

Molecular cloning and expression analysis of a vacuolar invertase gene, DoVIN2, from *Dendrobium officinale* Kimura et Migo

Boting Liu

Shaoguan University

Junqi Mai

Shaoguan University

Jieying Xie

Shaoguan University

Jiayi Chen

Shaoguan University

Lijun Meng

Harbin University of Commerce

Di Cui

Harbin University of Commerce

Zaohai Zeng

South China Agricultural University

Yuanlong Liu

South China Agricultural University

Xiang Li

Shaoguan University

Yu-jia Liu (✉ liuyj1206@sgu.edu.cn)

Shaoguan University <https://orcid.org/0000-0002-0406-8569>

Research Article

Keywords: *Dendrobium officinale*, vacuolar invertase, DoVIN2 gene, molecular cloning, expression analysis, abiotic stress

Posted Date: July 20th, 2023

DOI: <https://doi.org/10.21203/rs.3.rs-3120522/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

Abstract

Background

Dendrobium officinale Kimura et Migo, a traditional Chinese orchid herb famous for its great horticultural and medicinal value, grows in an adverse habitat and suffers from abiotic or biotic stresses. Sugar metabolism is a vital metabolic process in plants that involves acid invertase. Moreover, the participation of acid invertases in plant responses to environmental stress has also been unveiled. However, the function and structure of the vacuolar invertase gene in *Dendrobium officinale* remain unclear.

Methods and Results

In this study, the vacuolar invertase gene (*DoVIN2*) was cloned from the “DanXia” cultivar of *D. officinale* using homologous cloning. The cDNA sequence of *DoVIN2* was 1368 bp and included a 455-amino-acid protein with a sugar hydrolase domain. The *DoVIN2* protein did not possess a signal peptide or transmembrane region, was a hydrophilic stable protein, and contained 46 phosphorylation sites, with high conservation in evolution. The transient green fluorescent protein gene (*GFP*) expression results in rice mesophyll protoplasts demonstrated that *DoVIN2* was localized in the cell nucleus. The expression level of the *DoVIN2* gene differed significantly among different tissues, with the highest expression in flowers followed by stems, roots, and leaves. Several abiotic stress-responsive elements were present in the promoter region of the *DoVIN2* gene. Cold stress and abscisic acid (ABA) treatment upregulated *DoVIN2* gene expression, while the expression of *DoVIN2* was negatively correlated with polyethylene glycol (PEG) treatment duration. Additionally, the *DoVIN2* protein interacted with several enzymes closely associated with sugar metabolism, indicating that *DoVIN2* might play a role in abiotic stress responses by regulating the expression of these enzyme genes, thereby enhancing the tolerance of *D. officinale* to adverse conditions.

Conclusions

The *DoVIN2* gene are involved in various biological processes, including growth and development, stress response, and sugar metabolism. The *DoVIN2* might participate in the cold stress response through ABA signaling pathways, while also assuming a negative regulatory function in the response to drought stress. The result provided a basis for further exploring the function of the *DoVIN2* gene and a foundation for studying the response of *D. officinale* to abiotic stresses.

Introduction

Dendrobium officinale Kimura et Migo, a parasitic perennial herb belonging to the Orchidaceae family and *Dendrobium* Sw. genus, is an endangered medicinal plant that can tonify the stomach, generate body fluids, nourish yin, and clearing heat. It is commonly used to prevent and treat diseases such as fever-induced fluid depletion, deficient stomach yin, excessive yin fire, vision impairment, and muscle weakness. The pharmacological and component analysis results revealed that *Dendrobium* polysaccharides were important and effective active ingredients to measure the medicinal value of *D. officinale*, having abilities to enhance immunity, reduce blood sugar and lipids, combat tumors, combat fatigue, and improve intestinal function [1, 2]. Previous studies indicated

that the synthesis, transportation, and metabolism of *Dendrobium* polysaccharides involved multiple proteins or enzyme genes. In contrast, sucrose was the primary direct product of photosynthesis, serving as the main form of carbohydrate transportation in *D. officinale* [3]. In the *D. officinale* stems, sucrose transported from the leaves was mainly hydrolyzed and transformed by sucrose synthase (SUS) and invertase (INV) to form polysaccharides [4]. During sucrose decomposition, the reaction involving SUS was reversible and mainly directed toward sucrose synthesis. Conversely, INV irreversibly decomposed sucrose into glucose and fructose. In particular, INV played a more significant role than VIN in sucrose hydrolysis [5, 6].

In higher plants, the protein coding for INV belongs to a multi-gene family primarily categorized into three types based on the optimal pH values, solubility, and subcellular localization: cytoplasmic invertase (CINV), cell wall invertase (CWINV), and vacuolar invertase (VINV) [7]. Among these, CINV is neutral/alkaline invertase, and the optimal pH is 7.0–7.8. On the contrary, both CWINV and VINV are acid invertases (AINVs), exhibiting optimal activity at a pH range of 4.5–5.5. They can hydrolyze sucrose and other β -fructofuranoside oligosaccharides and are hence also referred to as β -fructofuranosidases [8]. Previous studies have indicated that AINV catalyzes the irreversible degradation of sucrose into glucose and fructose, providing a carbon source for polysaccharide synthesis and regulating plant cell expansion and carbohydrate composition in storage organs [9]. Among the three types, CWINV is known to bind to the cell wall as ions and is involved in important life processes such as sucrose unloading, signal transduction, and defense emergency response [10]. VINV, which accumulates in the acidic region of the vacuole as a soluble protein, plays important roles in plant cell osmotic regulation, tissue and organ differentiation, hormone responses, and stress resistance processes [11, 12].

VINV usually has at least two isoforms in most plants; the DNA or cDNA sequences encoding VINV genes have been isolated from various crops, such as *Arabidopsis thaliana* [13], carrots (*Daucus carota*) [14], and sweet sorghum (*Sorghum bicolor*) [15]. Numerous studies have shown that the number of VINV genes varies among different plants, and their expression exhibits temporal and spatial differences, which profoundly affects the transportation and accumulation of photosynthates as well as crop yield formation [16, 17]. For instance, Yao et al. discovered three VINV isoforms in cassava (*Manihot esculenta*), but only two displayed functionality [18]. In rice (*Oryza sativa*), two isoforms of VINV were found: *OsVIN1* and *OsVIN2*, and they exhibited different expression patterns [19]. *OsVIN1* showed high expression levels in the heading and flowering stages, whereas *OsVIN2* exhibited high expression levels from 3 days after heading to 3 days before heading [20]. The stem sugar content of sweet sorghum was negatively correlated with VINV, indicating that VINV was a key enzyme for regulating the stem sugar accumulation [15]. Furthermore, the VINV mutant (*sgs1*) in rice exhibited a reduction in grain size and weight due to the smaller cell size, resulting in a decrease in yield [7].

Additionally, the VINV gene plays an important regulatory role in promoting plant growth and accelerating the breakdown of sucrose and accumulation of polysaccharides [9, 12]. The overexpression of *CsINV5*, a VINV gene in tea (*Camellia sinensis*), in *A. thaliana* could promote the elongation of the main root and lateral roots by affecting glucose-mediated auxin signaling [21]. Wang et al. showed that the transcripts encoding VINV and 6-phosphofructokinase were downregulated in the high-sucrose accumulation internodes of sugarcane (*Saccharum officinarum*) [22]. Further studies demonstrated that the peptidyl-prolyl isomerases (*FKBP15-1* and *FKBP15-2*) negatively regulated the development of lateral roots by inhibiting the activity of VINV [23–25]. Numerous studies showed that VINV was closely related to biotic and abiotic stresses in plants besides their key role in plant growth and development [26, 27]. For example, VINV gene expression in the ovaries of maize (*Zea mays*) decreased under drought stress, but could recover to normal levels through sucrose supplementation [28]. High-

temperature stress led to reduced VINV activity in maize kernels, which hindered sucrose hydrolysis and reduced starch synthesis in the endosperm, resulting in decreased grain yield [29]. The activity of VINV in young fruit of heat-tolerant tomato (*Solanum lycopersicum*) cultivars was significantly higher than that in heat-sensitive cultivars, while their leaf photosynthetic capacity showed no difference [8].

