# Transcriptome and Metabolomic Analysis to Reveal the Browning Spot Formation of ‘Huangguan’ Pear

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**Research Article**

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# Transcriptome and metabolomic analysis to reveal the browning spot formation of 'Huangguan' pear

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**Abstract**:

**background**: Browning spot (BS) disorders seriously affect the appearance quality of 'Huangguan' pear and cause economic losses. Many studies on BS have mainly focused on physiological and biochemical aspects, and the molecular mechanism is unclear.

**Result:** In the present study, the structural characteristics of 'Huangguan' pear with BS were observed with SEM, the water loss and brown spot were evaluated, and transcriptome and metabonomics analyses were conducted to reveal the molecular mechanism of 'Huangguan' pear skin browning disorder. The results showed that the occurrence of BS was accompanied by a decrease in the wax layer and an increase in lignified cells. It appears that genes related to wax biosynthesis were down-regulated in BS, resulting in the decrease of wax layer in BS. Genes related to lignin were up-regulated at transcriptional level, resulting in upregulation of metabolites related to phenylpropanoid biosynthesis. Expression of calcium-related genes were upregulated in BS. The cold-induced genes *CS120, LTI65* and *RCI2B* may be the key genes that induce BS. In addition, the results demonstrated that exogenous $NaH_2PO_4 \cdot 2H_2O$ and ABA treatment could inhibit the incidence of BS during harvest and storage time by increasing the expression of wax-related genes and calcium-related genes and increasing plant resistance, whereas $GA_3$ may accelerate the incidence and index of BS in transcriptomics.

**Conclusions:** The results of this study would provide a basis for the molecular mechanism of BS formation and clarify the effects of different treatments on the incidence and molecular regulation of BS.

**Keywords**: 'Huangguan' pear, browning disorder, transcriptome, metabolomic, molecular mechanism

## 1. Background

'Huangguan' pear (*Pyrus bretschneideri* × *Pyrus pyrifolia*) is an early- and medium-maturing cultivar widely planted in northern China that has a high-quality and exquisite appearance after bagging[1]. However, browning spot (BS) disease often occurs at the surface of 'Huangguan' pear fruits after bagging before harvest or during storage[2]. The symptom of BS is a brown spot at first, and then the brown spot spreads irregularly from the disease spot to the surroundings and becomes darker during fruit maturation[2, 3]. Whole fruit browning may occur in the later stages of this disease. It's interesting that this disorder only affects the exocarp of pear fruit, and the flesh and core are not affected[4]. Multiple lesions are connected into a round, irregular shape or chicken claw shape. Therefore, BS disorder is also known as chicken-claw disease by orchardman in China, which causes a significant decrease in the commercial value of fruit for fruit farmers[5].

BS was first discovered in Xinji City, Hebei Province, in 1996. This disease mainly occurs on 'Huangguan' pears. However, a small number of green pear varieties, such as 'Lvbaoshi' (*P. pyrifolia* Nakai), 'Suisho' (*P. pyrifolia* Nakai), 'Xuehua' (*P. bretschneideri* Rehd.) and 'Xueqing' (*P. pyrifolia* Nakai), also experience BS[6]. It was reported that BS disease of 'Huangguan' pear is an important physiological disorder[7-9] that mainly occurs in bagged fruits at the mature stage and after low-temperature storage[10-12]. In general, BS disorder of 'Huangguan' pear is affected by many factors, such as environmental factors (continuous rainfall and low temperature weather[10], the use of chemical fertilizers[13]), preharvest factors (bagging times, type of fruit bags[3, 14], the use of swelling agents[15]), and postharvest factors (the duration of cooling period[10-12, 16], the storage temperature and the concentrations of $CO_2$ and $O_2$ [17-19]).

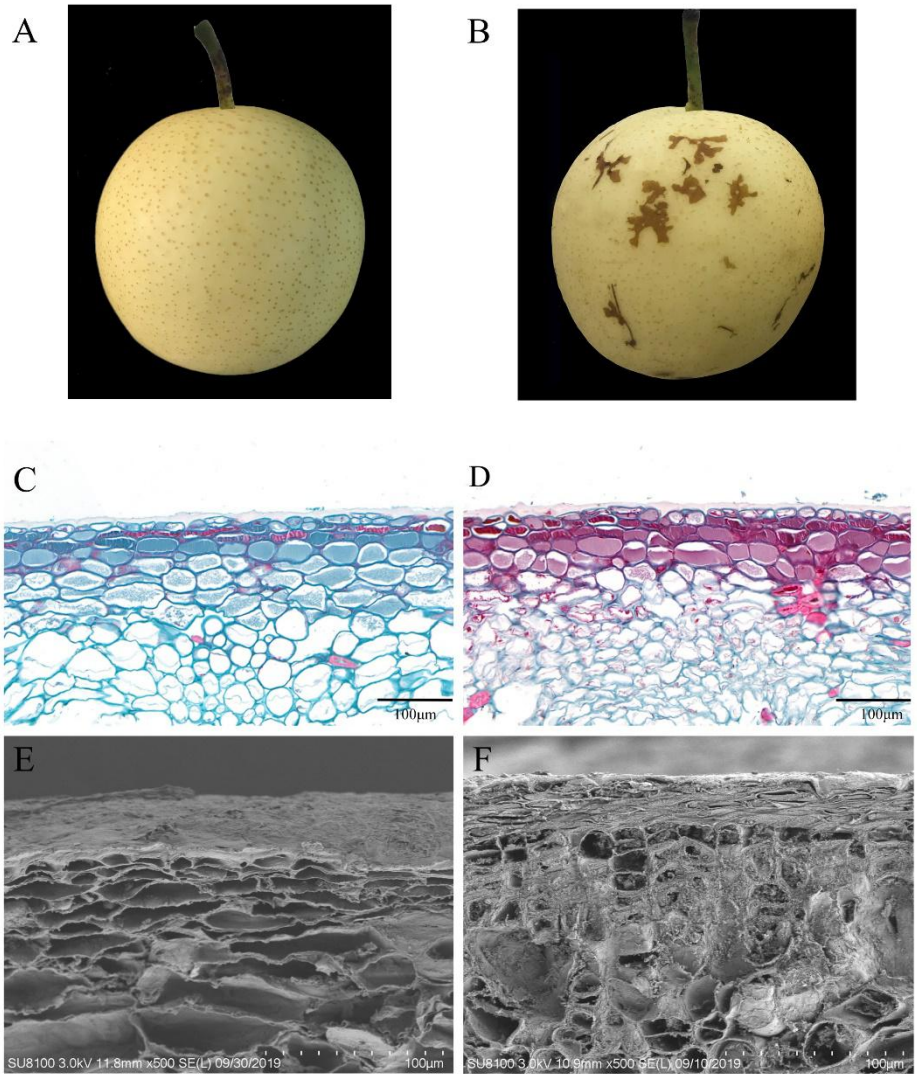
Some researchers believe that the thinning of the wax layer and skin cell wall of pears caused by bagging is the main cause of BS[14]. The adaptability of fruit exocarp to severe environmental changes is reduced, and the development of fruit exocarp is delayed after bagging. It has been reported that BS is closely related to calcium deficiency and phenolic dysregulation in pericarp tissue[7, 17, 20]. To date, research on BS disease has mainly focused on mineral nutrition (such as Ca[1, 3, 7, 9, 15, 21-25], Mg[3, 7], K[3, 7] and B[21]) and physiology and biochemistry[26, 27]. Additionally, the use of swelling agents may be one of the causes of BS[15]. Exogenous treatment with ethylene[2, 28], methyl jasmonate (MeJA)[29], 1-methylcyclopropene (1-MCP)[16] and $CaCl_2$[30] has been reported to affect the browning of postharvest 'Huangguan' pear. Recent reports have demonstrated that SA treatment can inhibit BS during cold storage. In addition, it has been reported that rapid postharvest cooling tends to increase BS, while slow cooling inhibits BS[10]. However, there are few studies on the exogenous treatment of phytohormones and molecular mechanisms that regulate BS processes in 'Huangguan' pear.

This study observed the changes at the site of BS and analyzed the molecular mechanism of BS at the transcriptomic and metabolomic levels. The incidence of BS after treatment with exogenous reagents [$NaH_2PO_4 \cdot 2H_2O$ (P), abscisic acid (ABA), gibberellin A3 ($GA_3$)] during harvest and storage was investigated. The key genes involved in exocarp formation were also analyzed after treatment, which would provide a basis for the molecular mechanism of BS and clarify the influence of different treatments on the molecular regulation of BS.

## Results

### Phenotype characteristics of BS disease of 'Huangguan' pear

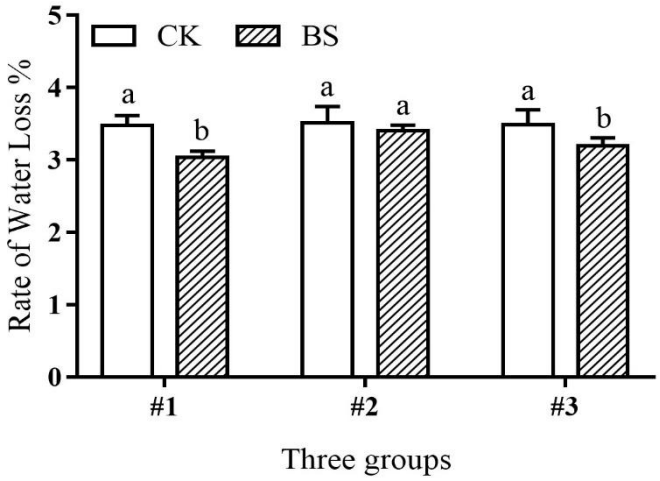
Compared with the normal pear skin (Fig. 1A) of ‘Huangguan’ pear, the BS-infected skin exhibits an irregular chicken claw shape, and the location of the disease is not fixed (Fig. 1B). BS is a physiological disease with slight depression in the affected area. Paraffin section observation revealed that the degree of lignification of the exocarp cells of BS parts was significantly higher than that of the normal parts (Fig. 1 B).C, D). Scanning electron microscopy (SEM) revealed a thick cuticular layer on the skin of the normal ‘Huangguan’ pear, but the BS part of ‘Huangguan’ pear skin consisted of layers of dead cells, and exocarp cells were more densely arranged (Fig. 1 B). E, F). Therefore, the occurrence




of BS may be caused by necrosis of the exocarp and hypodermal cortical tissues.

**Fig. 1 Phenotypes of normal ‘Huangguan’ pear (A) and ‘Huangguan’ pear with BS disease (B).** Observation of the paraffin sections of the normal part (C) and BS disease part (D) of ‘Huangguan pear’. SEM analysis of the normal part (E) and BS disease part (F) of ‘Huangguan’ pear.

Alternatively, we found many cracks on the fruit surface, while the cracks on the BS part disappeared (Additional file 1: Figure. S1). Thus, the experiment to detect RWL between CK and BS was conducted. After 10 d of storage at room temperature (25°C), the RWLs of the three groups of ‘Huangguan’ pear and ‘Huangguan’ pear with BS disease were calculated. The results showed that the RWLs of two groups (#1 and #3) of ‘Huangguan’ pear fruits were significantly higher than that of ‘Huangguan’ pear with BS disease (Fig. 2). The average RWLs of the CK and BS groups were 3.49%




and 3.19%, respectively. This means that BS lesions could inhibit water loss, which may be regulated by the layers of densely arranged dead cells at the fruit surface.

**Fig. 2 RWL of CK and BS of ‘Huangguan’ pear at 10 days of storage under room conditions after harvest.** The vertical bar indicates the standard error. The reported value is the mean ± SEM ($p > 0.05$). The ordinate represents three different groups, and each group has 10 fruits.

**Transcriptome and metabolome profiles of 'Huangguan pear' exocarp**

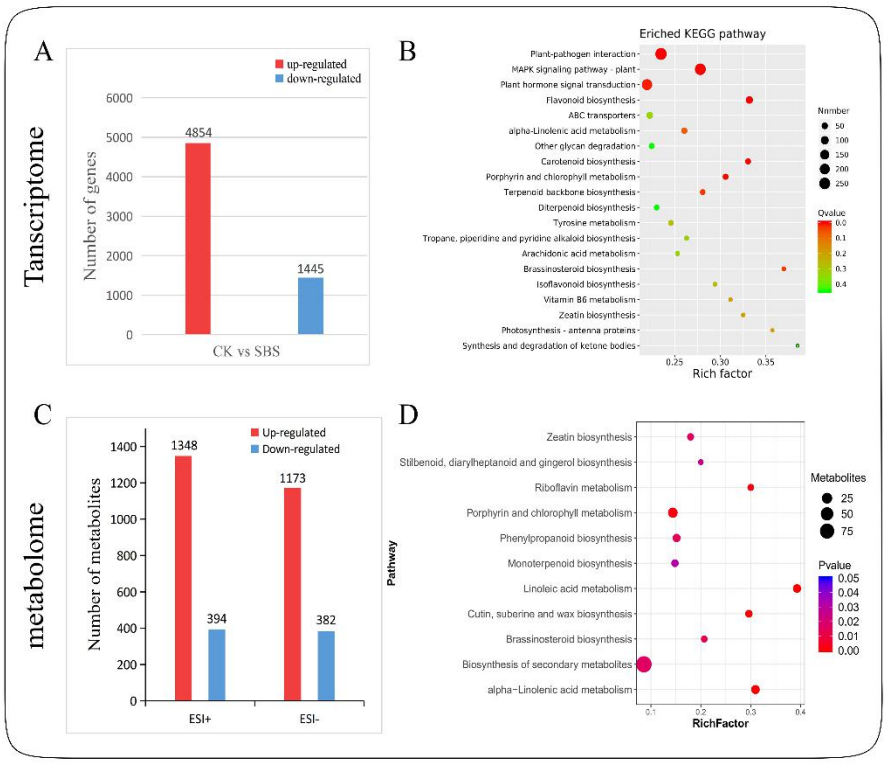
RNA-Seq generated 6.24 gigabytes (GB) of clean data of each sample from the 5 complementary DNA (cDNA) libraries. A total of 30,596 expressed genes were identified, including 1,212 new genes that were initially identified in this study. Successfully mapped reads ranged between 69.91% and 72.68%, and the average was 71.29% (Additional file 2: Table S1).

To compare control group (CK) and BS-infected group (BS) metabolites of 'Huangguan' pears, datasets obtained from a Xevo G2 XS QTOF high-resolution tandem mass spectrometer (Waters) in electrospray ionization positive ion mode (ESI+) and negative ion mode (ESI−) were subjected to principal component analysis (PCA). The results showed that metabolites from CK and BS were clearly separated in the score plots, where the first principal component (PC1) was plotted against the second principal component (PC2). (Additional file 1: Figure S2 A, B). PLS-DA (Plots from partial least squares discriminant) analyses were further used to check the metabolite differences between CK and BS (Additional file 1: Figure S2 C, D). These results showed significant biochemical differences between CK and BS.

**Transcriptome and metabolome differences between CK and BS**

Transcript analysis of the two comparison groups by DESeq2[31] identified 6299 DEGs, including 4854 induced and 1445 repressed DEGs in the BS pear exocarp (Fig. 3A). To classify the functions of DEGs between CK and BS, the assembled unigenes were annotated by using different protein databases (GO, KEGG) for homologous alignment. In the GO categories, DEGs were annotated in 1212 GO terms with 1480 unigenes in biological process, 1906 unigenes in cellular component, and 1609 unigenes in molecular function, which included terms such as metabolic process, membrane and

catalytic activity (Additional file 1: Figure S3). KEGG pathway annotation analysis showed that global and overview maps, carbohydrate metabolism, signal transduction and environmental adaptation were overrepresented (Additional file 1: Figure S4). KEGG enrichment analysis was further used to assess DEGs between CK and BS. We found seven significant pathways, including the MAPK signaling pathway (245), flavonoid biosynthesis (72), plant-pathogen interaction (267), carotenoid biosynthesis (38), porphyrin and chlorophyll metabolism (37), plant hormone signal transduction (216) and




brassinosteroid biosynthesis (17) (Fig. 3B). To further identify the functions of BS-related genes, we analyzed gene expression in those significantly enriched pathways. The numbers of up- and downregulated genes are listed in Table 1.

