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ORIGINAL PAPER

Comparative transcriptome analysis reveals an ABA-responsive regulation network associated with cell wall organization and oxidation reduction in sugar beet

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Abstract

Abscisic acid (ABA) is an essential phytohormone and plays a key role in root architecture and plant stress responses. However, the ABA signalling pathway and its regulatory network in sugar beet roots remain unclear. Here, we carried out a time course experiment and performed global transcriptome profiling via strand-specific RNA sequencing (ssRNA-seq) to evaluate the response of sugar beet plants to exogenous ABA. According to the expression patterns of 5625 differentially expressed transcription units (TUs), the ABA-responsive stages within 24 h were divided into the early (1 h), intermediate (6 h and 12 h) and late (24 h) stages. Gene Ontology (GO) analysis revealed that oxidation reduction (GO: 0055114) and cell wall organization (GO: 0071555) were enriched in all ABA-responsive stages. For oxidation reduction, genes encoding cytochromes, peroxidases (PODs) and 2-oxoglutarate and Fe(II)-dependent oxygenases (2OG-Fe(II)s) constituted the largest proportion. ABA-responsive xyloglucan endotransglucosylase/hydrolase (XTH), expansin A (EXPA), pectinesterase (PME), pectate lyase (PL) and cellulose synthase (CES) were also detected in terms of cell wall organization. By performing regulation prediction and co-expression analysis, we determined that three genes, one encoding an AP2 domain-containing transcription factor (TF) and two encoding Dof domain-containing TFs (*BVRB_4g074790, BVRB_8g180860* and *BVRB_9g211370*, respectively) may play an important role in the regulation of oxidation reduction and cell wall organization. Our profiling of ABA-responsive genes provides valuable information for understanding the molecular functions of regulatory genes and will aid in the future molecular breeding of sugar beet.

Keywords Sugar beet · Abscisic acid · Transcriptome · AP2 transcription factor · Dof transcription factor

Introduction

Sugar beet (*Beta vulgaris* L.) is a major sugar-producing crop species that is planted in at least 50 countries and accounts for approximately 30% of global sugar production (Zhang et al. 2016). In China, sugar beet has been cultivated for more than 100 years (Gui and Ji 2015). The main sugar beet planting area has shifted from Heilongjiang Province to Inner Mongolia and Xinjiang Region during the past 10 years because of the greater sunshine hours and greater cumulative temperature in these areas than in Heilongjiang Province. However, the low rainfall in arid and semi-arid regions results in additional irrigation costs and the potential risk of drought. In addition, soil salinity and cold limiting local sugar beet production. Thus, studying the regulation of cellular processes in response to stress to increase sugar beet stress tolerance is highly warranted.

Wang Xing and Zhi Pi contributed equally to this work.

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Abscisic acid (ABA), an isoprenoid phytohormone, was first isolated from cotton fruits, where it accelerated abscission (Ohkuma et al. 1963). The expression of a series of genes involved in various essential developmental processes, including seed dormancy and germination, root architecture modulation and stomatal regulation, is expressed or repressed in response to ABA (Sah et al. 2016). In addition to its involvement in developmental regulation, ABA acts as a key regulator of responses to abiotic stresses, such as drought, extreme temperature and salinity (Raghavendra et al. 2010; Cutler et al. 2010; Malaga et al. 2020). In *Arabidopsis thaliana*, a group of membrane proteins, namely, pyrabactin resistance 1 (PYR1), PYR-like protein (PYL) and regulatory component of ABA receptor (RCAR), are believed to be ABA receptors (Ma et al. 2009; Park et al. 2009). These proteins can sense and bind ABA molecules, inhibiting the enzymatic activity of type 2C protein phosphatases (PP2Cs) (Umezawa et al. 2009). Without dephosphorylation caused by PP2Cs, the downstream sucrose-non-fermenting kinase 1-related protein kinase 2 (SnRK2) is activated, which subsequently phosphorylates the bZIP transcription factor (TF, ABI5) and the B3 domain-containing TF (ABI3, Nakashima et al. 2009; Boudsocq et al. 2007; Soon et al. 2012). Phosphorylated ABI5 and ABI3 can recognize and bind to ABA-responsive element (ABRE) motifs within the promoter regions of downstream genes and trigger downstream ABA responses.

In addition to endogenous ABA, the exogenously applied ABA can enhance plant defences and tolerance. Many researchers have applied exogenous ABA to the shoots and roots of plants to protect them against drought (Tian and Li 2018; Wu and Liang 2017). In leaves, an increase in cytosolic Ca^{2+} in guard cells is induced by ABA, which down-regulates inward K^+ channels and leads to stomatal closure, which is beneficial for reducing plant transpirational water loss (Cousson 2003). In addition, it has been reported that exogenous ABA can stimulate the accumulation of reactive oxygen species (ROS) and enhance the activities of several antioxidative enzymes, such as superoxide dismutase (SOD) and ascorbate peroxidase (APX), in maize, leading to a reduction in membrane lipid peroxidation (Jiang and Zhang 2001). In rice roots, exogenous ABA can promote the elongation of root hairs through crosstalk with auxin biosynthesis and transport (Wang et al. 2017). The expression of several genes encoding aquaporins, which form water channels that allow water to cross cell membranes, can also be induced by exogenous ABA, increasing hydraulic conductivity (Ding et al. 2016).

Given the key role of ABA in plant development and the stress response, understanding the mechanism of the ABA regulatory pathway at the transcriptional level is highly warranted for sugar beet. In this study, a time course ABA treatment with parallel mock treatments was used to explore the regulatory mechanism via strand-specific RNA sequencing (ssRNA-seq) in response to exogenous ABA in sugar beet roots, which are the main organs of ABA synthesis and stress sensing (Puértolas et al. 2015). Our results suggested that oxidation reduction and cell wall organization were the main processes in response to exogenous ABA in sugar beet roots. Additionally, one AP2 domain- and two Dof domain-containing TFs (*BVRB_4g074790, BVRB_8g180860* and *BVRB_9g211370*, respectively) were identified as potential key genes involved in the ABA signalling pathway and in the regulation of oxidation reduction and cell wall organization.

Materials and methods

Plant materials and ABA treatments

Seeds of the sugar beet accession KWS9147 were sequentially sterilized with 70% (v/v) ethanol, 0.1% (w/w) mercuric chloride and 0.2% (w/w) thiram. After they germinated in vermiculite, the seedlings were transplanted into containers filled with half-strength modified Hoagland solution (pH 5.8) and grown in an artificial cultivation room under a 300 μmol m^{-2} s^{-1} light intensity and a 16 h photoperiod. The temperature and relative humidity were maintained at 24 ± 2 °C and 40 ± 5%, respectively. After 4 weeks of growth, the plants were transplanted into the nutrient solution supplemented with or without 100 mg/L ABA (approximately 0.38 mmol/L, Solarbio, Beijing, China) at 3 h after dawn. The roots of the sugar beet plants were then sampled at 1, 6, 12 and 24 h, after which they were stored at − 80 °C for further analysis. For each mock and ABA treatment, three biological replicates were independently treated, and each replicate contained 16 seedlings.

RNA extraction, library construction and Illumina sequencing

Total RNA was isolated with TRIzol reagent (Invitrogen, USA) and treated with TURBO DNase I (Ambion, USA) for 30 min to remove any DNA residue. RNA purification was subsequently performed with an RNeasy Plant Mini Kit (Qiagen, Germany). A NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biosystems) was used to prepare RNA sequencing libraries according to the manufacturer's instructions. For successful sequencing, the quality and size of the cDNA libraries were checked by an Agilent 2200 TapeStation system (Agilent Inc.). Last, a HiSeq X10 Sequencer (Illumina Inc.) was used to sequence the libraries according to a 150-cycle paired-end sequencing protocol.