Previously, we identified four members of the AINV gene family from the published genome of *D. officinale* [30, 31]. Further bioinformatics analysis indicated at least two genes encoding VINV in *D. officinale*. One of the VINV genes, such as *DOSAI*, was cloned from the Yunnan-grown *D. officinale*, and its expression patterns in roots, stems, and leaves of 1-, 2-, and 3-year-old plants were analyzed, as reported in a previous study [32]. However, no studies on the structural and functional characterizations of the gene or its expression characteristics in response to abiotic stress are available. Therefore, the present study involved cDNA amplification of *DOSAI* (now called *DoVIN2* in this study) based on the identification of the AINV gene family in *D. officinale* and its location on the chromosome, and was performed in the *D. officinale* “Danxia” cultivar using homologous cloning. The bioinformatics analysis and expression profiling were conducted to elucidate the characteristics of *DoVIN2*, including its differential expression across multiple tissues and in response to several abiotic stresses. Furthermore, a protein–protein interaction network for *DoVIN2* was constructed to facilitate the functional studies of this gene. The findings provided a strong foundation for further exploring the precise regulatory mechanisms of *DoVIN2* in the context of polysaccharide synthesis metabolism and abiotic stress response.

Materials and Methods

Plant materials and treatments

The *D. officinale* “DanXia” cultivar was obtained from the *Dendrobium* herb at the Engineering Technology Development Center in Shaoguan, China. The capsules were surface-disinfected, and the seeds were sown in half-strength Murashige and Skoog (MS) medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 20% potato flour. The seedlings were grown aseptically at 25°C ± 1°C with a 12/12-h light/dark cycle and 60–70% relative humidity in a growth chamber at Shaoguan University (Shaoguan, China). The seedlings were subcultured every 2 months until they reached a height of 6–8 cm (approximately 6 months old) and then subjected to various treatments. Six leaves from six separate seedlings were pooled as one sample for each treatment, and experiments were performed in triplicate.

The seedlings were subjected to various treatments: exogenous abscisic acid (ABA, 100 µM for 24 h), polyethylene glycol (PEG) 6000 (20% by mass for 24 h), and cold stress (4°C ± 1°C for 24 h). The control seedlings were grown in liquid half-strength MS medium. The leaves were harvested 0, 1, 3, 6, 9, 12, and 24 h after treatment, flash-frozen in liquid nitrogen, and stored at –80°C for subsequent expression analysis. Additionally, leaf, stem, root, and flower samples were collected from *D. officinale* plants in the flowering stage and treated similarly. Three biological replicates were collected for each sample.

RNA isolation and cDNA synthesis

Total RNA was extracted from the *D. officinale* leaves using MiniBEST Plant RNA Extraction Kit (9769, TaKaRa, Japan). The concentration and quality of RNA were detected using a NanoDrop 600 spectrophotometer (Nano-600, Jiapeng, Shanghai, China) and 1% agarose gel electrophoresis. The complementary DNA (cDNA) was synthesized by first-strand cDNA synthesis using PrimeScript RT Master Mix (RR036A, TaKaRa, Japan).

Identification and cloning of DoVIN2

The cDNA derived from *D. officinale* leaf samples was prepared as a template to clone the *DoVIN2* gene. The specific primers (Table S1) were designed using Primer Premier 5.0 software based on the open reading frame (ORF) sequence of the published *DOSA1* gene (Login No. KU598852) obtained from the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). The ORF sequence of *DoVIN2* was amplified by polymerase chain reaction (PCR). Thereafter, the amplified products were purified using a FastPure Gel DNA Extraction Mini Kit (AU1701, BioTeke, Beijing, China), subcloned into the pUCm-T vector (B522211, Sangon Biotech, Shanghai, China), and then sequenced (B522211, Sangon Biotech, Guangzhou, China).

Analysis of gene structure and protein properties

The exons and introns of *DoVIN2* were analyzed using the Gene Structure Display Server 2.0 (GSDS, <http://gsds.cbi.pku.edu.cn/>) through comparisons with the relevant coding DNA sequence and genomic DNA (gDNA). The promoter sequences, 2.0-kb upstream of the start codon, related to the *DoVIN2* gene were analyzed using PlantCARE database (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The theoretical molecular weight (Mw), isoelectric point (pI), and instability index (II) of the protein were calculated using the ExPasy database (<http://expasy.org/>). The protein domain of *DoVIN2* was analyzed using Conserved Domain Database (NCBI-CDD, <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The secondary and tertiary structures were predicted using Self-Optimized Prediction Method from Alignment (SOPMA, https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and the ExPaSy Swiss-Model (<http://swissmodel.expasy.org>), respectively. The transmembrane helix and signal peptide were predicted through the prediction of transmembrane helices in Hidden Markov Models Server (TMHMM v2.0, <https://services.healthtech.dtu.dk/service.php?TMHMM-2.0>) and SignalP 5.0 Server (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>). The subcellular localization of *DoVIN2* was predicted using the Cell-PLoc 2.0 (<http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/>) online tools. The phosphorylation sites were predicted using NetPhos 3.1 Servers (<https://services.healthtech.dtu.dk/service.php?NetPhos-3.1>).

Phylogenetic analysis and protein–protein interaction network

The VINV protein sequences of *A. thaliana* and other plants, comprising eight eudicots and six monocots (Table S2), used for multiple alignment and phylogenetic analyses were downloaded from the *Arabidopsis* Information Resource (<https://www.arabidopsis.org/>) and the Ensembl Plants database (<https://plants.ensembl.org/info/data/ftp/index.html>). DNAMAN software (version 6.0) was used to perform multiple comparisons of VINV proteins, and the phylogenetic analysis was performed with MEGA 11 using the neighbor-joining (NJ) algorithm and the bootstrap test carried out with 1000 replicates. The potential interaction proteins for DoVIN2 were aligned to the latest STRING v11.5 available online database (<https://cn.string-db.org/>) to predict the relationships.

Subcellular localization of DoVIN2

The *DoVIN2* coding sequence without the termination codon was inserted into the pC1300s vector using specific primers (Table S1) containing the cauliflower mosaic virus (CaMV) 35S promoter and a *GFP* gene, generating the *35S–DoVIN2–GFP* fusion gene. The clone was verified by DNA sequencing at Sangon. The mesophyll protoplasts were isolated from the fresh leaves of 2–3-week-old Nipponbare rice seedlings and used to transfect an empty vector (*35S::pC1300s–GFP*) and the constructed *35S::DoVIN2–GFP*. The mesophyll protoplast isolation and

transfection system was as described by Chen et al. [33]. GFP fluorescence was visualized and photographed using a laser scanning confocal microscope (Olympus, FluoView FV1200).

Expression profiling of DoVIN2 in different tissues and organs

The raw RNA-seq reads of flower buds (SRR4431603), gynostemium (SRR4431596), labellum (SRR4431602), sepals (SRR4431597), leaves (SRR4431601), stems (SRR4431600), white parts of roots (SRR4431598), and green root tips (SRR4431599) were downloaded from the NCBI Sequence Read Archive for the tissue expression profiling of *DoVIN2* gene [31]. The reads of all the samples were aligned to the NCBI *Dendrobium* reference genome through HISAT package [34]. The expression levels were calculated and normalized as fragments per kilobase per million mapped fragments (FPKM). The heat map was drawn through the eFP Browser function of TBtools [35].

Transcriptomic analysis

The sequencing procedures were completed by BGI Tech Co., Ltd. (Shenzhen, China), including RNA extraction, library construction, and sequencing. Illumina Hiseq 2500/Miseq (Illumina, USA) was used as the sequencing platform. The adapter sequences and low-quality sequences produced in sequencing were removed using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC program (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The FPKM for mRNA expression evaluation was calculated using StringTie [35]. The DEGseq R package (version 1.12.0) was used to identify significantly differentially expressed genes ($\log_2$ -based fold-changes > 1 ; $\log_2$ -based fold-changes < -1) in response to cold stress, PEG, and ABA treatments.

Expression analysis of DoVIN2 under different conditions

The real-time quantitative reverse transcription–PCR (qRT-PCR) amplification was performed using TB Green Premix Ex Taq (Tli RNaseH Plus) Kit (RR820A, TaKaRa, Japan) on an Applied Bio-Rad CFX Connect Real-time System (BIORAD, USA). The qRT-PCR reaction mixture (20 μ L) was prepared to analyze the expression of the *DoVIN2* gene under different conditions following the manufacturer's protocols, including different organs (leaves, roots, stems, and flowers) and treatments (cold, PEG, and ABA). The *DoACTIN* gene was used as an internal control [36]. All reactions were performed in triplicate in each experiment, and three biological repeats were conducted. The primers used in the qRT-PCR experiments are listed in Table S1. The relative expression levels of *DoVIN2* were calculated using the normalized expression method ($2^{-\Delta\Delta C_T}$ method) [37].

