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Multi-omics analysis of the regulatory network in winter buds of 'Cabernet Sauvignon' grapevine from dormancy to bud break

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Summary

Winter dormancy and bud break are crucial to the viability, adaptability and yield of fruit trees, but not all metabolic activities or regulatory factors involved in maintaining and breaking dormancy are known. Here, winter buds, spanning from natural dormancy to bud break, were collected from 'Cabernet Sauvignon' grapevines maintained outdoors or forced indoors. The transcriptomes, proteomes and plant hormone contents were analysed across several bud stages. The winter buds presented three main stages, dormancy, dormancy release and bud development, whether grown in or outdoors. Weighted Correlation Network Analysis (WGCNA) and Gene Ontology (GO) analysis of the omics data revealed that the different stages were enriched for different biological processes. Analysis of the differentially expressed genes (DEGs) identified seven candidate genes that may affect grape dormancy and bud break. Transient transformation of these seven genes showed that *VvDOGL4*, *VvAGL65* and *VvMARD* could promote maintenance of winter bud dormancy in grapevine. Subcellular localization showed that these three proteins all located to the nucleus, and yeast two-hybrid screening showed that they may interact with proteins related to plant hormone signal transduction, respiration, energy metabolism and transcription regulation to affect winter bud break in grapevine. Overall, these findings contribute to a better understanding of the regulatory dynamics of bud dormancy in a perennial fruit crop and lay a foundation for exploring key genes and regulatory mechanisms that can be manipulated to improve fruit quality and yields as the global climate shifts growing regions.

INTRODUCTION

To adapt to the low temperatures and water availability during the winter season, woody perennials in boreal and temperate zones often enter dormancy and resume growth in the warm spring. Photoperiod and temperature are the main environmental signals that regulate this seasonal synchronization of developmental transitions in the annual growth cycle (Singh *et al*., 2018). In most woody plants, short days (SD) induce or accelerate growth arrest and dormancy of branches, while long days (LD) have the opposite effect (Azeez *et al*., 2021). Once dormancy is established, a prolonged period of low temperature is required to meet the plant's chilling requirement, enabling it to resume growth under suitable conditions.

In grapevine, winter buds enter physiological dormancy in response to short days and low temperatures through regulatory networks that include photosensors, cell cycle genes, oxidative stress, carbohydrate metabolism and phytohormones (Beauvieux *et al*., 2018; Kuehn *et al*., 2009; Vergara *et al*., 2017). These endodormant winter buds are insensitive to environmental signals outside of their chilling requirement and do not germinate under otherwise favourable conditions unless there is a signal to break dormancy. Physical treatment methods such as high temperature and hypoxia or chemical treatment methods such as hydrogen cyanamide (HC), lime nitrogen and plant hormones can change the physiological state of dormant buds. Multiple metabolic pathways such as oxidative stress and antioxidant, glycolysis, pyruvate metabolism, anaerobic respiration-related, carbohydrate metabolism and phytohormones are involved at this time (Sudawan *et al*., 2016; Vergara *et al*., 2012; Zheng *et al*., 2018a).

Several genes that influence the transition of buds from dormancy to bud break have been identified in various species. In poplar, the transcription factor genes *SHORT VEGETATIVE PHASE-LIKE* (*SVL*) and *TCP18* are down-regulated by low temperature and mediate bud rupture by acting on gibberellin (GA) and abscisic acid (ABA) pathways (Rajesh Kumar Singh *et al*., 2019). The AP2/ERF family transcription factor EARLY BUD-BREAK 1 (EBB1) is a positive regulator of bud break and negatively regulates *SVL* expression, while EBB3 is a temperature-responsive and epigenetic positive regulator of bud break that is directly linked to cell cycle activation during the spring (Azeez *et al*., 2021). *EBB1* also triggers bud break in peach trees by regulating hormone metabolism, cell cycle and cell wall modification (Zhao *et al*., 2020). In grapes, transgenic vines overexpressing the ABA 8′ -hydroxylase *VvA8H-CYP707A4* showed an increase in ABA catabolism and higher bud break (Zheng *et al*., 2018b). The transcription factor *VvWRKY37* is highly expressed in dormant buds and is induced by exogenous ABA, and its ectopic overexpression in poplar significantly delayed bud break (Wang *et al*., 2022).

A working model of artificially induced bud dormancy release proposes that hydrogen cyanamide (HC) and heat shock can disturb cytochrome pathway in mitochondria, leading to respiratory and oxidative stress and the induction of related physiological indicators and genes (Ophir *et al*., 2009; Zheng *et al*., 2015). These strategies are selectively induced by organisms to cope with energy crisis under low oxygen conditions. Furthermore, these changes may affect the interaction between ethylene and abscisic acid, thereby removing the inhibition of abscisic acid on meristem activity and restoring growth (Khalil-Ur-Rehman *et al*., 2020; Ophir *et al*., 2009; Zheng *et al*., 2018a).

Although grape bud dormancy has been extensively studied, details on the complex and intricate molecular network regulating the release of grape bud dormancy remains limited. In this study, winter buds of 'Cabernet Sauvignon' were collected multiple times over winter from field-grown grapevines and from cuttings brought indoor, covering all stages from dormancy to bud break. Multi-omics evaluation of RNA transcript, protein and phytohormone levels were employed to detect changes in the metabolic pathways regulating winter bud reactivation in grapevine over time. Transient transformation of candidate genes into winter buds confirmed their roles in the mechanisms regulating grapevine winter bud dormancy and break in grapevine.

RESULTS

Winter bud morphology significantly changes after the bleeding stage

Field-grown winter buds were imaged at 10 developmental stages [100 days before bud break (BB), 80 days before BB, 65 days before BB, 40 days before BB, 28 days before BB, 14 days before BB, 7 days before BB, bud swell (BS), bud downy (BD) and BB] by stereoscope and indoor-grown winter buds were imaged at 4 developmental stages (0, 6, 12 days and BB) (Figure 1c, d). The internal structure of winter bud is shown in Figure 1a, with three internal buds shown.

Significant changes were observed following the bleeding stage (14 days before BB) in the field samples (Figure 1d), and no significant physical changes were observed externally or internally beyond 2 weeks before bud break (BB). At 14 days before BB, the main buds were enlarged and the inflorescence primordia began to develop. During the three stages of budding, more rapid changes occurred, with the outer buds of the winter buds expanded significantly, scales open up, and the bud axes beginning to elongate at bud swell (BS), and broken scales, spheroidal buds, and extension of the inner bud axis of the primary bud further extended by the bud downy stage (BD). The structure of the future shoots was already clearly visible inside the winter buds at bud break (BB). The development of winter buds maintained indoors was essentially the same as in the field, with some winter bud scales slightly dehiscent at 12 days. Internal changes in the winter buds became evident, followed by the onset of sprouting (Figure 1c).

Molecular changes over three stages from dormancy to bud break in grape winter buds

Winter buds of 'Cabernet Sauvignon' growing in the field were collected from dormancy to bud break during the 2020–2021 winter season, with increased sampling frequency after the bleeding stage (14 days before BB). A total of 12 groups of samples were subjected to RNA sequencing, resulting in the detection of 32 230 expressed genes, of which 27 965 genes had an FPKM value greater than 0. Transcriptome samples from winter buds on cuttings of grapevine taken indoors to force the transition from dormancy to bud break were also collected every 2 days (with day 0 the day the branches were taken from the field to the indoors), with a total of 9 groups gathered, which generated 32 118 expressed genes, of which 27 630 genes had an FPKM value greater than 0. The indoor and field DEGs were essentially identical. To verify the results, DEGs were randomly selected to detect the transcript levels using RT-qPCR (Figure S2). The RT-qPCR results were basically consistent, indicating that the transcriptome data were credible.

Pearson correlation coefficients were calculated between the 27 indoor samples (Figure 1e) and the 36 field samples (Figure 1g), with the stronger correlation between samples in darker colours in the heatmap (Figure 1e, g). The 0 day samples from the indoors set were not considered as a separate stage. Principal component analysis (PCA) plots of both transcriptomes showed that each respective PC1 contributed more than 60% of the variance (Figure 1f, h). This combination of PCA, Pearson correlation and phenotypic changes indicated that the transcriptomes from both the field and indoor samples exhibit three distinct stages of transition from dormancy to bud flush: the dormant stage, the dormancy release stage and the bud development stage. In the field, the timing of the three stages was divided as follows: 100–80 days before BB, 50–19 days before BB and 14 days to BB. Indoors, the three stages were 0–2 days, 4–10 and 12–14 days. Comparison of the number of DEGs between the samples reflecting the similarity between samples, also indicated that the winter buds experienced three stages (two major changes) from dormancy to growth and development (Figure S3).

WGCNA and GO enrichment analyses highlight the active biological processes of the dormancy stages

The field and indoor transcriptome datasets were analysed by Weighted Gene Co-Expression Network Analysis (WGCNA) to observe trends of each transcript over time and to identify key genes that may regulate the transition of winter buds from dormancy to activity in grapevine (Figure S4). The WGCNA filtered 18 815 DEGs from the field transcriptome data into eight modules (Figure S4a, grey module not displayed), with the transcripts in each module showing similar trends in expression levels over time. For each module, the inflection points were consistent with the three developmental stages in the previous section, that is, 80 and 14 days before BB, as the dividing line. The distribution of genes across the eight colour-coded modules is as follows: 1211 in yellow, 313 in red, 8472 in turquoise, 431 in green, 1401 in brown, 6890 in blue, 97 in black and 760 in grey (Figure 2d). The turquoise and blue modules contained 81.65% of the genes, with transcripts in the turquoise module gradually increasing over time and those in the blue decreasing during bud transition. Two other sets of modules, yellow versus green and red versus brown, also showed roughly opposite trends. Genes within the black module were essentially highly expressed only during the bleeding stage, and genes in the grey module were essentially stably expressed with no significant changes detected.