Analysis of ssRNA-seq datasets

Trimmomatic was used to clean the raw data (raw reads) in FASTQ format by filtering and removing adaptor-only reads, removing reads containing > 10% poly-N, and removing low quality reads to ensure PHRED quality scores ≥ 20 (Peng et al. 2017). The Q20, Q30, and GC contents of the high-quality clean data were calculated by FastQC. The *B. vulgaris* genome sequence (RefBeet 1.2.2), and annotation files were then downloaded from the Ensembl plant database. Index files were constructed by HISAT2 for all sugar beet chromosomes and scaffold sequences (Kim et al. 2015; Dohm et al. 2014), and HISAT2 was used to align the ssRNA-seq reads to the genome sequences of the sugar beet chromosome and scaffold sequences; the following parameter settings were used: "--rna -strandness RF". The fragments per kilobase of transcript per million fragments mapped (FPKM) of the assembled transcripts was calculated and normalized by Cufflinks, Cuffcompare, HTseq-count and DESeq2 in accordance with global normalization parameters (Anders et al. 2014; Love et al. 2014; Quinn and Chang 2016). Last, Pearson correlation coefficients between the samples were calculated to evaluate the reproducibility of the biological replicates.

Differential expression analysis

To identify ABA-responsive genes, we used DESeq2 to perform differential expression analysis based on the negative binomial distribution (Anders and Huber 2010). Only the transcription units (TUs) that met the following restrictions were considered differentially expressed: (1) the normalized expression fold change was greater than 2, and (2) the Benjamini–Hochberg adjusted *P* value/false discovery rate (FDR) was less than 0.05. We executed a custom Perl script to summarize and integrate the results from DESeq2. The difference in TUs between the ABA and mock treatments at each sampling time point was then calculated and used for principal component analysis (PCA) to understand different ABA-responsive stages. Gene Ontology (GO) annotation and enrichment analysis

To determine the enriched terms at different ABA-responsive stages, we employed the Singular Enrichment Analysis (SEA) tool in the AgriGO toolkit (http://bioinfo.cau.edu.cn/agriGO/) for GO enrichment analysis (Tian et al. 2017). The gene annotation file of *B. vulgaris* was downloaded from the Ensembl plant database (release 42; http://plants.ensembl.org) and selected as the background. The default parameters of the advanced options were used. The rich factor of the significantly enriched terms was calculated and presented in a bubble chart. To determine the families or superfamilies involved in oxidation reduction and cell wall organization, HMMER web server (version 2.40.0) was used to perform an hmmscan against the Pfam database; the gathering threshold was used (Potter et al. 2018). The top hits were used for further classification.

TF binding site prediction and regulatory network construction

To predict the TFs involved in ABA-responsive TUs associated with oxidation reduction and cell wall organization, regulation prediction was first performed by the PlantTFDB 5.0 (Jin et al. 2017). The potential promoter region of ABA-responsive TUs in the sugar beet genome was set from − 2000 bp to + 100 bp. With a threshold *P* value of less than 1E−5, the candidate TFs were predicted and screened. The Pearson correlation coefficients between the candidate TFs and ABA-responsive TUs were then calculated on the basis of the FPKM in the mock and ABA treatments. Only the candidate TFs whose Pearson correlation coefficient was > 0.5 and whose significant changes happened before those of target genes were considered potential upstream regulators. Last, Cytoscape (version 3.7.1) was used to visualize the regulatory network and calculate the betweenness and closeness centrality (Kohl et al. 2011).

Quantitative RT-PCR

The RNA samples were treated with 1 μg of DNase I and were reverse transcribed by oligo(dT) primers and SuperScript III (Invitrogen). cDNA was analysed by quantitative PCR via SYBR Premix Ex Taq (Takara) in a Bio-Rad CFX96 real-time PCR system. qRT-PCR was performed in triplicate for each cDNA sample; the annealing temperature was 60 °C, there were a total of 40 cycles of amplification. The actin transcript was used to normalize the expression levels. The primers used are listed in Table S1. Three biological replicates were independently analysed for each mock and ABA treatment. The results were subjected to one-way analysis of variance and compared at $P < 0.05$.

Results

ssRNA-seq of sugar beet roots under ABA treatment

The sugar beet accession KWS9147 is one of the most widely distributed cultivars in China. We grew 4-week-old KWS9147 plants and transplanted the seedlings to a nutrient solution that contained 100 mg/L ABA. The sugar beet roots were sampled for RNA extraction at 1, 6, 12 and 24 h under ABA treatment. In parallel with the ABA treatments, seedlings transplanted into nutrient solution that lacked ABA were used as mock treatments (Fig. 1a). To confirm the time point-specific response of the sugar beet

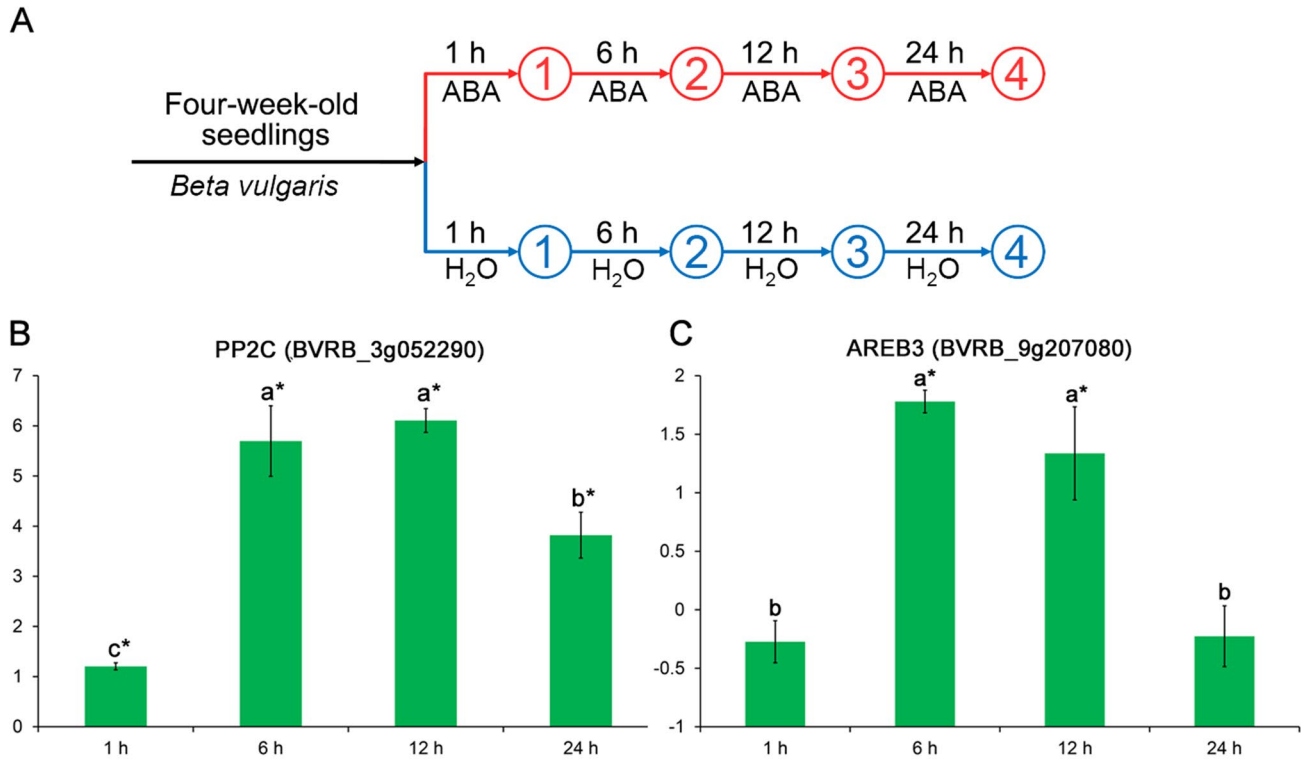

Fig. 1 Exogenous ABA was applied to sugar beet roots. **a** Schematic of the ABA and mock treatments. Four-week-old sugar beet seedlings were transferred to a nutrient solution that contained 100 mg/L ABA. The roots were isolated at 1, 6, 12 and 24 h under ABA treatment (red line). Seedlings growing in the nutrient solution that lacked exogenous ABA constituted the mock treatment (blue line). **b** Expression pattern of *BvPP2C*. **c** Expression pattern of *BvAREB3*. The corresponding mock samples served as controls. The expression fold change was log2 transformed. The bars indicate the standard error of the values of three repeats, the asterisks indicate significant differences ($P < 0.05$) between the ABA treatment and the corresponding mock, and the letters represent significant differences ($P < 0.05$) among ABA treatments. (Color figure online)

roots in response to exogenous ABA, two well-known ABA-responsive genes (*BvPP2C* and *BvAREB3*) were selected as markers (Umezawa et al. 2009; Zong et al. 2016). The expression of *BvPP2C* was up-regulated twofold at 1 h after ABA treatment surpassed 60-fold within 12 h (Fig. 1b). The expression of *BvAREB3* was also induced specifically at 6 and 12 h after ABA treatment (Fig. 1c). These results suggested the existence of a transcriptomic response to ABA in sugar beet roots.