Statistical analysis

A one-way analysis of variance was performed using Student *t* test with a significance level of 0.05 in GraphPad Prism v9.5 (GraphPad Software, Inc., Chicago, USA, www.graphpad.com) to determine the differences between values. All of the data analyses were performed for three biological repeats. The values shown in the figures represent the average values of three repeats. The sample variability was given as the standard deviations (SDs) of the mean.

Results

Isolation of cDNA from DoVIN2 and sequence analysis

The full-length cDNA fragments of *DoVIN2* were isolated from *D. officinale* leaves. The cDNA sequence of *DoVIN2* was 1368 bp in length, and the gene encoded 455 amino acids (Fig. 1a). A comparison of the *DoVIN2* gene to the public genomic sequence of *D. officinale* showed that the gene was located on chromosome 8, spanning four introns and three exons (Fig. 1b). Four nucleotide differences were identified, consisting of two synonymous substitutions and two nonsynonymous mutations, by aligning with the reference sequence (Fig. S1).

The bioinformatics analysis results revealed that DoVIN2 belonged to the glycoside hydrolase family 32 and possessed a conserved glycosyl hydrolase domain (Glyco_32) of the AINV gene family (Fig. 2a). The DoVIN2 protein had a molecular formula of $C_{2277}H_{3473}N_{603}O_{691}S_{12}$, with a theoretical Mw of 50.74 kDa, pI of 5.32, and II of 37.87. Furthermore, it was a hydrophilic protein lacking both transmembrane regions and signal peptides (Figs. 2b, S2, and S3). The secondary structure of DoVIN2 protein was primarily composed of random coils, accounting for 53.41%, followed by extended strands at 28.35% and alpha helixes at 11.43% (Fig. 2c). Its tertiary structure had a close resemblance to beta-fructofuranosidase from *Vanilla planifolia*, with a high similarity of 74.39% (Fig. 2d). Further, the DoVIN2 peptide chain contained 46 phosphorylation sites, including 25 serine (Ser) sites, 14 threonine (Thr) sites, and 7 tyrosine (Tyr) sites (Fig. 2e).

Homology and phylogenetic analysis of DoVIN2 protein

A comparative analysis was conducted on 23 full-length amino acid sequences from 6 monocot plant species, including rice, sweet sorghum, maize, pineapple (*Ananas comosus*), banana (*Musa acuminata*), and barley (*Hordeum vulgare*), as well as 20 full-length amino acid sequences from 9 eudicot plant species, comprising *A. thaliana*, tomato, pepper (*Capsicum annuum*), grape (*Vitis vinifera*), beet (*Beta vulgaris*), soybean (*Glycine max*), lucerne (*Medicago truncatula*), potato (*Solanum tuberosum*), and pomelo (*Citrus grandis*), totaling 43 VINV proteins, to further illustrate the evolutionary relationship between DoVIN2 in *D. officinale* and that in other species (Fig. 3). The results showed that VINV members from different species were clustered into two subgroups in the evolutionary tree based on their taxonomic classification. All monocot plant VINV members were clustered into the monocot subgroup, whereas all eudicot plant VIN members were clustered into the eudicot subgroup. This indicated that plant VINVs were highly conserved during evolution. Furthermore, it suggested that plant VINVs had evolved and executed their biological functions before the differentiation of monocot and eudicot plants. In particular, DoVIN2 belonged to the monocot subgroup and was found to cluster with MaVIN1 from bananas on a separate branch, indicating that they had a close relationship and might execute similar biological functions.

Subcellular localization of DoVIN2

The subcellular localization of a protein is crucial for the execution of its biological functions in the cell. The subcellular localization prediction results indicated that *DoVIN2* might be localized to the vacuole. However, the rice mesophyll protoplasts containing the 35S::DoVIN2-GFP fusion protein showed fluorescence in the nucleus only, whereas 35S::pC1300s-GFP was found throughout the cell (Fig. 4). The result showed that DoVIN2 protein was localized to the nucleus.

Expression analysis of DoVIN2 in different tissues

The expression profile of *DoVIN2* was performed by reanalyzing the RNA-seq data from eight tissues, including flower buds, sepal, gynostemium, labellum, leaves, stems, white roots (equivalent to mature zone), and green root tips, to investigate the potential functional region of *DoVIN2* gene in different types of plant tissues in *D. officinale*.

[31]. The relative expression levels of the *DoVIN2* gene varied significantly across different tissues in *D. officinale* (Fig. 5a). Its expression was relatively high in flowers, with the highest expression level in sepals, followed by styles, flower buds, and labellum; however, the expression level in leaves was the lowest. These results suggested that the *DoVIN2* gene might play crucial roles in the formation and development of reproductive organs in *D. officinale*. The qRT-PCR analysis further validated the differences in the expression of *DoVIN2* across different tissues in *D. officinale* “DanXia” cultivar (Fig. 5b). The results showed that the *DoVIN2* expression level was the highest in flowers, which was 3.0-fold higher, followed by stems and roots with expression levels 2.9-fold and 2.0-fold higher, than that in leaves. These findings indicated that the *DoVIN2* gene might play an important role in the development of flower organs in *D. officinale*.

Expression analysis of DoVIN2 under abiotic stresses

PlantCARE analysis demonstrated that the 2.0-kb upstream promoter sequences of the *DoVIN2* gene contained several elements in response to abiotic stresses, such as low temperature, drought, dehydration, osmotic pressure, environmental stress, and ABA responsiveness (Table 1). Therefore, the expression patterns of the *DoVIN2* gene were investigated in response to cold stress, PEG, and ABA treatments. The transcriptome analysis revealed that *DoVIN2* expression levels increased over time in response to both cold stress and ABA treatment. The most significant change was observed under cold stress, with peak expression occurring after 6 h. In contrast, *DoVIN2* expression did not change significantly in response to PEG treatment (Fig. 6). These results suggested that the *DoVIN2* gene was induced by cold stress and ABA treatment and might play a role in abiotic stress response processes.

Table 1
Cis-acting elements analyze in the promoter of DoVIN2 promoter

No.	Regulated element	Motif sequence	Amount	Biological Function
1	AAGAA-motif	GAAAGAA	2	abscisic acid responsiveness
2	ABRE	AACCCGG	1	abscisic acid responsiveness
3	LTR	CCGAAA	3	low-temperature responsiveness
4	DRE core	GCCGAC	1	dehydration responsiveness
5	STRE	AGGGG	4	osmotic responsiveness
6	ARE	AAACCA	2	anaerobic induction element
7	MYB	CAACAG/CAACCA	3	environmental stress responsiveness
8	MYC	CAATTG	1	environmental stress responsiveness

The qRT-PCR analysis was used to validate the expression patterns of *DoVIN2* in response to cold stress, PEG, and ABA treatments. The results showed that *DoVIN2* expression was significantly induced by cold stress and ABA treatments, with expression levels 5.9-fold and 4.0-fold higher than those before treatment after 6 h, respectively (Fig. 7a and 7c). These findings suggested that *DoVIN2* might participate in cold stress response processes through the ABA signaling pathway in *D. officinale*. In contrast, *DoVIN2* expression decreased over time in response to PEG treatment, reaching a significantly different level after 3 h and continuing until 24 h of treatment (Fig. 7b). This suggested that *DoVIN2* might play a negative regulatory role in response to PEG treatment. Overall, these

results indicated that *DoVIN2* might serve as a functionally diverse gene with different response mechanisms to various abiotic stresses.

Protein–protein interaction network of DoVIN2 in *D. officinale*

The protein–protein interaction network was analyzed using the STRING website to further investigate the biological function of DoVIN2 in polysaccharide biosynthesis and abiotic stress response (Fig. 8). The results showed that DoVIN2 interacted with several enzymes involved in sugar synthesis and metabolism, including alpha-galactosidase (α -GAL), beta-glucosidase (BGLU4), sucrose phosphatase (SPP2), sucrose synthase (SUS4 and SUS5), fructokinase (PfkB2 and PfkB5), and hexokinase (HXK1, HXK2, and HXK3) (Table S3). These findings suggested that DoVIN2 might function together with these enzymes to regulate the expression of related enzyme genes and sugar metabolism pathways during abiotic stress. This might increase the polysaccharide content inside the cell to maintain osmotic stability and reduce or eliminate stress-induced damage to plants.