For the indoor samples, the WGCNA identified a total of 19 341 DEGs, organized into 9 modules (Figure S4b). These independently colour-coded modules of expression trends were yellow (comprising 1473 DEGs), red (891), turquoise (7401),

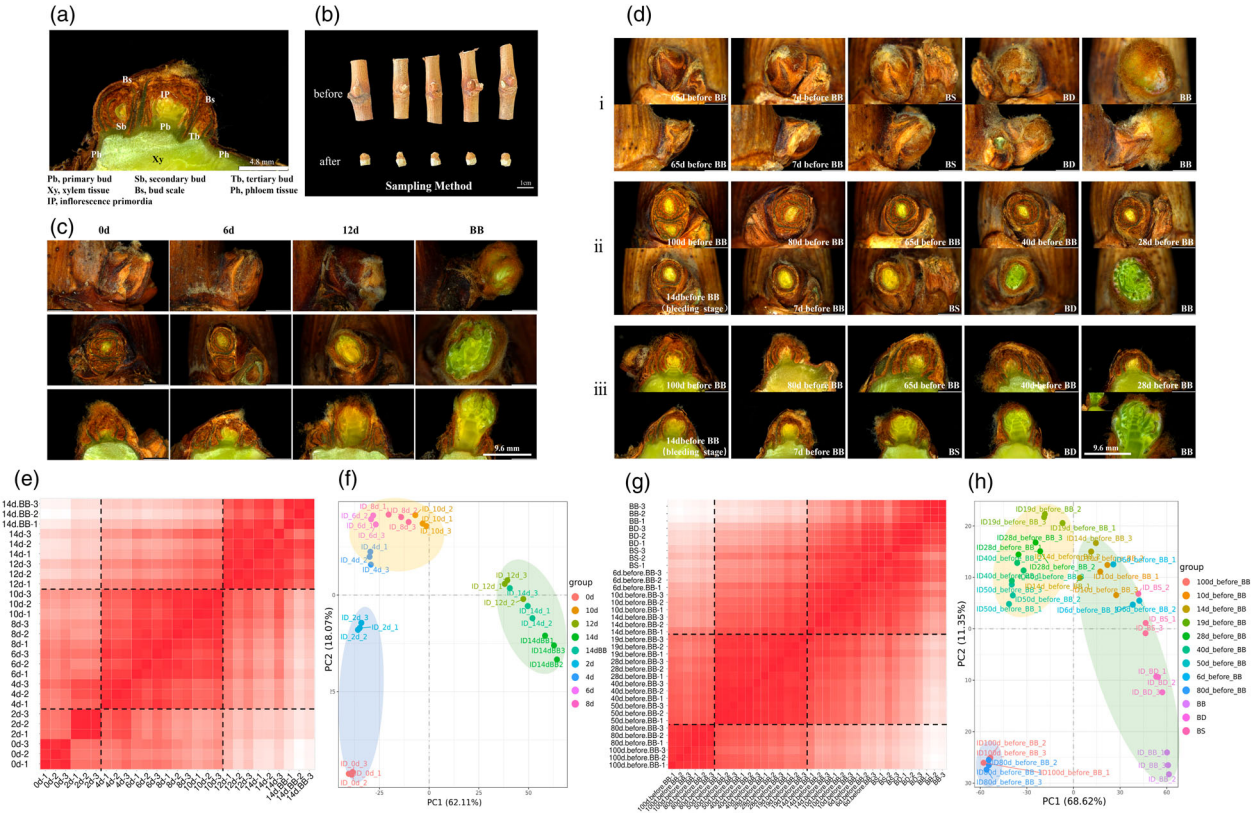

Figure 1 Transition from dormancy to activity of grape winter buds. (a) Internal tissues of winter buds. (b) Winter buds and their surrounding tissues were taken for various omics tests. (c) Indoor samples were branches removed from field-grown grapevines and taken indoor for transition from dormancy. Buds were collected every 2 days from day 0 (day the branch was removed) to bud break over 14 days. The figure shows samples of 0, 6, 12 days and bud break (BB). (d) Winter bud development in field conditions, i: front and side views of winter buds at five times: 65 days before BB, 7 days before BB, bud swell (BS), bud downy (BD) and BB, other sampling times are not shown since there were no obvious changes in the appearance of winter buds between those periods; ii, iii: transverse and lateral views of field-grown winter buds of the 10 groups of samples. (e-h) Statistical analyses of transcriptome data derived from indoor (e and f) and outdoor (g and h) samples. Pearson correlation coefficient plots between (e) 27 indoor samples and (g) 36 field samples. The highly correlated sample sets indicate the three stages of winter bud dormancy, as indicated by dotted lines. Principal component analysis (PCA) between the indoor and field samples, with the three colour blocks representing the three stages of winter bud dormancy.

green (1173), magenta (381), blue (3108), pink (427), brown (3102) and black (739) (Figure 2d). Among these nine modules, genes in the yellow, green and pink presented similar transcriptional trends, gradually increasing and then decreasing during bud development, but the time points at which expression began to increase were different. Genes in the red and turquoise modules both gradually increased, but the genes in the red module had high expression at 0 day. The genes in blue module showed gradually declining expression. The other modules contained genes with narrow expression windows, with the genes in the brown module only highly expressed at 0 day, in the black at 2 days and in the magenta at both 0 day and 2 days, respectively (Figure S4b). The modules derived from the indoor and field datasets were compared to see how many DEGs were shared between the modules showing similar expression trends (Figure 2d). This allows comparison of the modules despite the colour coding.

Based on the WGCNA, the red, turquoise and blue modules of the field transcriptome (and the corresponding indoor pink, turquoise and brown modules) were further analysed (Figure 2). According to their expression trends, it was speculated that the three modules contained key genes that promoted bud break, bud development and bud dormancy. Subsequently, GO annotations were determined for these modules (Figure 2a–c, and Table S2).

The red module of the field transcriptome was hypothesized to promote bud break and contained only 313 genes (Figure 2a) and just 23 enriched GO pathways. The enriched biological processes (BP) included aminoglycan catabolic process, chitin metabolic process, carbohydrate derivative catabolic process, polysaccharide catabolic process, response to jasmonic acid, response to fatty acid and carbohydrate catabolic process. Cellular component (CC) and molecular function (MF) categories showed significant enrichment of genes related to secretory vesicles and chitinase (Table S2). These pathways are mainly associated with cell wall metabolism, representing significant mobilization of a large number of cell wall-related pathways before bud break. The GO analysis of the pink module of the indoor transcriptome, also hypothesized to promote bud break, indicated enrichment for a large number of pathways (Table S3). The significantly enriched ($p < 0.05$) BPs included tropism, hydrotropism, response to blue light, mRNA transcription and regulation of developmental

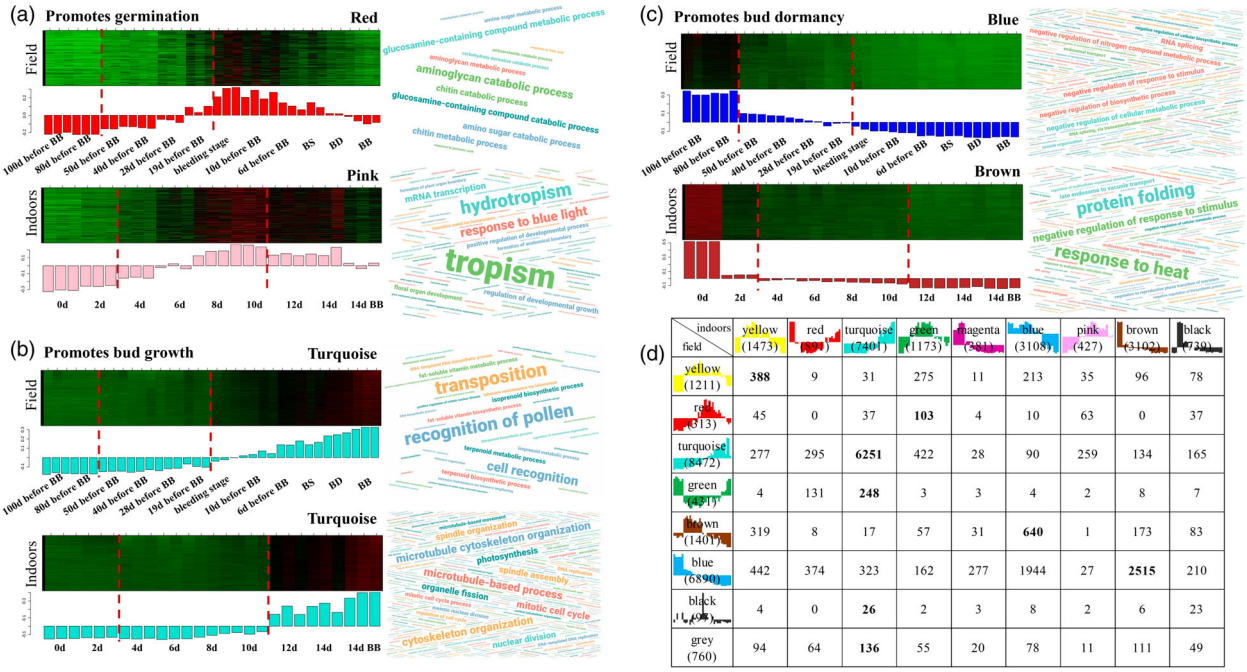

Figure 2 GO enrichment analyses of processes that change between the stages of dormancy at the transcriptional level. (a–c) The modules from the field and indoor transcriptomes are divided for putative promotion of bud break, bud growth and development and bud dormancy, respectively. The top half of each panel shows the clustered heatmap of the genes within the module, with high expression in red and low expression in green; the bottom half shows the expression pattern of the module eigenvalues in different samples. The word cloud on the right shows the results of the differential gene GO enrichment analysis, based on significantly enriched Biological Process items with the value of $p < 0.05$, with larger font size indicating more significant changes. Red dashed lines are used to indicate the time points of the three stages of bud dormancy. (d) Number of DEGs shared between the transcriptome modules. The mini-bar plot in each column and row head shows the expression patterns of genes in this module. The numbers in brackets are the number of DEGs in this module, and the numbers in the table are the number of shared DEGs between the module pairs.

