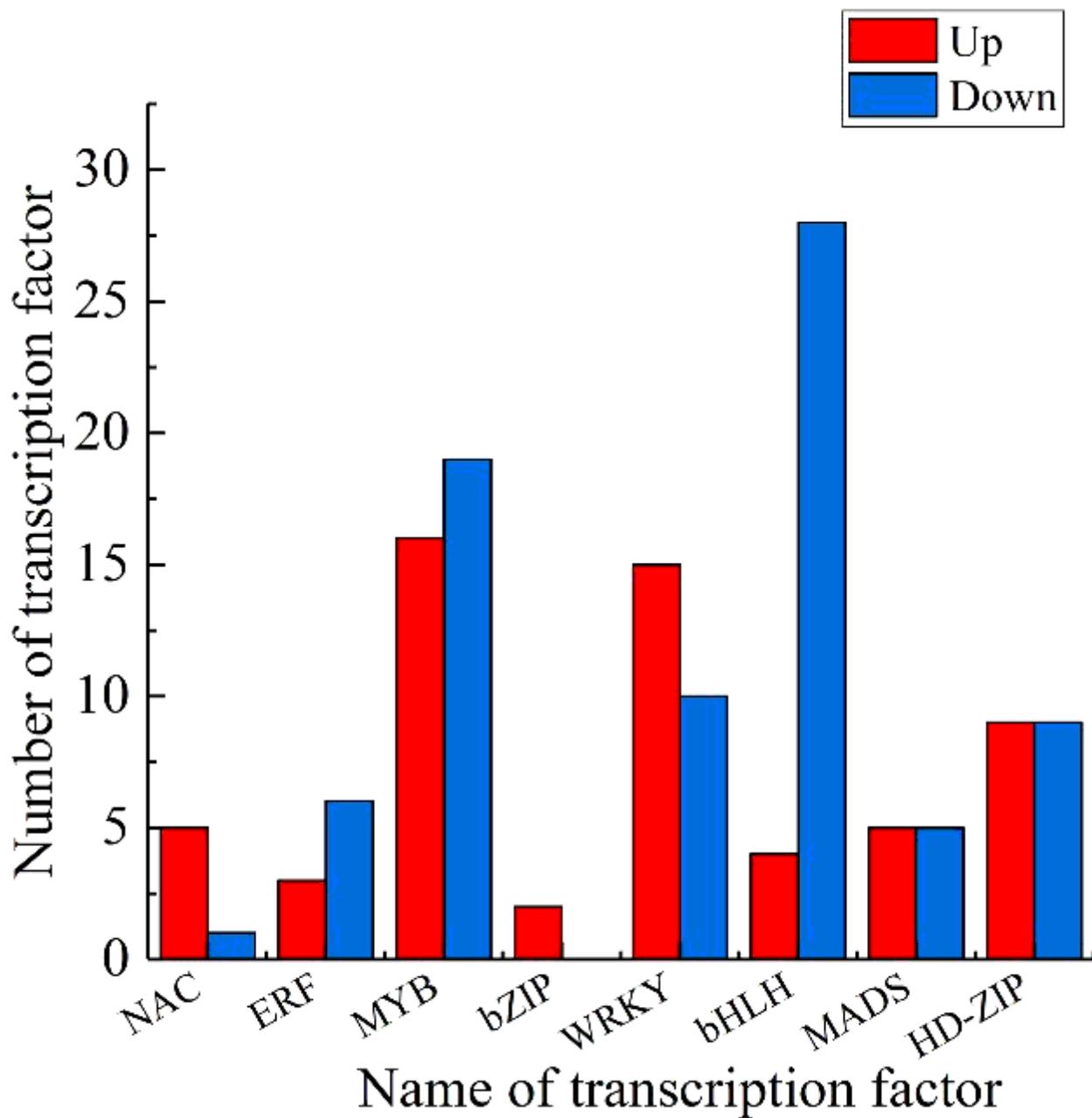


Figure 6

Eight major transcription factors differentially expressed in abscising and non-abscising carpopodium.

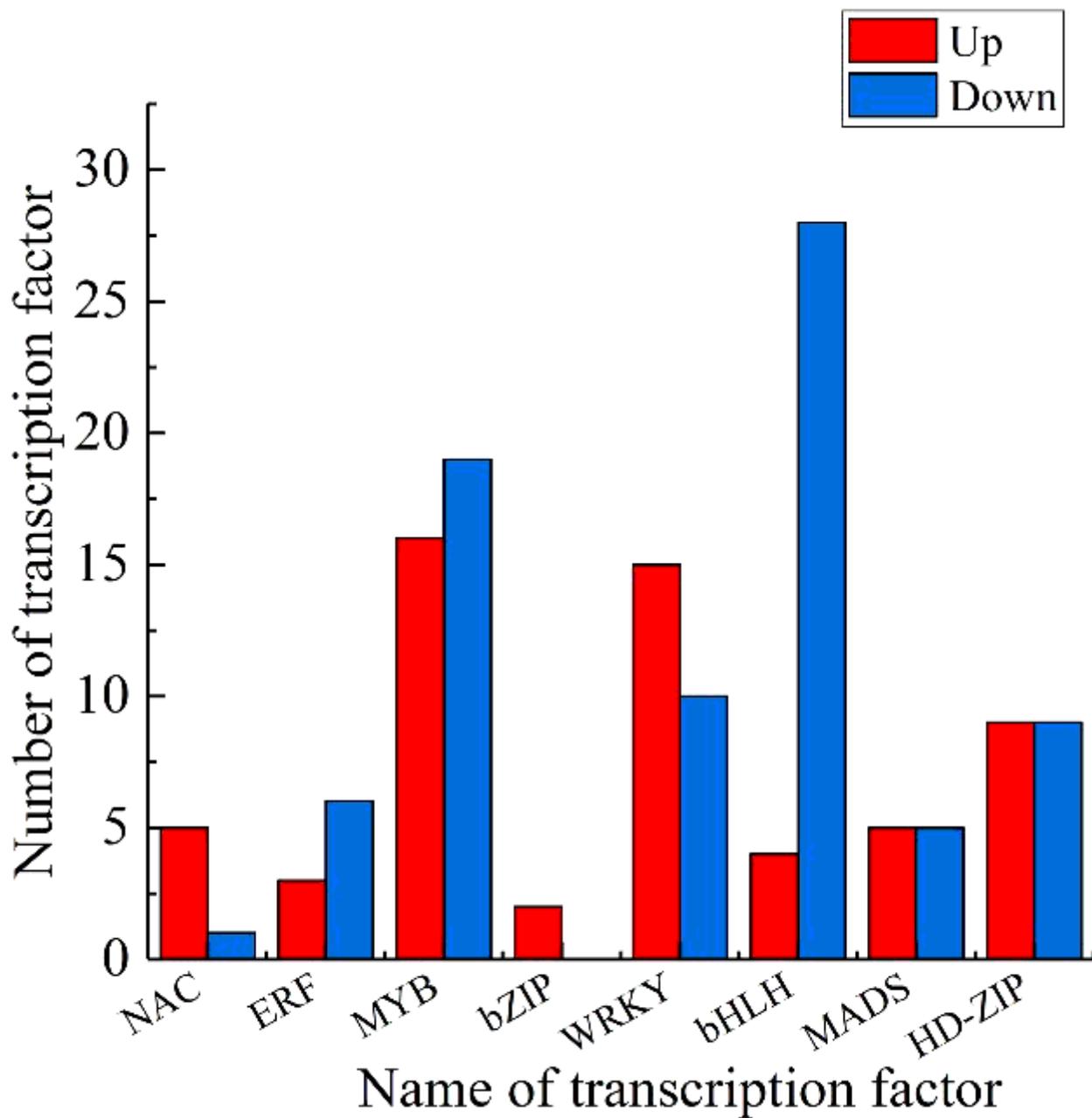


Figure 6

Eight major transcription factors differentially expressed in abscising and non-abscising carpodium.