To explore the ABA-responsive genes expressed in sugar beet roots, we carried out ssRNA-seq profiling to evaluate the expression patterns of the sugar beet transcriptome and deposited the raw data in the Sequence Read Archive (PRJNA594791). After the data were filtered, an average of 46,500,554 clean reads was obtained in each sample. Nearly 90% of the ssRNA-seq reads were mapped to the sugar beet genome (RefBeet 1.2.2) by HISAT2 (Kim et al. 2015; Dohm et al. 2014). According to a pipeline consisting of Cufflinks, SAMtools, HTseq-count and DESeq2 with strand-specific parameters, we assembled 21,819 TUs representing 16,827 annotated and 3510 unannotated genes (Anders et al. 2014; Love et al. 2014; Quinn and Chang 2016). The expression levels of the TUs were then calculated by counting the unique reads among them. The ratio between the read counts derived from these TUs and the total reads mapped on the genome reached 94.07% in our datasets, indicating that our RNA experiment was highly accurate at detecting the transcriptional direction of genes (Figure S1). To evaluate the reproducibility of the data, Pearson correlation coefficients were calculated for the samples. All the correlation coefficients among the replicates were greater than 0.9, indicating that our ssRNA-seq data were highly confident (Fig. 2a). A total of 13,899 expressed TUs were subsequently filtered and used for further analysis, for which the FPKM values were more than 3 in at least 1 sample (Table S2). Of these TUs, 12,862 representing 12,404 genes were previously annotated, and 1037 TUs were located at unannotated loci.

Next, we investigated the differentially expressed TUs in response to ABA by pairwise differential expression analysis to evaluate the differences between the ABA- and mock-treated samples at different time points. According to the common criteria (fold change > 2 and P value < 0.05),

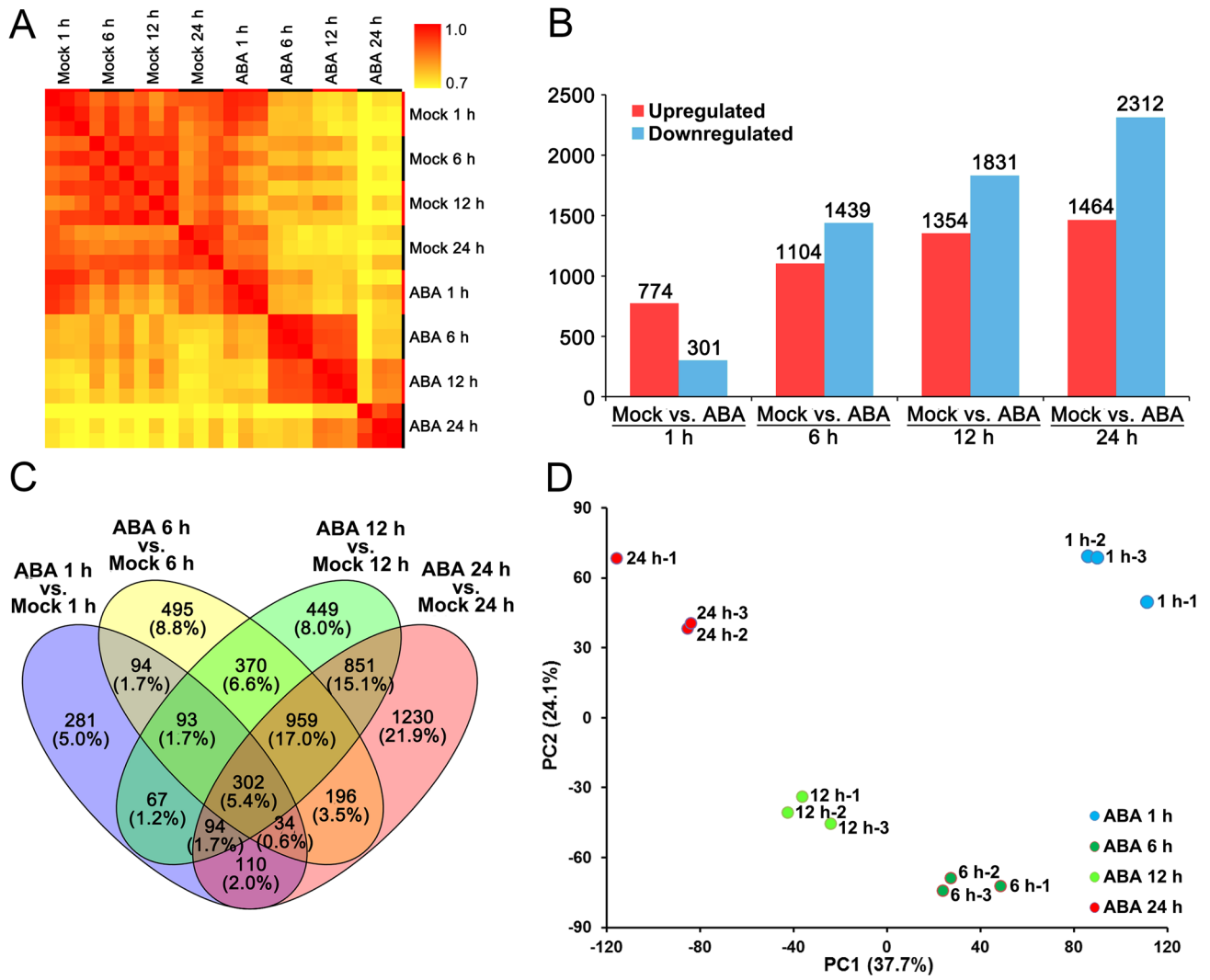

Fig. 2 Transcriptome analysis of sugar beet roots under ABA and mock treatments. **a** Heat map of Pearson correlation coefficients among samples. **b** Up-regulated and down-regulated differentially expressed TUs in all pairwise comparisons. **c** Venn diagram showing the differentially expressed TUs at each time point. **d** PCA of differentially expressed TUs at each time point. The ABA-responsive stages within 24 h were divided into the early (1 h), intermediate (6 h and 12 h) and late (24 h) stages. Three biological replicates are shown in the same point colour and labelled by Arabic numerals

a total of 5625 differentially expressed TUs were identified, representing 4903 annotated genes and 629 unannotated genes (Table S3). There were 1075, 2543, 3185 and 3775 differentially expressed TUs at 1, 6, 12 and 24 h under ABA treatment, respectively (Fig. 2b). The expression levels of 774 and 301 differentially expressed TUs were up-regulated and down-regulated, respectively, within 1 h after ABA treatment. Under ABA treatment for 6 h, 1104 up-regulated and 1439 down-regulated differentially expressed TUs were detected. The number of up- and down-regulated differentially expressed TUs subsequently increased to 1354 and 1831, respectively, after 12 h of ABA treatment. At 24 h of ABA treatment, we identified 1464 and 2312 differentially expressed TUs whose expression was up- and down-regulated in the roots, respectively. Among these differentially expressed TUs, only 302, accounting for 5.4%, were detected throughout the whole ABA treatment (Fig. 2c). The differentially expressed TUs after 1 h of ABA treatment shared a low level of overlap (no more than 10%) with those after the other durations after ABA treatment. The overlap between 6 h and 12 h, 6 h and 24 h and 12 h and 24 h accounted for 30.6%, 26.5% and 39.2%, respectively. These results indicated that the expression of a large number of ABA-responsive genes was regulated specifically at different time points. To determine the time point specificity of differentially expressed TUs in response to ABA, the difference in FPKM values between the ABA-treated and mock samples at each time point was subjected to PCA

(Fig. 2d). Two principal components explained most of the information from the treatments, with a total cumulative variance of 61.8%. On the basis of the normalized variables of each ABA treatment, three stages, namely, the early (1 h), intermediate (6 h and 12 h) and late (24 h) stages, were distinguishable within 24 h of ABA treatment.