**Fig. 3 Significant DEG and DEM analysis between CK and BS.** (A) Column chart of DEGs; red represents upregulated DEGs, and blue represents downregulated DEGs. (B) KEGG enrichment

analysis of DEGs between CK and BS. The number of genes in each pathway is equal to the dot size. The dot color represents the q-value. The smaller the q-value, the redder the dot. (C) Numbers of upregulated (red) and downregulated (blue) metabolites. (D) KEGG enrichment analysis of differential metabolites. The number of DEMs in each pathway is equal to the dot size. The dot color represents the P-value. A redder point represents a smaller P-value.

Table 1 The top 7 enriched pathways of DEGs in BS

| Pathway name | Type | Down | Pathway ID | Q-value |
|---|---|---|---|---|
| MAPK signaling pathway - plant | Environmental Information Processing | 34 | ko04016 | 4.86E-12 |
| Flavonoid biosynthesis | Metabolism | 21 | ko00941 | 1.47E-06 |
| Plant-pathogen interaction | Organismal Systems | 22 | ko04626 | 1.14E-05 |
| Carotenoid biosynthesis | Metabolism | 12 | ko00906 | 0.001625608 |
| Porphyrin and chlorophyll metabolism | Metabolism | 13 | ko00860 | 0.008369283 |
| Plant hormone signal transduction | Environmental Information Processing | 71 | ko04075 | 0.008369283 |
| Brassinosteroid biosynthesis | Metabolism | 3 | ko00905 | 0.02783563 |

These were selected with an FDR adjusted Q-value< 0.05

We characterized the exocarp of ‘Huangguan’ pear metabolomic changes in the BS disease part. A total of 8829 and 8646 ions were identified in ESI+ and ESI− modes, respectively. After filtering low-quality ions that had RSD > 30%, 8432 and 7887 ions were retained in ESI+ and ESI− modes, respectively. Then, we identified differential metabolites between CK and BS, and we detected 1742 and 1555 differential icons in BS, including 1348 and 1173 upregulated icons and 394 and 382

136 downregulated icons in ESI+ and ESI− modes, respectively (Fig. 3C). In addition, 1581 and 781
137 differentiated metabolites were categorized into 96 and 74 KEGG pathways in ESI+ and ESI− mode,
138 respectively (Additional file 3: Table S2). KEGG enrichment analysis of differentiated metabolites
139 (removing the duplicated ions in ESI+ and ESI− mode) showed that the pathways biosynthesis of
140 secondary metabolites, porphyrin and chlorophyll metabolism, cutin suberin and wax biosynthesis,
141 phenylpropanoid biosynthesis, alpha-linolenic acid metabolism and brassinosteroid biosynthesis were
142 the most abundant (Fig. 3D). In addition, the up- and downregulation of differential metabolites are
143 listed in Table 2.

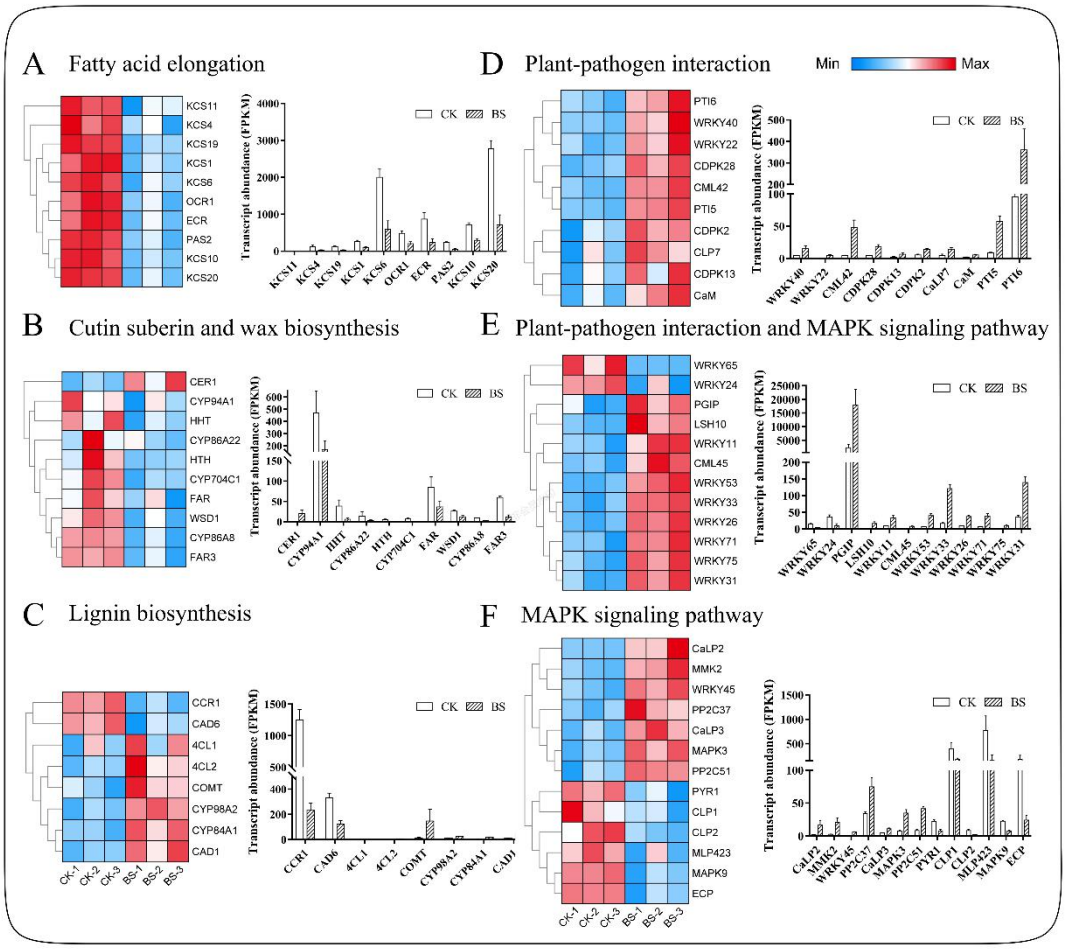
144 Table 2 Enriched KEGG pathways of differential metabolites between CK and BS

| Pathway | Count | Up | Down | Pathway ID |
|---|---|---|---|---|
| Biosynthesis of secondary metabolites | 228 | 173 | 55 | map01110 |
| Phenylpropanoid biosynthesis | 31 | 22 | 9 | map00940 |
| Porphyrin and chlorophyll metabolism | 29 | 21 | 8 | map00860 |
| Flavonoid biosynthesis | 18 | 16 | 2 | map00941 |
| Brassinosteroid biosynthesis | 17 | 15 | 2 | map00905 |
| Carotenoid biosynthesis | 16 | 13 | 3 | map00906 |
| Linoleic acid metabolism | 16 | 14 | 2 | map00591 |
| alpha-Linolenic acid metabolism | 13 | 12 | 1 | map00592 |
| Cutin, suberine and wax biosynthesis | 11 | 11 | 0 | map00073 |

145 **Analysis of DEGs and DEMs between CK and BS**

146 The phenotypic characteristics and metabonomics analysis of the pericarp indicated that the cutin
147 suberin and wax biosynthesis pathway and lignin biosynthesis may be involved in the formation of BS.
148 The fatty acid elongation pathway is upstream of cutin suberin and wax biosynthesis[32]. At the
149 transcriptome level, we found that 10 DEGs in the fatty acid elongation pathway were downregulated

in BS, including *KCS11*, *KCS4*, *KCS19*, *KCS1*, *KCS6*, *OCR1*, *ECR*, *PAS2*, *KCS10* and *KCS20* (Fig. 4A). Additionally, in the cutin suberin and wax biosynthesis pathways, 9 DEGs were downregulated,




including *CYP94A1*, *HHT*, *CYP86A22*, *HTH*, *CYP704C1*, *FAR*, *WSD1*, *CYP86A6*, and *FAR3,* while 11 DEMs in this pathway were all upregulated. (Fig. 4B, Table 2). Six genes involved in lignin biosynthesis were upregulated, including *4CL2*, *CAD1*, *CYP84A1*, *4CL1*, *CYP98A2*, and *COMT1*, and two genes were downregulated, including *CAD6* and *CCR1* (Fig. 4C), resulting in the upregulation of the metabolites in the phenylpropanoid biosynthesis pathway (Table 2).

**Fig. 4 Significant DEGs between CK and BS.** Heatmap of DEGs involved in fatty acid elongation (A), cutin suberin and wax biosynthesis (B), lignin biosynthesis (C), plant-pathogen interaction (D)，

both plant-pathogen interaction and MAPK signaling pathway (E), MAPK signaling pathway (F). Red represents upregulation, and blue represents downregulation.

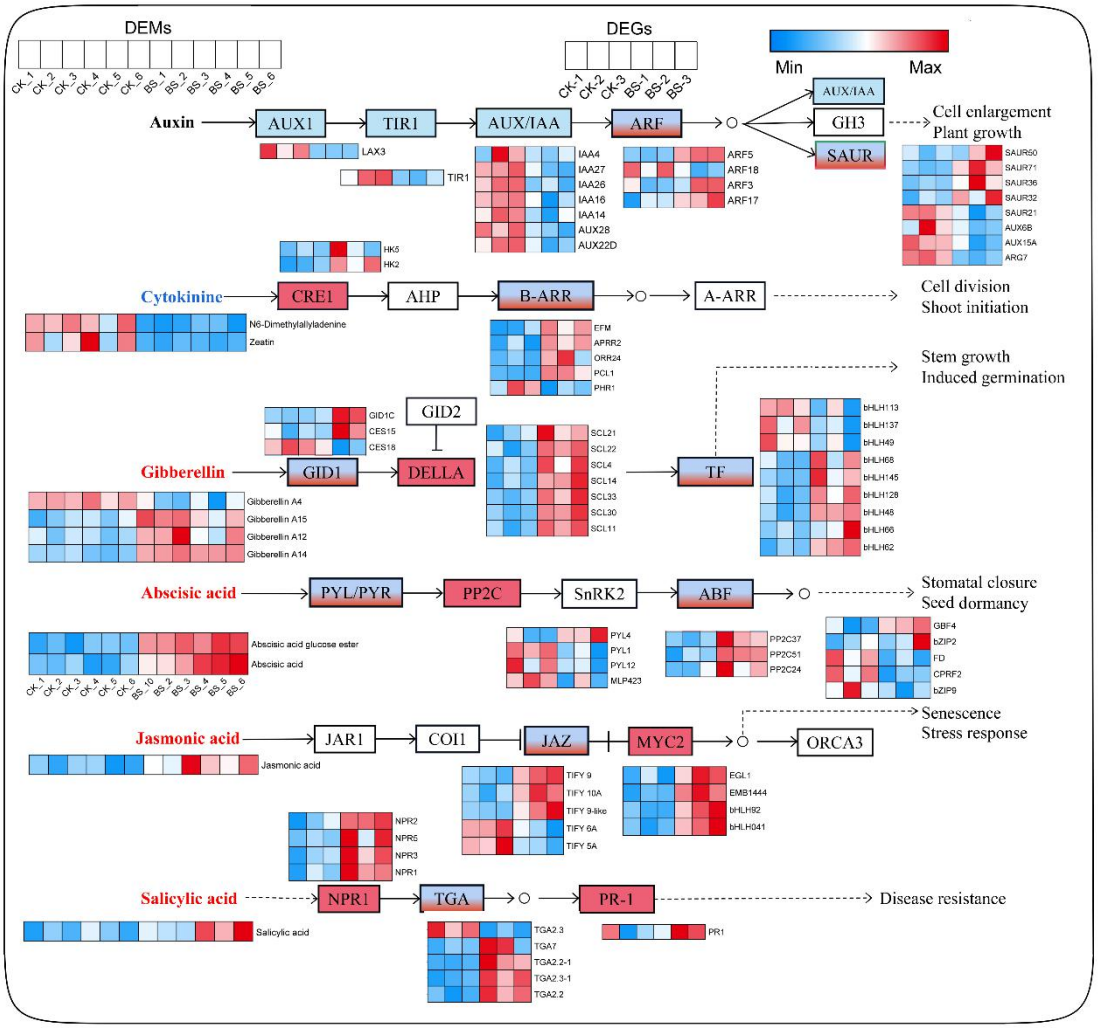
Transcriptome analysis revealed that plant-pathogen interactions (PPI) and the MAPK signaling pathway (MAPK) are also key pathways associated with BS. We detected 12 DEGs involved in both PPI and MAPK, including *PGIP, LSH10, CML45* and 9 WRKY family TFs (Fig. 4D). In the PPI pathway, we found that 10 genes were upregulated in BS, including three calcium-related proteins, CaM, CML42, and CaLP7; two WRKY family TFs, *WRKY40* and *WRKY22*; two pathogenesis-related genes, *PTI5* and *PTI6*; and three CDPK family genes, *CDPK28*, *CDPK2* and *CDPK13*. In the MAPK signaling pathway, we found that *CaLP2*, *MMK2 WRKY45*, *PP2C37*, *CaLP3*, *MAPK3*, and *PP2C51* were upregulated, and *PYR1*, *CLP1*, *CLP2*, *MLP423*, *MAPK9*, and *ECP* were downregulated. Detailed information is listed in Additional file 4: Table S3.

Through metabolomics, we found that there was no significant difference in auxin (IAA) content between CK and BS. In and cytokinin (CTK), it appears that N6-Dimethylallyladenine and zeatin were reduced in BS, while gibberellin (GA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) were all up-regulated in BS. In addition, we identified 216 DEGs involved in plant hormone signal transduction transcriptome analysis (Fig. 5). In the auxin, CTK and GA pathways related to cell growth and division, we identified 55, 15, and 37 DEGs, respectively. Our results indicated that BS-related pathways interact with auxin regulation through the transcription of *AUX/IAA, IAA4*, *IAA27*, *IAA26*, *IAA16*, *IAA14*, *AUX28* and *AUX22D* and were downregulated in BS. In the CTK pathway, two genes were upregulated in *CER1,* including *HK5* and *HK2,* and four genes were upregulated in *B-APR*, including EFM, APRR2, *ORR24* and *PCL1.* In the GA pathway, *GID1C* and *CES15* were upregulated

180 in *GID1*, and seven genes were upregulated in *DELLA*, including *SCL21*, *SCL22*, *SCL4*, *SCL14*, *SCL33*,
181 *SCL30,* and *SCL11*. Additionally, nine TFs in the bHLH family were identified, of which six were
182 upregulated and three were downregulated. In the ABA, JA and SA pathways related to the stress
183 response, 18, 23 and 12 DEGs were identified, respectively. In the ABA pathway, three genes
184 upregulated in *PP2C* were identified, including *PP2C37, PP2C51,* and *PP2C24.* In the JA pathway,
185 four genes upregulated in *MYC2,* including *EGL1, EMB1444, bHLH92,* and *bHLH041,* were identified.
186 In the SA pathway, four *NPR1* genes, four *TGA* genes and the *PR-1* gene were also upregulated in BS.
187 Detailed information on all genes involved in plant hormone signal transduction is listed in Additional

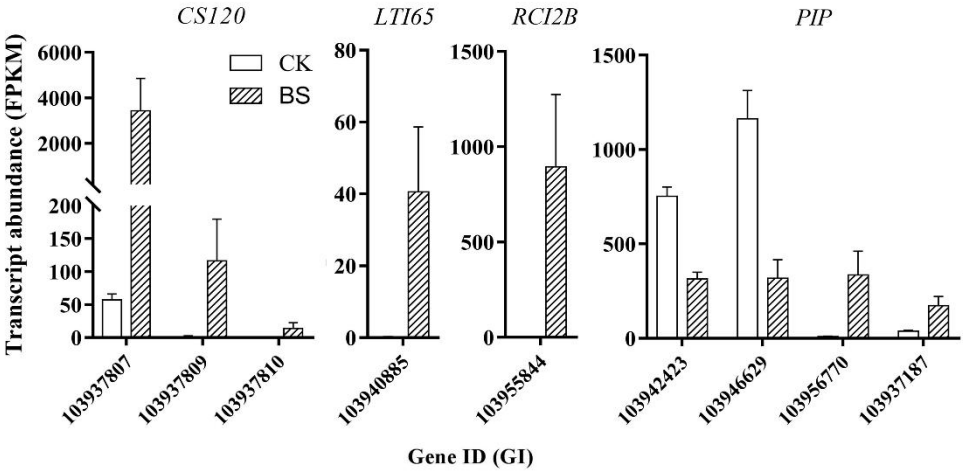
file 4：Table S4.