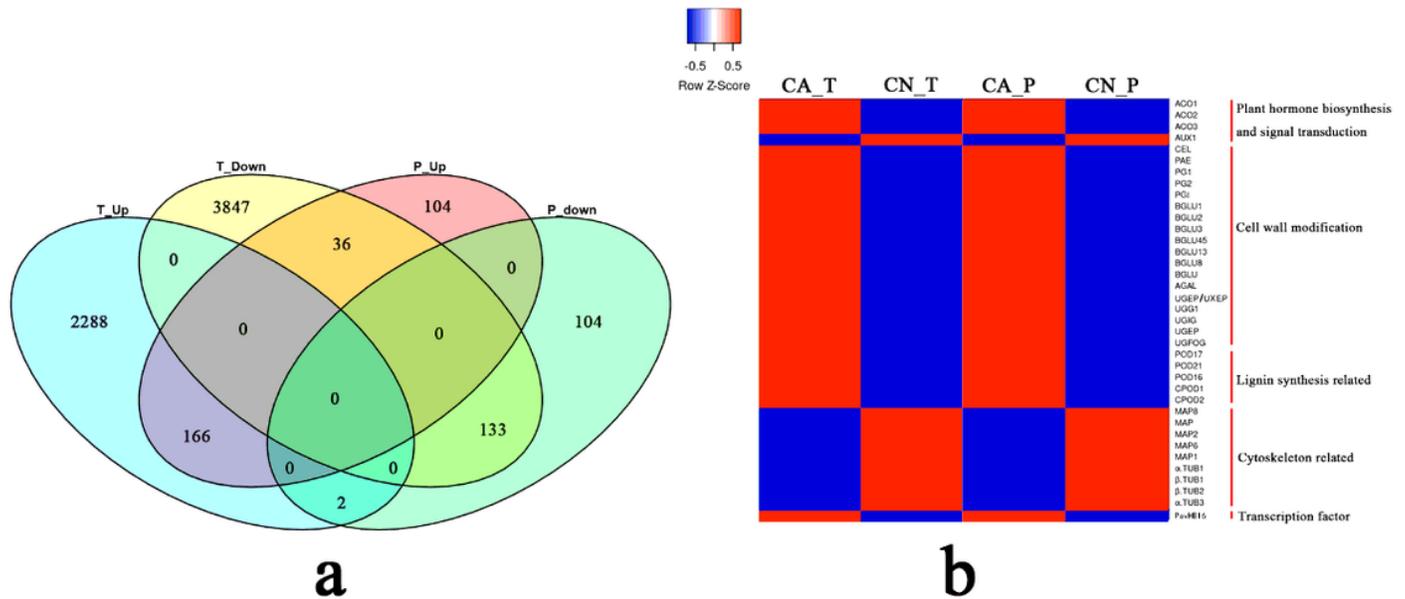


Figure 7

Venn (a) and expression (b) analysis of DEGs and DAPs between abscising carpodium and non-abscising carpodium. T_Up: the up-regulated genes in the transcriptome; T_Down: the down-regulated genes in the transcriptome; P_Up: the up-regulated genes in the proteome; P_Down: the down-regulated genes in the proteome.