Functional annotation and enrichment analyses of different ABA-responsive stages

To explore the biological functions of the ABA-responsive genes, we used AgriGO to analyse the genes at different ABA-responsive stages. There were 43, 65 and 63 terms enriched in the early, intermediate and late stages, respectively. A total of 19 enriched terms were shared across all ABA-responsive stages. In addition, there were 17, 14 and 11 unique terms enriched in the early, intermediate and late stages. On the basis of the FDR values and subordinate relationships, 5 unique and 10 universal terms were selected as representative GO terms for each ABA-responsive stage (Fig. 3).

In the early stage, the biological process terms, including dephosphorylation (GO: 0016311), regulation of RNA metabolic process (GO: 0051252), response to chitin (GO: 0010200) and response to carbohydrate stimulus (GO: 0009743), were uniquely enriched, suggesting that ABA perception and signalling transduction occurred within 1 h after ABA treatment (Fig. 3a). Functional genes associated with ion transport and catalytic activities were found to be significantly enriched in the intermediate stage (Fig. 3b). For example, genes encoding transporters and enzymes, which are associated with transmembrane transport (GO: 0055085), transition metal ion binding (GO: 0046914), transferase activity (GO: 0016740) and carbohydrate phosphatase activity (GO: 0019203), were enriched in this stage. In the late stage, we found that the structural constituent of ribosome (GO: 0003735), translation (GO: 0006412), and ribosome (GO: 0005840) were first enriched and presented

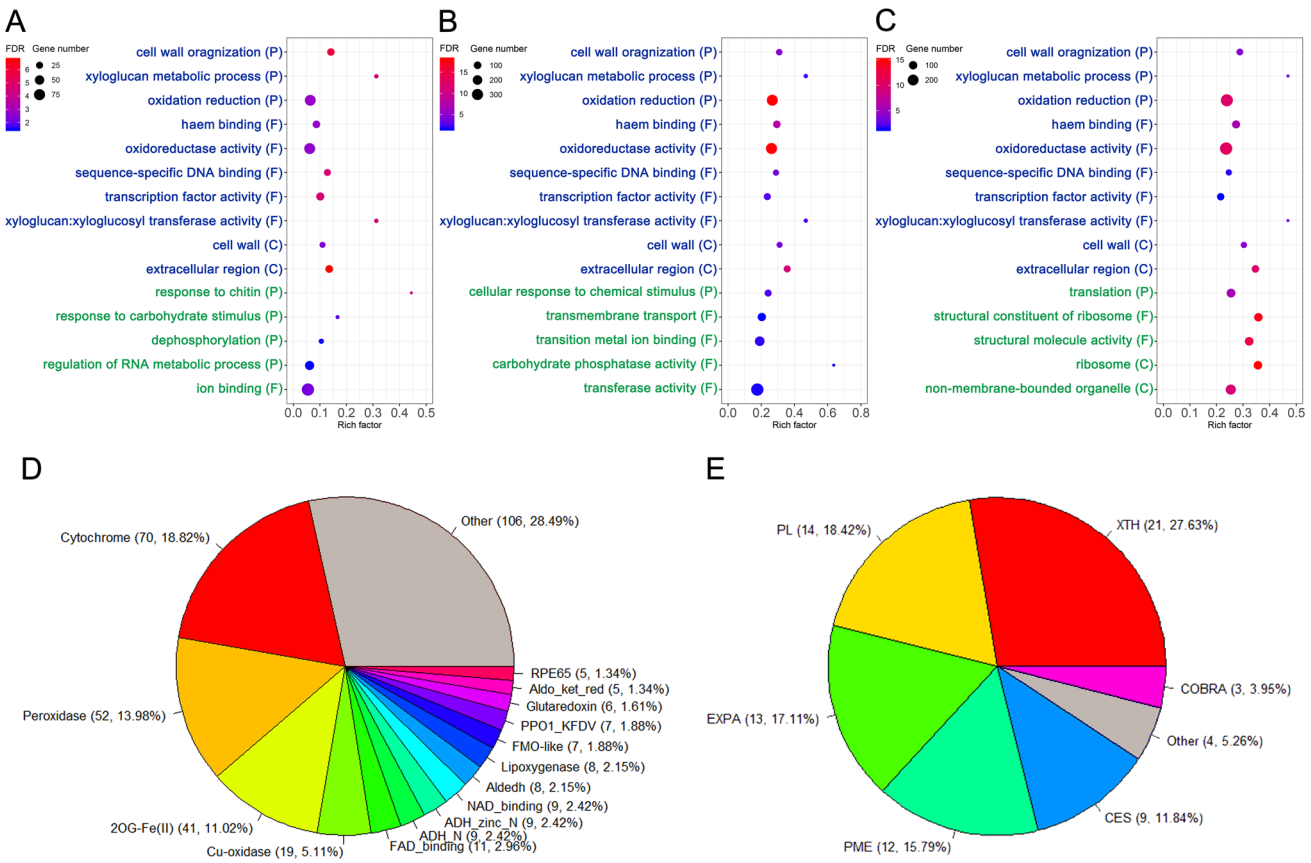

Fig. 3 AgriGO analysis of ABA-responsive genes in the early, intermediate and late stages. **a** GO terms significantly enriched in the early stage (1 h). **b** GO terms significantly enriched in the intermediate stage (6 h and 12 h). **c** GO terms significantly enriched in the late stage (24 h). The blue letters indicate GO terms universally enriched in the three stages, and the green letters indicate GO terms uniquely enriched in the early, intermediate or late stage. The letters P, F and C in brackets represent biological process, molecular function and cell component, respectively. **d** Pie chart of oxidation reduction (GO: 0055114). The category "Other" consisted of families with fewer than five members. The numbers shown in the brackets represent the number of members and percentages, respectively. **e** Pie chart of cell wall organization (GO: 0071555). (Color figure online)

low FDR values (less than 1E−6), indicating increased translation of ABA-responsive genes (Fig. 3c). The unique terms supported the division of the ABA-response process into early, intermediate and late stages, when ABA signal transduction, functional gene expression and increased translation occurred in sugar beet roots.

The universal terms generally had a lower FDR-adjusted *P* value than did the unique terms, suggesting that these terms were processed mainly in response to exogenous ABA. From the biological process category, oxidation reduction (GO: 0055114), cell wall organization (GO: 0071555) and xyloglucan metabolic process (GO: 0010411), were universally enriched at all stages. Totals of 70, 293 and 263 differentially expressed TUs implicated in oxidation reduction were detected in the early, intermediate and late stages, respectively. The FDR-adjusted *P* values of oxidation reduction, which were the most significantly enriched term, reached 1.9E−3, 9.3E−19 and 9.4E−11 in the early, intermediate and late stages, respectively. Additionally, 76 and 21 differentially expressed TUs were associated with cell wall organization and the xyloglucan metabolic process, respectively. Notably, both cell wall organization and the xyloglucan metabolic process were child terms of cell wall organization or biogenesis (GO: 0071554), and 21 xyloglucan metabolism TUs were also associated with cell wall organization. These results showed that exogenous ABA had an impact on the cell wall in sugar beet roots. In the molecular function category, heme binding (GO: 0020037), oxidoreductase activity (GO: 0016491) and xyloglucosyl transferase activity (GO: 0016762) were significantly enriched and closely associated with the enriched biological process terms. In addition, sequence-specific DNA binding (GO: 0043565) and TF activity (GO: 0003700) presented low FDR adjusted *P* values in the early stage, indicating an ABA signalling in sugar beet roots. From the cellular component category, enriched terms, including the cell wall (GO: 0005618) and extracellular region (GO: 0005576), were universally identified at all ABA-responsive stages, which was in line with enrichment of cell wall organization and the xyloglucan metabolic process.