**Fig. 5 Significant DEGs and DEMs involved in plant hormone signal transduction between CK and BS.** Red represents upregulation, and blue represents downregulation.




It has been reported that BS is associated with a sudden drop in temperature. Cold exercise or slow cooling are commonly used in production to reduce the incidence of BS[10-12]. We identified three cold-shock protein CS120-like (*CS120*) genes (gene ID：103937809, 103937810, 103937807) and one low-temperature-induced 65 kDa protein-like isoform X1 (*LTI65,* gene ID： 103940885) that were significantly upregulated in BS (Fig. 6). Hydrophobic protein RCI2B (*RCI2B,* gene ID: 103955844)

has been proven to be a cold-induced gene[33] that is upregulated in BS. Aquaporin is a membrane protein that was originally characterized as a water channel through which $H_2O$ could permeate biological membranes[34]. Four DEGs in aquaporin PIP (gene ID: 103946629, 103942423, 103937187, 103956770), *PIP1-4* and *PIP2-8* were upregulated, while *PIP2-2* and *PIP2-5* were downregulated in BS.




| Gene ID | Annotation |
|---|---|
| 103937807 | cold-shock protein CS120-like |
| 103937809 | cold-shock protein CS120-like |
| 103937810 | cold-shock protein CS120-like isoform X1 |
| 103940885 | low-temperature-induced 65 kDa protein-like isoform X1 |
| 103955844 | hydrophobic protein RCI2B |
| 103942423 | probable aquaporin PIP1-4 |
| 103946629 | probable aquaporin PIP2-8 |
| 103956770 | aquaporin PIP2-2 |
| 103937187 | probable aquaporin PIP2-5 |

**Fig. 6 Transcript abundance of significant DEGs between CK and BS.** The error bars are the means ± SEM of three biological repeats.

**Transcription factors (TFs) involved in BS formation**

TFs are important regulators that activate or repress the expression of both coding and noncoding genes to influence or control many biological processes[35]. In our analysis of the transcriptome data,

206 we detected 423 differentially expressed TFs between CK and BS, including 341 upregulated and 82

207 downregulated TFs. The AP2-EREBP, MYB and WRKY families were the most abundant TF families

208 between CK and BS, followed by the bHLH, NAC, C2H2, and HSF families (Table 3).

209 Table 3 Differentially expressed transcription factors (TFs) between CK and BS

| TF family | Number | Up | Down | Description |
|---|---|---|---|---|
| AP2-EREBP | 53 | 40 | 13 | Ethylene-responsive transcription factor |
| MYB | 49 | 43 | 6 | MYB-related protein |
| WRKY | 46 | 44 | 2 | WRKY DNA -binding domain |
| bHLH | 35 | 21 | 14 | Helix-loop-helix DNA-binding domain |
| NAC | 26 | 24 | 2 | NAC domain-containing protein |
| C2H2 | 17 | 16 | 1 | Zinc finger protein |
| HSF | 16 | 16 | 0 | Heat stress transcription factor |
| GRAS | 15 | 15 | 0 | scarecrow-like protein |
| LOB | 13 | 10 | 3 | LOB domain-containing protein |
| MADS | 10 | 8 | 2 | SRF-type transcription factor |
| G2-like | 10 | 9 | 1 | myb-related protein |
| C3H | 9 | 8 | 1 | Zinc finger CCCH domain-containing protein |
| mTERF | 9 | 8 | 1 | mTERF domain-containing protein |
| C2C2-Dof | 9 | 6 | 3 | dof zinc finger protein |
| TCP | 7 | 5 | 2 | Circadian rhythm - plant |
| FHA | 7 | 5 | 2 | FHA domain-containing protein |
| C2C2-GATA | 7 | 4 | 3 | GATA-binding protein |
| Tify | 7 | 5 | 2 | jasmonate ZIM domain-containing protein |
| C2C2-CO-like | 7 | 6 | 1 | zinc finger protein CONSTANS |
| ABI3VP1 | 6 | 3 | 3 | B3 domain-containing protein |
| Trihelix | 6 | 5 | 1 | trihelix transcription factor |
| OFP | 6 | 3 | 3 | isoleucyl-tRNA synthetase |
| FAR1 | 5 | 5 | 0 | zinc finger SWIM domain-containing protein |
| ARF | 5 | 3 | 2 | auxin response factor |

| | | | |
|---|---|---|---|
| other TFs | 43 | 29 | 14 |
| total | 423 | 341 | 82 |

**Coexpression network of BS-related genes**

In our transcriptome analysis, we found that genes associated with wax, lignin, calcium, plant hormone signal transduction, as well as cold-induced gens. were the key genes for BS formation. We performed coexpression network analysis to illuminate the collaboration between those genes. Coexpression network analyses with transcriptome data showed that GA signal and IAA signal genes were classified into different coexpression clusters with wax, lignin biosynthesis and calcium-related genes (Fig. 7). We found that *bHLH137*, *bHLH128*, *IAA14* and *IAA27* were coexpressed with multiple genes involved in fatty acid elongation, cutin, suberin and wax biosynthesis, lignin biosynthesis, MAPK and PPI. This indicates that the formation of BS may be regulated by plant hormone signals, especially IAA and GA signals.

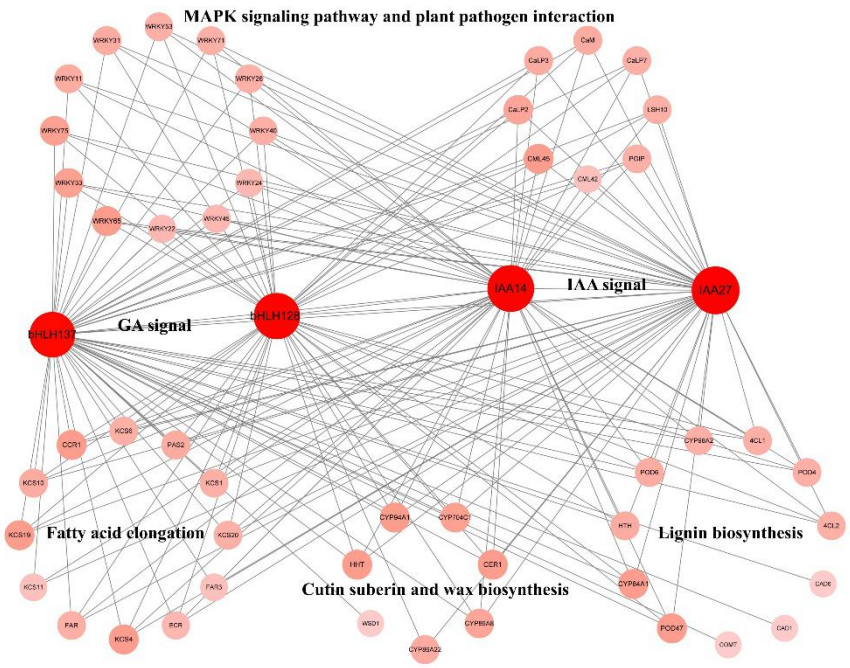
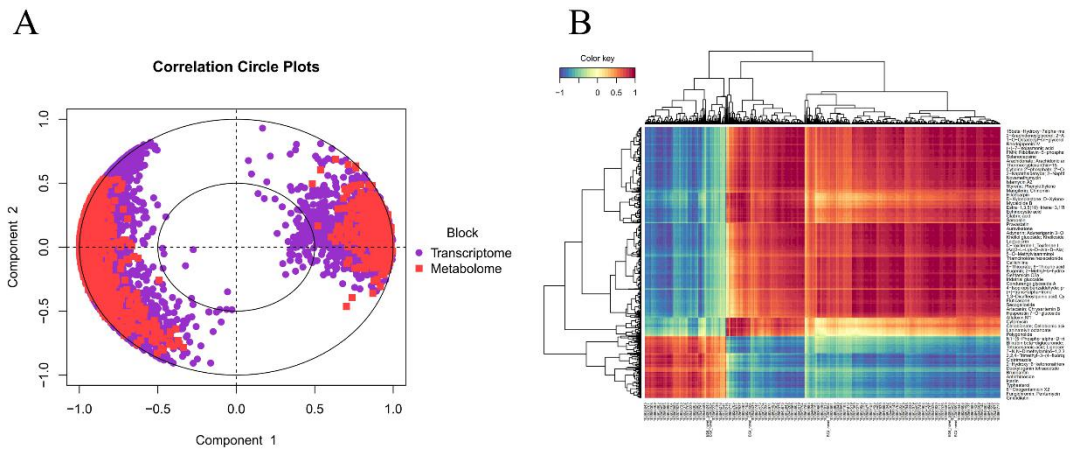



**Fig. 7 Coexpression network of genes involved in BS formation.** Detailed information on the genes

is listed in Additional file 4: Table S3 and Table S4.

**Combined analysis of the metabolome and transcriptome**

MixOmics[31] multifunctions were used for multivariable dimensionality reduction to explore the relationship between transcriptomics and metabolomics (Fig. 8A). The block.splsda function in mixOmics was used to analyze differential genes and differential metabolites, and plotVar and circosPlot functions were used to visualize the results. We found that there was a closely related correlation between DEGs and DEMs. Regularized canonical correlation analysis (rCCA)[36] was used to measure the degree of correlation between genes and metabolites (Fig. 8B). Approximately 75% of




DEGs and DEMs had a positive correlation, and approximately 25% of them had a negative correlation.

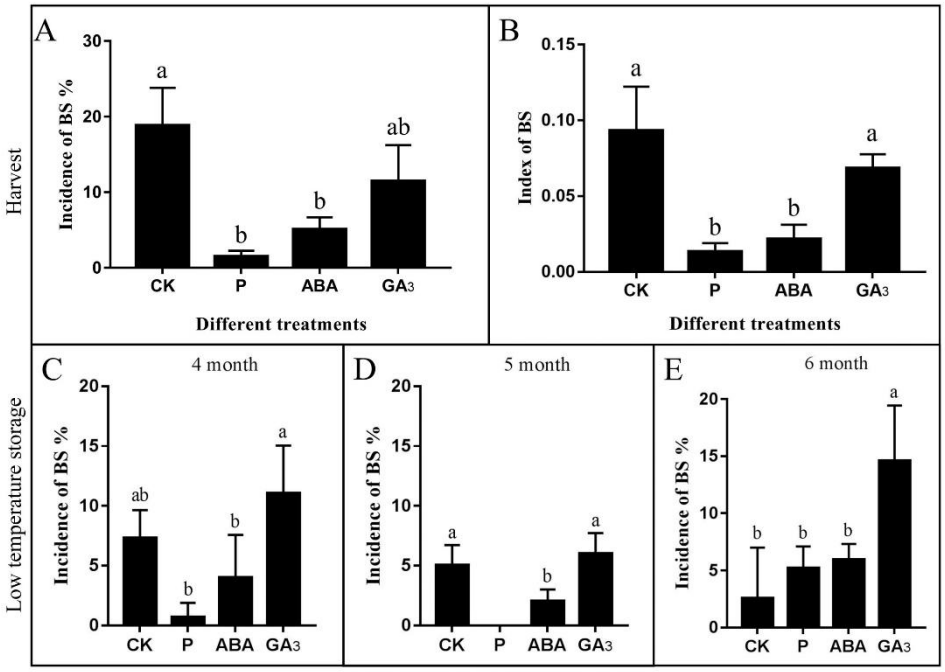
**Fig. 8 Combined analysis of the metabolome and transcriptome between CK and BS.** (A) Concentric diagram of the correlation of DEGs and DEMs between CK and BS. Each point in the circle represents a gene, and each square represents a metabolite. If the angle between the DEG and DEM is an acute angle, the correlation is positive. If the angle is the deltoid angle, it is negatively

correlated. In general, variables far away from the center of the circle are more closely related. (B) Heatmap cluster of DEGs and DEMs. Each row represents a DEM, and each column represents a DEG. Blue represents a negative correlation, and red represents a positive correlation.

**Effects of P, ABA and $GA_3$ treatments on BS**

Treatments with P, ABA, and $GA_3$ were performed to investigate their effects on the BS of 'Huangguan' pear (Fig. 9A, B). P and ABA treatments can significantly reduce the incidence and index of BS. The incidence and index of BS treated with $GA_3$ was higher than those of other treatments. The results showed that P treatment has the best inhibitory effect on BS disorder, and ABA also has a certain inhibitory effect on BS, while $GA_3$ treatment was able to promote the occurrence of BS.

In addition, we investigated the BS incidence of 'Huangguan' pears with different treatments during storage (Fig. 9C, D, E). We found that P treatment effectively inhibited BS at 4 and 5 months of storage (Fig. 9C, D). ABA treatment inhibited BS at 5 months of storage, while there was no significant difference in other time periods compared with CK (Fig. 9D). The incidence of BS was higher after $GA_3$ treatment during storage, indicating that $GA_3$ may cause the occurrence of BS after



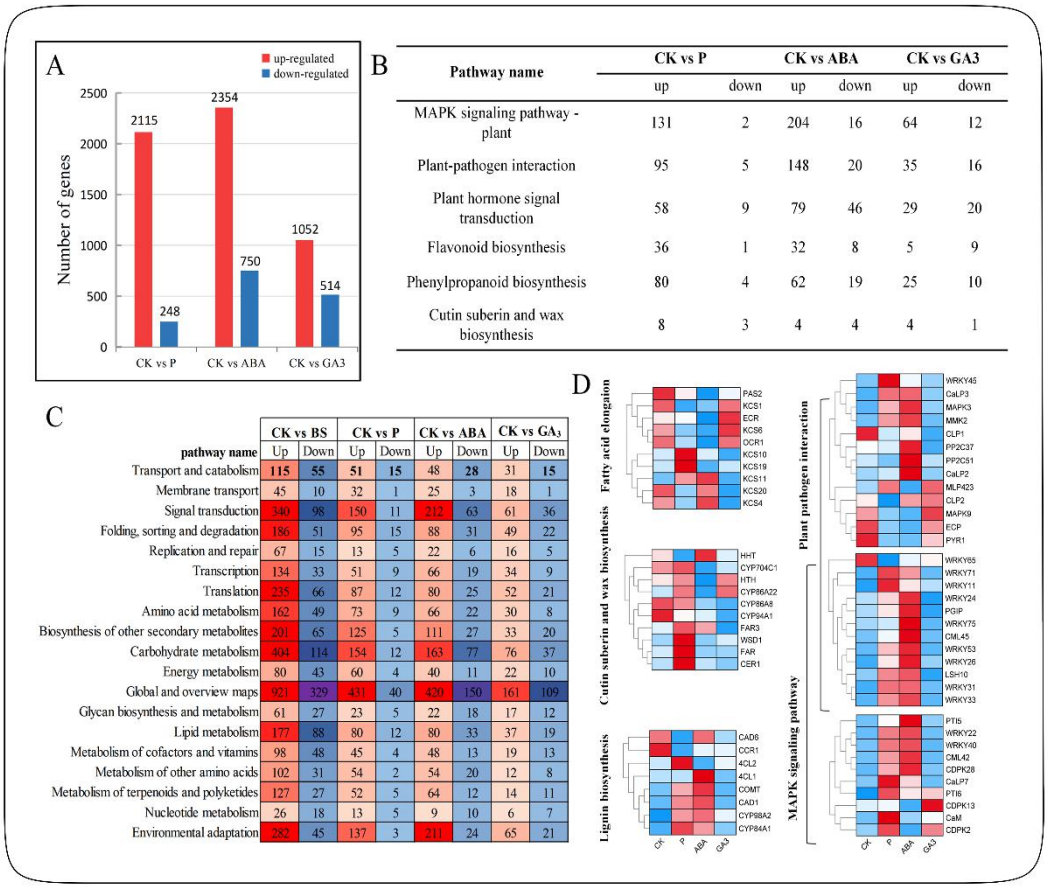
low-temperature storage (Fig. 9C, D, E).