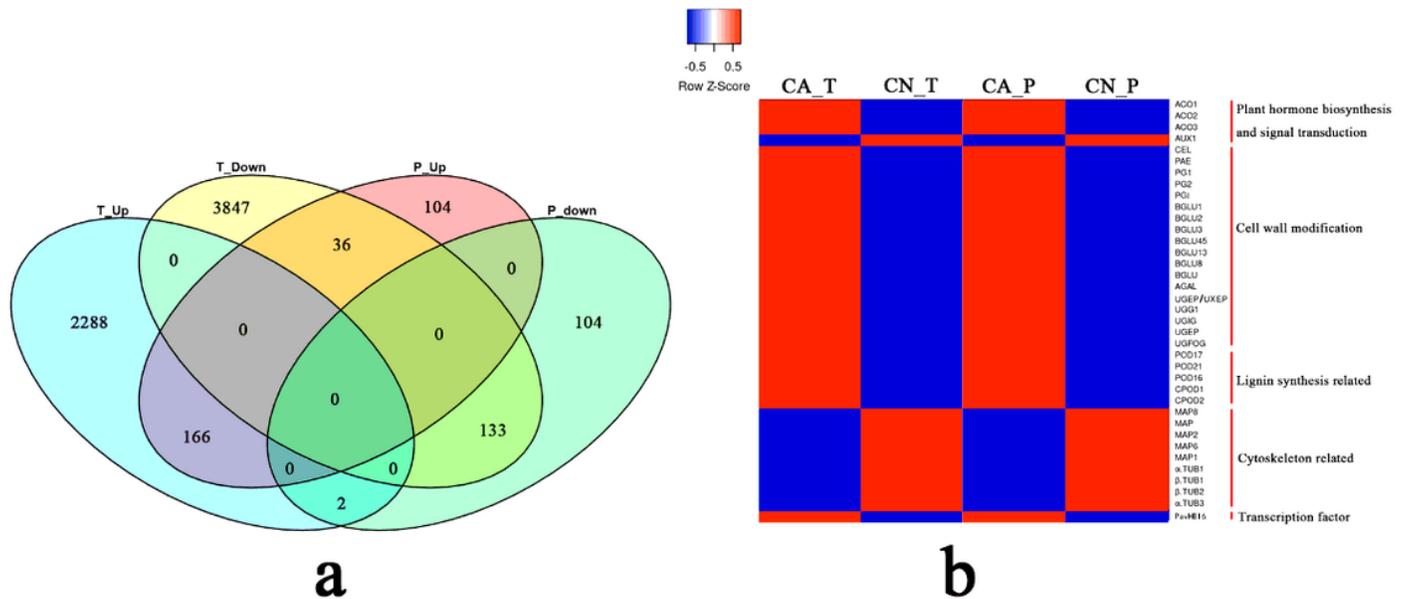


Figure 7

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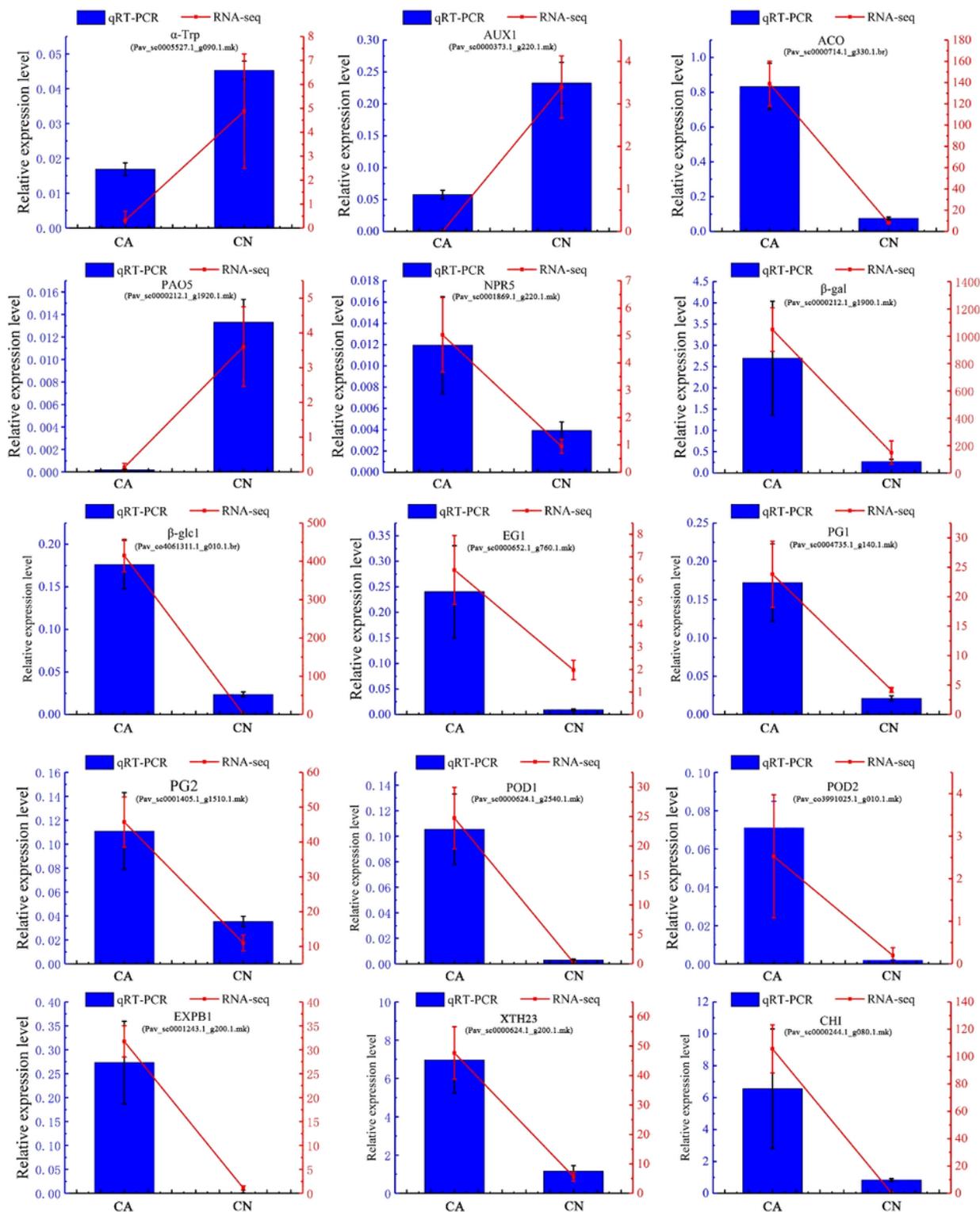


Figure 8

Relative expression levels of 15 DEGs in abscising carpodium and non-abscising carpodium. α -Trp: tryptophan synthase alpha chain; AUX1: auxin influx carrier; ACO: 1-Aminocyclopropane-1-Carboxylate Oxidase; PAO5: polyamine oxidase 5; NPR5: Non-expressor of PR5; β -gal: beta-galactosidase; β -glc1: beta-glucanase; EG1: endoglucanase; PG: polygalacturonase; POD: peroxidase; EXPB1: expansin-like B1; XTH23: xyloglucan endotransglucosylase/hydrolase 23; CHI: endochitinase.