On the basis of the above results, oxidation reduction and cell wall organization with low FDR adjusted *P* values were considered important ABA-responsive processes. We subsequently used hmmscan to determine what gene families or superfamilies are mainly implicated in oxidation reduction and cell wall organization. With respect to oxidation reduction, 372 differentially expressed TUs were categorized into more than 40 gene families (Fig. 3d and Table S4). The cytochrome superfamily comprised 65 cytochrome P450 (CYP) and 5 cytochrome bf561 (CYB561) constituting the largest proportion (18.82%), followed by peroxidase (POD, 13.98%) and 2-oxoglutarate and Fe(II)-dependent oxygenase (2OG-Fe(II), 11.02%). With respect to cell wall organization, 10 families comprising 76 differentially expressed TUs were identified (Table S5). Xyloglucan endotransglucosylase/hydrolase (XTH, 27.63%), pectate lyase (PL, 18.42%), expansin A (EXPA, 17.11%), pectinesterase (PME, 15.79%) and cellulose synthase (CES, 11.84%) were identified as members representing the main families involved in more than 90% of differentially expressed TUs (Fig. 3e). The expression patterns of these ABA-responsive TUs were then analysed by hierarchical clustering, the results of which are shown in Figures S2 to S9. Briefly, TUs involved in oxidation reduction could be clustered into groups with multiple variation tendencies, while several groups containing a large number of down-regulated TUs were found in terms of cell wall organization.

Regulation prediction of genes involved in oxidation reduction and cell wall organization in *B. vulgaris*

We performed a TF regulation prediction via the PlantTFDB (version 5.0, database last modified 2019-10-11) and calculated Pearson correlation coefficients between the expression of TFs and target genes to determine which ABA-responsive TFs might be responsible for the changes in the expression of genes involved in oxidation reduction and cell wall organization. A total of sixty-three ABA-responsive TFs were identified as potential regulators; these TFs had potential binding sites in the promoters of target genes, were co-expressed with target genes and responded to exogenous ABA no later than target genes did. A transcriptional regulatory network comprising 207 nodes and 302 edges was generated with Cytoscape (Figure S10 and Table S6). There were 20 TFs including 4 ERFs, 3 Dofs, 3 HSFs, 3 MYBs, 3 WRKYs, 2 C2H2s, 1 bZIP and 1 HD-ZIP implicated in 105 pairs of interactions in early stage (Fig. 4). Of these, the Dof domain-containing TFs constituted approximately 60% of potential interactions (62 of 105 pairs), followed by ERFs, which were involved in 16 pairs of interactions. Notably, we found that most ABA-responsive EXPAs might be transcriptionally regulated by Dof domain-containing TFs at 1 h after ABA treatment. In the intermediate stage, another 35 TFs from 16 families were detected in response to exogenous ABA and were responsible for 176 pairs of interactions (Fig. 4). The MYB, ERF, C2H2, NAC and WRKY families, which contained 7, 4, 3, 3 and 3 ABA-responsive TUs, respectively, were the major TF families and their members regulated 30, 26, 19, 8 and 5 target genes, respectively, during this stage. Additionally, owing to their recognition of the promoters of 53 target genes, AP2 domain-containing TFs, especially *BVRB_4g074790*, also played a central role in the ABA response. Last, only 8 new TFs associated with 21 pairs of interactions were detected in the late stage (Fig. 4).

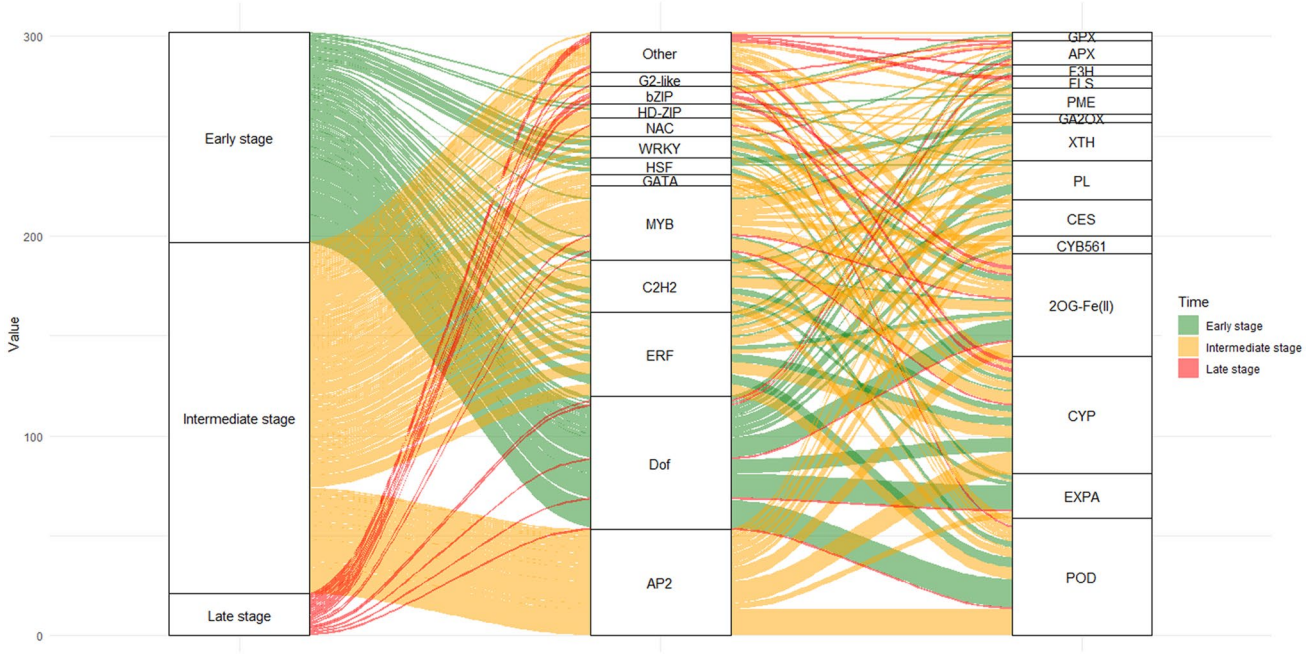

Fig. 4 Potential TF regulation distribution across the three ABA-responsive stages. The columns present the number of potential interactions, and the lines indicate the interaction pairs between TFs and target genes. The line colour indicates the earliest time when a significant change in TF expression was detected. (Color figure online)

According to betweenness and closeness centrality, three TFs, namely *BVRB_4g074790*, *BVRB_8g180860* and *BVRB_9g211370*, were identified at the centre of the network and had 51, 26 and 24 first neighbours, respectively (Fig. 5a). *BVRB_4g074790* is homologous to *BABY BOOM* (*At5g17430*) in *Arabidopsis*, which encodes a protein containing two repeated AP2/ERF domains. The targets of *BVRB_4g074790* were associated mainly with oxidation reduction and included 14 PODs, 13 CYPs and 8 2OG-Fe(II)s (Fig. 5b). The expression change of most PODs was positively correlated with *BVRB_4g074790* expression, while a negative correlation with *BVRB_4g074790* expression was commonly observed for CYP expression. Down-regulation of *BVRB_4g074790* expression differentially impacted the subsequent expression of 2OG-Fe(II)s. In addition, several cell wall organization TUs, including 5 CESs, 4 XTHs, 3 EXPAs, 3 PLs and 1 PME, might be regulated by *BVRB_4g074790*. *BVRB_8g180860* and *BVRB_9g211370* are separately homologous to *At1g29160* and *At1g51700*, each of which contains a Dof-type zinc finger. The expression of 2 Dofs significantly increased in the early stage and was negatively correlated with the expression change of most neighbours. Among the neighbours of *BVRB_8g180860*, the TUs whose expression was down-regulated were identified mainly as PODs, EXPAs, CYPs, PMEs and PLs. *BVRB_9g211370* preferentially repressed the expression of EXPAs and PODs (Fig. 5c). In particular, a total of 8 EXPAs, constituting 61% of ABA-responsive EXPAs, might be regulated by *BVRB_9g211370* (Fig. 5d).