growth. Additionally, pathways related to the formation of various organs and tissues, such as flower organs, anthers, stamens, meristem, shoot axis, epidermis and phloem, were also enriched, as were hormone-related pathways, including gibberellin, ethylene, cytokinin and steroid hormones, and other pathways such as photoperiodism, cellular response to heat and ion homeostasis. The number of genes within the pink module of the indoor transcriptome was higher (427) and more GO pathways were enriched, with numerous genes for growth and morphogenesis showing increased transcription. The two modules together showed enriched carbohydrate derivative catabolism, polysaccharide catabolism, response to jasmonic acid, response to fatty acid and carbohydrate catabolism.

The turquoise modules from both the field and indoor samples showed similar progressive up-regulation after dormancy release, with transcripts reaching peak expression in swelling buds, leading to the hypothesis that these modules contain genes related to the promotion of shoot growth and development (Figure 2b). Both modules contained the highest number of genes among their datasets. The BPs significantly enriched in the turquoise module of the field dataset included transposition, recognition of pollen, cell recognition, terpenoid biosynthetic and metabolic processes, isoprenoid biosynthetic and metabolic processes, and fat-soluble vitamin biosynthetic and metabolic processes (mainly vitamins K and E), as well as telomere maintenance, mitosis, chromosome separation and cell cycle-related pathways (Table S2). The turquoise module of the indoor dataset contained more BPs and was significantly enriched for more diverse biological processes such as cell cycle, cytoskeleton, microtubules, photosynthesis, cell wall, chloroplasts, meiosis and gibberellins, in addition to mitosis-related ones (Table S3). Unlike the pathways enriched within the module promoting bud break (i.e. cell wall and organ tissue-building), the module promoting bud development exhibited more cell cycle and cell division-related processes, suggesting that before bud break cells begin to swell, whereas after bud break the cells tended to grow and develop through cell division.

Finally, there is a module associated with bud dormancy (Figure 2c). In the blue module of the field dataset, a large number of negatively regulated processes, such as negative regulation of stimulus response, cellular metabolic process, nitrogen compound metabolic process, biosynthetic process, DNA-templated transcription and signal transduction, were significantly enriched. In addition, light signal pathways such as circadian rhythm, response to blue light, response to red or far-red light, photoperiodism, response to ultraviolet light, response to light intensity, as well as numerous plant hormone-related pathways like jasmonic acid, gibberellic acid, abscisic acid, ethylene and other responses to abiotic stimuli were significantly enriched (Table S2). The brown module of the indoor dataset was similar to the field module in that a large number of negative regulatory processes, and light signalling-related pathways were enriched. The difference was that only abscisic acid and salicylic acid were significantly enriched among the plant hormone signalling pathways within the indoor transcriptome (Table S3).

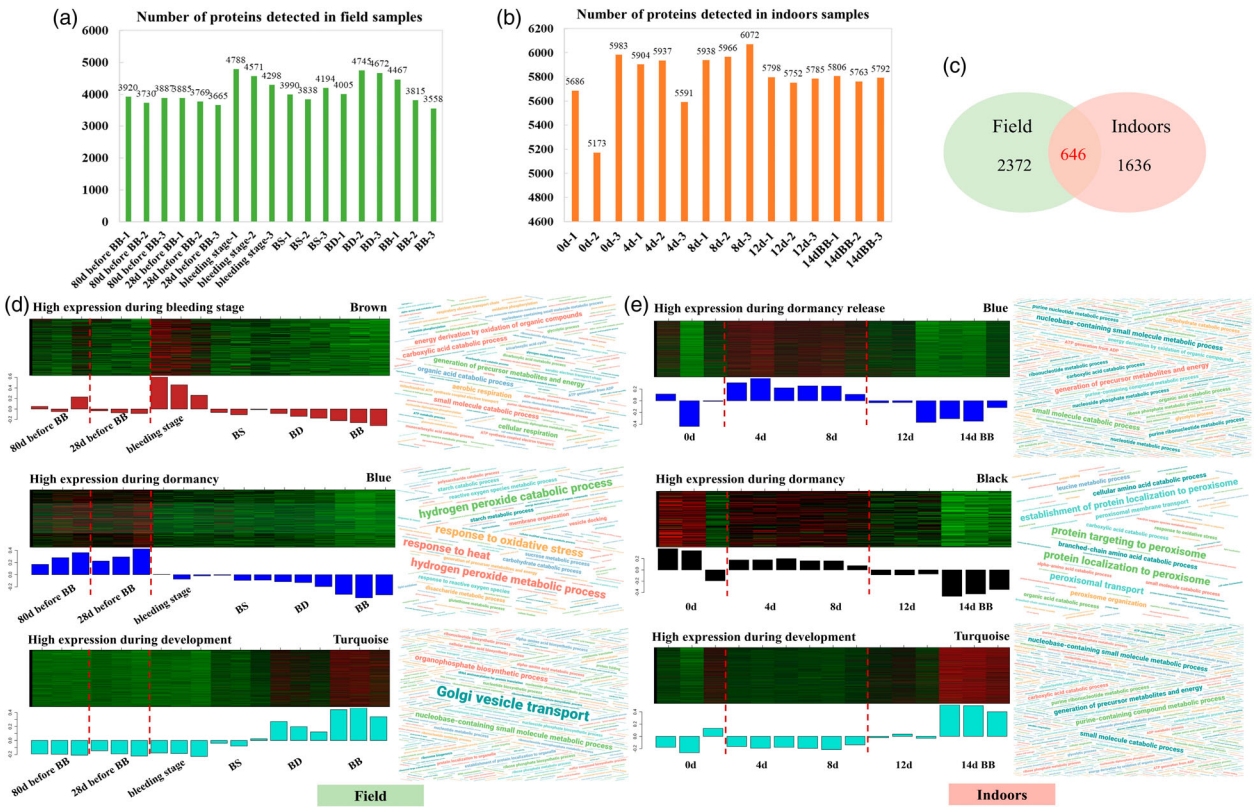

Figure 3 Proteomics of winter buds in grapes from dormancy to bud break. (a and b) The number of proteins detected in the proteome of each sample from the field (a) and from indoors (b), respectively. (c) Venn diagram of the number of proteins showing significantly different levels between field and indoor samples. (d and e) Some of the modules obtained from WGCNA of field and indoor proteome datasets, respectively. The top half of each panel shows the clustering heatmap of proteins within the module, with high expression in red and low expression in green; the bottom bar chart shows the expression patterns of module eigenvalues in different samples. The word cloud to the right shows the results of the differential protein GO enrichment analysis, made by selecting BP items that were significantly enriched with a $p < 0.05$ within the results, with the larger font size indicating more significant enrichment. Red dashed lines indicate the three stages of bud dormancy in the bar chart.

It is noteworthy that the genes in brown module of the field data and in the yellow module of the indoor data were highly expressed only during the dormancy release period. These two modules were significantly enriched in RNA splicing, various protein modifications, abscisic acid signalling, response to alcohol, autophagy, GPI anchor metabolic process, auxin metabolic process, hormone biosynthesis and metabolism. (Tables S2 and S3). The unique components of the indoor yellow module included cytokinin, abscisic acid, ethylene-related signalling pathways, active oxygen metabolism, photoperiod, flowering, leaf development and ageing and various negative regulations. The brown module from the field dataset contains some amino acid catabolism and synthesis, response to heat, response to environmental stimuli, peroxisome and red light signal processes, indicating that the indoor environment caused different responses in the winter buds at the dormancy release stage.

In summary, a multitude of cell metabolism, synthesis and signal transduction processes were negatively regulated in grapevine winter buds during the dormancy stage, while an enrichment of transcripts related to light signals, plant hormone signals and abiotic stimulation facilitated the ability of the winter buds to endure the winter. In the dormancy release phase, RNA splicing regulation, abscisic acid signalling pathway, hormone synthesis and metabolism, reaction to alcohol and other pathways became more active. Before bud break, numerous cell wall-related pathways, formation of various organs and tissues, hormone signals and various plant tropisms became active. After bud break, genes associated with mitosis and the cell cycle play a major role, indicating that cells began to grow rapidly through division.