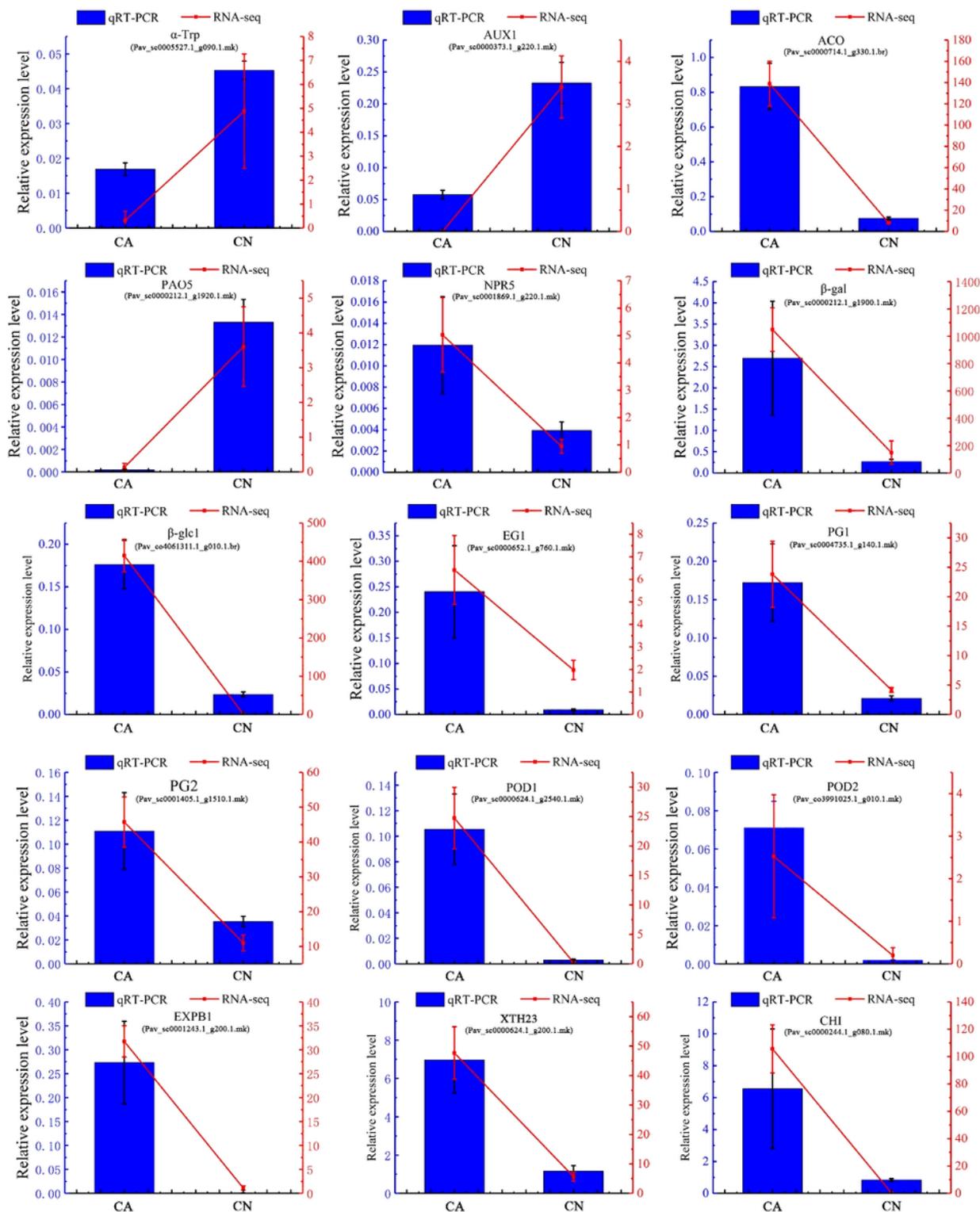


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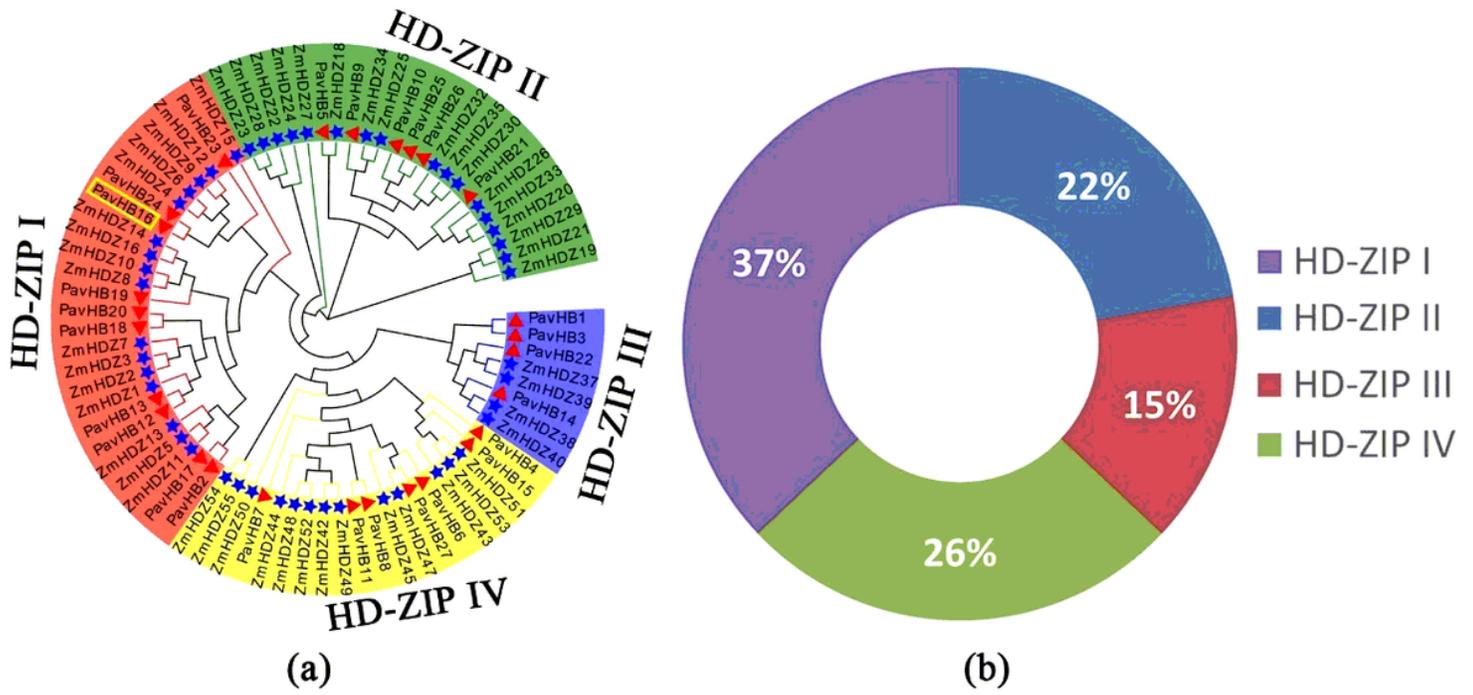


Figure 9

Unrooted phylogenetic tree (a) and proportions of various HD-ZIP groups in sweet cherry.