**Fig. 9 The incidence of BS disorder after different treatments in 'Huangguan' pears.** (A) Incidence of BS disorder treated with exogenous P, ABA, and $GA_3$. (B) Index of BS disorder treated with exogenous P, ABA, and $GA_3$. Incidence of BS disorder with different treatments after 4 (C), 5 (D), and 6 (E) months of storage. The error bars are the means ± SEM of three biological repeats. ($P \leq 0.05$).

**Transcriptomics analysis of pear exocarp after P, ABA, and $GA_3$ treatments**

We analyzed the changes at the transcription level of pear exocarp to explore the effects of different treatments on the occurrence of BS. After P treatment, 2363 DEGs were identified, including 2115 upregulated genes and 248 downregulated genes. A total of 3104 DEGs occurred after treatment with ABA, including 2354 upregulated genes and 750 downregulated genes. $GA_3$ treatment caused 1566 DEGs, including 1052 upregulated genes and 514 downregulated genes (Fig. 10A). To classify the functions of DEGs after different treatments, KEGG annotation analysis was carried out and showed that global and overview maps, carbohydrate metabolism, signal transduction and environmental adaptation were overrepresented (Fig. 10C). Furthermore, we identified expression of genes involved in BS formation (Fig 10B). We found that the expression of wax biosynthesis related genes were upregulation after P treatment, such as *KCS10*, *KCS19*, *KCS11*, *FAR3*, *WSD1*, *CER1*. Similarly, ABA treatment also increased the expression of wax related genes, including *KCS11*, *KCS20*, *KCS 4*, *FAR3*. What's more, treatment with P and ABA increased the expression of many genes involved in PPI and MAPK, including calcium-related genes (*CaM*, *CaLP3*, *CaLP2*, *CaLP7*, *CML42*, *CML45*) and WRKY TFs (*WRKY71*, *WRKY11*, *WRKY24*, *WRKY75*, *WRKY53*, *WRKY26*, *WRKY22*,

*WRKY40*), which may improve the plant's resistance to disease. However, the effect of $GA_3$ treatment was not obvious. These results are consistent with the previous incidence of BS after three treatments.




**B**

| Pathway name | CK vs P | | CK vs ABA | | CK vs GA3 | |
|---|---|---|---|---|---|---|
| | up | down | up | down | up | down |
| MAPK signaling pathway - plant | 131 | 2 | 204 | 16 | 64 | 12 |
| Plant-pathogen interaction | 95 | 5 | 148 | 20 | 35 | 16 |
| Plant hormone signal transduction | 58 | 9 | 79 | 46 | 29 | 20 |
| Flavonoid biosynthesis | 36 | 1 | 32 | 8 | 5 | 9 |
| Phenylpropanoid biosynthesis | 80 | 4 | 62 | 19 | 25 | 10 |
| Cutin suberin and wax biosynthesis | 8 | 3 | 4 | 4 | 4 | 1 |

**C**

| | CK vs BS | | CK vs P | | CK vs ABA | | CK vs $GA_3$ | |
|---|---|---|---|---|---|---|---|---|
| pathway name | Up | Down | Up | Down | Up | Down | Up | Down |
| Transport and catabolism | **115** | **55** | **51** | **15** | 48 | **28** | 31 | **15** |
| Membrane transport | 45 | 10 | 32 | 1 | 25 | 3 | 18 | 1 |
| Signal transduction | 340 | 98 | 150 | 11 | 212 | 63 | 61 | 36 |
| Folding, sorting and degradation | 186 | 51 | 95 | 15 | 88 | 31 | 49 | 22 |
| Replication and repair | 67 | 15 | 13 | 5 | 22 | 6 | 16 | 5 |
| Transcription | 134 | 33 | 51 | 9 | 66 | 19 | 34 | 9 |
| Translation | 235 | 66 | 87 | 12 | 80 | 25 | 52 | 21 |
| Amino acid metabolism | 162 | 49 | 73 | 9 | 66 | 22 | 30 | 8 |
| Biosynthesis of other secondary metabolites | 201 | 65 | 125 | 5 | 111 | 27 | 33 | 20 |
| Carbohydrate metabolism | 404 | 114 | 154 | 12 | 163 | 77 | 76 | 37 |
| Energy metabolism | 80 | 43 | 60 | 4 | 40 | 11 | 22 | 10 |
| Global and overview maps | 921 | 329 | 431 | 40 | 420 | 150 | 161 | 109 |
| Glycan biosynthesis and metabolism | 61 | 27 | 23 | 5 | 22 | 18 | 17 | 12 |
| Lipid metabolism | 177 | 88 | 80 | 12 | 80 | 33 | 37 | 19 |
| Metabolism of cofactors and vitamins | 98 | 48 | 45 | 4 | 48 | 13 | 19 | 13 |
| Metabolism of other amino acids | 102 | 31 | 54 | 2 | 54 | 20 | 12 | 8 |
| Metabolism of terpenoids and polyketides | 127 | 27 | 52 | 5 | 64 | 12 | 14 | 11 |
| Nucleotide metabolism | 26 | 18 | 13 | 5 | 9 | 10 | 6 | 7 |
| Environmental adaptation | 282 | 45 | 137 | 3 | 211 | 24 | 65 | 21 |

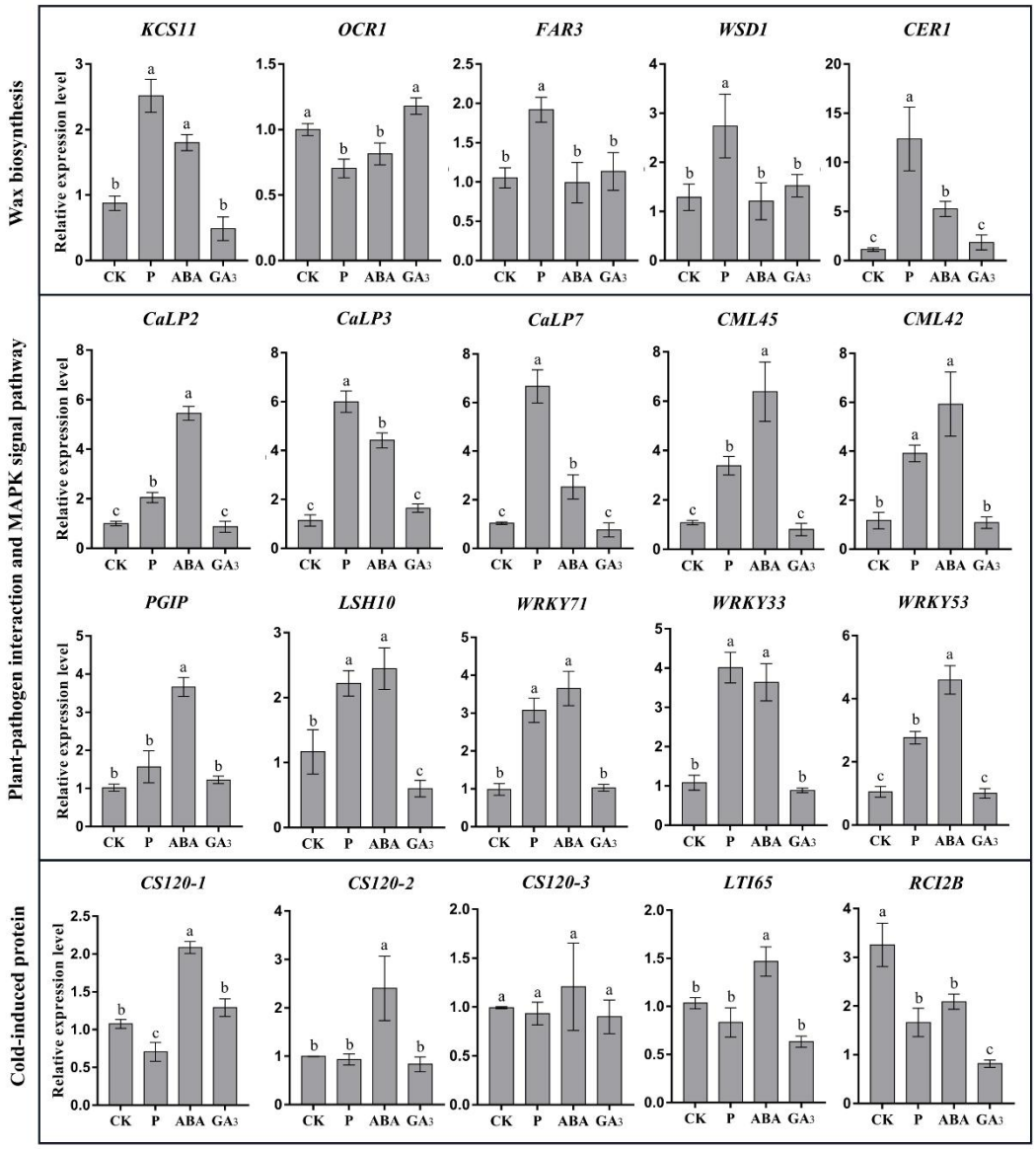
**Fig. 10 Significant DEGs between the CK-BS, CK-P, CK-ABA, and CK-$GA_3$ comparison groups.** (A) Column chart of DEGs. (B) The up- and downregulation of genes involved in BS-related pathways. (C) KEGG annotation of DEGs. (D) Expression of genes involved in BS related pathway after treatments. Red represents upregulation, and blue represents downregulation.

## Gene expression analysis by q-RT-PCR after treatment

Previous studies have shown that reduction of the wax layer may be one of the causes of BS. Therefore, we analyzed the expression of five wax-related genes in the pericarp of 'Huangguan' pear after different treatments (Fig. 11). We found that *KCS11*, *FAR3*, *WSD1*, and *CER1* were upregulated

after P treatment. ABA treatment improved the expression of *KCS11* and *CER1*. The expression of *OCR1* was downregulated after P and ABA treatment. However, there was no significant difference of those genes after $GA_3$ treatment. In addition, it is reported that BS is related to calcium deficiency in the peel[1, 7, 20]. We identified five calcium-related genes, including *CaLP2*, *CaLP3*, *CaLP7*, *CML45*, and *CML42*, which were all upregulated after P and ABA treatment. However, there was no significant change after $GA_3$ treatment (Fig. 11). Additionally, we detected five genes involved in both PPI and MAPK that can be activated by various biological and abiotic stresses[11], including *PGIP*, *LSH10* and three WRKY family TFs: *WRKY53*, *WRKY71*, *WRKY33*. Among them, the expression of *LSH10, WRKY53, WRKY71,* and *WRKY33* increased to different degrees after P and ABA treatment. The expression of *PGIP* was increased after ABA treatment. However, $GA_3$ treatment did not affect the expression of these genes and even had a persistent effect (Fig. 11). These results are consistent with transcriptome data.

Furthermore, the expression of five cold-induced genes were detected, including *CS120-1* (gene ID: 103937807), *CS120-2* (gene ID: 103937809), *CS120-3* (gene ID: 103937810), *LTI65* and *RCI2B.* Results show that ABA treatment could increase the expression of *CS120-1*, *CS120-2* and *LTI65,* while *CS120-1* and *LTI65* were downregulated after P treatment. The expression of *RCI2B* was decreased after all tree treatments. The results show that ABA treatment may improve the adaptability of fruit to chilling injury, while the effect of P and ABA treatment on the expression of cold-related genes was not obvious.




299 **Fig. 11 q-RT-PCR verification of genes related to BS after different treatments.** The error bars are

300 the means ± SEM of three biological repeats.

301 **The regulatory network of BS formation**

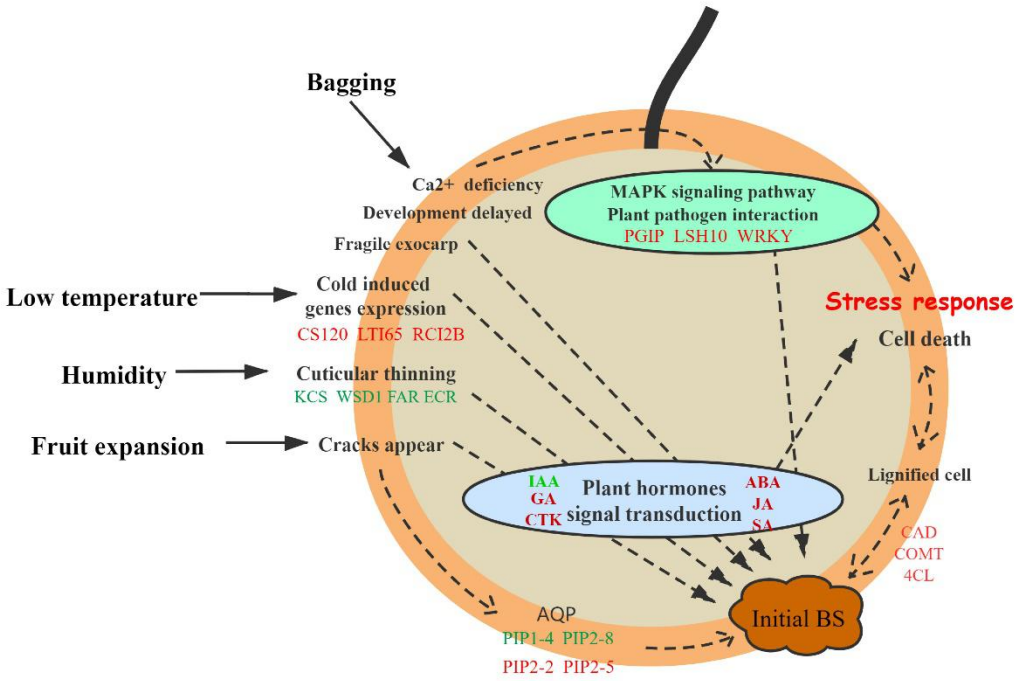
302 According to our investigation and research, we believe that many factors result in BS, especially

303 the low temperature. The possible regulatory network is shown in Fig. 12. The development of fruit

304 exocarp is delayed, and the concentration of $Ca^{2+}$ is reduced after bagging. The fragile peel cannot

305 withstand swelling when the fruit enlarges. The peel is stretched when the temperature drops, cracks

appear, and the expression of low temperature-induced genes is increased, which causes a series of defensive reactions through PPI and MAPK pathways. In addition, the high humidity conditions in bags cause cuticular thinning of the pear exocarp, which may cause cracks on the fruit surface. Then,




dead cells accumulate near those cracks, which ultimately become BS.

**Fig. 12 A proposed model of BS formation in 'Huangguan' pear fruits**. The red color represents upregulation and green color represents downregulation. The detailed gene information can be viewed in Additional file 4: Table S3, Table S4 and Fig. 6. IAA, Auxin; GA, gibberellic acid; CTK, cytokinin; ABA, abscisic acid; JA, jasmonic acid; SA, Salicylic acid.

## Discussion

### The influencing factors of BS of 'Huangguan' pear

BS disease is the main disease of 'Huangguan' pear and primarily occurs in bagged fruits at the

mature stage. However, a small amount of BS disorder has also been found on unbagged fruits, although the shape of the disease is mostly circular and not consistent with that of bagging (Additional file 1: Figure S5). Therefore, bagging may not be the only cause of BS. We observed that the onset of BS was characterized by a close arrangement of lignified dead cells accompanied by a significant reduction in epidermal wax (Fig. 1). Through transcriptomic analysis, it appears that the expression of wax-related genes in BS was decreased, while the expression of lignin-related genes was increased (Fig. 4), which was consistent with the observed phenotypic phenomenon. However, the cause of this phenomenon is still unclear.

It has been reported that BS is associated with sudden cold temperatures[10, 11, 16]. BS has been considered as a chilling injury symptom in cold-stored 'Huangguan' pear[28]. Studies have shown that 'Huangguan' pear are susceptible to BS disorder a few days after low temperature storage[2]. It has been reported that MeJA can improve chilling resistance of eggplant (*Solanum melongena* L.), which also can inhibit browning disorder[29, 37, 38]. It indicated that BS may cause by low temperature. We detected four low temperature-induced genes by transcriptomics that were highly expressed in BS but barely expressed in the normal pericarp, including three *CS120*, *LTI65* and *RCI2B* genes (Fig. 6). It was reported that most protein synthesis is inhibited when the temperature drops abruptly, which is significantly lower than its normal physiological temperature, while cold-shock proteins (CSPs) increase dramatically[39]. *LTI65* and *RCI2B* were proven to be induced by low temperature in *Arabidopsis thaliana*[33, 40]. Li. et al.[11] studied the effect of cold exercise treatment on 'Huangguan' pear, and the results showed that cold exercise effectively inhibited fruit peel brown spot and had no obvious effect on storage quality. Based on these findings, low temperature is indeed one of the causes

of BS.