Proteomic changes in regulatory pathways between the different stages of bud dormancy

Based on the transcriptome results, six sets of samples, 80 days before BB, 28 days before BB, 14 days before BB (bleeding stage), BS, BD and BB, from the field trial and five sets of samples, 0, 4, 8, 12 and 14 days BB, from the indoor trial were selected for proteomic analyses. Mass spectrometric analysis of individual winter bud samples from different periods in the field detected between 3558 and 4788 proteins (Figure 3a), of which 2372 proteins were significantly differentially expressed ($p < 0.05$), and each of the five groups of indoor samples detected between 5173 and 6072 proteins (Figure 3b), of which 1636 proteins were significantly differentially expressed. Only 646 significantly differentially expressed proteins were common to both field and indoor conditions (Figure 3c). The number of proteins detected in the indoor samples remained relatively consistent and was higher

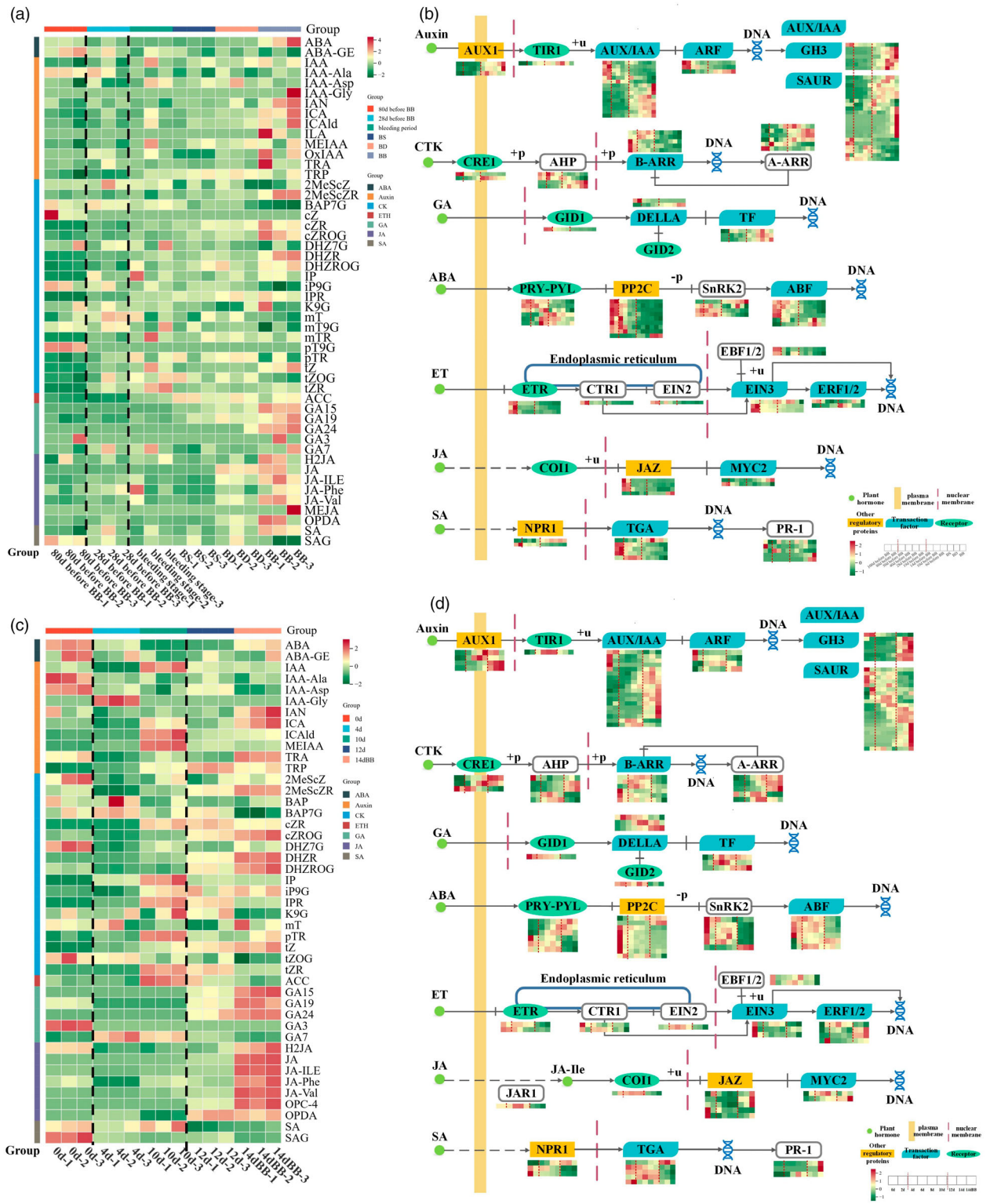

Figure 4 Changes in plant hormone content and signal transduction pathway gene expression. (a and c) Heat maps of hormone content in field (a) and indoor (c) samples, respectively. The three stages of bud dormancy are divided by black dashed lines. (b and d) Heat maps of hormone signal transduction pathway-related gene expression in field (b) and indoor (d) samples, respectively. Selected genes were identified using KEGG pathway analysis. The three stages of bud dormancy are outlined in the heat map beneath the gene expression profile using red dashed lines.

overall, whereas the number of proteins in the field samples slightly increased after the bleeding stage, potentially indicating that the metabolic activity of the plant also began to rise.

The proteome datasets were also subjected to WGCNA, which divided the proteins into nine modules for the indoor dataset, with only three modules showing regular trends (Figure S5b), and

into three modules for the field differentially expressed proteins (Figure S5a). GO enrichment analyses were performed on a total of six key modules between the field and indoor sets (Figure 3d, e; Table S4). In the field-grown bud proteome, the proteins in the brown module (472 proteins), which were highly expressed during the bleeding stage, were enriched in a large number of pathways related to respiration and energy metabolism, such as generation of precursor metabolites and energy, energy derivation by oxidation of organic compounds, cellular respiration and glycolytic process, indicating that numerous pathways related to energy metabolism were initiated during the bleeding stage, marking the increased activity of the bud. The blue module contained 703 proteins, predominantly expressed before the bleeding stage and then decreasing, and the enriched processes involved response to oxidative stress, hydrogen peroxide catabolic process, response to heat, reactive oxygen species metabolic process and starch metabolic process, which help the vines survive the long dormant period. The turquoise module contained the highest number of proteins (2832), with the expression of these proteins gradually increasing at the end of dormancy, and was enriched for Golgi vesicle transport, cellular amino acid biosynthetic process, nucleotide biosynthetic process, regulation of translation, vitamin metabolic and biosynthetic process, photosynthesis and protein localization, indicating that the expression of various proteins began to increase as the buds began to grow.

The blue module of the indoor set contained 1476 proteins, which were highly expressed during the dormancy release period and were mainly involved in small molecule catabolic process, cellular respiration, energy derivation by oxidation of organic compounds, photosynthesis, lignin biosynthetic process and other pathways. The 196 proteins in the black module were highly expressed during dormancy followed by a decline, similar to the field blue module, and were significantly enriched in a variety of peroxisome-associated pathways, as well as processes related to reactive oxygen species, oxidative stress and carboxylic acid metabolism processes. Additionally, the black module included several proteins involved in protein folding, transmembrane transport and localization, which aligned with but were not as enriched as, the transcriptome results. Finally, the turquoise module contained 2358 proteins, which were highly expressed during the budding phase. Like the turquoise module from the outdoor data, this module was similarly enriched in pathways related to the synthesis of various types of amino acids, proteins, nucleotides, chlorophyll, vitamins and other substances (Table S5), all contributing to the rejuvenation of the plant.

Overall, there are direct differences observed between the proteome and transcriptome analyses, with the proteome exhibiting fewer modular divisions and less regularity, but there are also areas of agreement. The modular analyses of the two omics showed that during dormancy and before the bleeding stage, the pathways related to various types of oxidative stress, the peroxisome, as well as protein transport and localization are more active in the vine. Before bud break, various small molecule catabolism- and morphogenesis-related pathways are activated, while after bud break, pathways related to amino acid, protein and vitamin synthesis are activated in large numbers to satisfy the growth and developmental needs of the plant. However, there are still large differences between the proteomes and transcriptomes, which may be caused by post-transcriptional regulation.

Phytohormonal changes during winter bud development

Plant hormones are crucial mediators of bud development. An LC–MS/MS platform was used to quantitatively detect the content of eight types of plant hormones [auxin, cytokinin (CK), abscisic acid (ABA), jasmonate (JA), salicylic acid (SA), gibberellin (GA), ethylene (ETH) and strigolactone (SL)] in winter bud samples from the field (six groups) and indoors (five groups) (Figure 4). However, SL was not detected in the actual test. The row clustering heat map of hormone content is shown in Figure S6. Between the two conditions, a greater number of different hormones were detected in the samples taken from the field. Furthermore, KEGG enrichment analysis of the changes in the expression of genes related to plant hormone signal transduction were demonstrated using a heat map (Figure 4), showing the complexity of phytohormone involvement in bud development.