Discussion

In plants, sucrose is the main storage energy source produced by photosynthesis, and the rate of its synthesis and metabolism is crucial for the growth, development, and biological yield of plants [38, 39]. AINV is a key enzyme in plants that is closely related to sucrose metabolism [12, 40]. In recent years, numerous functional VINV genes have been identified in various species. These genes played a significant role in the transport and accumulation of photosynthetic products and had substantial effects on plant morphology, signal transduction, and stress resistance [7, 27]. Previous studies demonstrated that the VINV gene family in plants was ancient, conserved, and relatively small, encoding proteins with various isoforms [41, 42]. In this study, the *DoVIN2* was cloned from the *D. officinale* “Danxia” cultivar, which exhibited the highest expression level under cold stress as detected using RT-PCR. The sequence analysis revealed four nucleotide differences between the DoVIN2’s ORF and the reference sequence, including two synonymous substitutions and two nonsynonymous mutations (Fig. S1). The results indicated that the DoVIN2 protein sequence and structure were highly conserved. The phylogenetic analysis showed that DoVIN2 clustered with VINV members from other monocotyledonous plants, suggesting that plant VINVs were highly conserved in evolution [41]. Notably, DoVIN1 and DoVIN2 clustered within the monocot subgroup, but they did not branch together and DoVIN1 seemingly appeared earlier than DoVIN2 (Fig. 3). This suggested that the VINV gene family members in *D. officinale* had diverged significantly during evolution and might have different regulatory mechanisms. This finding was consistent with previous studies of VINVs in sweet sorghum and rice [43, 44].

Signal peptides and transmembrane helices not only determine the intracellular localization of proteins but also dictate whether a protein is a secreted protein [45]. The bioinformatics analysis predicted that the DoVIN2 protein lacked both signal peptide and transmembrane regions (Figs. S2 and S3), and was most likely localized to the vacuole. This suggested that DoVIN2 was a non-transmembrane and nonsecretory vacuolar membrane protein. However, transient GFP expression analysis in rice mesophyll protoplasts revealed that DoVIN2 was localized in the cell nucleus (Fig. 4). This finding was consistent with a previous study by Zheng et al., in which the glycosyltransferase QUASIMODO1 (QUA1) was predicted to be located in the Golgi apparatus but was found to be localized in the chloroplast using GFP analysis [46]. Further investigation showed that QUA1 regulated plant salt and drought stresses by affecting chloroplast Ca^{2+} signaling. Given the close correlation between cold stress and organelle structure and function, it is possible that DoVIN2 might regulate vacuolar stability through nuclear signal regulation [47]. This might involve promoting the transfer of DoVIN2 from the nucleus to the vacuole to maintain

cell osmotic pressure during a response to certain abiotic stress stimuli. Additionally, phosphorylation is a fundamental mechanism for regulating protein activity and function in cellular signal transduction pathways [48]. The bioinformatics analysis predicted that the DoVIN2 peptide chain contained 46 phosphorylation sites, including serine, threonine, and tyrosine residues (Fig. 2). These findings suggested that DoVIN2 might play an essential regulatory role in multiple biological processes and molecular functions.

The tissue-specific expression of genes provides valuable evidence for exploring gene function. The temporal and spatial expression characteristics of VINV in different developmental stages and various plant organs play important roles in morphology and growth [40, 41]. Previous studies reported that the VINV gene in *A. thaliana* regulated the elongation of roots [9], whereas the VINV gene (*SAI-1*) in sweet sorghum was specifically expressed in the stems [15]. In this study, the *DoVIN2* expression levels were found to vary significantly among different tissues of *D. officinale*, with the highest expression detected in flower organs (Fig. 5). This was consistent with the expression pattern of the *Lycium barbarum* VINV gene (*LbSAI*), indicating a crucial role of these genes in plant reproductive structure formation and development [49]. These findings provided further evidence for the conservation and functional diversity of the VINV gene family. Furthermore, previous studies demonstrated that VINV family genes played an important role in plant response to abiotic stress [27, 30]. In the present study, *DoVIN2* expression was upregulated in response to cold stress and ABA treatment, with similar expression patterns and peak expression after 6 h (Fig. 7). This suggested that *DoVIN2* might participate in cold stress response processes through the ABA signaling pathway, although the specific regulatory mechanisms and genes involved remain unclear. In contrast, the *DoVIN2* expression was downregulated in response to PEG treatment, possibly due to the involvement of negative feedback regulation mechanisms. Thus, we hypothesized that *DoVIN2* might adopt different coping mechanisms in response to cold and drought stresses.

Additionally, the protein–protein interaction network analysis revealed that DoVIN2 was closely linked with the enzymes involved in sucrose hydrolysis and utilization, as well as polysaccharide synthesis and accumulation (Fig. 8). These enzymes played crucial roles in multiple biological processes related to sugar synthesis and metabolism, including fructose metabolic process, starch biosynthetic process, hexose metabolic process, and cellular glucan metabolic process [50]. Therefore, we hypothesized that *DoVIN2* might function as a regulatory gene by forming an expression regulation network through interactions with these enzymes to jointly regulate polysaccharide synthesis and metabolism. In response to abiotic stresses, *DoVIN2* might coordinate the regulation of related enzyme gene expression and sugar metabolism through its protein–protein interaction network to increase total sugar accumulation in plants. However, the specific regulatory mechanisms remain unclear. In the present study, we successfully cloned *DoVIN2*. However, its function was still unambiguous and needed to be further explored by genetic transformation.

Conclusions

This study provided evidence that the *DoVIN2* gene might play a significant role in the regulation of plant growth, besides being involved in plant response to abiotic stresses in *D. officinale*. It was essential to perform further overexpression or gene-knockout experiments to examine and verify the functions of the *DoVIN2* gene. In summary, this study provided a platform and informative insights on the VINV genes in *D. officinale* for future studies.

Declarations

Data availability statements

The datasets supporting our conclusions of the current study are included in the manuscript and additional file. The genome sequence of *D. officinale* were downloaded from the NCBI database. The tissue expression profiling analysis used already published raw sequence data of *D. officinale* were obtained from NCBI-BioProject PRJNA348403. The generated sequencing data in this study were deposited in NCBI's SRA database. The raw sequence data of *D. officinale* in response to abiotic stress are available in NCBI-BioProject database under the accession number PRJNA943399, PRJNA949800 and PRJNA949802, respectively.

Ethics approval and consent to participate We declare that no wild plants were collected or used in this study. All experimental research was conducted in accordance with relevant institutional, national, and international guidelines and legislation. Permission was obtained for the collection of the *D. officinale* “DanXia” cultivar from Prof. Baiyin Yu of the

Dendrobium herb at the Engineering Technology Development Center in Shaoguan, China.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Acknowledgements

This research was supported by grants from the National Science Foundation of China (No. 32000266), Basic and Applied Research Projects of Guangdong Province (No. 2020A1515011438), Key Areas Funding in Ordinary Universities of Guangdong Province (No. 2022ZDZX4044), the Open Fund of the Guangdong Provincial Key Laboratory of Utilization and Conservation of Food and Medicinal Resources in Northern Region (No. FMR2022002Z), Special Funding for Science and Technology Innovation Strategy of Guangdong Province (No. pdjh2023b0477), Innovation and Entrepreneurship Training Program for Undergraduate of Shaoguan University (No. Sycxcy2023083).

Authors' contributions

Boting Liu, Xiang Li, and Yujia Liu designed and coordinated the research; Boting Liu, Junqi Mai, Jieying Xie and Jiayi Chen performed the main experiments. Lijun Meng and Di Cui participated in some of the experiments and data collection. Boting Liu, Yuanlong Liu and Yujia Liu analyzed all the data. Boting Liu, Xiang Li, and Yujia Liu wrote and revised the manuscript. All authors reviewed and approved the manuscript.

Supplementary Information

Additional file 1: Fig. S1. cDNA sequence of *DoVIN2* gene and deduced amino acid sequence. The red arrow represents a base difference between *DoVIN2* gene and the reference sequences.

Additional file 2: Fig. S2. Predicted transmembrane helix domain architectures of *DoVIN2* protein.

Additional file 3: Fig. S3. Predicted signal peptide of *DoVIN2* protein.

Additional file 4: Table S1. The primers used for gene-specific clone and qRT-PCR analysis of *DoVIN2*.

Additional file 5: Table S2. List of VINV protein sequences used in this study.

Additional file 6: Table S3. Function annotation of DoVIN2-interacting proteins.