Calcium deficiency in the pericarp is also responsible for BS[1, 3, 7, 9, 15, 21-25]. Studies have shown that the water-soluble and total $Ca^{2+}$ contents in both the skin and flesh tissue and the total $Ca^{2+}$ content only in the skin of fruits with BS were significantly lower than those of fruits without BS[1]. Alternatively, stress can not only induce calcium signaling but also induce the expression of calcium-binding proteins in plant [41]. Ferguson believes that the imbalance of $Ca^{2+}$ contents lead to metabolic disorders, resulting in physiological diseases[42]. In our study, the expression of calcium-related genes in the pericarp of the infected and unaffected pericarp was analyzed by transcriptome analysis. We detected six calcium-related genes that were upregulated in BS, including *CaLP2*, *CaLP3*, *CML45*, *CML42*, *CaLP7*, and *CaM*. They were involved in PPI and the MAPK pathways. In addition, studies have shown a close relationship between $Ca^{2+}$ and aquaporin (AQP) activity[43]. The regulation of $Ca^{2+}$ on AQP activity is mainly achieved through CDPK[44]. Certain environmental factors, such as drought, low temperature, light exposure and nutritional deficiency, can promote the expression of the AQP gene[45, 46]. We detected four AQP genes that showed differential expression, including *PIP1-4*, *PIP2-8*, *PIP2-2*, and *PIP2-5* (Fig. 6). The AQP genes may affect BS by regulating the calcium concentration.

The MAPK signaling pathway was the most significantly enriched pathway in the CK-BS comparison group. It is associated with various physiological, developmental and hormonal responses[47]. Molecular and biochemical studies have revealed that MAPK activation correlates with stimulatory treatments such as low temperature, drought, wounding, pathogen infection, hyper and hypo-osmolarity, and reactive oxygen species[48-52]. Genes involved in both PPI and MAPK

pathways have been detected. *PGIP* was proven to change the composition of the degradation products in the cell wall of pear fruit and increase the content of pectin monomer to induce the disease resistance of plants[53], which was upregulated in BS. WRKY family TFs have been proven to be involved in the plant defense response[54]. We detected 12 WRKY family TFs that showed differential expression (Fig. 4). Therefore, BS disease may be a manifestation of fruit responses to adverse environments.

Plant hormone signal transduction also plays a critical role in the formation of BS. Hormonal cues regulate many aspects of plant growth and development, facilitating the ability of plant to respond to environmental changes systemically[55]. We found that genes involved in IAA were downregulated, while genes involved in GA and CTK were upregulated. It has been proven that cold temperatures inhibit plant growth by reducing auxin accumulation[56]. Alternatively, a study has shown that low temperature induces an increase in $GA_3$ sensitivity[57]. We predict that low temperature causes the differential expression of plant hormone signaling pathway genes, which indicates that low temperature might be the most important cause of BS.

Furthermore, the humidity in fruit bags may also be a factor affecting BS. Studies have shown that wax is influenced by temperature, light intensity and humidity[58]. Additionally, it was reported that high humidity inhibits wax synthesis[59]. In addition to wax, there are reticular or strip cracks on the fruit surface, which are caused by the continuous expansion of flesh cells during the development stage, leading to epidermal expansion and cracking. Some studies have found that these cracks are easily affected by external environmental factors[60]. These cracks may be the cause of BS. Under the action of AQP, brown spots are formed in pear fruits. Humidity may be a critical impact factor on BS formation.

**Effects of different treatments on BS of 'Huangguan' pear**

Key differentially expressed genes in BS were screened by transcriptome analysis. With different treatments, it was found that P and ABA significantly inhibited the incidence of BS. Then, the expression of key genes at the transcriptional level after treatments was analyzed. The results showed that P treatment could improve the expression of the wax-related genes *WSD1* and *FAR*, resulting in the thicker cuticle. The expression of calcium-related genes *CaLP3*, *CML45*, *CML42*, *CaLP7*, and *CaM*, were upregulated, which could alleviate calcium deficiency in the fruit exocarp. Additionally, P treatment improved the expression of genes involved in both PPI and MAPK pathways, including *LSH10*, *WRKY53*, *WRKY71*, *WRKY33*, *WRKY31*, *WRKY26*, and *WRKY11*, which improved the adaptability of fruit in response to adverse environments, which inhibiting the incidence of BS.

ABA treatment also had a certain inhibitory effect on BS. ABA has been reported to control the expression of wax synthesis genes and prevent leaf water loss[61]. However, it is a major hormone involved in the plant response to stress. In our results, we found that ABA treatments can increase the expression of the calcium-related genes *CaLP2*, *CaLP3*, *CML45*, *CML42*, and *CaLP7*, and adaptability of fruits might be improved by increasing the expression of *PGIP*, *LSH10*, *WRKY53*, *WRKY71*, *WRKY75*, *WRKY33*, *WRKY31*, *WRKY26*, *WRKY24*, and *WRKY11*. In general, ABA treatment may roughen the exocarp and improve the disease resistance of the fruit.

**Conclusion**

This study describes the occurrence of BS was accompanied by the reduction of the wax layer and the tight accumulation of dead cells with lignification. At the transcriptional level, genes related to wax synthesis were greatly down-regulated, genes related to suberin and lignin biosynthesis were greatly

up-regulated, genes related to calcium and low temperature were up-regulated. In addition, the difference of endogenous hormone content between CK and BS was the decrease of CTK and the increase of ABA, JA, GA and SA, which was consistent with the expression trend of their signaling transduction related genes except CTK. We also found that P and ABA treatments inhibited BS to varying degrees while $GA_3$ treatment may promote it. The expression levels of key genes involved in BS formation after different treatments were consistent with the morbidity results. Those results provide a theoretical basis for the molecular mechanism of 'Huangguan' pear browning spot disease.

## Methods

### Plant materials and treatment

Ripe 'Huanguan' pears (CK) and 'Huangguan' pears with BS disorder (BS) were harvested from an orchard in the gardening field of Dangshan County, Suzhou City, Anhui Province, during the harvest season in 2018. Treatments were carried out by spraying $NaH_2PO_4 \cdot 2H_2O$ (0.2%, Sigma 04269), ABA (100 μM, Sigma A1049), and $GA_3$ (300 mg/L, Sigma G8040) on 'Huangguan' pears at 10, 20, and 30 days after full bloom (DAFB). The reagent treatments are all commonly used in fruit bags during production. Each treatment had three biological replicates, and each tree contained approximately 120 treated fruits.

Pears were immediately transported to the laboratory at Anhui Agricultural University (Hefei, China) after harvest. The 0.5 mm thickness exocarp was dissected from the fruit skin with a double-sided blade. Six biological replicates for metabolic profiling were collected randomly from the CK and BS of 'Huangguan' pear exocarp. Three biological replicates of CK, BS and different hormone treatments were used for RNA sequencing (RNA-Seq). The collected fruit samples were frozen in

liquid nitrogen immediately and then stored at -80°C.

**Observation of paraffin sections and scanning electron microscopy of pear exocarp**

After removing the dirt on the fruit surface, a 0.6 cm × 0.7 cm piece was cut on the pear surface with a double-sided blade and then fixed in FAA solution immediately. A 3 mm tissue block was cut with a sharp blade and then fixed in electron microscope fixing solution. The preparation of paraffin sections and electron microscope secyions were conducted at Servicebio (WUHAN) Biotechnology Co., Ltd.

**Pear postharvest water loss measurement**

'Huangguan' pear fruits with BS disease of the same size stored at room conditions at 25°C were used in the experiment, and normal 'Huangguan' pear fruits were used as a control. The rate of water loss (RWL) was calculated using the formula RWL (%) = $(FW_{t1}-FW_{t2})/FWt1\times100\%$ ($FW_{t1}$ = weight of the fruit at a certain storage time t1, and $FW_{t2}$ = weight of the fruits at a certain storage time t2)[32]. Each group had 10 fruits, and three independent biological replicates were performed.

**Evaluation of brown spot disorder**

According to the coverage rate of spots on the surface of pears, the incidence is divided into 4 levels[29]: 0 for no browning, 1 for 1~10%, 2 for 11%~20%, 3 for 21%~30%, and 31%~100%. The index of BS was calculated based on the formula $index = \frac{\sum(\text{number of fruit} \times \text{level})}{\text{total fruit number} \times 4}$[1].

**Metabolite statistical analysis**

An advanced Xevo G2-XS QTOF mass spectrometer (Waters, UK) was used for data acquisition, and commercial software Progenesis QI (version 2.2) (Waters, UK) and the BGI metabolomics

software package metaX[62] were used for mass spectrometry data analysis (Filtering out ions with relative standard deviation (RSD) greater than 30%), while identification was based on the KEGG database. The project uses variable importance in projection (VIP) values of the first two principal components in the multivariate PLS-DA model, combined with fold change (FC) and q-values of univariate analysis to choose differentially expressed metabolites (DEMs) (VIP > 1 and FC >1.2 or < 0.833 and with an adjusted q-value < 0.05 were considered significant).

**Transcriptome analysis of the pear exocar**p

Total RNA was purified from plant tissues by ethanol precipitation and CTAB-PBIOZOL reagent according to the instructions. DNA nanoballs were loaded into the patterned nanoarray, and single-end 50-base reads were generated on the BGISeq500 platform (BGI-Shenzhen, China). Reads with low quality, connector contamination and a proportion of N > 5% were removed before data analysis to ensure the reliability of the results. The selected clean reads were mapped to the reference genome of Chinese white pear (*Pyrus bretschneideri*)[63]. Gene expression level was calculated based on the fragments per kilobase of transcript per million mapped reads (FPKM), which were further used to analysis the differentially expressed genes (DEGs)[64]. Transcripts with fold change (FC) values > -2 (upregulated) or < -2 (downregulated) and with an adjusted *P*-value <0.001 were considered significant.

**Gene expression analysis by qRT-PCR**

Quantitative real-time PCR (qRT-PCR) was applied to evaluate the transcription levels of genes associated with BS under different treatments. Total RNAs were extracted from collected plant materials using the Trizol kit (TIANGEN) as instructed by the manufacturer. qRT-PCR was conducted

with the SYBR Green (TOYOBO, SHANGHAI) and carried out in an optical 48-well plate using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, California). Three biological replicates were performed to ensure the reliability of the data.

**Additional file 1： Figure S1:** show the pericarp surface differences between CK and BS. **Figure S2:** show the PCA score plot derived from metabolite ions. **Figure S3:** show the GO enrichment analysis of DEGs between CK and BS. **Figure S4:** KEGG enrichment analysis of DEGs between CK and BS. **Figure S5:** show the phenotypes of BS in unbagged 'Huangguan' pear.

**Additional file 2:** list the number of reads based on RNA-Seq data.

**Additional file 3:** list the number of Differential metabolites between CK and BS.

**Additional file 4:** list the detailed information of genes involved in BS formation.

**List of abbreviations**

BS: Browning spot; MeJA: Methyl jasmonate; 1-MCP: 1-methylcyclopropene; P: $NaH_2PO_4 \cdot 2H_2O$; ABA: Abscisic acid; GA3: gibberellin A3; CTK: Cytokinin; SEM: Scanning electron microscopy; IAA: Auxin; GA: gibberellin; JA: Jasmonic acid; SA: Salicylic acid; DAFB: Days after full bioom; DEG: Differentially expressed gene; DEM: Differentially expressed metabolite; FPKM: Fragments per kilobase of transcript per million mapped reads；FC: Fold change; RWL: The rate of water loss; PCA: Principal component analysis; PLS-DA: Plots from partial least squares discriminant; ESI+: Electrospray ionization positive ion mode; ESI−: Electrospray ionization negative ion mode; TFs: Transcription factors; VIP: Variable importance in projection; PPI: Plant-pathogen interactions; MAPK: MAPK signaling pathway; AQP: Aquaporin; qRT-PCR: Quantitative real-time PCR

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**Author's contributions**

QW and WH conceived and designed the study. XNC and DZY conducted treatments experiment. JF and JCL collected fruits and prepared for RNA. LWZ, PL and ZFY contributed to the data analysis. XYW, BJ and LL prepared the figures and tables. XYW conducted the qRT-PCR verification. WQ wrote the manuscript and HW revised the manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare that they have no competing interests

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**Availability of data and materials**

Data sets supporting the results of this article (BioProject: PRJNA682706) is currently being submitted to the National Center for Biotechnology Information and additional information will be added once available. (https://www.ncbi.nlm.nih.gov/sra/PRJNA682706)

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

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# Figures

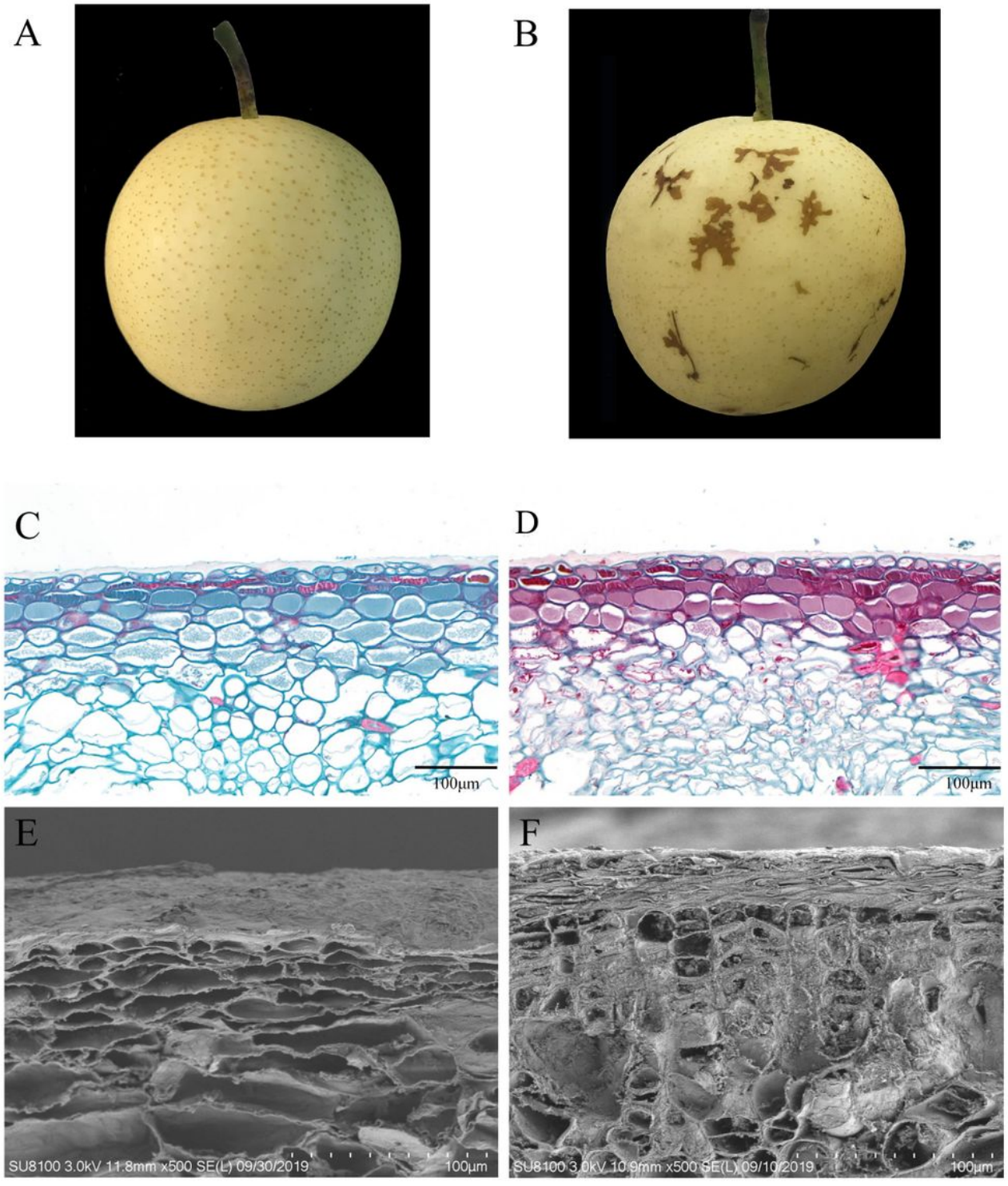



**Figure 1**

Phenotypes of normal ‘Huangguan’ pear (A) and ‘Huangguan’ pear with BS disease (B). Observation of the paraffin sections of the normal part (C) and BS disease part (D) of ‘Huangguan pear’. SEM analysis of the normal part (E) and BS disease part (F) of ‘Huangguan’ pear.