The contents of auxins in field and indoor winter buds basically continued to increase, with the peak of IAA content detected in indoor samples at 10 days. Some of the synthesized precursors of IAA, such as TRP, TRA and IAN, also exhibited a gradual increase in content (Figure 4). At the transcriptome level, the auxin internal carrier *AUX1*, the auxin receptor *TIR1* and the auxin early response genes *AUX/IAA*, *GH3* and *SAUR* all showed continuous increases in expression after the bleeding stage, indicating that the auxin signal was strengthened to positively regulate the activation of grapevine winter buds.

Abscisic acid plays a significant role in the maintenance of bud dormancy. In this study, it was observed that the content of ABA in both indoor and field conditions decreased initially and then increased in germinating buds. In the ABA signalling pathway, the binding of ABA to the PYR/PYL receptors inhibits the activity of the protein phosphatase PP2C. The expression levels of ABA receptor *PYR/PYL* genes showed a mix of increases and decreases. *PP2C* and *ABF* genes were gradually down-regulated, with a few showing an increasing trend. This suggests that ABA plays a role in promoting or maintaining dormancy during the dormant period, while the content rises again after bud break, synergizing with other hormones to finely regulate plant growth and development.

Cytokinin was the most diverse hormone detected in winter buds, with a total of 21 related metabolites detected in samples from the field and 18 from indoor samples (Figure 4). One of them, trans-zeatin (tZ), is the predominant active form of cytokinin. tZ tended to increase in both sets of winter buds and was higher in the breaking buds. However, regarding the cytokinin signal transduction pathways, the expression of *A-ARR*, a negative regulator of the cytokinin signalling pathway (To *et al*., 2007), was elevated after the bleeding stage, while the expression of the transcription factor *B-ARR* was high during the dormancy and dormancy release periods.

The roles of gibberellins in breaking bud dormancy often vary depending on the species and the timing of exogenous application (Rinne *et al*., 2011; Zheng et al., 2018a; Zhuang *et al*., 2013). Five gibberellins, GA_3, GA_7, GA_{15}, GA_{19} and GA_{24}, were identified in both field and indoor samples, all of which generally exhibited an increasing trend. Gibberellin signal transduction is mediated by its receptor GID1, repressor DELLA and the F-box protein GID2, while TFs regulate GA content. The transcriptome data showed that *GID1* and *2* gradually decreased, *DELLA* partially increased and the selected *TF*s generally increased. These results indicated that in winter buds the increase of GA content encouraged the DELLA

protein to gradually allow GA to regulate the growth of winter buds in a homeostatic manner.

Only one ethylene-related component, 1-aminocyclopropane-carboxylic acid (ACC), was identified in winter buds. ACC, a precursor of ethylene synthesis, essentially mirrors the levels of ethylene in plants. ACC reached its peak in field-grown winter buds as the buds were about to break (BS) and was essentially stable thereafter. On the other hand, ACC peaked at 10 days in indoor samples and declined thereafter (Figure 4). This suggested that ethylene may play a significant role before bud break. The positive regulators of ethylene, *EIN2*, *3* and *ERF1*, were largely and consistently down-regulated, but the negative regulators, *CTR* and *EBF1/2*, were also down-regulated, showing complex expression changes underlying the role of ethylene in bud dormancy and break.

Among all the samples, jasmonic acid generally tended to rise abruptly at a later stage (Figure 4). In sweet cherry, during flower bud dormancy and release, the content of methyl JA in flower buds increased significantly, indicating that JA may also play an important role in dormancy release (Ionescu *et al*., 2017). JA and its amino acid conjugates were highly expressed in sprouting buds, and genes encoding components of their signalling pathway were more highly expressed in the indoor transcriptome.

Two types of salicylic acid-related components, SA and salicylic acid 2-*O*-*β*-glucoside (SAG), were identified. SAG can be converted into SA to exert its effects and consistently showed a decreasing trend. SA showed a gradual increase in the field samples, while it decreased in the indoor samples (Figure 4). The TGA transcription factors are regulators of the *PR-1* gene, which can be highly induced by SA (Kesarwani *et al*., 2007). Generally, the expression of *TGA* was gradually down-regulated in both field and indoor samples. The *PR-1* gene showed diverse trends both indoors and in the field.

Although various types of phytohormones and their signalling pathway-associated DEGs showed diverse forms of changes, these changes generally followed a certain pattern as the winter buds progressed from dormancy to bud break to further growth, suggesting that there is a complex crosstalk among these phytohormones that synergistically affects the growth and development of plants.

Screening for key genes affecting dormancy and bud break

To identify key genes influencing winter bud dormancy and activation in grapevine, genes expressed in both field and indoor transcriptomes were initially selected. Subsequently, genes with FPKM values over 80 at their peak expression level and $|\log_2 FC|$ values over 2 (focusing on $\log_2 FC$ values for the comparisons of 100 days before BB vs BB or 100 days before BB vs 14 days before BB in the field transcriptome, and 0 vs 14 days BB or 0 vs 12 days indoors) were selected. Finally, the literature was reviewed to determine whether the functional annotations of any of the screened genes were related to bud activation or dormancy, thereby identifying candidate genes for further study. The functions of these candidate genes were then preliminarily verified by transient transformation experiments. The final selection of seven candidate genes were involved in phytohormone signalling (ethylene, ABA, gibberellin), seed dormancy, cell wall modification and transcription regulation (Table 1).

Single-bud cuttings from pruned annual branches of 'Cabernet Sauvignon' grapevines were used for transient overexpression and silencing of candidate genes, followed by observation of the

Table 1 Candidate genes affecting dormancy/bud break in grapes

Gene ID	Trends in the field transcriptome	Trends in the indoor transcriptome	Gene length	Gene description	Gene name
VIT_01s0150g00120			1245 bp	Ethylene-responsive transcription factor ERF114	VvERF114
VIT_11s0016g00690			892 bp	Mediator of ABA regulated dormancy	VvMARD
VIT_07s0031g02670			896 bp	Protein DOG1 like 4, seed dormancy control	VvDOGL4
VIT_17s0000g06360			771 bp	Expansin-A1	VvEA1
VIT_17s0000g06340			1614 bp	Agamous-like MADS-box protein AGL65	VvAGL65
VIT_18s0001g14270			339 bp	Gibberellin regulated protein 1	VvGAST1
VIT_00s0309g00090			967 bp	Expansin-like B1	VvEB1

Note: The mini line plots in the table are based on the FPKM value of the genes in the outdoor and indoor transcriptomes.

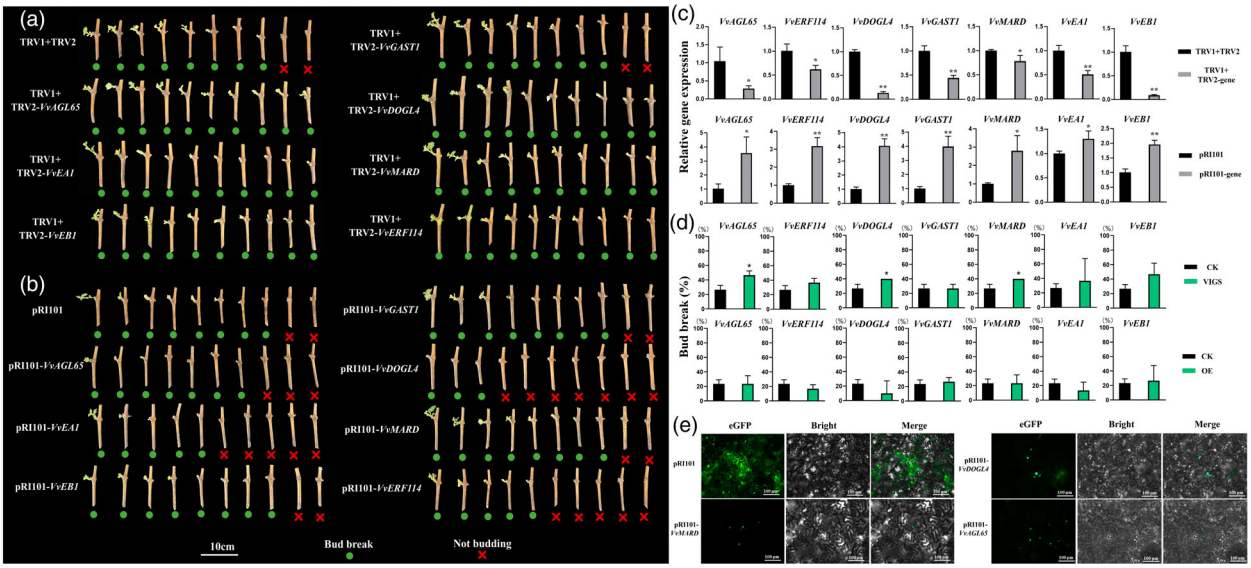

Figure 5 Candidate gene functional verification. (a and b) Transient silencing and overexpression of seven candidate genes in winter buds of 'Cabernet Sauvignon', respectively. (c and d) Transcript levels of targeted genes in the transiently silenced (c) and overexpressing (d) winter buds, respectively. (e) Subcellular localization of VvMARD, VvDOGL4 and AGL65 proteins in tobacco leaves transiently expressing the mentioned proteins fused to GFP.

winter buds (Figure 5a–d). RT-qPCR demonstrated that all candidate genes were significantly down-regulated or up-regulated in winter buds during the transient transformation experiments (Figure 5c). Among the silenced genes, there was no significant change in the bud flush rate after silencing *VvGAST1*, while the rate increased after the silencing of the other genes, with *VvAGL65*, *VvDOGL4* and *VvMARD* silencing treatments showing a significant increase (Figure 5a, d). Among the overexpressed genes, no significant change was observed after the overexpression of *VvEB1*, *VvGAST1* and *VvMARD*, whereas the activation rate decreased after the overexpression of the other genes (Figure 5b, d). The above results indicated that *VvGAST1* may have no obvious function in bud dormancy, whereas *VvERF114*, *VvMARD*, *VvDOGL4*, *VvEA1*, *VvAGL65* and *VvEB1* may inhibit bud activation and promote dormancy.