References

1. Sun L, Chen X, Wu C, Guo S (2020) Advances and prospects of pharmacological activities of *Dendrobium officinale* Kimura et Migo polysaccharides. *Acta Pharm Sin* 55:2322–2329. <https://doi.org/10.16438/j.0513-4870.2020-0126>
2. Chen W, Wu J, Li X, et al (2021) Isolation, structural properties, bioactivities of polysaccharides from *Dendrobium officinale* Kimura et. Migo: a review. *Int J Biol Macromol* 184:1000–1013. <https://doi.org/10.1016/j.ijbiomac.2021.06.156>
3. Li Q, Li B, Guo S (2016) Advance in molecular biology of *Dendrobium* (Orchidaceae). *China J Chin Mat Med* 41:2753–2761. <https://kns.cnki.net/kcms/detail/11.2272.r.20160727.1414.004.html>
4. Li M, Chen T, Gao T, et al (2015) UDP-glucose pyrophosphorylase influences polysaccharide synthesis, cell wall components, and hyphal branching in *Ganoderma lucidum* via regulation of the balance between glucose-1-phosphate and UDP-glucose. *Fungal Genet Biol* 82:251–263. <https://doi.org/10.1016/j.fgb.2015.07.012>
5. Barratt DHP, Derbyshire P, Findlay K, et al (2009) Normal growth of *Arabidopsis* requires cytosolic invertase but not sucrose synthase. *Proc Natl Acad Sci U S A* 106:13124–13129. <https://doi.org/10.1073/pnas.0900689106>
6. Yang J, Meng H, Yang S, et al (2012) Correlation between soluble polysaccharide and sucrose metabolic enzymes in *Dendrobium officinale*. *J West China Forestry Sci* 41:62–67. <https://doi.org/10.16473/j.cnki.xblykx1972.2012.02.009>
7. Xu X, Ren Y, Wang C, et al (2019) *OsVIN2* encodes a vacuolar acid invertase that affects grain size by altering sugar metabolism in rice. *Plant Cell Rep* 38:1273–1290. <https://doi.org/10.1007/s00299-019-02443-9>
8. Ruan Y, Jin Y, Yang Y, et al (2010) Sugar input, metabolism, and signaling mediated by invertase: roles in development, yield potential, and response to drought and heat. *Mol Plant* 3:942–955. <https://doi.org/10.1093/mp/ssq044>
9. Sergeeva LI, Keurentjes JJB, Bentsink L, et al (2006) Vacuolar invertase regulates elongation of *Arabidopsis thaliana* roots as revealed by QTL and mutant analysis. *Proc Natl Acad Sci U S A* 103:2994–2999. <https://doi.org/10.1073/pnas.0511015103>
10. Su T, Wolf S, Han M, et al (2016) Reassessment of an *Arabidopsis* cell wall invertase inhibitor *AtCIF1* reveals its role in seed germination and early seedling growth. *Plant Mol Biol* 90:137–155. <https://doi.org/10.1007/s11103-015-0402-2>
11. Verhaest M, Lammens W, Le Roy K, et al (2006) X-ray diffraction structure of a cell-wall invertase from *Arabidopsis thaliana*. *Acta Crystallogr D Biol Crystallogr* 62:1555–1563. <https://doi.org/10.1107/S0907444906044489>
12. Jain R, Singh SP, Singh A, et al (2017) Soluble acid invertase (SAI) activity and gene expression controlling sugar composition in sugarcane. *Sugar Tech* 19:669–674. <https://doi.org/10.1007/s12355-017-0511-0>
13. Haouazine-Takvorian N, Tymowska-Lalanne Z, Takvorian A, et al (1997) Characterization of two members of the *Arabidopsis thaliana* gene family, *At beta fruct3* and *At beta fruct4*, coding for vacuolar invertases. *Gene*

197:239–251. [https://doi.org/10.1016/s0378-1119\(97\)00268-0](https://doi.org/10.1016/s0378-1119(97)00268-0)

14. Unger C, Hardegger M, Lienhard S, Sturm A (1994) cDNA cloning of carrot (*Daucus carota*) soluble acid beta-fructofuranosidases and comparison with the cell wall isoenzyme. *Plant Physiol* 104:1351–1357. <https://doi.org/10.1104/pp.104.4.1351>
15. Liu Y, Dun B, Zhao X, et al (2013) Correlation analysis between the key enzymes activities and sugar content in sweet sorghum (*Sorghum bicolor* L. Moench) stems at physiological maturity stage. *Aust J Crop Sci* 7:84–92
16. Liu Y, Nie Y, Han F, et al (2014) Allelic variation of a soluble acid invertase gene (*SAI-1*) and development of a functional marker in sweet sorghum [*Sorghum bicolor* (L.) Moench]. *Mol Breeding* 33:721–730. <https://doi.org/10.1007/s11032-013-9988-8>
17. Sturm A (1996) Molecular characterization and functional analysis of sucrose-cleaving enzymes in carrot (*Daucus carota* L.). *J Exp Bot* 47 Spec No:1187–1192. https://doi.org/10.1093/jxb/47.Special_Issue.1187
18. Yao Y, Wu X, Geng M, et al (2014) Cloning, 3D modeling and expression analysis of three vacuolar invertase genes from cassava (*Manihot Esculenta* Crantz). *Molecules* 19:6228–6245. <https://doi.org/10.3390/molecules19056228>
19. Ji X, Van den Ende W, Van Laere A, et al (2005) Structure, evolution, and expression of the two invertase gene families of rice. *J Mol Evol* 60:615–634. <https://doi.org/10.1007/s00239-004-0242-1>
20. Ji X, Van den Ende W, Schroeven L, et al (2007) The rice genome encodes two vacuolar invertases with fructan exohydrolase activity but lacks the related fructan biosynthesis genes of the *Pooideae*. *New Phytol* 173:50–62. <https://doi.org/10.1111/j.1469-8137.2006.01896.x>
21. Qian W, Xiao B, Wang L, et al (2018) *Cs/INV5*, a tea vacuolar invertase gene enhances cold tolerance in transgenic *Arabidopsis*. *BMC Plant Biol* 18:228. <https://doi.org/10.1186/s12870-018-1456-5>
22. Wang J, Zhao T, Wang W, et al (2019) Culm transcriptome sequencing of Badila (*Saccharum officinarum* L.) and analysis of major genes involved in sucrose accumulation. *Plant Physiol Biochem* 144:455–465. <https://doi.org/10.1016/j.plaphy.2019.10.016>
23. Wang J, Sun W, Kong X, et al (2020) The peptidyl-prolyl isomerases FKBP15-1 and FKBP15-2 negatively affect lateral root development by repressing the vacuolar invertase VIN2 in *Arabidopsis*. *Planta* 252:52. <https://doi.org/10.1007/s00425-020-03459-2>
24. Wang L, Chen M, Zhu F, et al (2019) Alternative splicing is a *Sorghum bicolor* defense response to fungal infection. *Planta* 251:14. <https://doi.org/10.1007/s00425-019-03309-w>
25. Wang X, Chen Y, Jiang S, et al (2020) *PpINH1*, an invertase inhibitor, interacts with vacuolar invertase PpVIN2 in regulating the chilling tolerance of peach fruit. *Hortic Res* 7:168. <https://doi.org/10.1038/s41438-020-00389-8>
26. Li R, Hou Z, Zou H, et al (2018) Inactivation kinetics, structural, and morphological modification of mango soluble acid invertase by high pressure processing combined with mild temperatures. *Food Res Int* 105:845–852. <https://doi.org/10.1016/j.foodres.2017.12.018>
27. Chen L, Liu X, Huang X, et al (2019) Functional characterization of a drought-responsive invertase inhibitor from maize (*Zea mays* L.). *Int J Mol Sci* 20:4081. <https://doi.org/10.3390/ijms20174081>
28. McLaughlin JE, Boyer JS (2004) Sugar-responsive gene expression, invertase activity, and senescence in aborting maize ovaries at low water potentials. *Ann Bot* 94:675–689. <https://doi.org/10.1093/aob/mch193>
29. Cheikh N, Jones R (2006) Heat stress effects on sink activity of developing maize kernels grown in vitro. *Physiol Plantarum* 95:59–66. <https://doi.org/10.1111/j.1399-3054.1995.tb00808.x>