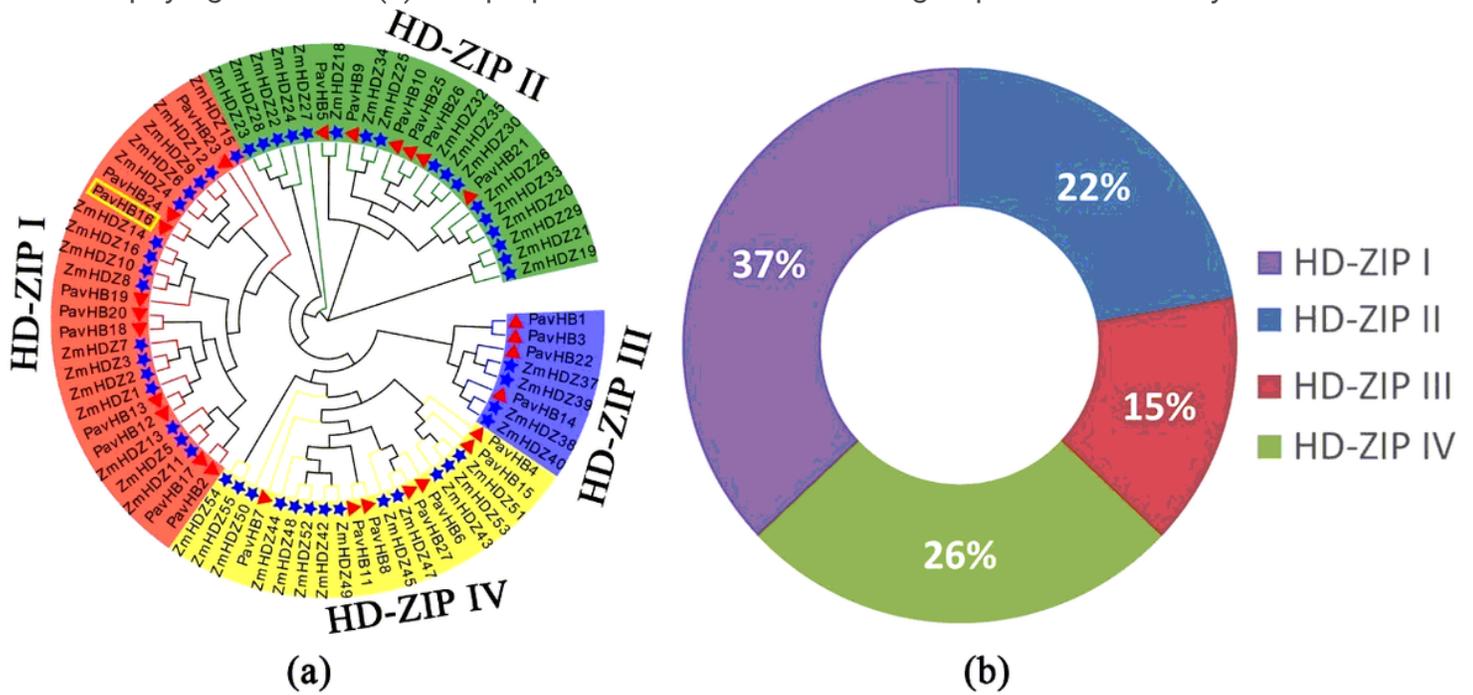


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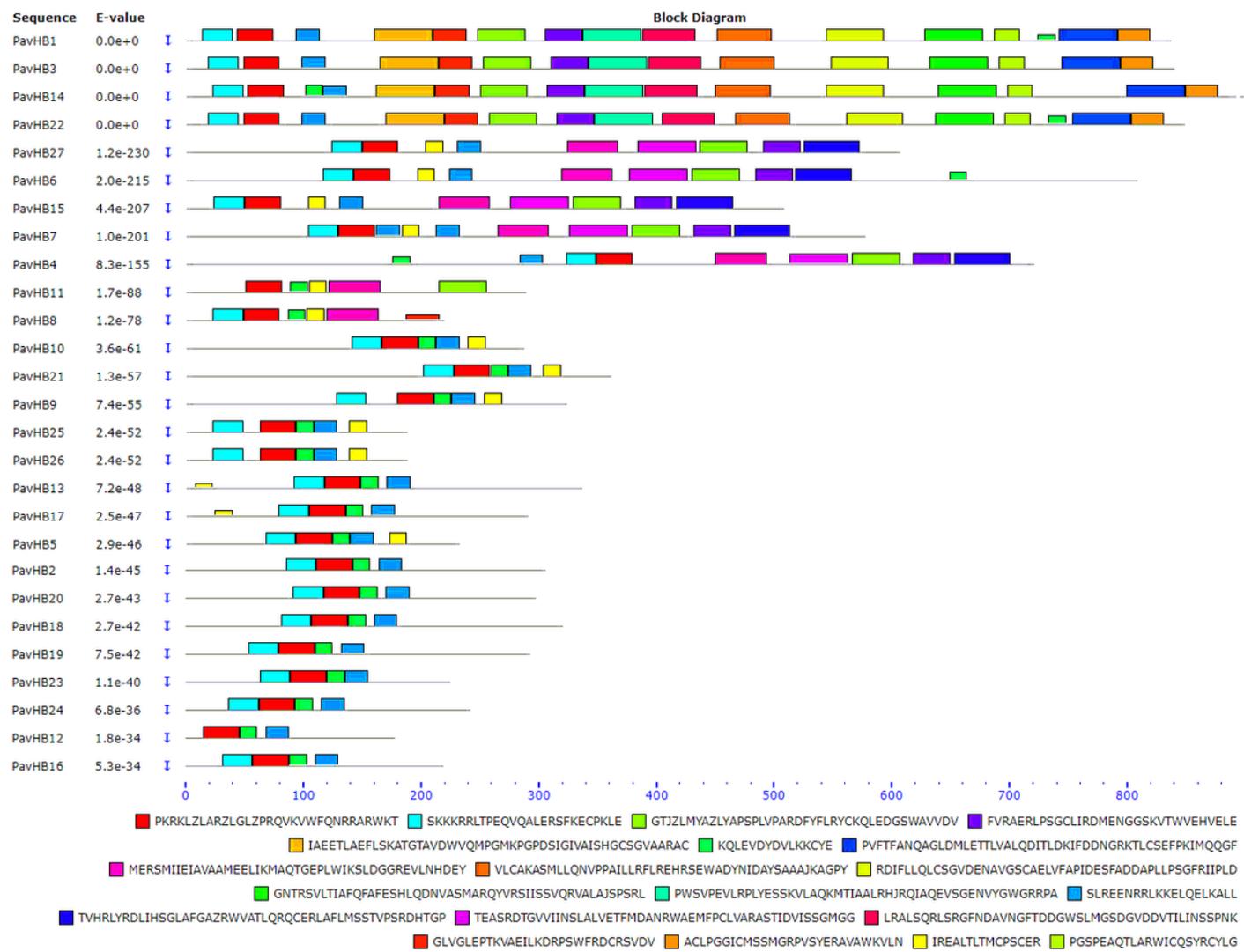


Figure 10

Common motifs of HD-ZIP family proteins in *Prunus avium*.