qRT-PCR-based experimental validation of select ABA-responsive genes

To verify the transcriptome profiling results, we used qRT-PCR to determine the expression levels of a representative group of ABA-responsive genes implicated in signal transduction, oxidation reduction and cell wall organization (Fig. 6). As mentioned above, *BvPP2C* and *BvAREB3* are well-known ABA-responsive regulators (Umezawa et al. 2009; Zong et al. 2016). Both qRT-PCR and ssRNA-seq revealed that the expression of *BvPP2C (BVRB_3g052290)* was rapidly induced by exogenous ABA in the early stage, after which the expression reached a high level during the intermediate and late stages. The expression of *BvAREB3* was first found to be significantly up-regulated in the intermediate stage, while its expression decreased to its original level in the late stage. In contrast, exogenous ABA played a negative role in the AP2 domain-containing TF (*BVRB_4g074790*). These results were also consistent with the ssRNA-seq results. With respect to the expression of the two Dof domain-containing TFs (*BVRB_8g180860* and *BVRB_9g211370*), significant up-regulation was detected by both qRT-PCR and ssRNA-seq at 1 h after ABA treatment, after which the expression decreased with time. In association with the oxidation reduction term, three *BvCYP*s

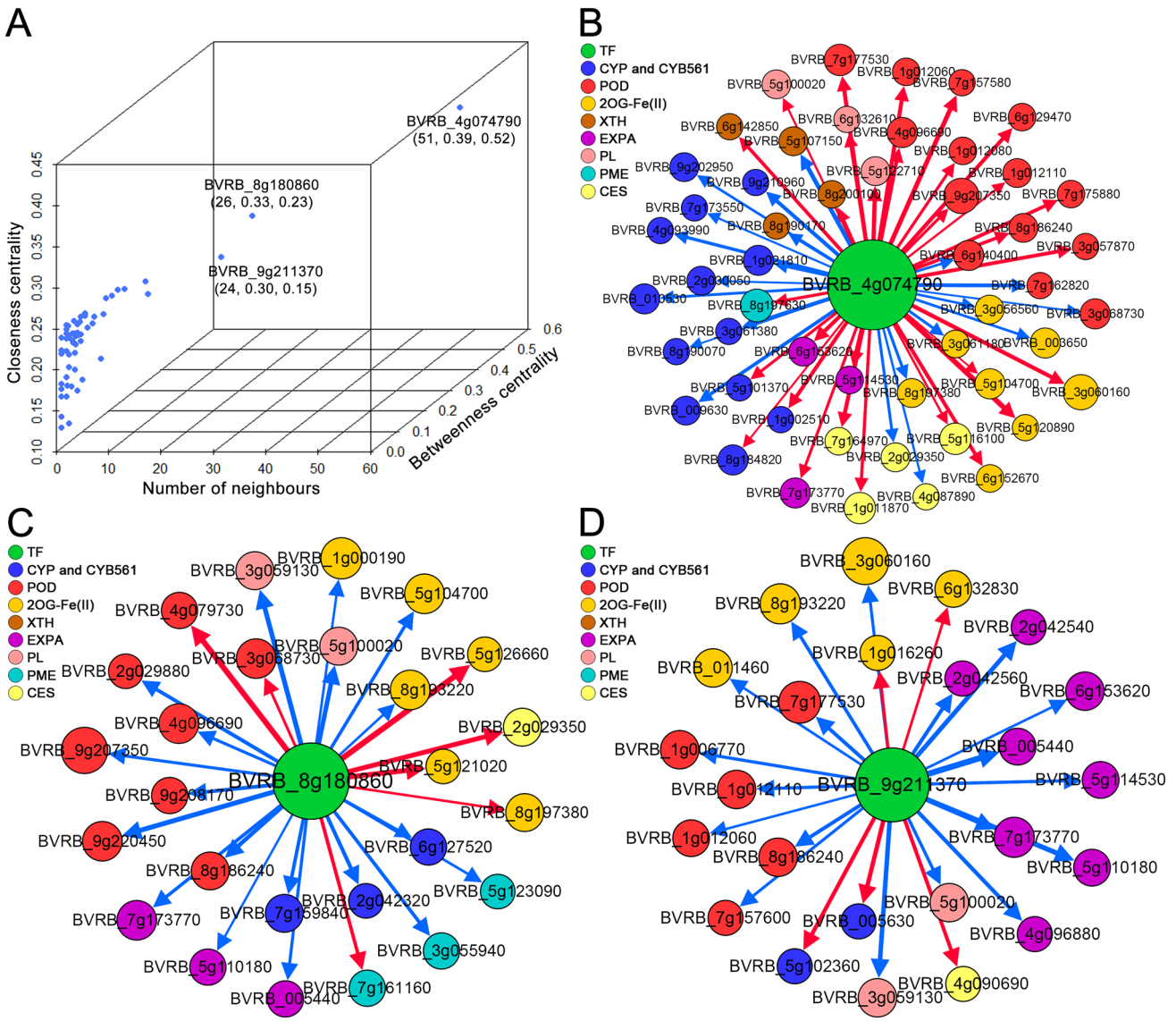

Fig. 5 Important TF regulatory subnetworks in sugar beet. **a** Closeness and betweenness centrality of ABA-responsive TFs. **b** Subnetwork of *BVRB_4g074790*. **c** Subnetwork of *BVRB_8g180860*. **d** Subnetwork of *BVRB_9g211370*. The red and blue arrows represent positive and negative correlations between the expression patterns of TFs and targets, respectively. The width of the lines was determined by the strength of the Pearson correlation coefficient. The size of circles was determined by the number of neighbours. (Color figure online)

were selected. Up-regulated expression of *BvCYP94* during all ABA-responsive stages was revealed by qRT-PCR and ssRNA-sEq. However, the results of qRT-PCR and ssRNA-seq were not strictly equal with respect to the expression of *BvCYP72* at 1 h and *BvCYP707* at 24 h. Both the qRT-PCR and ssRNA-seq results supported the hypothesis that exogenous ABA plays a negative role in *BvCYP72* expression and a positive role in *BvCYP707* expression. With respect to the cell wall organization term, the same results by which the expression of the three selected genes was significantly down-regulated in response to exogenous ABA occurred when both methods were used. In summary, the qRT-PCR results of the selected genes were generally consistent with those revealed by ssRNA-seq, although the expression fold change was not strictly consistent for all genes.

Discussion

ABA is especially vital for plants in the response to environmental stresses, including drought, cold and soil salinity (Raghavendra et al. 2010; Sah et al. 2016; Malaga et al. 2020). Many studies have focused on the regulation of ABA application to different crop species (Ding et al.