The three genes that exhibited more pronounced phenotypes in the transient transformation experiments, namely *VvAGL65*, *VvDOGL4* and *VvMARD*, were selected for additional preliminary experiments. Transient expression in tobacco leaves as fusions to GFP showed that VvAGL65, VvDOGL4 and VvMARD were localized in the nucleus (Figure 5e). Proteins that interact with each of these three proteins were screened by the yeast two-hybrid system (Table S6). It was found that VvMARD may interact with four auxin signalling-related proteins, five gibberellin signalling-related proteins, one abscisic acid signalling-related proteins, two cell wall dilation proteins, sixteen transcription factors and other proteins. The candidate VvAGL65-interacting proteins included proteins related to brassinosteroid, auxin, ethylene, jasmonic acid and other plant hormone signalling pathways, as well as germin-like proteins, lipid metabolism-related proteins, heat shock proteins and tubulin proteins. VvDOGL was found to bind to more proteins involved in respiration, carbohydrate metabolism and various types of transport, along with two abscisic acid syntheses, two peroxidase enzymes and proteins involved in abscisic acid, growth hormone and ethylene signalling pathways.

Distinction between indoor and outdoor omics datasets

The differences in the omics datasets from the indoor and outdoor winter buds are noteworthy. The genes detected in the field transcriptome included all of the genes detected indoors, but an additional 112 genes were detected exclusively outdoors (Table S7), all exhibiting low expression levels (the highest-expressed FPKM value was 86). Of these genes, 79 lacked annotated functions while 33 had annotations. Among the 33 genes with functional annotation (their expression patterns are shown in Figure S7), the most highly expressed gene, with an FPKM value of 49, was a HOP family protein (novel.1489), which showed high expression in the dormancy and pre-swelling phases. The primary role of proteins in this family is to act as synergistic molecular chaperones mediating the interaction between the heat shock proteins HSP90 and HSP70. The HOP family has been reported in *Arabidopsis thaliana* to play an important role in long-term acquired heat tolerance in plants (Fernandez-Bautista *et al.*, 2018). In addition, a protein S-acyltransferase gene (novel.1569) and a GPI (glycosylphosphatidylinositol) ethanol-amine phosphate transferase gene (novel.1557) were stably and highly expressed in outdoor winter buds (6 < FPKM <12). Protein S-acylation modification, as the only reversible lipid modification, plays a crucial role in physiological processes such as plant cell wall synthesis, intracellular localization of proteins, translocation, sorting and downstream signal transduction (Tian *et al.*, 2022). GPI anchoring is a conserved post-translational modification in eukaryotes and is associated with plasma membrane anchoring of bound proteins. A cell cycle-related gene (novel.1558) was expressed only after the bleeding stage, with peak expression at bud break (FPKM of 30), and cell cycle-related genes have also been found to be associated with budding in poplar (Azeez *et al.*, 2021). Furthermore, a chitinase gene (novel.1548) with high expression (FPKM >10) during dormancy was identified.

The genes in the black module from the WGCNA of the field transcriptome were only highly expressed during the bleeding

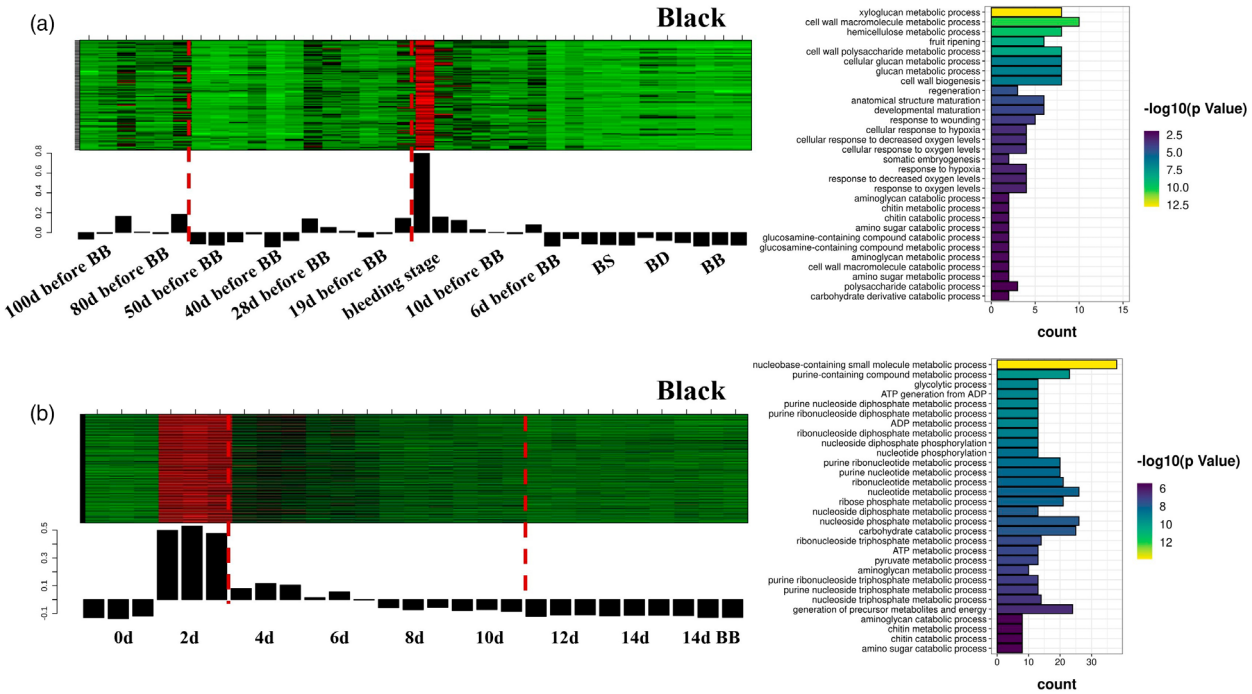

Figure 6 Unique transcriptome modules from field and indoor buds. (a) A module of genes that are highly expressed only during the bleeding period in the field transcriptome and (b) a module that is highly expressed only 2 days after relocation to the indoors. The histograms on the right show the top 30 terms significantly enriched under BP ontology obtained from GO enrichment analyses. The colour indicates significance, and the length of the column indicates the number of genes enriched under this term.

stage (Figure 6a; Table S2), whereas the indoor grapevine cuttings did not show the 'bleeding' phenomenon. A number of genes associated with xyloglucan metabolism, cell wall macromolecule metabolism, hemicellulose metabolism, fruit ripening, cell wall polysaccharide metabolism, cellular glucan metabolism, cellular responses to oxygen levels, and abscisic acid, gibberellin and salicylic acid signalling pathways were significantly enriched within this module.

Similarly, another module (black module) included genes that were only highly expressed for 2 days after moving field branches indoors and then not expressed (Figure 6b; Table S3). GO enrichment analysis of these genes revealed that the main processes were nuclease-containing small molecule metabolism, glycolysis and ribonucleoside diphosphate metabolism. Additionally, carbohydrate, chitin, pyruvate, hydrogen peroxide, cell wall correlation and other catabolic processes, as well as the cell response to oxygen level, chemical pressure and stomata were also significantly enriched. The results indicated that when the dormant winter buds were suddenly subjected to conditions conducive to bud break, pathways such as nucleotide metabolism, energy metabolism and oxidative stress were mobilized to adapt to the environmental changes.

DISCUSSION

Division of dormancy stages

Previous studies on the division of bud dormancy into stages were predominantly based on observing the physiological conditions of the plants, with a lack of research at the molecular level. In this study, a multi-omics approach was used on grapevine winter buds in natural field and indoors to delineate the transition from dormancy to bud break into three distinct stages, each with unique enrichment of specific gene expression and metabolic pathways. The results elucidated the changes in plant hormone signal transduction, cell cycle, cell wall metabolism, starch and sucrose metabolism, oxidative stress, respiratory stress and other metabolic pathways during dormancy release (Figure 7a) and identified several candidate genes (Figure 7b) that may affect winter bud activation in grapevine. This preliminary screen lays the groundwork for more detailed analyses of dormancy and bud release mechanisms in perennial woody crops like grapevine.

Close observation of winter buds using a stereomicroscope showed that there were no significant changes on the exterior or in the interior of the dormant buds before the bleeding stage. During the bleeding stage, the primary bud inside the winter bud began to expand significantly as stored water and nutrients were transported upwards along the xylem and about 2 weeks later the winter buds opened. This study found that the bleeding stage was an important dividing point between winter bud dormancy and bud break, with pathways related to cell wall modification and metabolism significantly enriched at this time. There were significant differences in gene expression before and after bleeding the phase.