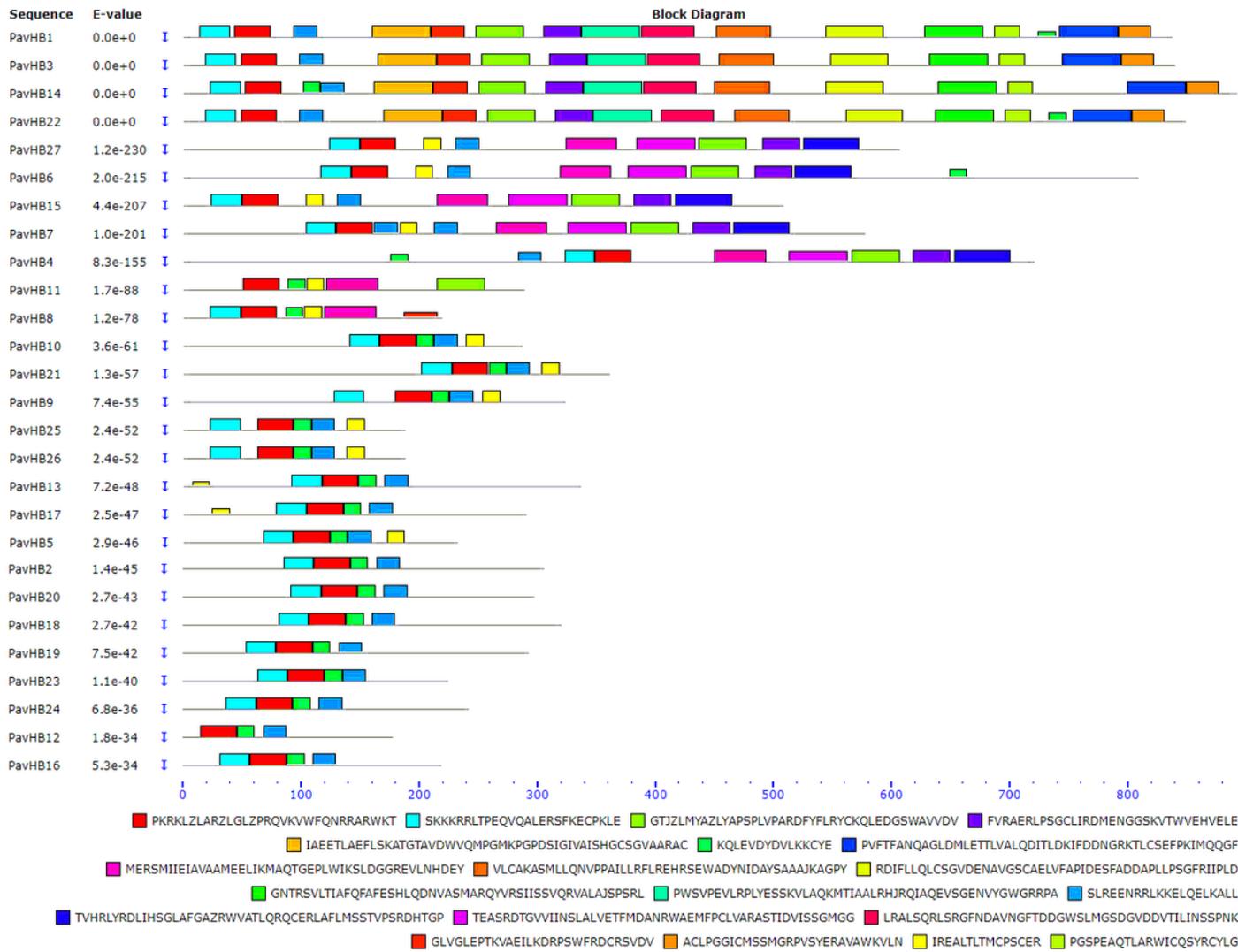


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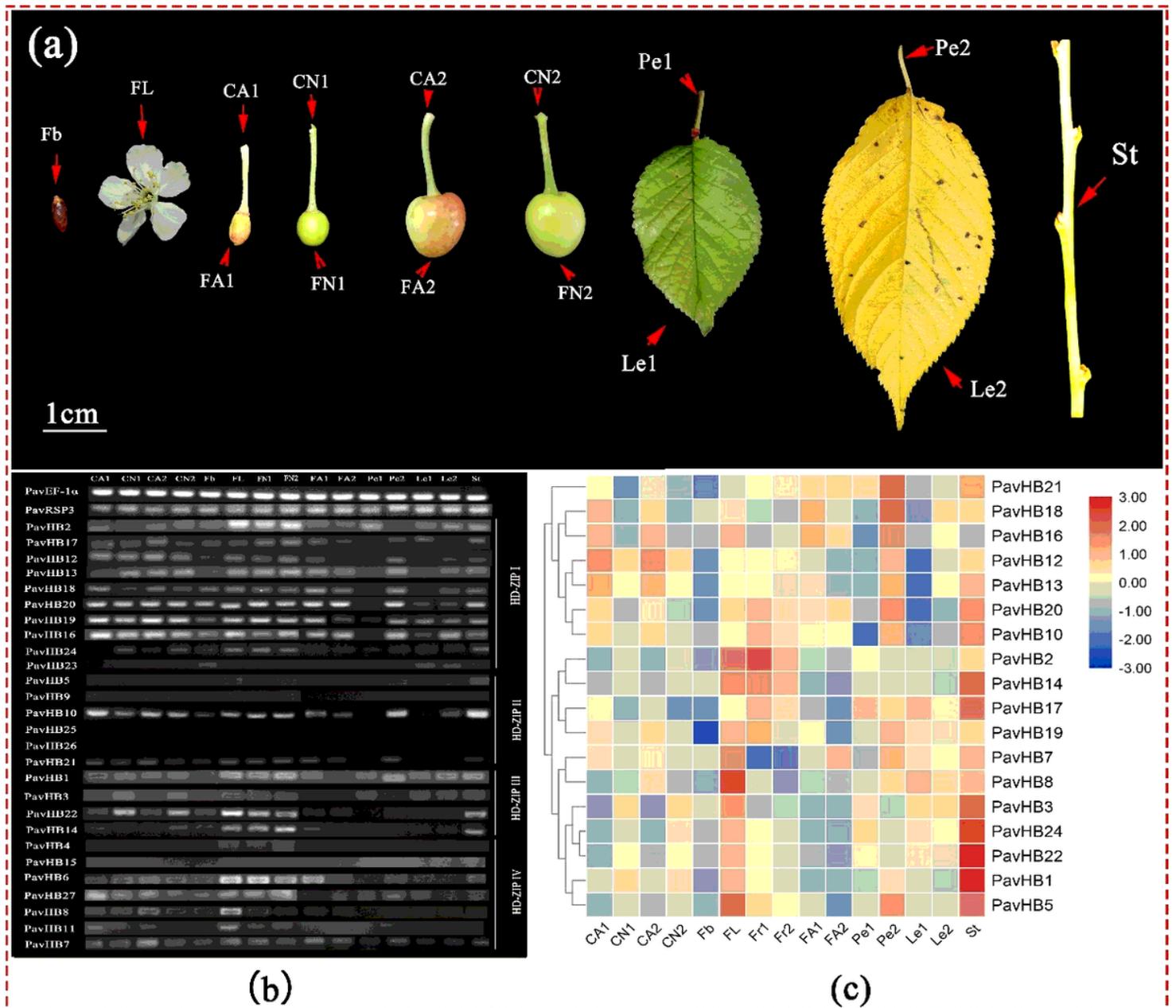


Figure 11

Gene expression levels in different tissue. (a) Different tissue of sweet cherry; (b) The semiquantitative of HD-ZIP gene family in different tissue (the original gel images were shown in Additional file 8); (c) The heatmap representing the expression patterns of HD-ZIP gene family. Fb: Flower bud; FL: Flower; CA1: Abscising carpodium in the first stage; CN1: Non-abscission carpodium in the first stage; FA1: Abscising fruit in the first stage; FN1: Non-abscising fruit in the first stage; CA2: Abscising carpodium in the second stage; CN2: Non-abscission carpodium in the second stage; FA2: Abscising fruit in the second stage; FN2: Non-abscising fruit in the second stage; Pe1: Young leaf petiole; Pe2: Old leaf petiole; Le1: Young leaf; Le2: Old leaf; St: Stem.