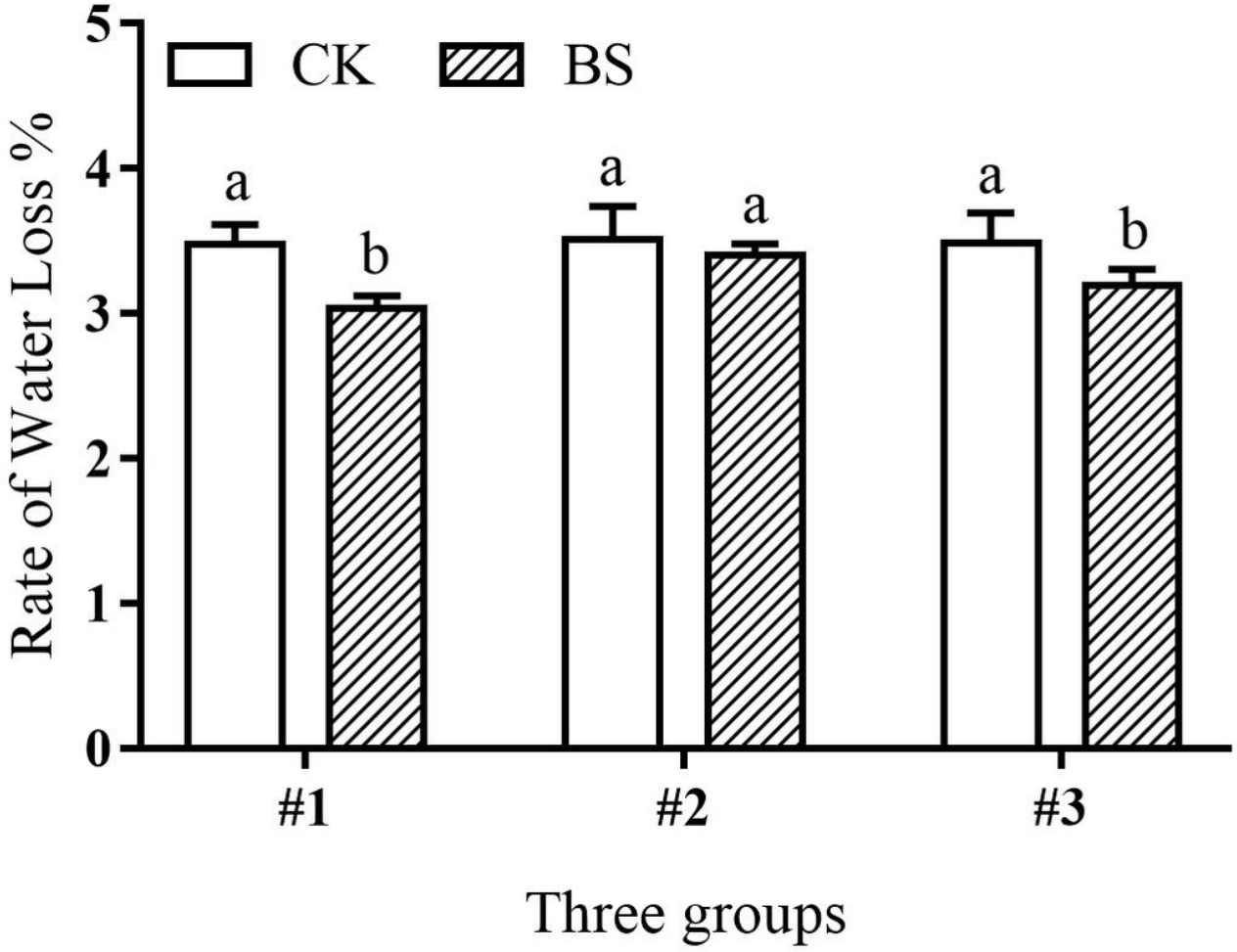



**Figure 2**

RWL of CK and BS of ‘Huangguan’ pear at 10 days of storage under room conditions after harvest.

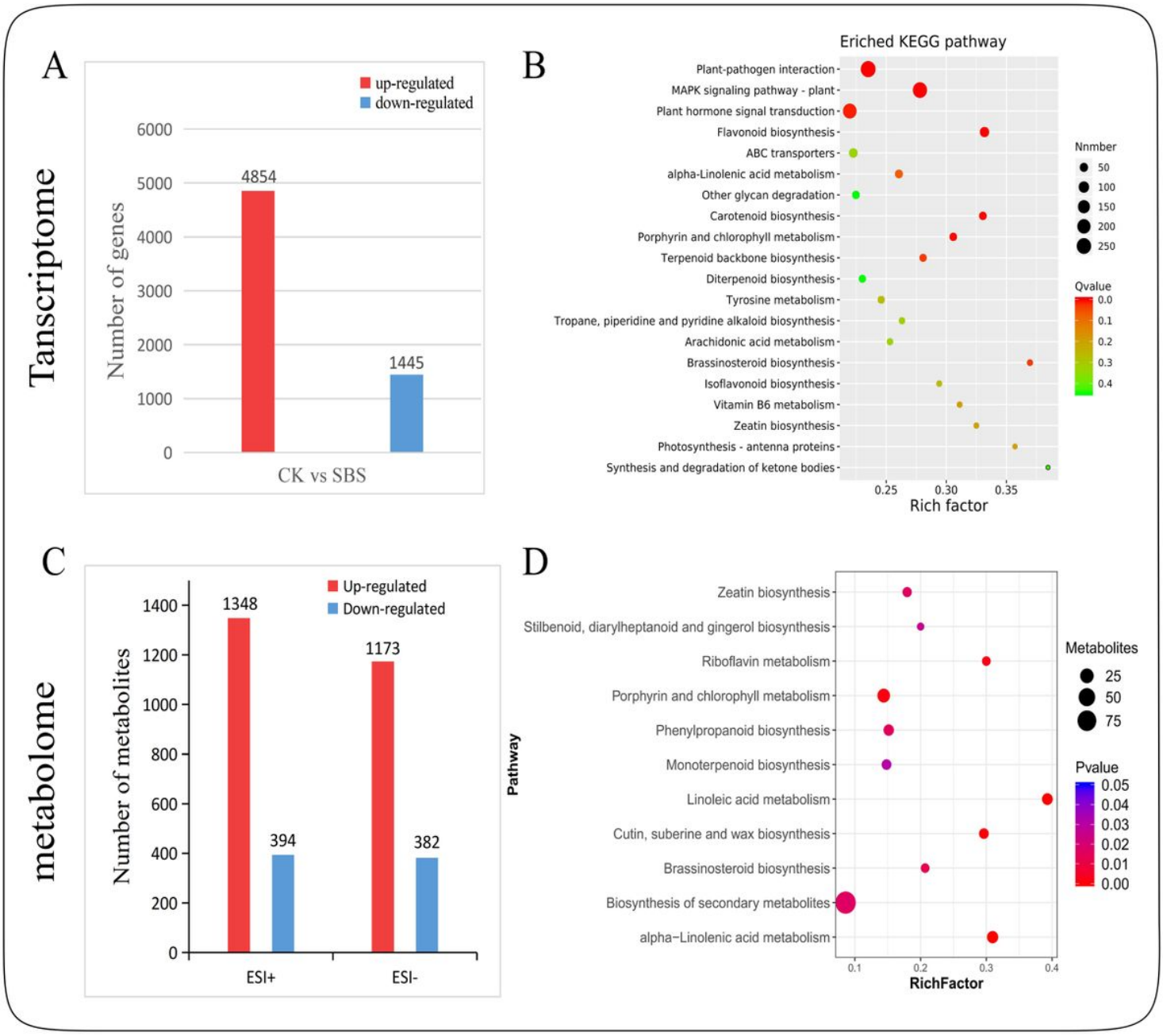
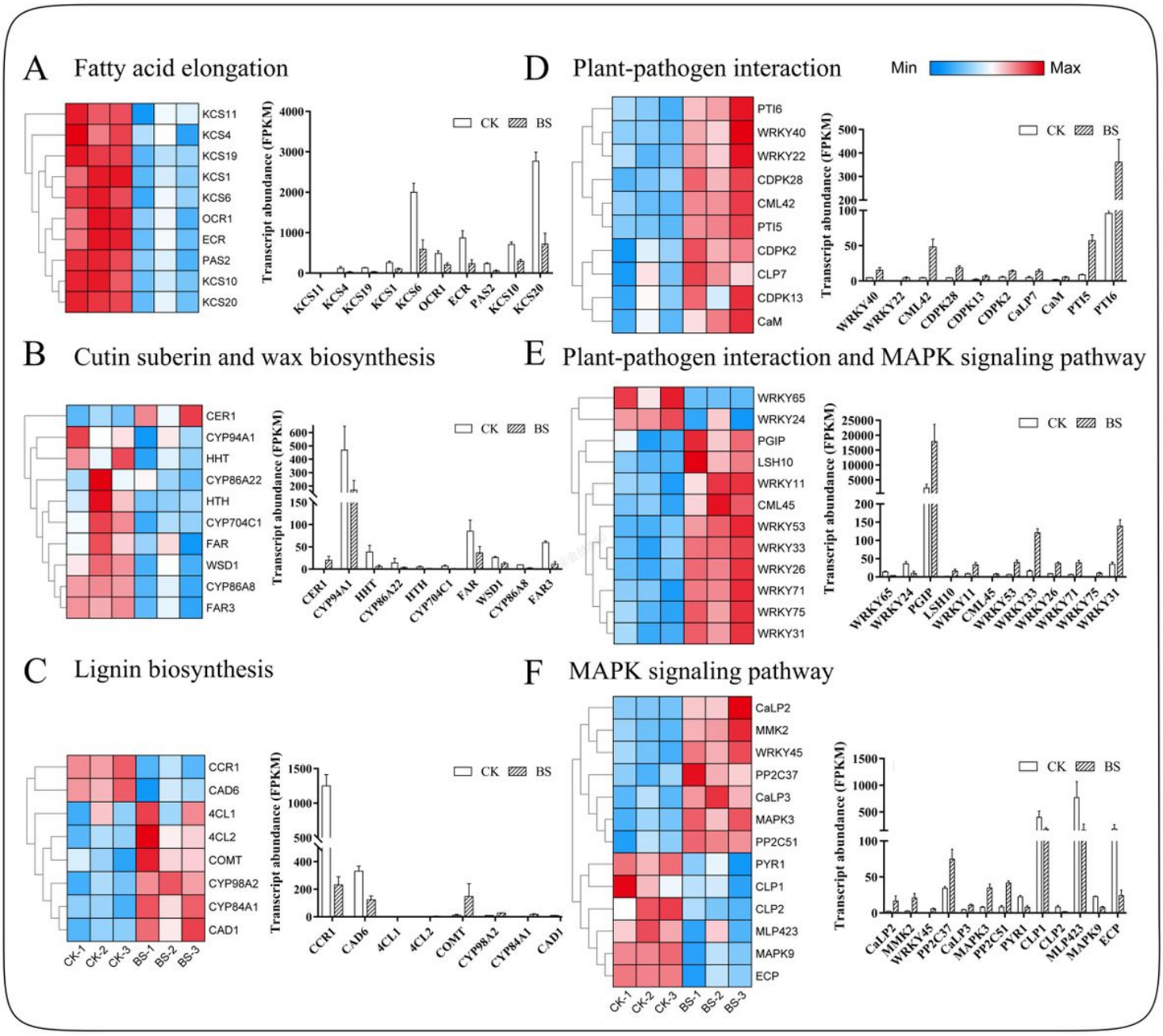


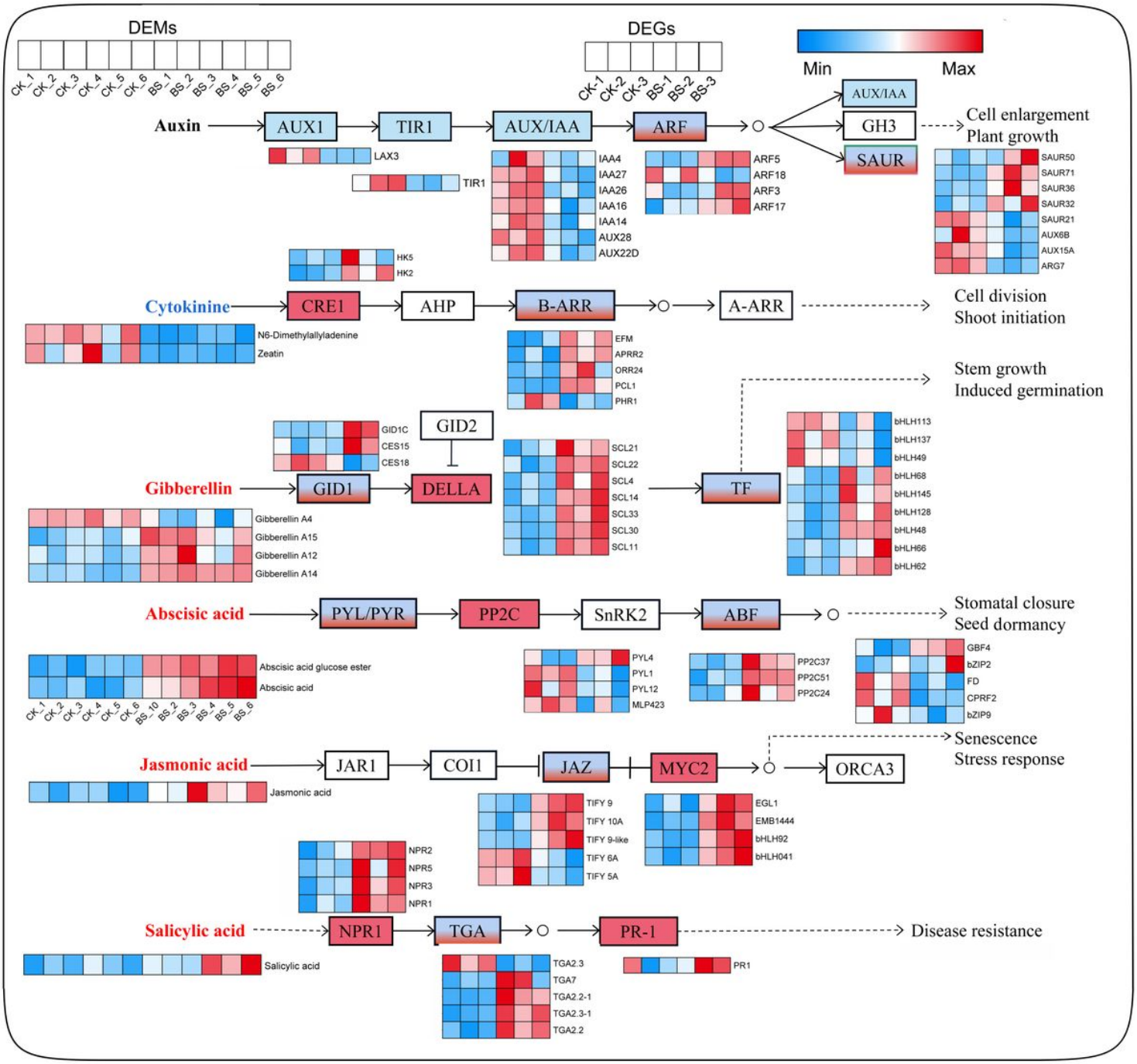

**Figure 3**

Significant DEG and DEM analysis between CK and BS. (A) Column chart of DEGs; red represents upregulated DEGs, and blue represents downregulated DEGs. (B) KEGG enrichment analysis of DEGs between CK and BS. The number of genes in each pathway is equal to the dot size. The dot color represents the q-value. The smaller the q-value, the redder the dot. (C) Numbers of upregulated (red) and downregulated (blue) metabolites. (D) KEGG enrichment analysis of differential metabolites. The number of DEMs in each pathway is equal to the dot size. The dot color represents the P-value. A redder point represents a smaller P-value.