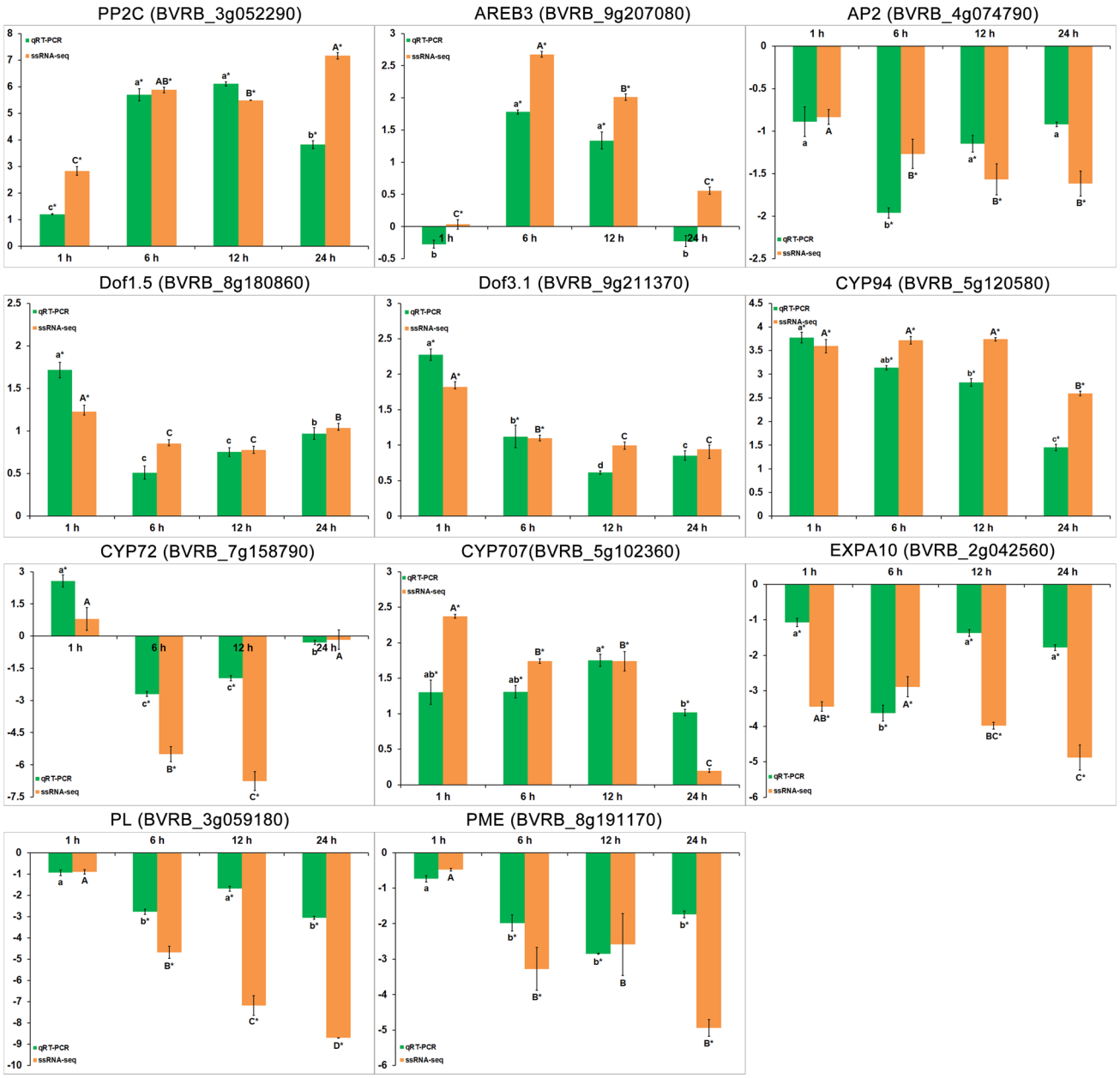

Fig. 6 Expression patterns of select genes as revealed by qRT-PCR and ssRNA-sEq. The corresponding mock samples served as controls. The expression fold changes were log2 transformed. The bars show the standard errors of three replications, and the asterisks represent significant differences ($P < 0.05$) between the ABA treatment and the corresponding mock treatment. The lowercase letters represent significant differences ($P < 0.05$) among ABA treatments as revealed by qRT-PCR, and the uppercase letters represent significant differences ($P < 0.05$) among ABA treatments as revealed by ssRNA-seq

2016; Tian and Li 2018; Wu and Liang 2017). Previous studies in sugar beet have suggested that ABA stimulates sucrose uptake and increases resistance to biotic and abiotic stresses (Saftner and Wyse 1984; Schmidt et al. 2010; Pospíšilová and Baťková 2004). However, the ABA regulatory mechanism in sugar beet roots has not yet been elucidated. Here, by using ssRNA-seq, we comprehensively analysed sugar beet roots after ABA treatment. We found that a large number of CYPs, PODs and 2OG-Fe(II)s in the roots responded to exogenous ABA and were responsible for oxidation reduction enrichment (GO: 0055114). In addition, cell wall organization (GO: 0071555) involving XTHs, EXPAs, CESs, PLs and PMEs was overrepresented.

ABA-responsive TUs involved in oxidation reduction

Previous studies have shown that CYPs, which compose a large and ancient superfamily, are largely involved in phytohormone biosynthesis and degradation and in secondary metabolite biosynthesis (Wei and Chen 2018; David and Danièle 2011). The *CYP707* encodes an ABA 8'- hydroxylase, a key enzyme involved in the oxidative degradation of ABA (Kushiro et al. 2014). Compared with that in wild-type *Arabidopsis*, the ABA content in the *cyp707* mutant dramatically accumulates. The induction of CYP707 genes by exogenous ABA and environmental stresses were normally detected in *Arabidopsis* and *Prunus avium* (Saito et al. 2004; Leng et al. 2018). However, CYP707s may not function as inhibitors of ABA accumulation because the endogenous ABA content can also typically increase in response to exogenous ABA and environmental stresses (Yang et al. 2011; Jia et al. 2002). In our study, the expression of *BvCYP707*s was also up-regulated in sugar beet roots, which could be considered to indicate ABA turnover and homeostasis.

The interplay between ABA and other phytohormones, such as gibberellic acid (GA), brassinosteroid (BR) and jasmonic acid (JA), to regulate growth and enhance stress resistance has been well documented (Shu et al. 2018; Ha et al. 2016; Avramova 2017). In our study, up-regulation of *BvCYP701* was detected in the late stage. The expression of *BvCYP88* first decreased but then increased over time. Previous studies have shown that CYP701 and CYP88 participate in three successive oxidations of ent-kaurene, producing GA (Helliwell et al. 2001; Dana et al. 2010). We also found that a *BvCYP714*, which belongs to subfamily A and plays a role in GA inactivation, was repressed by ABA (Zhang et al. 2011). Consistent with these results, the total GA concentration significantly increased at 3 days after ABA treatment in oriental melon (Kim et al. 2016). In addition to *CYP*s, the expression of two 2OG-Fe(II) genes, which encodes gibberellin 2-beta-dioxygenases, also increased in response to exogenous ABA, the process of which plays a central role in the homeostatic regulation of endogenous GA levels (Huang et al. 2010). These results gave us a transcriptional clue as to how ABA interacts with endogenous GA. Our results also suggested that exogenous ABA is involved in BR biosynthesis and activation. The expression of *BvCYP85*, which encoding protein catalyses C-6 oxidation in BR biosynthesis, fluctuated substantially after ABA treatment (Shimada et al. 2001). In addition, the expression of several CYP734 genes indicated BR activation and regulation of BR homeostasis (Thornton et al. 2010). Considering the similarity in functions between ABA and JA, it is presumably important for ABA-treated sugar beets to balance fitness and metabolic costs associated with sustained defence responses by the attenuation of JA signalling. Unsurprisingly, a number of hydroxylases (CYP94) involved in the oxidation of jasmonoyl-L-isoleucine (JA-Ile) were significantly up-regulated after ABA treatment, suggesting the negative control of JA-Ile levels (Kitaoka et al. 2014). Taken together, this evidence indicates that exogenous ABA-induced alterations of phytohormone homeostasis are largely dependent on the expression of CYP and 2OG-Fe(II) genes.

In addition to CYPs and 2OG-Fe(II)s associated with phytohormone metabolism, we also detected the up-regulated expression of *BvCYP73 and BvCYP98*, which encode trans-cinnamate 4-monooxygenase and phenolic ester 3'-hydroxylase, respectively, catalysing two beginning steps of flavonoid biosynthesis (Blee et al. 2001; Schoch et al. 2001). In the down-stream pathway, the expression of another 5 *2OG-Fe(II)*s (3 flavanone 3-hydroxylase (F3H) and 2 flavonol synthases (FLS)) was also regulated by exogenous ABA. The expression of all the *BvF3H*s was up-regulated in response to exogenous ABA, implying the accumulation of dihydroflavonols which are intermediates shared between flavonol and anthocyanidin biosynthesis (Han et al. 2017). Considering the repression of FLSs by exogenous ABA, the inhibition of flavonol biosynthesis might be inhibited, while the accumulation of intermediates would benefit anthocyanidin biosynthesis. These results are also consistent with those of a recent study showing that exogenous ABA promotes anthocyanin accumulation in table grapes (Koyama et al. 2018).