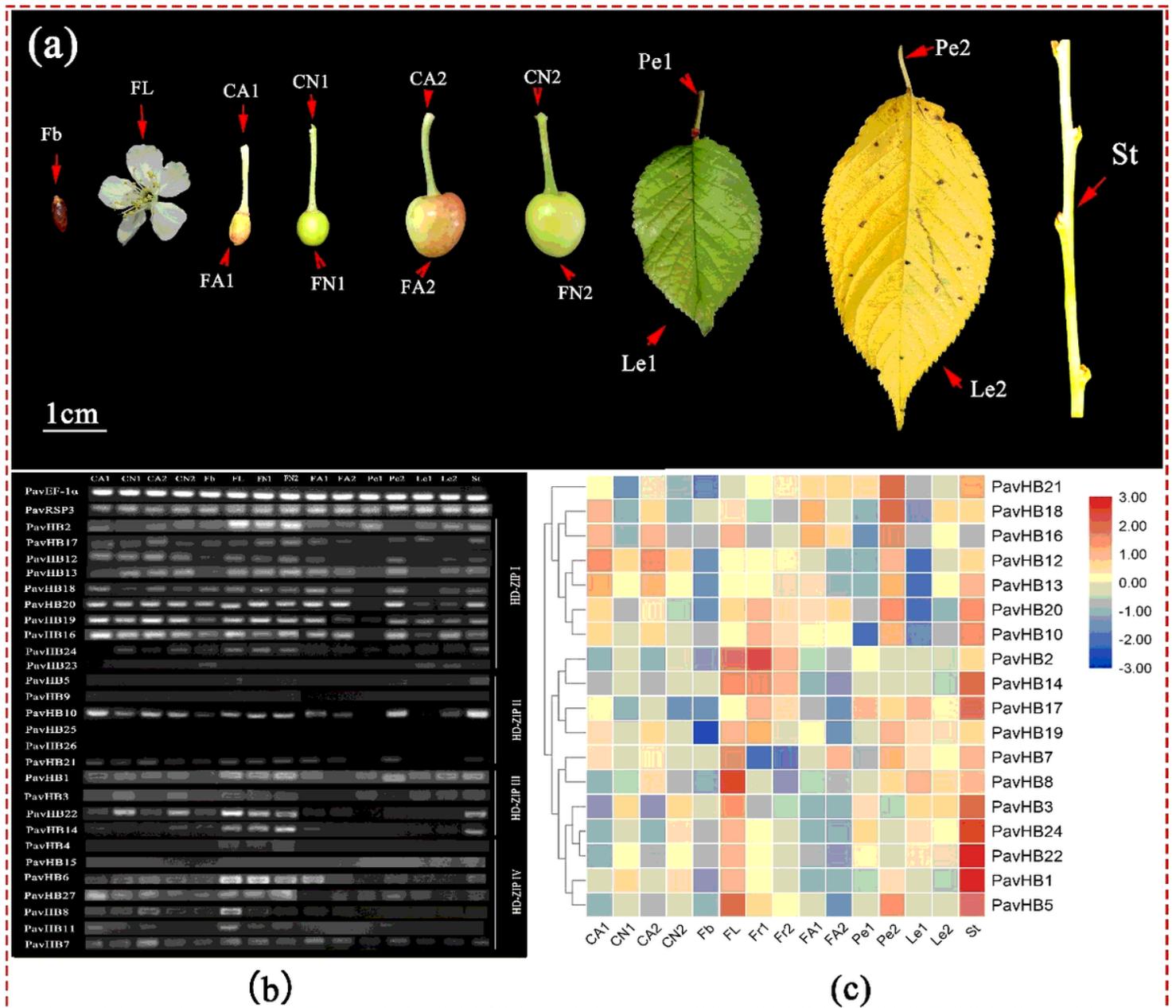


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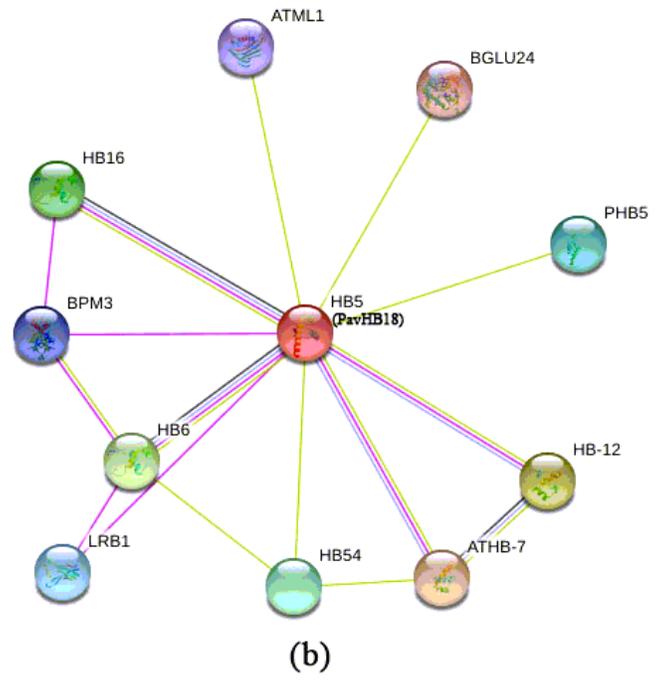
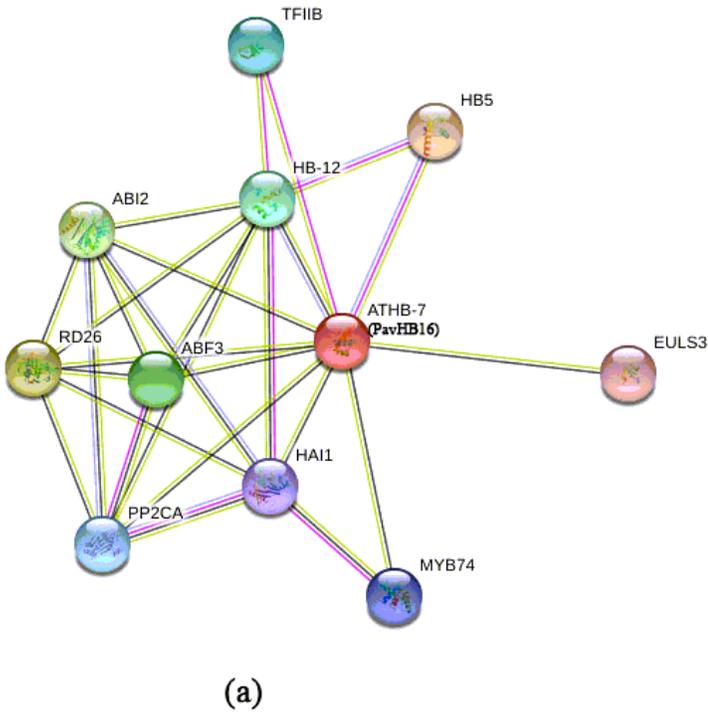


Figure 12

Interaction networks of PavHB16 (a) and PavHB18 (b) according to the orthologs in Arabidopsis.