The bleeding stage is thusly named as the spring flow of nutrients escapes wounds in the bud prior to flushing. When winter buds were forced to break indoors, the detached single-bud grapevine branches did not show the phenomenon of 'bleeding' and it only took about 2 weeks under the right conditions to complete the germination process that takes several months in natural conditions. The indoor transcriptome indicated that pathways such as nucleotide metabolism, energy metabolism and oxidative stress responded to the sudden change from a winter

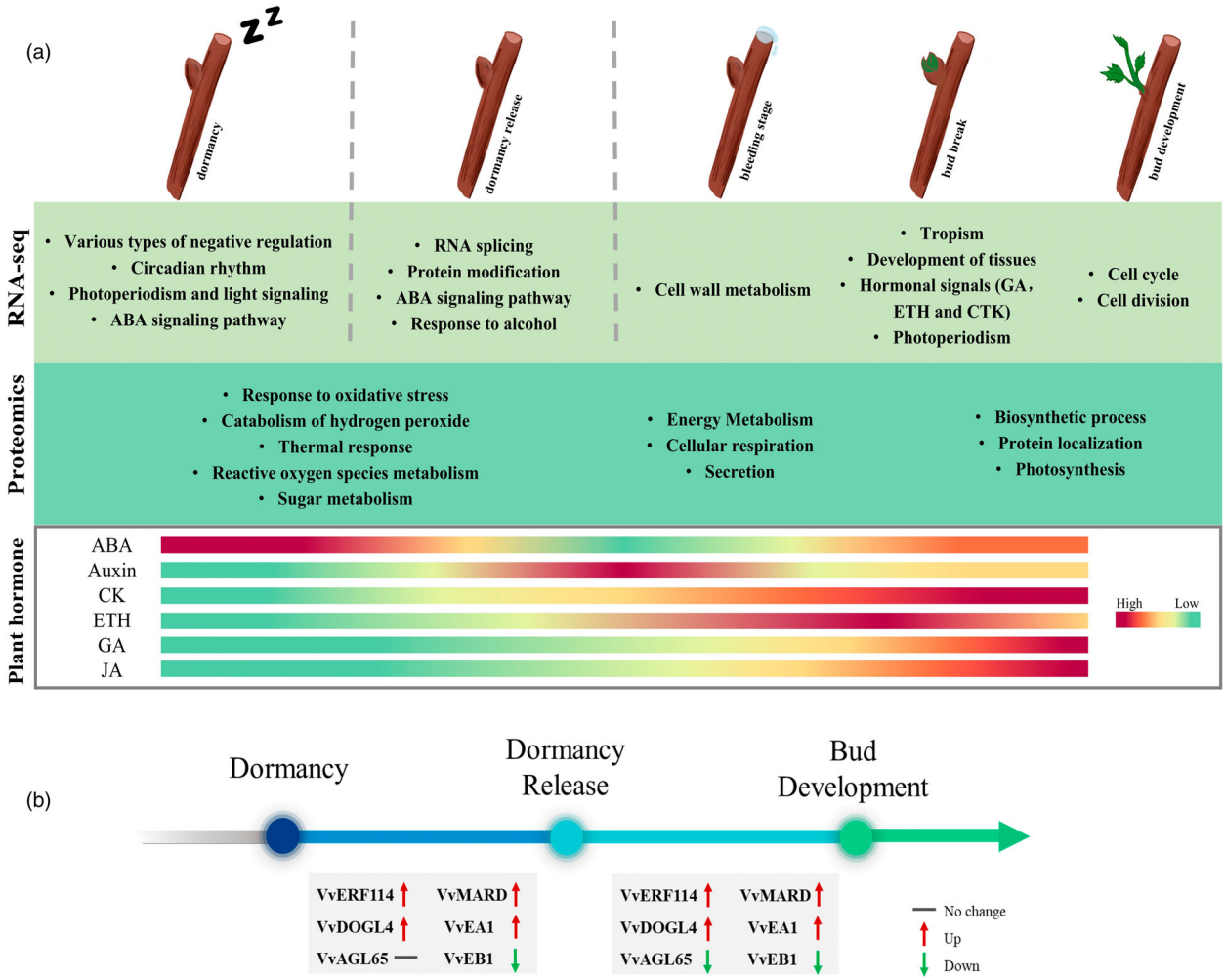

Figure 7 Multi-omics analysis of the regulatory network of grapevine winter buds from dormancy to bud break. (a) Changes in the transcriptome, proteome and plant hormone levels in winter buds from dormancy to bud break in grapevines (*Vitis vinifera* L. 'Cabernet Sauvignon'). The red and green bars indicate the level of the corresponding plant hormone, with the content gradually decreasing from red to green. The three stages of bud dormancy are divided by dotted lines. (b) Schematic representation of the changes in expression of the six candidate genes investigated in this study during the three stages of bud dormancy to bud break in winter buds.

environmental to conditions suitable for sprouting. However, further studies are needed to explore the underlying reasons for the differences in bud break between indoor and outdoor conditions.

Metabolic pathways that regulate dormancy and reactivation of grapevine winter buds

Many studies have investigated the regulation of bud dormancy, and numerous regulatory pathways have been found to breaking bud dormancy. Phytohormones, including ABA, GA, ethylene, auxin and cytokinin, have been shown to be important in bud dormancy (Baba *et al.*, 2011; Roman *et al.*, 2016; Signorelli *et al.*, 2018; Vergara *et al.*, 2017; Zheng *et al.*, 2018a, 2018b). Transcriptomic analysis of dormant buds revealed that genes related to brassinolide, salicylic acid and jasmonic acid were also differently regulated during dormancy (Howe *et al.*, 2015). Based on the fluctuations in hormone levels and the omics analyses in this study, it is clear that ABA, ethylene, cytokinin and GA play more significant roles in the bud dormancy and bud break mechanisms in grapevine. ABA plays an important role during dormancy, while the other three hormones are more active before and after bud break. Interestingly, a relatively high concentration of ABA was detected in both indoor and outdoor opening buds, particularly in field samples, indicating that ABA may be also required in the nascent tissues after breaking to finely regulate plant growth and development in concert with other hormones. All types of hormones showed a distinct change with the release of winter bud dormancy, suggesting that different hormones interact with each other and participate in the regulation of seasonal bud dormancy.

Based on the results of the multi-omics, seven candidate genes were selected for preliminary functional studies using transient transformation experiments. Genes associated with hormone regulation, seed dormancy, cell wall expansion and MADS-box transcription factors were identified as potential inhibitors of bud break. The genes *VvAGL65*, *VvMARD* and *VvDOGL4* exhibited more pronounced effects in the transient transformation experiments and were selected for further preliminary regulatory network research.

Multiple members of the MADS-box transcription factor family can regulate bud dormancy (Hao *et al.*, 2024; Lempe *et al.*, 2024;

Lloret *et al*., 2021; Voogd *et al*., 2022). A group of MADS transcription factors known as Dormancy-Associated MADS-BOX (DAM) is believed to control temperature-mediated bud dormancy. These transcription factors are encoded by genes analogous to *Short Vegetative Phase* (*SVP*) from *Arabidopsis* (Falavigna *et al*., 2021). The expression of these genes is up-regulated with accumulation of low temperature and rapidly down-regulated once the chilling requirement is satisfied (Lloret *et al*., 2021; Zhao *et al*., 2018), and these genes also correlated with epigenetic changes to control bud break (Voogd *et al*., 2022).

The *Mediator of ABA regulated dormancy* (*MARD*) gene was first discovered in *Arabidopsis* in 2004 and is characterized as a proline-rich N-terminal zinc finger protein (He and Gan, 2004). The expression of *MARD1* can be up-regulated by ABA, and the seeds of mutant *mard1* exhibit reduced dormancy, and can germinate in complete darkness (He and Gan, 2004). *MARD* is highly expressed in seeds and involved in ABA signalling (Bogamuwa and Jang, 2016). Furthermore, it has been found to be related to flower induction in saffron and lotus (Kuang *et al*., 2024; Renau-Morata *et al*., 2021).

The *Arabidopsis DOG1* (*delay of germination 1*) gene is a major gene controlling the seed dormancy quantitative trait locus (QTL). DOG1-related proteins are functionally conserved in other plants and are key factors promoting seed dormancy, through temperature, GA, ABA, kinase and other signalling pathways (Carrillo-Barral *et al*., 2020).

Cell wall-associated genes have been screened in numerous studies on bud break, with some relevant regulatory genes capable of directly binding to expansion-related proteins. The bud break-promoting factor PpEBB1 in peach can directly interact with the cell wall modification-related protein PpEXBL1 to promote bud break (Zhao *et al*., 2020). Administration of hydrogen cyanamide (HC) to promote dormancy release also caused cell wall degradation and modified up-regulation of DEGs, and the temporary oxidative stress induced by HC subsequently led to increased cell activity and cell wall loosening (Tang *et al*., 2019). In walnut, the thin-walled cells of the internal dormant bud scale became even thinner during activation, indicating that cell wall polysaccharides were mobilized by hemicellulase and pectinase and that cell wall hydrolases were activated during dormancy release (Gholizadeh *et al*., 2021). The main metabolic and cellular events in the woody layer of grape during bud break also include cell division, cell wall metabolism and sugar mobilization (Noronha *et al*., 2021).

There are many other pathways involved in the regulation of bud break, including epigenetics (Falavigna *et al*., 2019), light sensing (Smith, 2000), nitrogen metabolism (Sudawan *et al*., 2016), lipid metabolism (Zhuang *et al*., 2015), plasmodesmata (Xu *et al*., 2016) and membrane binding channel proteins (Yooyongwech *et al*., 2008). These pathways do not operate in isolation, but instead they interact and regulate each other, forming an intricate dormancy-emergence mechanism. Extensive experimental work is still required to further explore these mechanisms, particularly for the identification and characterization of key regulatory genes.