The accumulation of ROS, including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), typically occurs in response to exogenous ABA (Jiang and Zhang 2001). In sugar beet roots, exogenous ABA induced the expression of calcium-dependent NADPH oxidase (*BVRB_4g075730*) which is responsible for ROS production (Kwak et al. 2003). Interestingly, there was no significant change in *BvSOD* expression in response to ROS production, suggesting that O_2^- accumulates and functions as a secondary messenger in ABA signalling. In *Arabidopsis*, direct evidence at cellular biological and molecular genetic levels has shown that ROS produced by NADPH oxidase act as rate-limiting secondary messengers in ABA signalling (Kwak et al. 2003). A previous study on the effect of ABA on the antioxidant system in developing wheat grains showed that ABA can increase the activity of POD, which possibly contributes to improved tolerance against oxidative damage (Asthir et al. 2009). In our study, a large number of *BvPOD*s were found to respond to ABA. However, their expression patterns were quite different (Figure S3), implying that exogenous ABA displayed preferential regulation of *BvPOD*s. Another study also showed that POD isoforms responded differently at the protein level in ABA-treated mung bean roots (Das and Kar 2017). According to the well-annotated TUs, the expression of all three *APX*s was repressed, while the expression of all three *GPX*s up-regulated by exogenous ABA. This

phenomenon provided insight into how exogenous ABA regulates ROS scavenging in sugar beet roots.

ABA-responsive TUs involved in cell wall organization

Expansins refer to a family of nonenzymatic proteins that characteristically cause loosening and expansion of plant cell walls by disrupting noncovalent bonds between cellulose microfibrils and matrix glucans (Cosgrove 2000). In *B. vuglaris*, up-regulated expression of *BvEXPA* was reported to be associated with the formation of lateral roots and root hairs (Gil et al. 2018). Our transcriptomic study revealed that ABA played a rapid and universal negative role in *BvEXPA* expression. The expression of 13 *BvEXPA*s decreased by 5-fold to 100-fold within 24 h after ABA treatment, of which the down-regulation of 7 *BvEXPA*s rapidly reached more than threefold at 1 h after ABA treatment, which might have inhibited cell expansion in sugar beet roots.

Pectin, cellulose and xyloglucan are essential components of cell walls. Numerous enzymes associated with cell wall metabolism were detected in response to ABA. PLs and PMEs catalyse two important steps of pectin degradation, contributing to the eliminative cleavage of pectin and play a role in the demethylesterification of cell wall pectin (Yadav et al. 2009). In our study, owing to down-regulation of most *PL*s and *PME*s, exogenous ABA drastically inhibited pectin degradation. Exogenous ABA also impacts the expression of *BvCES*s and *BvXTH*s, which encoding proteins catalyse polymerization of uridine 5′-diphosphate glucose into cellulose and the religation of xyloglucan polymers (Campbell and Braam 1999; Richmond and Somerville 2000). CESs and XTHs from different families exhibit different preferences for principal polysaccharides during cell wall formation and modification (Hyles et al. 2017; Campbell and Braam 1999). The different changes in *BvCES*s and *BvXTH*s expression induced by exogenous ABA could be considered modulation of cell wall elements. On the basis of the above results, we proposed that the expansion of the cell wall would be slowed and that the cell wall was strengthened by exogenous ABA via constituent reorganization of pectin, cellulose and xyloglucan.

Important regulators involved in ABA-responsive pathways

One desirable question is which TFs are the main hubs for the regulation of ABA-responsive TUs involved in oxidation reduction and cell wall organization. Benefitting from the increase of ChIP-seq and DNase-seq data, TF regulation prediction based on TF binding motifs and regulatory elements has become possible. In addition to physical interactions, owing to direct regulation at the transcriptional level, co-expression should be detected between up-stream TFs and their targets. Considering that different TFs sharing the same targets may affect this phenomenon, we did not set a high level of correlation coefficient as a threshold in this study. On the basis of the above mentioned, we employed PlantTFDB-based and co-expression analysis to screen important regulators in response to exogenous ABA. Among 63 ABA-responsive TFs, one novel AP2 domain containing TF (*BVRB_4g074790*) and 2 Dof domain-containing TFs (*BVRB_8g180860* and *BVRB_9g211370*) were identified as potential hub regulators.

In *Arabidopsis*, TFs with AP2 domains, such as ABI4 and ABR1, are widely known to function in the regulation of organ development, including lateral root inhibition and floral organ development, and increase resistance to drought, salt and cold stress (Shu et al. 2018; Pandey et al. 2005; SöDerman et al. 2000). According to the sequence alignment results, *BABY BOOM* (*BBM*, At5g17430) was the closest homologue to *BVRB_4g074790*. Ectopic expression of *BBM* in *Arabidopsis* and *Brassica* resulted in hormone-free regeneration of explants, and alterations in seedling morphology (Boutilier et al. 2002). By regulating phytohormone homeostasis, cell growth and oxidation resistance, *BVRB_4g074790* may also play an important role in root growth and stress tolerance. Among its targets, CYP707 and GA2OX are involved in ABA and GA homeostasis (Saito et al. 2004; Thornton et al. 2010). CYP86 acts on saturated and unsaturated fatty acids with chain lengths ranging from 12 to 18 carbons during cell elongation (Rupasinghe and Duan 2010). CYP78 plays a role in regulating directional growth and promotes organ growth (Eriksson et al. 2010). In addition to growth regulating genes, 13 ABA-responsive *BvPOD*s may be regulated by *BVRB_4g074790*. This could partially explain why exogenous ABA preferentially regulated *BvPOD*s expression.

Dof proteins that contain a conserved C2C2 finger structure are plant-specific TFs involved in the regulation of gene expression in processes such as seed germination and defence mechanisms (Lijavetzky et al. 2003). In our study, the expression of two Dof domain-containing TFs was rapidly up-regulated by exogenous ABA within 1 h during which time the expression of the well-known ABA-responsive regulator (*BvAREB3*) was not significantly altered (Choi et al. 2000). This implied that two Dof domain-containing TFs are components in a different ABA-responsive pathway. In addition to targets such as PODs and 2OG-Fe(II)s, EXPAs were noteworthy for being regulated mainly by *BVRB_9g211370*, and their expression significantly changed in the early ABA-responsive stage. These results suggested that cell expansion might be reduced by exogenous ABA via a Dof-mediated pathway. Consistent with our speculation, a Dof domain-containing

TF (*AtDOF5.4/OBP4*) is cloned in *Arabidopsis* and negatively regulates cell expansion via repression of *EXPA*s and *XTH*s (Xu et al. 2016).

Conclusion

In this study, 5625 TUs representing 4903 annotated genes and 629 unannotated genes were transcriptionally identified to participate in ABA-responsive pathways. These genes were responsible for the significant enrichment of 43, 65 and 63 GO terms in the early, intermediate and late stages. Among these terms, oxidation reduction and cell wall organization were the most significantly enriched ones in all the ABA-responsive stages. With respect to oxidation reduction, CYPs, PODs and 2OG-Fe(II)s constituted the largest proportion, with ABA-responsive TUs possibly contributing to phytohormone homeostasis and secondary metabolite biosynthesis. XTHs, EXPAs, PMEs, PLs and CESs were also detected in response to exogenous ABA, implying regulation of cell expansion and reorganization of cell wall constituents. Beneficial for TF binding site prediction and co-expression analysis, one AP2 domain- and two Dof domain-containing TFs (*BVRB_4g074790, BVRB_8g180860* and *BVRB_9g211370*, respectively) were identified as potential hub genes for the regulation of ABA-responsive oxidation reduction and cell wall organization. Our study on the profile of ABA-responsive genes provides new insights into the molecular functions of regulatory genes and will aid future molecular breeding of sugar beet.

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Author contributions WX, YZ, MW and DL performed the experimental work. XL, ZP and QW participated in the bioinformatics and statistical analysis. ZP, JL and WX wrote and edited manuscript. ZW conceived and directed the overall concept of this work.

Data availability The raw data of ssRNA-seq was deposited in Sequence Read Archive (PRJNA594791).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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