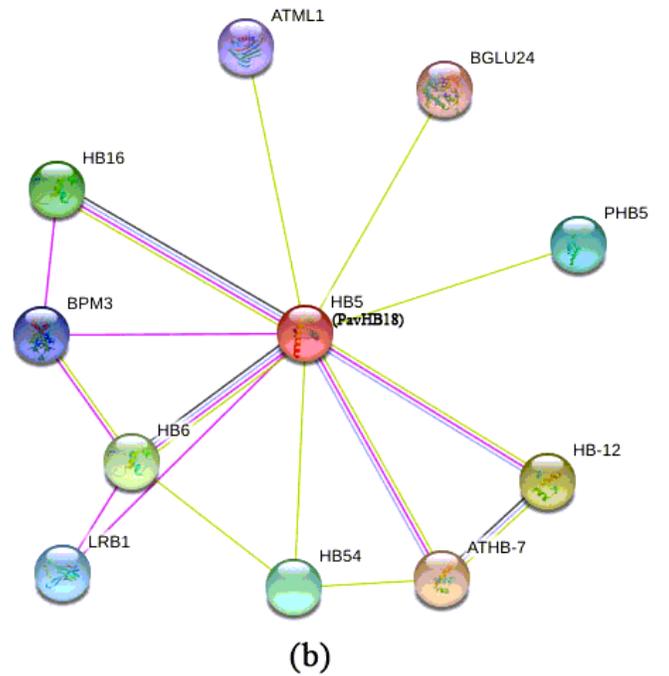
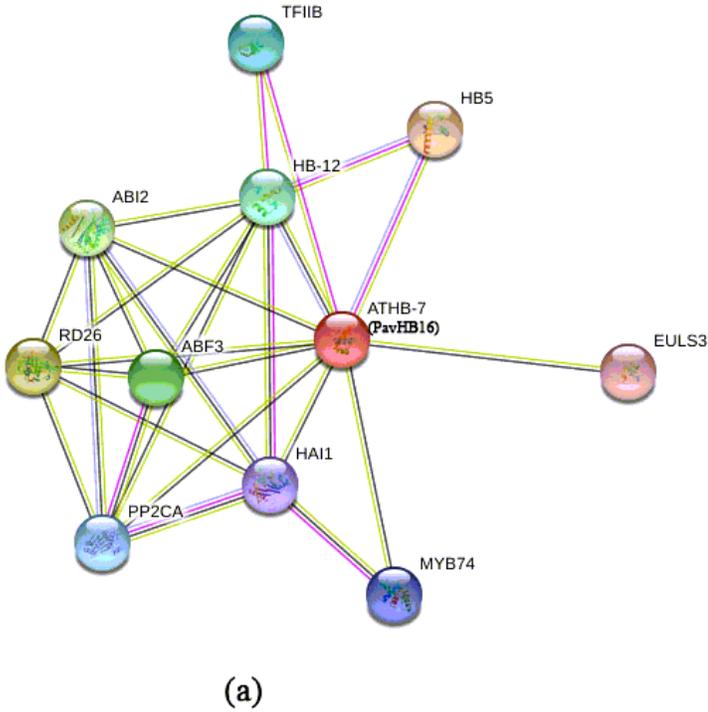


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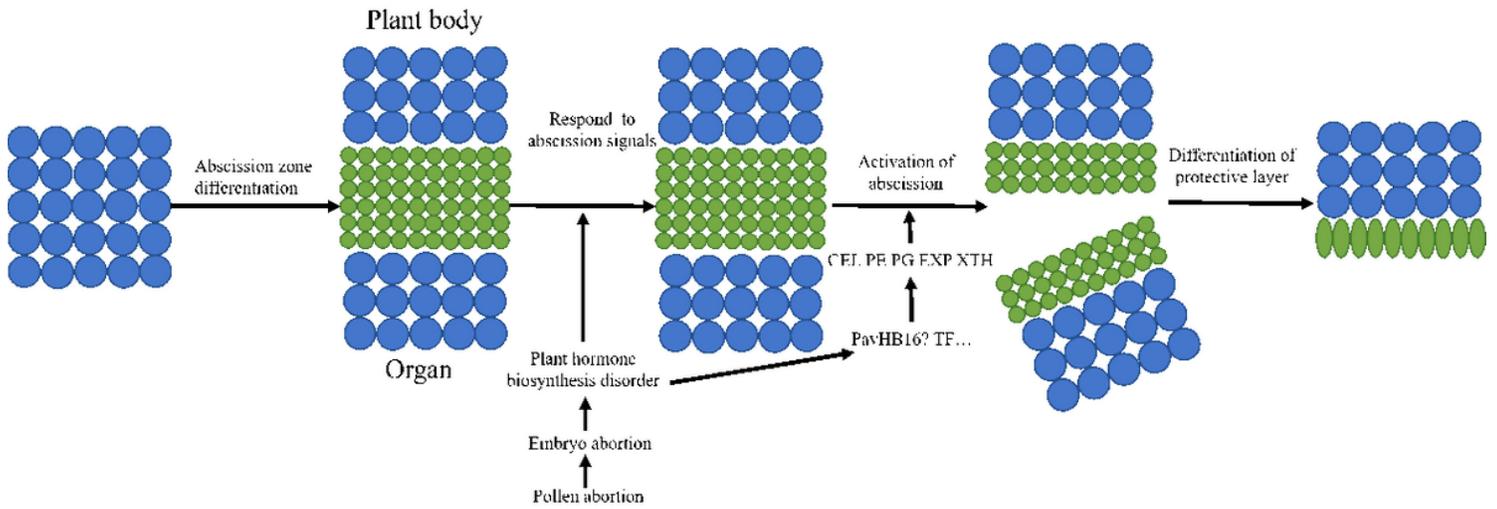


Figure 13

Model of fruitlet abscission in sweet cherry.

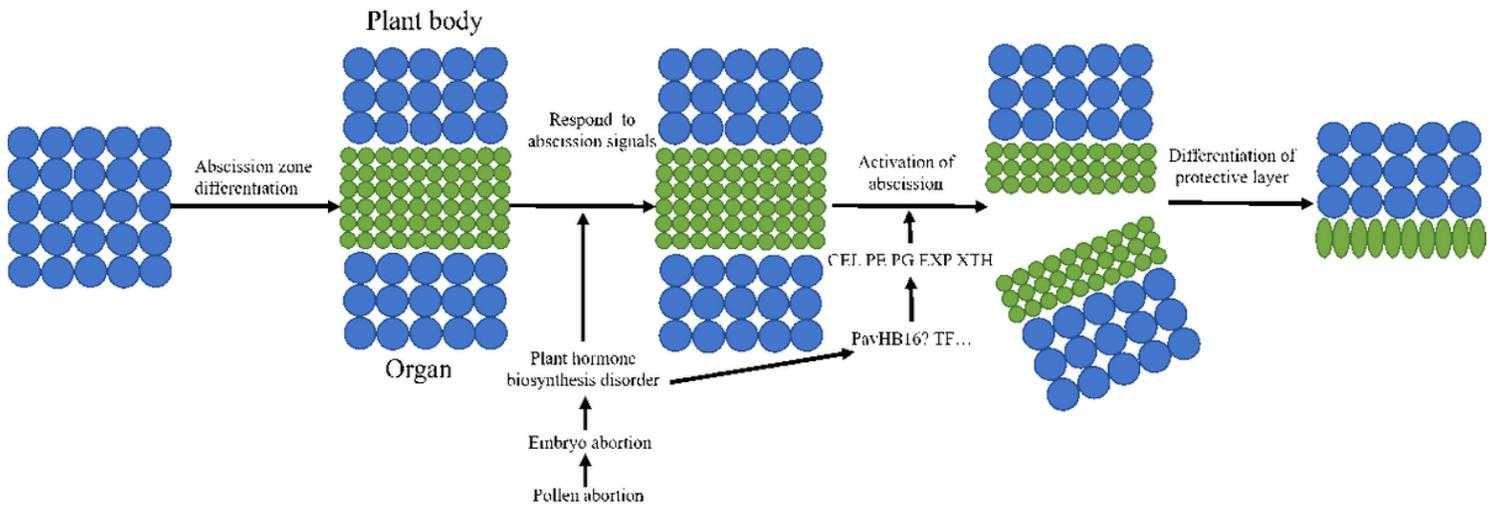


Figure 13

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