30. Liu B, Tang Y, Li L, et al (2022) Identification of acid sucrose invertase gene family in *Dendrobium catenatum* Lindl. and its expression analysis under low temperature. Jiangsu Agr Sci 50:33–42. <https://doi.org/10.15889/j.issn.1002-1302.2022.24.005>
31. Zhang G, Xu Q, Bian C, et al (2016) The *Dendrobium catenatum* Lindl. genome sequence provides insights into polysaccharide synthase, floral development and adaptive evolution. Sci Rep 6:19029. <https://doi.org/10.1038/srep19029>
32. Meng H, Zhang W, Lu B, et al (2017) Cloning and expression analysis of soluble acid invertase gene from *Dendrobium officinale*. J South China Agric Univ 38:81–85. <https://kns.cnki.net/kcms/detail/44.1110.S.20170110.1424.044.html>
33. Chen S, Tao L, Zeng L, et al (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol 7:417–427. <https://doi.org/10.1111/j.1364-3703.2006.00346.x>
34. Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. Nat Methods 12:357–360. <https://doi.org/10.1038/nmeth.3317>
35. Pertea M, Pertea GM, Antonescu CM, et al (2015) StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat Biotechnol 33:290–295. <https://doi.org/10.1038/nbt.3122>
36. Yuan Y, Zhang J, Liu X, et al (2020) Tissue-specific transcriptome for *Dendrobium officinale* reveals genes involved in flavonoid biosynthesis. Genomics 112:1781–1794. <https://doi.org/10.1016/j.ygeno.2019.10.010>
37. Livak K, Schmittgen T (2000) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ Ct method. Method Methods 25:. <https://doi.org/10.1006/meth.2001>
38. Zhang B, Tolstikov V, Turnbull C, et al (2010) Divergent metabolome and proteome suggest functional independence of dual phloem transport systems in cucurbits. Proc Natl Acad Sci U S A 107:13532–13537. <https://doi.org/10.1073/pnas.0910558107>
39. Wang L, Zheng Y, Ding S, et al (2017) Molecular cloning, structure, phylogeny and expression analysis of the invertase gene family in sugarcane. BMC Plant Biol 17:109. <https://doi.org/10.1186/s12870-017-1052-0>
40. Juárez-Colunga S, López-González C, Morales-Elías NC, et al (2018) Genome-wide analysis of the invertase gene family from maize. Plant Mol Biol 97:385–406. <https://doi.org/10.1007/s11103-018-0746-5>
41. Shen L, Qin Y, Qi Z, et al (2018) Genome-wide analysis, expression profile, and characterization of the acid invertase gene family in pepper. Int J Mol Sci 20:15. <https://doi.org/10.3390/ijms20010015>
42. Chen Z, Gao K, Su X, et al (2015) Genome-wide identification of the invertase gene family in populus. PLoS One 10:e0138540. <https://doi.org/10.1371/journal.pone.0138540>
43. Chi Y, Wilson K, Liu Z, et al (2020) Vacuolar invertase genes *SbVIN1* and *SbVIN2* are differently associated with stem and grain traits in sorghum (*Sorghum bicolor*). Crop J 8:299–312. <https://doi.org/10.1016/j.cj.2019.06.012>
44. Deng X, Han X, Yu S, et al (2020) *OsINV3* and its homolog, *OsINV2*, control grain size in rice. Int J Mol Sci 21:2199. <https://doi.org/10.3390/ijms21062199>
45. Sinha S, Aradhyam GK (2019) Identification and characterization of signal peptide of Mitofusin1 (*Mfn1*). Biochem Biophys Res Commun 509:707–712. <https://doi.org/10.1016/j.bbrc.2018.12.165>
46. Zheng Y, Liao C, Zhao S, et al (2017) The glycosyltransferase QUA1 regulates chloroplast-associated calcium signaling during salt and drought stress in *Arabidopsis*. Plant Cell Physiol 58:329–341. <https://doi.org/10.1093/pcp/pcw192>

47. Cui X, Wang Y, Wu J, et al (2019) The RNA editing factor *DUA1* is crucial to chloroplast development at low temperature in rice. *New Phytol* 221:834–849. <https://doi.org/10.1111/nph.15448>
48. Needham EJ, Hingst JR, Parker BL, et al (2022) Personalized phosphoproteomics identifies functional signaling. *Nat Biotechnol* 40:576–584. <https://doi.org/10.1038/s41587-021-01099-9>
49. Wang L, Zhao H, Wang Y, et al (2014) Cloning and tissues expression analysis of soluble acid invertase gene from *Lycium barbarum* L. *North Hortic* 86–90. https://kns.cnki.net/kcms2/article/abstract?v=jJlyIHAC3foFNLM1W1bE5xM1UAaK1hMR-jDIIXH3TKNGJ6UCThrxK0hutLve0fscfHqWbnOy_PkpekIdNzt3Ro_y4_6IMwCmFZYBJz0vGTPvHQZq3TtgI8qz-JADfq&uniplatform=NZKPT&language=CHS
50. Malek JA, Mathew S, Mathew LS, et al (2020) Deletion of beta-fructofuranosidase (invertase) genes is associated with sucrose content in *Date palm* fruit. *Plant Direct* 4:e00214. <https://doi.org/10.1002/pld3.214>

Figures

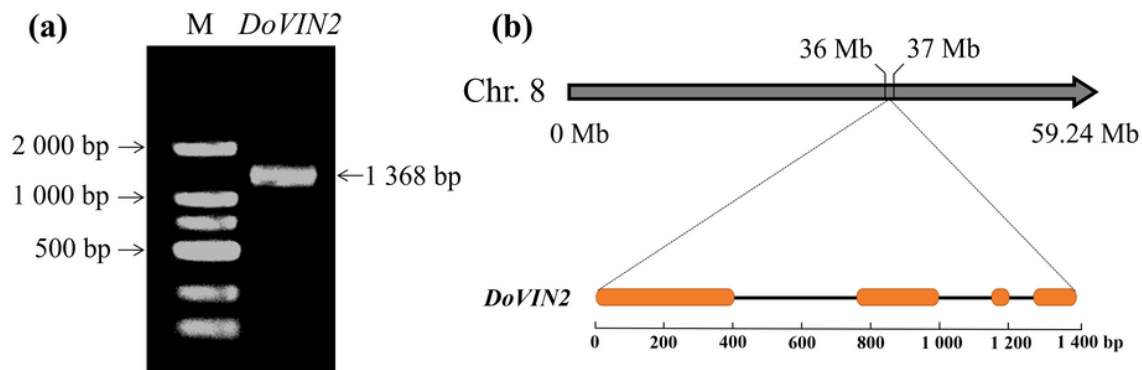


Figure 1

cDNA cloning of the *DoVIN2* gene. (a) PCR amplification electrophoresis of *DoVIN2* gene. M represents DNA marker 2000. (b) Gene structure and chromosome location of *DoVIN2* gene.