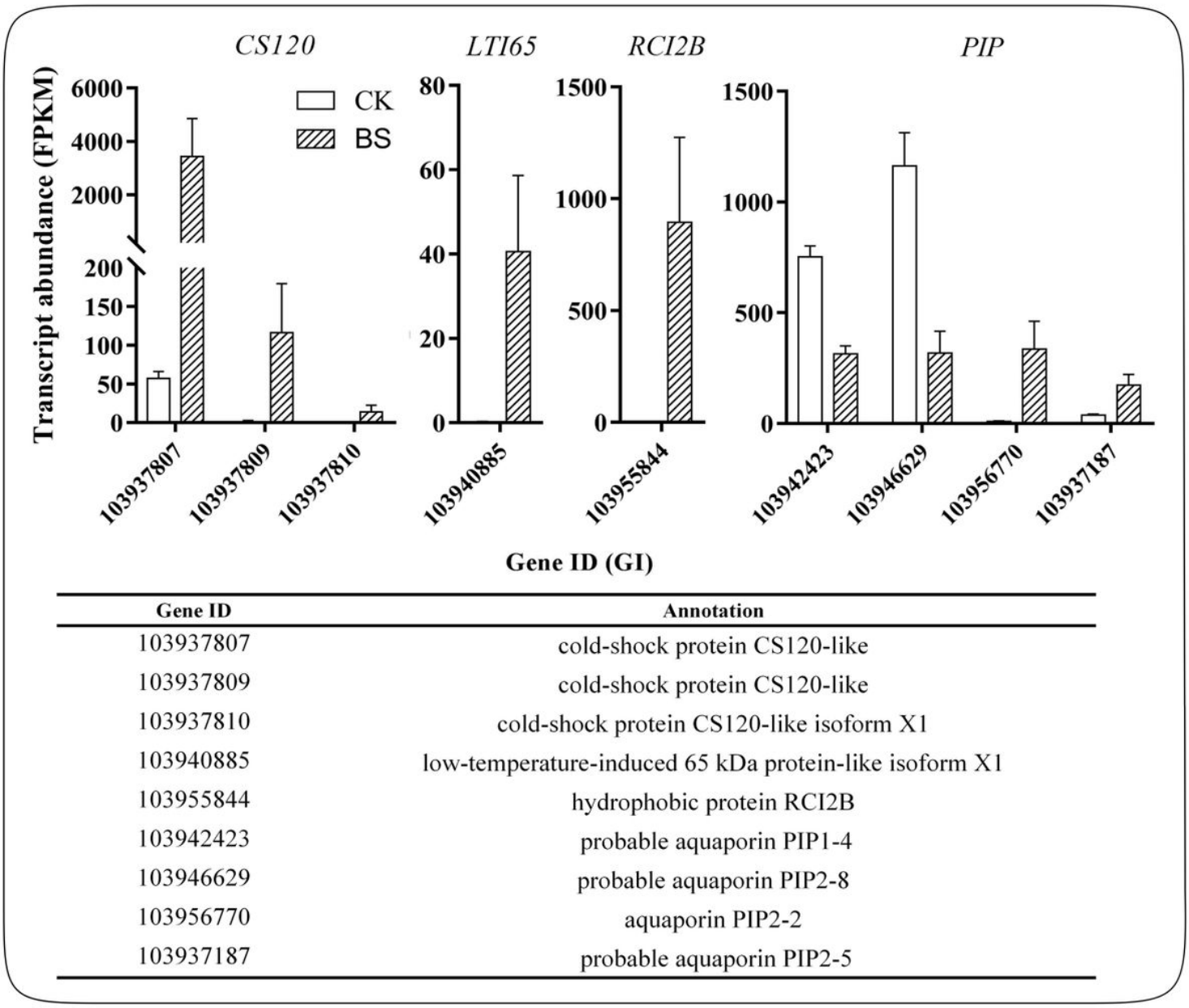

## Figure 4

Significant DEGs between CK and BS. Heatmap of DEGs involved in fatty acid elongation (A), cutin suberin and wax biosynthesis (B), lignin biosynthesis (C), plant-pathogen interaction (D)，both plant-pathogen interaction and MAPK signaling pathway (E), MAPK signaling 159 pathway (F). Red represents upregulation, and blue represents downregulation.




## Figure 5

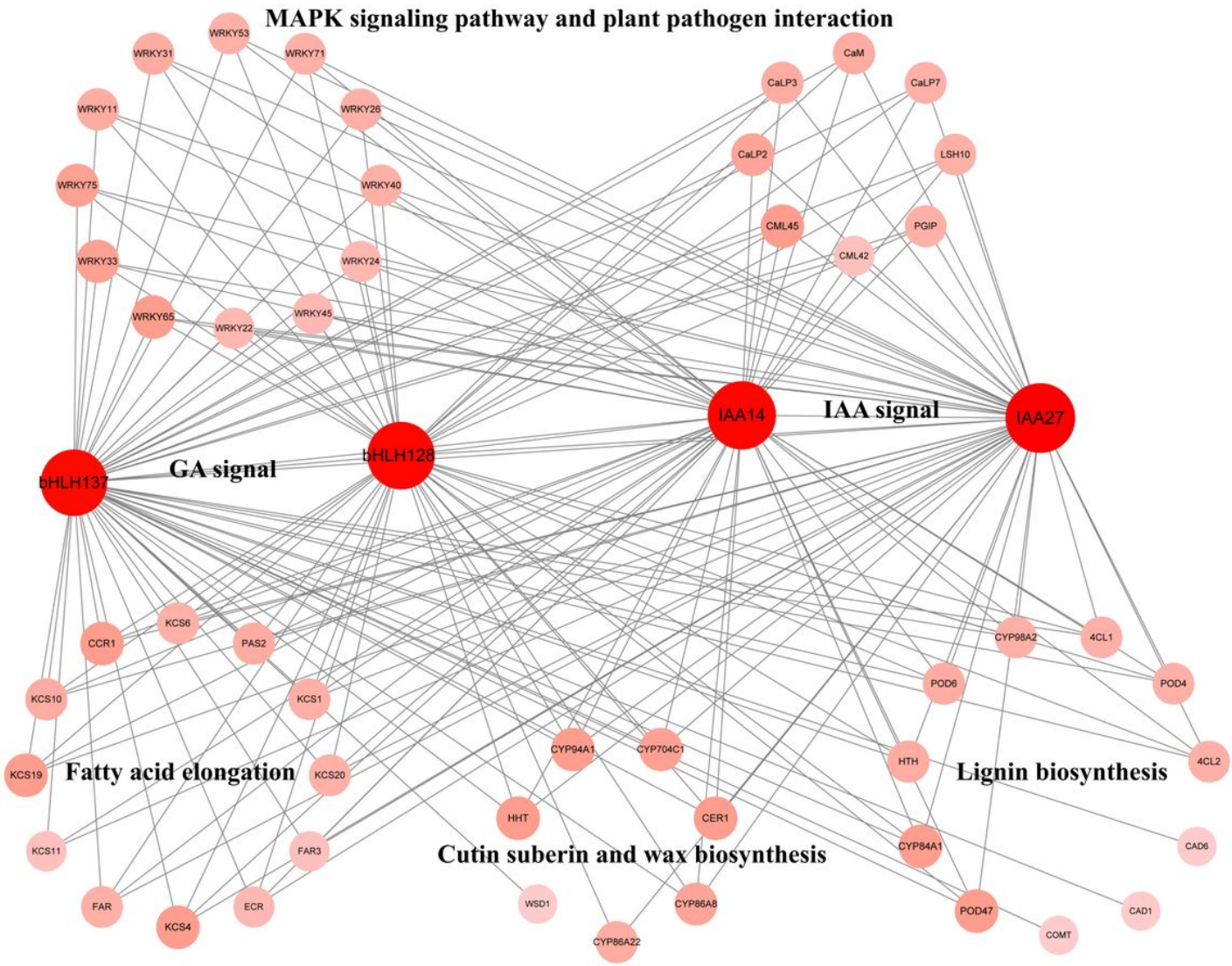
Significant DEGs and DEMs involved in plant hormone signal transduction between CK and BS. Red represents upregulation, and blue represents downregulation.




| Gene ID | Annotation |
|---|---|
| 103937807 | cold-shock protein CS120-like |
| 103937809 | cold-shock protein CS120-like |
| 103937810 | cold-shock protein CS120-like isoform X1 |
| 103940885 | low-temperature-induced 65 kDa protein-like isoform X1 |
| 103955844 | hydrophobic protein RCI2B |
| 103942423 | probable aquaporin PIP1-4 |
| 103946629 | probable aquaporin PIP2-8 |
| 103956770 | aquaporin PIP2-2 |
| 103937187 | probable aquaporin PIP2-5 |

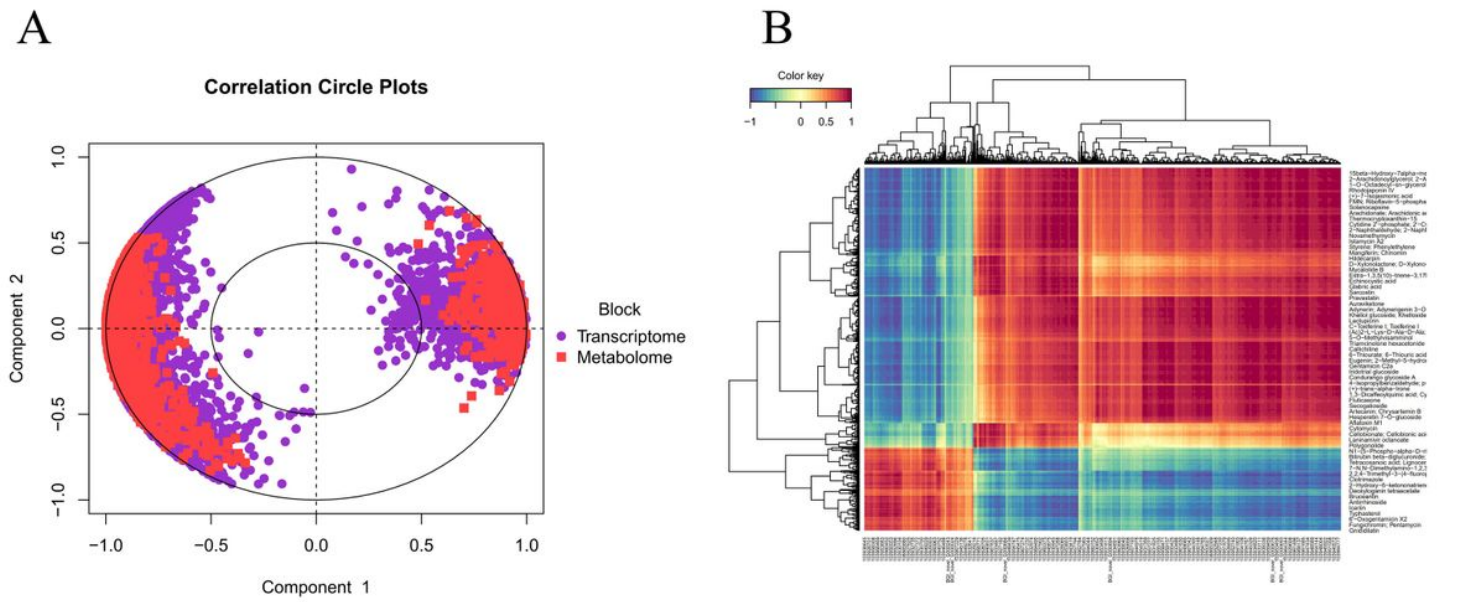
## Figure 6

Transcript abundance of significant DEGs between CK and BS. The error bars are the means ± SEM of three biological repeats.



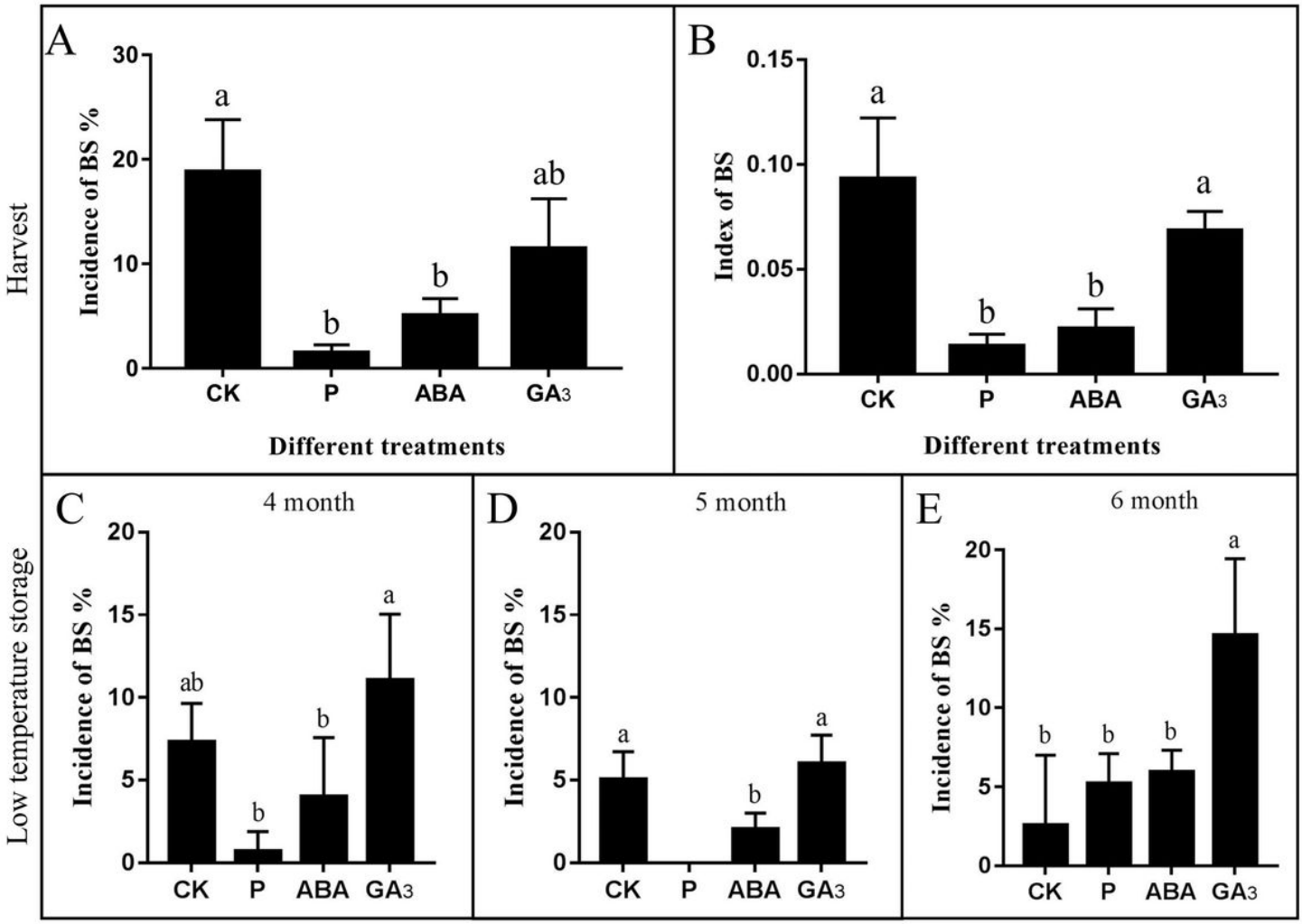

## Figure 7

Coexpression network of genes involved in BS formation. Detailed information on the genes is listed in Additional file 4: Table S3 and Table S4.