This study observed that winter buds in grapevine undergo three principal stages from dormancy to budding: dormancy, dormancy release and bud development. Transcriptomics revealed that numerous processes of cellular metabolism, biosynthesis and signal transduction were negatively regulated during winter bud dormancy, and that light, phytohormone and abiotic stimulus signals were enriched. In contrast, pathways related to RNA regulation, abscisic acid signalling, hormone synthesis and metabolism, and response to alcohol were more active during the dormancy release phase. After bud break, genes related to mitosis and cell cycle play a major role, and cells begin to grow more rapidly through division. Seven genes were identified from the transcriptome that may affect winter bud dormancy or release. Preliminary functional verification using the transient transformation test showed that *VvDOGL4*, *VvAGL65* and *VvMARD* may have roles inhibiting the release of winter buds from dormancy in grapevine. Yeast two-hybrid screening showed that these three proteins may interact with proteins related to phytohormone signalling, respiratory energy metabolism and transcription. This study provides strong support for continued exploration of the key genes and their regulatory mechanisms that govern dormancy maintenance and bud break in the woody perennial crop grapevine, especially as global temperatures change and growing regions shift.

MATERIALS AND METHODS

Plant material

Dormant buds and dormant branches were harvested from 'Cabernet Sauvignon' grapevines (*Vitis vinifera* L.; planted in 2018) growing at the Cao Xin Grapevine Demonstration Base of Northwest A&F University, Yangling Agricultural Demonstration Zone, Shaanxi Province. Plant row spacing is 1.5 m × 3 m, in a north–south direction, with conventional management. Samples were collected in the winter of 2020–2021. Daily temperatures and day lengths at the time of sample collection are shown in Figure S1.

Transcriptome sequencing and analysis

To collect field-grown transcriptome samples, annual branches were pruned, with four buds retained from each annual branch. Winter buds and their surrounding tissues at the top of the branch were collected at each sampling, and the outermost scales and part of the downy hairs of the winter buds were removed (Figure 1b). Vaseline was applied to the wound after collection. More frequent sampling was performed after the bleeding stage until bud break (bud exposed green tissue). A total of 12 groups of samples were collected for transcriptome sequencing: 100 days before BB, 80 days before BB, 50 days before BB, 40 days before BB, 28 days before BB, 19 days before BB, 14 days before BB (bleeding stage), 10 days before BB, 6 days before BB, bud swell (BS), bud downy stage (BD) and bud break (BB), with three biological replicates per group. Samples were snap frozen in liquid nitrogen and stored at −80 °C until use.

To collect indoor transcriptome samples, uniformly long, healthy annual branches were collected from the site of the field trial and trimmed into cuttings containing a single bud. These cuttings were placed in sandy soil and watered every 2 days. They were cultured in an incubator at 25 °C, with a 16 h/8 h light cycle and 40% humidity. Bud samples were collected every 2 days in the same way as field sampling. Active and non-active buds were collected at 14 days, and a total of 9 groups of samples were collected for transcriptome sequencing, with three biological replicates in each group.

After total RNA was extracted from each sample, mRNA was enriched using oligo (dT) magnetic beads, and libraries were constructed according to the NEB common library construction method. The libraries were assayed using an Agilent 2100

bioanalyzer and sequenced using Illumina. An index of the reference genome was constructed, and paired-end clean reads were compared to the reference genome using HISAT2 v2.0.5. Feature Counts (1.5.0-p3) were used to calculate the number of reads mapped to each gene. Differential expression analysis between two compared groups was performed using DESeq2 software (1.20.0). The above transcriptome-related tests were performed by Beijing Novozymes Technology Co., Ltd.

Proteome sequencing and analysis

Six groups of field samples, namely 80 days before BB, 28 days before BB, bleeding stage (14 days before BB), BS, BD and BB, and five groups of indoor samples, 0, 4, 8, 12 and 14 days BB, were selected, for a total of eleven groups of samples, for the proteomic assay. The enzymolyzed peptide samples were analysed by mass spectrometry using a label-free quantitative method. The above proteome-related tests were performed by Beijing Novozymes Technology Co., Ltd.

Plant hormone content

Five groups of indoor samples, 0, 4, 10, 12 and 14 days BB, and six groups of field samples, 80 days before BB, 28 days before BB, bleeding stage (14 days before BB), BS, BD and BB, were used to extract plant hormones. The extraction and detection of plant hormones from bud samples were carried out by Jiaxing Metware Biotechnology Co., Ltd., using LC–MS/MS.

Winter bud development profile

Within 3 h after collection, fresh winter buds were dissected with the blade of a cryotome and photographed with a fluorescence stereomicroscope (Leica M205FCA), to observe exterior and interior changes of the winter buds.

Transient transformation of grape winter buds

The experimental method of Zhao *et al*. (2023) was referenced and modified. Cuttings harbouring a single bud were pruned from annual branches of 'Cabernet Sauvignon'. The coding sequences of the target genes were amplified and cloned into the pTRV2 vector for silencing and the pRI101 vector for overexpression. EHA105 *Agrobacterium* cells transformed with the pTRV1 and pTRV2 or the pRI101 constructs were resuspended in infiltration solution (10 mM $MgCl_2$, 10 mM MES, pH = 5.8, 150 μM AS). A wound about 2–3 mm deep was made about 1 cm above and below the winter bud of the cuttings, and then, the cuttings were immersed in the *Agrobacterium* solution containing the target constructs and incubated under vacuum for 10 min. The treated cuttings were incubated normally in fresh water under a temperature range of 18–22 °C and a light/dark cycle of 16 h/8 h. Winter bud samples were collected 5 days after infection for gene expression detection and 15 days after infection for bud observation. Transformation with the silencing vectors was done at a 1:1 ratio of pTRV1 and pTRV2.

Yeast two-hybrid assays

A cDNA library from 'Cabernet Sauvignon' available in the laboratory was screened for proteins that interact with the seven candidate proteins identified in the omics analyses. The coding sequences of each corresponding gene were amplified and cloned into the pGBKT7 bait vector. After verifying that bait vectors did not self-activate in yeast, the Y2H Gold pGBKT7-Bait constructs were transformed with the grape cDNA library to screen for positive clones. After sequencing the positive clones, Y2H backcrosses were performed for verification.

Subcellular localization

The coding sequence of each target gene without the terminator was inserted into the pRI101-eGFP vector to obtain a eukaryotic expression vector encoding a fusion of the target protein and eGFP. Recombinant vectors were transformed into *Agrobacterium* EHA105, and positive colonies were identified and cultured. Leaves of *Nicotiana benthamiana* were infiltrated with the transformed *Agrobacterium*. Green fluorescent protein signals were observed using fluorescence microscopy 3–5 days after infiltration.

RT-qPCR

Total RNA was extracted using an RNA extraction kit (Tiangen, Beijing, China), and cDNA was obtained using a HiScript II Q RT SuperMix kit (Vazyme, Nanjing, China). The Quant Studio 6 platform (Life Technologies, USA) was used for RT-qPCR analysis. The reaction system was prepared according to the instructions of ChamQ SYBR qPCR Master Mix (Vazyme, Nanjing, China). Three biological replicates were performed. Gene primers for RT-qPCR are shown in Table S1. *VvACTIN7* was used as an internal reference gene, and the relative expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method.

Data processing

SPSS 20 (IBM, USA) was used for univariate analysis of variance (ANOVA) and Duncan multivariate test ($p < 0.05$). GraphPad Prism 8 and Excel were utilized for image drawing and data analysis. Advanced heatmap, WGCNA and GO enrichment analyses were completed using the Metware Cloud Platform (https://cloud.metware.cn).

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Author contributions

Y.F. and K.Z. conceived and designed the experiments. L.C., J.J. and D.W. performed the experiments. L.C. and K.C. analysed the data and wrote the manuscript. Y.F. and K.Z. revised the manuscript.

Data availability statement

All the raw sequencing data generated for this project have been deposited in the National Genomics Data Center (NGDC) Genome Sequence Archive (GSA) (https://ngdc.cncb.ac.cn/gsa/) with BioProject number PRJCA034424 (transcriptome) and PRJCA034442 (proteome).

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Temperature and daylength on the days of sample collection.
Figure S2 Transcriptome validation by RT-qPCR for 15 selected genes from the outdoor (a) and indoor (b) datasets.
Figure S3 The numbers of differentially expressed genes (DEGs) in the field (a) and indoor (b) transcriptomes.
Figure S4 Transcriptome WGCNA modules with expression heatmaps and profiles.
Figure S5 Proteomic WGCNA modules with expression heatmaps and profiles.
Figure S6 Heat map of changes of plant hormone content during dormancy and bud break of winter bud.
Figure S7 Heatmap of expression of genes expressed only in the field with functional annotation.
Table S1 Primers used for RT-qPCR validation.
Table S2 GO enrichment (field transcriptome).
Table S3 GO enrichment (indoor transcriptome).
Table S4 GO enrichment (field proteome).
Table S5 GO enrichment (indoor proteome).
Table S6 Candidate interacting proteins (by Y2H).
Table S7 Genes expressed only in the field.