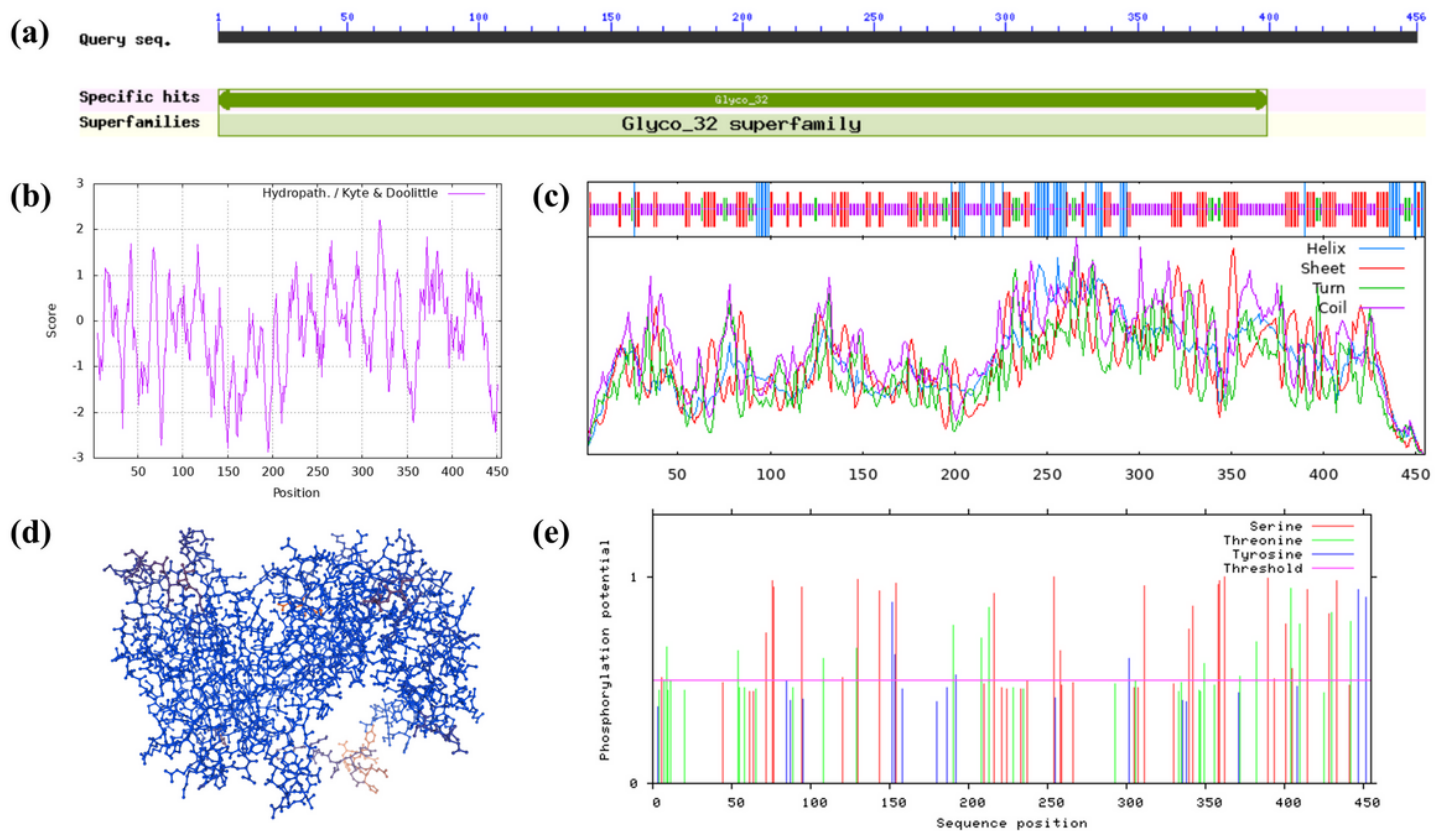


Figure 2

Prediction and analysis of characteristics of the DoVIN2 protein. (a) Analysis of the protein domain found in DoVIN2. (b) Prediction of the hydropobicity of DoVIN2 protein. (c) Analysis of the secondary structure of DoVIN2 protein. (d) Tertiary structure prediction of DoVIN2 protein. (e) Identification of predicted phosphorylation sites on the DoVIN2 protein.

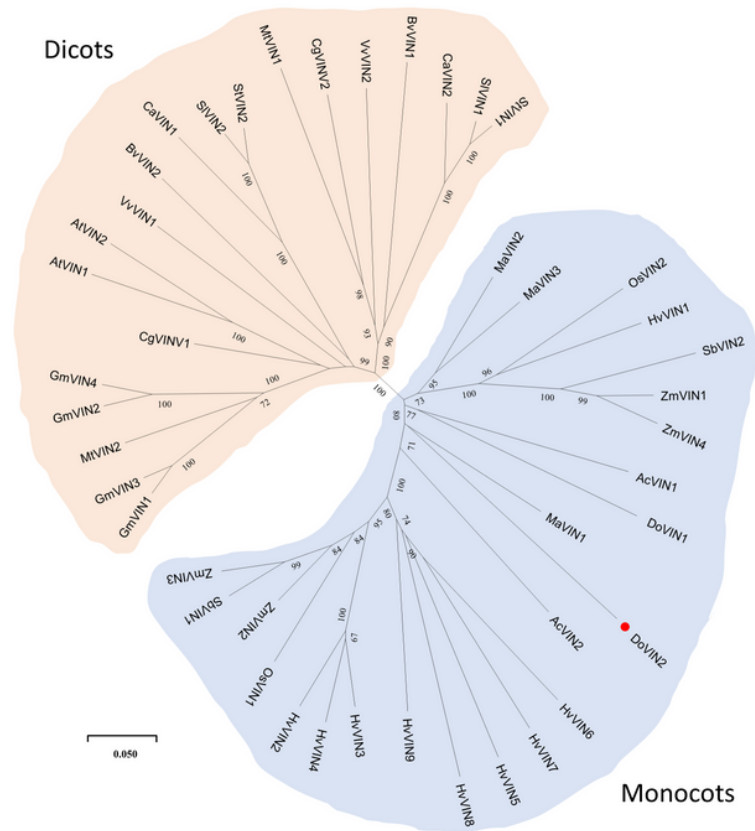


Figure 3

Phylogenetic tree based on VINV protein amino acid sequence of different species. The phylogenetic tree was constructed using the NJ method with 1000 bootstrap replicates. The red point represents DoVIN2 protein, and the blue and orange zones represent monocot and dicot plants, respectively.

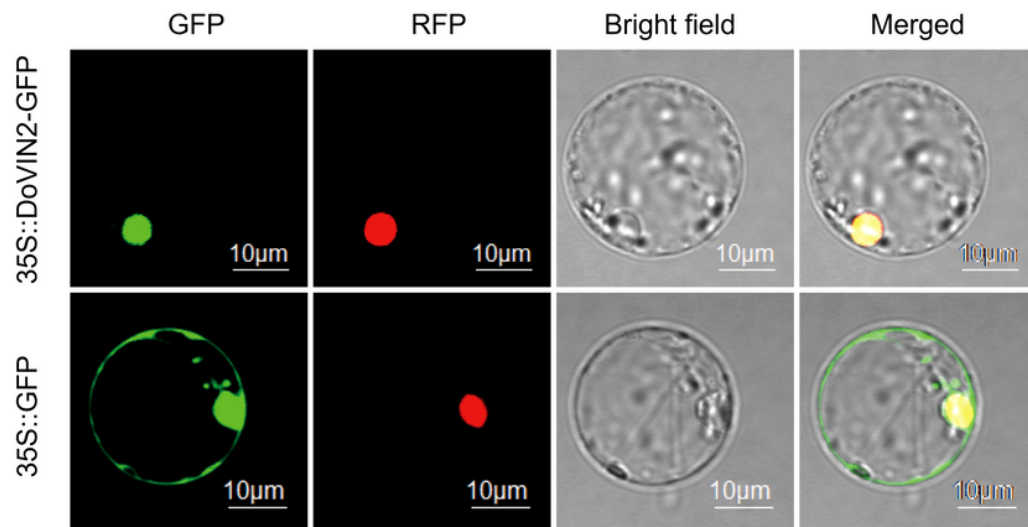


Figure 4

Subcellular localization of DoVIN2. Bars indicate a length of 10 μ m.

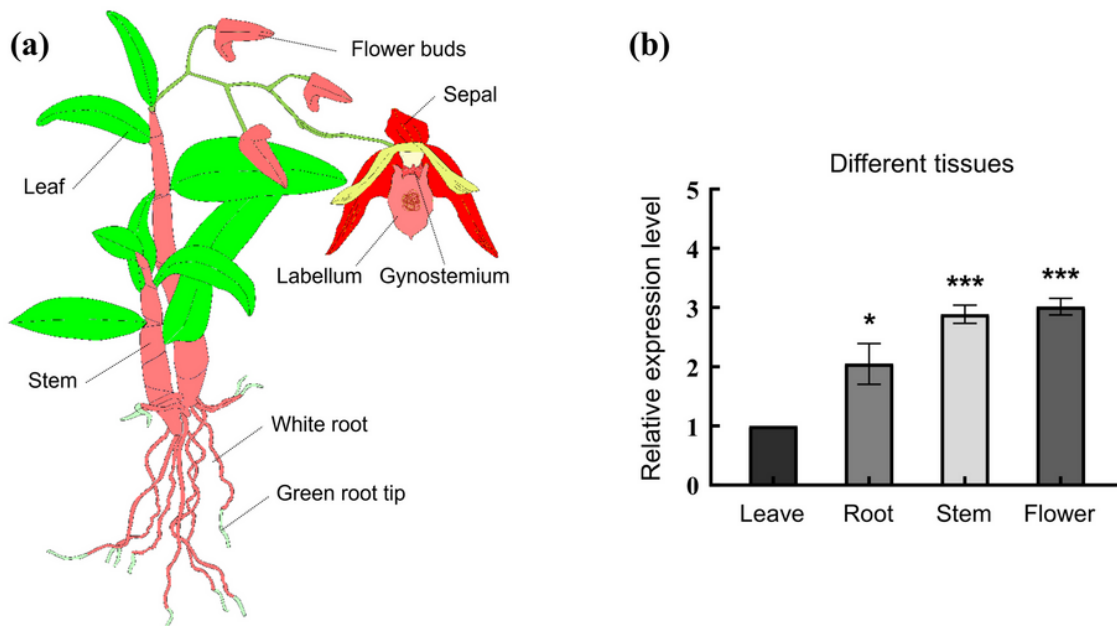


Figure 5

Expression level analysis of *DoVIN2* gene in different tissues of *D. officinale*. (a) Tissue-specific expression pattern of *DoVIN2* in eight tissues: flower buds, sepals, gynostemium, labellum, leaves, stems, white roots, and green root tips. The heatmap was generated using the TBtools software; the color bar represents the expression level of the *DoVIN2* gene, and its value was denoted by $\log_2$ (FPKM value). (b) qRT-PCR analysis of the relative expression of the *DoVIN2* gene. The mean $\pm$ standard deviations (SDs) of three biological replicates are presented, and the significant differences were analyzed using the *t* test. *, a significant difference at $P < 0.05$; *** a significant difference at $P < 0.001$.