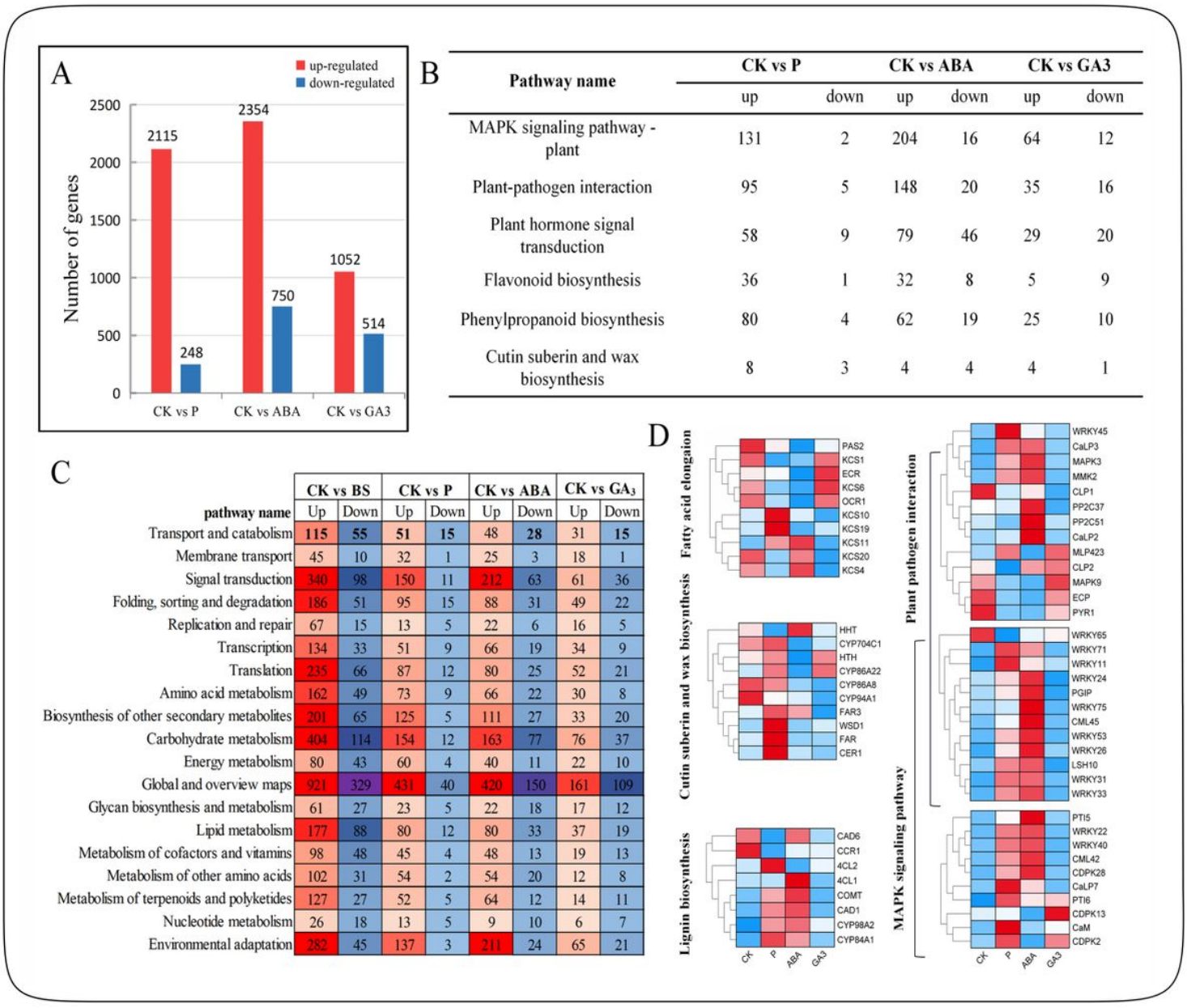

## Figure 8

Combined analysis of the metabolome and transcriptome between CK and BS. (A) Concentric diagram of the correlation of DEGs and DEMs between CK and BS. Each point in the circle represents a gene, and each square represents a metabolite. If the angle between the DEG and DEM is an acute angle, the correlation is positive. If the angle is the deltoid angle, it is negatively correlated. In general, variables far away from the center of the circle are more closely related. (B) Heatmap cluster of DEGs and DEMs. Each row represents a DEM, and each column represents a DEG. Blue represents a negative correlation, and red represents a positive correlation.




## Figure 9

The incidence of BS disorder after different treatments in ‘Huangguan’ pears. (A) Incidence of BS disorder treated with exogenous P, ABA, and GA3. (B) Index of BS disorder treated with exogenous P, ABA, and GA3. Incidence of BS disorder with different treatments after 4 (C), 5 (D), and 6 (E) months of storage. The error bars are the means ± SEM of three biological repeats. ($P \leq 0.05$).

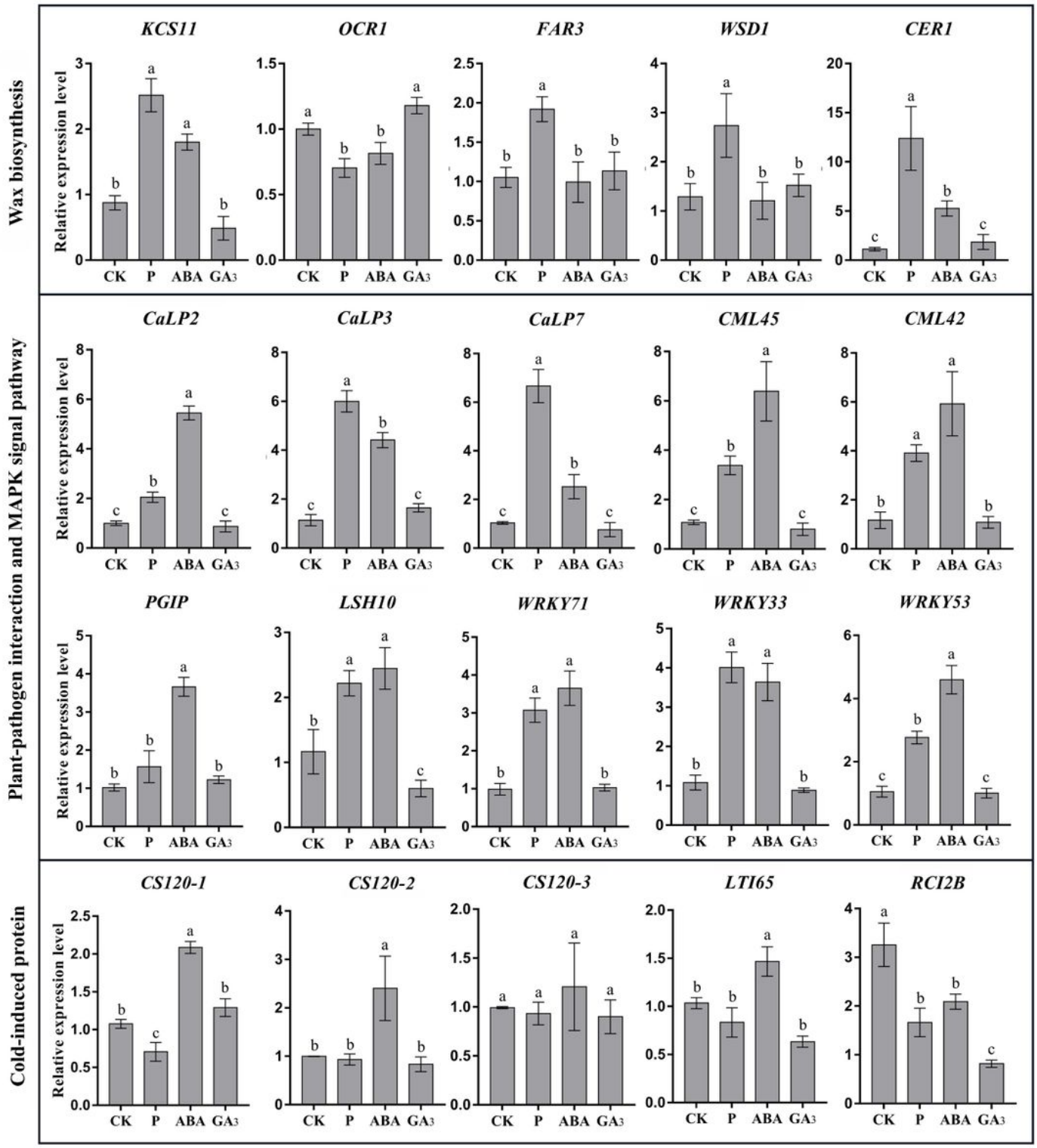



| Pathway name | CK vs P | | CK vs ABA | | CK vs GA3 | |
|---|---|---|---|---|---|---|
| | up | down | up | down | up | down |
| MAPK signaling pathway - plant | 131 | 2 | 204 | 16 | 64 | 12 |
| Plant-pathogen interaction | 95 | 5 | 148 | 20 | 35 | 16 |
| Plant hormone signal transduction | 58 | 9 | 79 | 46 | 29 | 20 |
| Flavonoid biosynthesis | 36 | 1 | 32 | 8 | 5 | 9 |
| Phenylpropanoid biosynthesis | 80 | 4 | 62 | 19 | 25 | 10 |
| Cutin suberin and wax biosynthesis | 8 | 3 | 4 | 4 | 4 | 1 |

| | CK vs BS | | CK vs P | | CK vs ABA | | CK vs $GA_3$ | |
|---|---|---|---|---|---|---|---|---|
| pathway name | Up | Down | Up | Down | Up | Down | Up | Down |
| Transport and catabolism | **115** | **55** | **51** | **15** | 48 | **28** | 31 | **15** |
| Membrane transport | 45 | 10 | 32 | 1 | 25 | 3 | 18 | 1 |
| Signal transduction | 340 | 98 | 150 | 11 | 212 | 63 | 61 | 36 |
| Folding, sorting and degradation | 186 | 51 | 95 | 15 | 88 | 31 | 49 | 22 |
| Replication and repair | 67 | 15 | 13 | 5 | 22 | 6 | 16 | 5 |
| Transcription | 134 | 33 | 51 | 9 | 66 | 19 | 34 | 9 |
| Translation | 235 | 66 | 87 | 12 | 80 | 25 | 52 | 21 |
| Amino acid metabolism | 162 | 49 | 73 | 9 | 66 | 22 | 30 | 8 |
| Biosynthesis of other secondary metabolites | 201 | 65 | 125 | 5 | 111 | 27 | 33 | 20 |
| Carbohydrate metabolism | 404 | 114 | 154 | 12 | 163 | 77 | 76 | 37 |
| Energy metabolism | 80 | 43 | 60 | 4 | 40 | 11 | 22 | 10 |
| Global and overview maps | 921 | 329 | 431 | 40 | 420 | 150 | 161 | 109 |
| Glycan biosynthesis and metabolism | 61 | 27 | 23 | 5 | 22 | 18 | 17 | 12 |
| Lipid metabolism | 177 | 88 | 80 | 12 | 80 | 33 | 37 | 19 |
| Metabolism of cofactors and vitamins | 98 | 48 | 45 | 4 | 48 | 13 | 19 | 13 |
| Metabolism of other amino acids | 102 | 31 | 54 | 2 | 54 | 20 | 12 | 8 |
| Metabolism of terpenoids and polyketides | 127 | 27 | 52 | 5 | 64 | 12 | 14 | 11 |
| Nucleotide metabolism | 26 | 18 | 13 | 5 | 9 | 10 | 6 | 7 |
| Environmental adaptation | 282 | 45 | 137 | 3 | 211 | 24 | 65 | 21 |

## Figure 10

Significant DEGs between the CK_BS, CK-P, CK-ABA, and CK-GA3 comparison groups. (A) Column chart of DEGs. (B) The up- and downregulation of genes involved in BS-related pathways. (C) KEGG annotation of DEGs. (D) Expression of genes involved in BS related pathway after treatments. Red represents upregulation, and blue represents downregulation.




## Figure 11

q-RT-PCR verification of genes related to BS after different treatments. The error bars are the means ± SEM of three biological repeats.

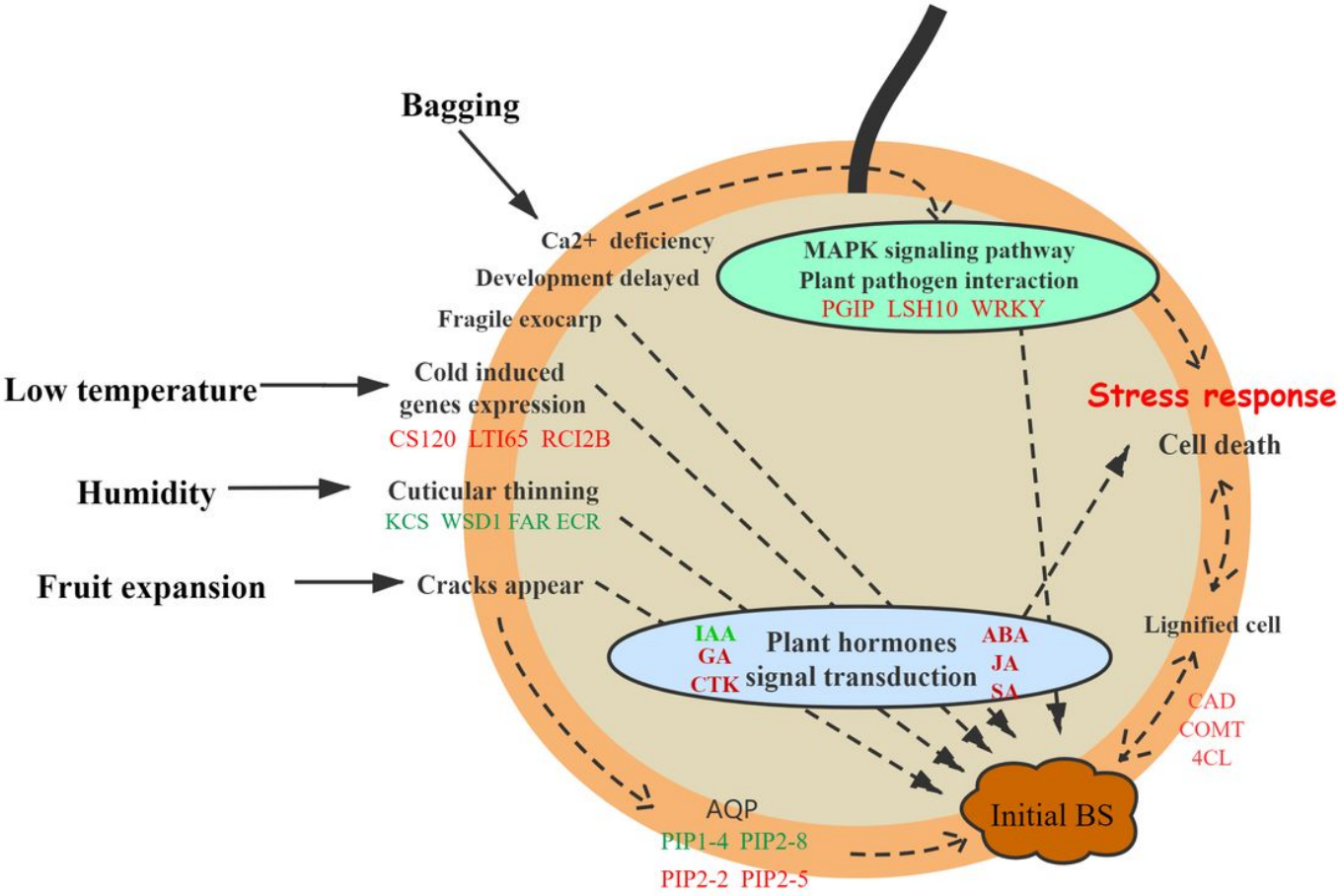



**Figure 12**

A proposed model of BS formation in ‘Huangguan’ pear fruits. The red color represents upregulation and green color represents downregulation. The detailed gene information can be viewed in Additional file 4: Table S3, Table S4 and Fig. 6. IAA, Auxin; GA, gibberellic acid; CTK, cytokinin; ABA, abscisic acid; JA, jasmonic acid; SA, Salicylic acid.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.doc
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- Additionalfile3.xlsx
- Additionalfile4.xlsx