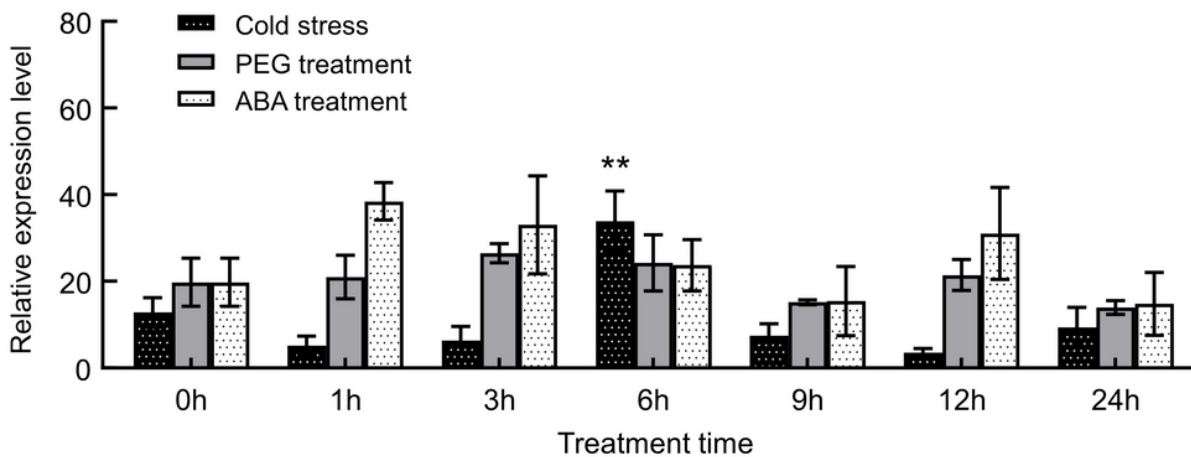


Figure 6

Expression analysis of the *DoVIN2* gene under cold stress, PEG, and ABA treatments. The x-axis indicates different treatment times, including 0, 1, 3, 6, 9, 12, and 24 h. The y-axis represents the relative expression level, and its value is denoted by the FPKM value. The values are the mean of three biological replicates, and SDs are indicated by error bars. Significant differences were analyzed using the *t* test. ** Significant difference at $P < 0.01$.

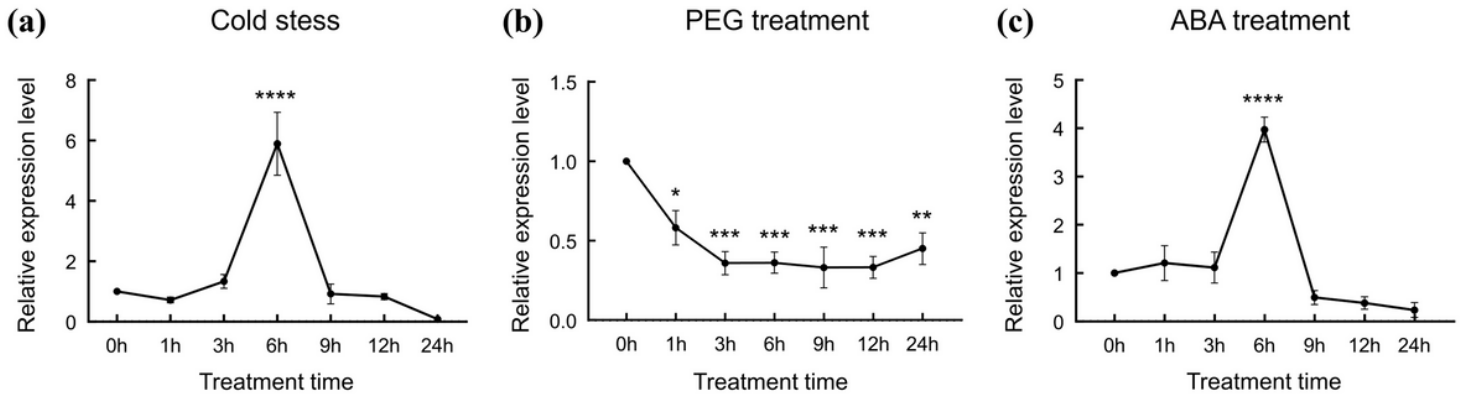


Figure 7

qRT-PCR analysis of the relative expression of the *DoVIN2* gene of *D. officinale* in response to abiotic stresses. The x-axis indicates different treatment times, including 0, 1, 3, 6, 9, 12, and 24 h, under cold stress (a), PEG treatment (b), and ABA treatment (c). The y-axis represents the relative expression level. The values are the mean of three biological replicates, and SDs are indicated by error bars. Significant differences were analyzed using the *t* test. **** and ***, Significant differences at $P < 0.001$; **, significant difference at $P < 0.01$; *, significant difference at $P < 0.05$.

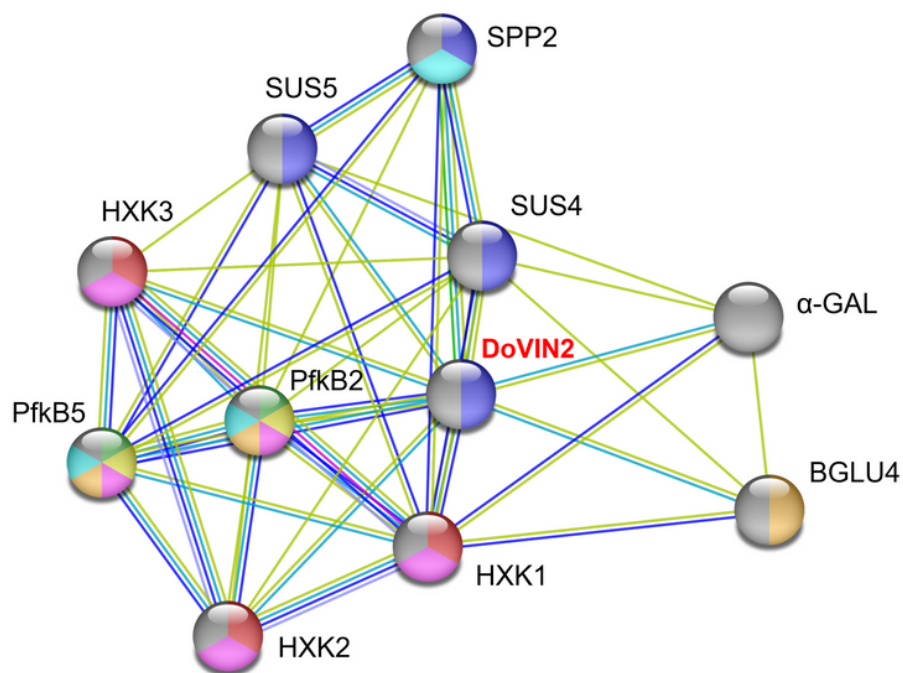


Figure 8

Protein–protein interaction network of DoVIN2 in *D. officinale*. The network was generated using the latest STRING v11.5. Each node represented an interacting protein. The color of the nodes meant that they were involved in various biological processes. Red indicates cellular glucose homeostasis, green indicates the fructose metabolic process, yellow represents the starch biosynthetic process, purple represents the hexose metabolic process, orange represents the cellular glucan metabolic process, gray represents the carbohydrate metabolic process, dark blue represents the sucrose metabolic process, and light blue represents cellular carbohydrate biosynthetic process. The edge (the connecting line) indicates the interaction between two proteins, and the number of edges between them is a measure of their interaction. Multiple edges show strong interaction, whereas fewer edges show weak interaction.

Supplementary Files

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

- [FigureS1.tif](#)
- [FigureS2.tif](#)
- [FigureS3.tif](#)
- [TableS1.xlsx](#)
- [TableS2.xlsx](#)
- [TableS3.xlsx](#)