

Walnut phosphatase 2A proteins interact with basic leucine zipper protein JrVIP1 to regulate osmotic stress response *via* calcium signaling

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Abstract

Walnut is an important economic tree species that is susceptible to osmotic stress. Scientific cultivation management is an important way to improve the yield and quality of walnuts, which requires understanding the regulatory mechanisms in response to osmotic stress. Therefore, in this study, 15 protein phosphatase 2A (PP2A) genes were identified from the walnut transcriptome (named *JrPP2A01~15*) and their potential function responses to osmotic stress were elucidated. The open reading frame (ORF) of *JrPP2A01~15* ranges from 651 to 1,764 bp in length, the molecular weight of the encoded proteins are 24.15–65.61 kDa, and the theoretical isoelectric points are 4.80–8.37. These *JrPP2As* were unevenly distributed on 10 chromosomes and divided into five groups based on the composition of conserved domains, motifs, and exon/intron organizations. The five groups are *JrPP2AAs*, *JrPP2AB's*, *JrPP2AB's*, *JrPP2AB5's*, and *JrPP2ACs*, including 1, 5, 2, 3, and 4 members, accordingly. The *cis*-elements in *JrPP2As*' promoters were involved in responses to hormone and abiotic stress. Most *JrPP2A* genes, excluding *JrPP2A01*, *JrPP2A02*, *JrPP2A05*, *JrPP2A06*, and *JrPP2A13*, could be induced significantly by PEG₆₀₀₀, NaCl, CaCl₂ and ABA. *JrPP2A02*, *JrPP2A05*, *JrPP2A07*, *JrPP2A09*, and *JrPP2A14*, could independently interact with a bZIP transcription factor JrVIP1. Moreover, overexpression of *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* could significantly decrease ROS accumulation while increasing calcium (Ca) uptake exposed to PEG₆₀₀₀ and NaCl stresses, which was mediated by exogenous CaCl₂ and ABA. These results suggested that *JrPP2A* genes play potential key roles in walnut response to drought and salt-inducing osmotic stress involving Ca- and ABA-dependent signaling pathways.

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Introduction

Walnut is one of the most important economic oil tree species and plays a vital role in rural revitalization and poverty alleviation in China^[1]. Currently, the planting area of walnut trees ranks first among the four dried fruits (walnuts, almonds, cashews, and hazelnuts). However, the various stresses caused by unstable environmental changes can affect the yield and quality of walnuts, especially drought, abnormal temperature changes, and soil salinization are the main abiotic stimuli restricting the development of the walnut industry in the northwest region of China, which is the main production area. During severe 'late spring cold', all newly sprouted tender branches could be frozen; long-term drought and high temperature can cause different types of diseases and burns, and the commodity rate decreases; saline-alkali soil directly affects the growth and quality of walnuts^[1,2]. To survive in poor environments, plants have evolved a variety of sophisticated strategies to alleviate damage *via* various pathways, such as releasing stress signals, regulating related genes' expression, physiological responses^[3]. In regulation mechanism, plant transcription factors (TFs), such as MYB (myeloblastosis), ERF (ethylene-responsive element binding factor), WRKY (containing the WRKYQK protein domain), NAC (NAM/ATAF/CUC domain), bHLH (basic helix-loop-helix), perform an important role in

transmitting stress-induced signals and coordinating the functional genes' expression in cells^[4,5]. In signal transduction networks of developmental processes and stress conditions, plant protein kinases and protein phosphatases play key functions^[6]. Reactive oxygen species (ROS) signaling is a prominent pathway for plants to respond to stress^[1, 5]. Protein phosphatase 2A (PP2A), a group of serine/threonine (Ser/Thr) phosphatases, was verified to be involved in the ROS pathway to regulate metabolic changes and cell death^[7]. Clearly, identification of key factors bound to stress response, such as PP2A, is an important basis for revealing the molecular mechanism of walnut response to osmotic stress.

PP2A exists as a trimer made up of three subunits (A, B and C) in eukaryotic cells. The A, B and C subunits are the structural/scaffold, regulatory, and well-conserved catalytic subunit, accordingly^[8]. Subunit A is a complex formed by a series of 15 helical HEAT repeats, each repeat consists of approximately 40 leucine residues, which provide a scaffold for the binding of B and C; Subunit C is strongly conserved among different species^[9]. Subunits A and C shape the core enzyme which interacts with B-subunit to produce the holoenzyme; Subunit B is the 'specificity unit' that determines the specificity of PP2A holoenzyme^[10]. The B subunit could be further classified into three sub-classes, they are B', B'' and B''' (B55)^[11]. Genes encoding PP2A subunits have been identified and characterized in

some plant species. In *Arabidopsis thaliana*, the A, B and C subunit of PP2A contains three, 17 and five members, accordingly^[12]. In *Hevea brasiliensis*, four, 24 and eight members of A, B and C subunit were identified^[13]. In other species, the B subunit was also discovered to contain the most members of the three^[8–11]. There are differences in the quantity of the PP2A family among different species, but the B subunit group covers most members, which may be related to the functions of various types of subunits.

PP2A proteins are reported to be involved in several notable biological processes, such as stress-related signaling, which includes abundant hormone-related signal transduction pathways and enzymes^[7–12]. In *Arabidopsis*, PP2A dephosphorylation and auxin efflux proteins' correct orientation are necessary for auxin polar transport^[14]; the PP2A B subunit B' α and B' β activate brassinosteroid signaling by dephosphorylating the BZR1 TF in the nucleus^[15]; the RCN1, a PP2A A subunit gene, was reported to be involved in the regulation of MeJA/ABA/ethylene signaling^[16]. In rice, the expression of all catalytic subunit genes (*OsPP2A-1-5*) was significantly up-regulated by salinity stress^[17]. In wheat, over-expression of *TaPP2AC-1* in transgenic tobacco enhanced drought tolerance through drought-responsive signal transduction pathways^[18]. While the expression of *TaPP2Ac-4B* and *TaPP2Ac-4D* negatively regulated the defense response to *R. cerealis* infection may modulate the expression of certain pathogen-response (PR) and ROS-scavenging-related genes^[19]. These studies indicated that PP2As have diverse functions in plant stress response, and the walnut PP2A family deserves deep attention for revealing the molecular mechanism of stress adaptation.

In recent years, the diverse roles of PP2A in herbaceous plants, especially its positive roles in response to some abiotic stresses, have attracted widespread attention. However, little is known about the identification and characterization of PP2A genes in woody plants. In this study, we identified the walnut PP2A gene family members and further analyzed their phylogenetics, gene structure, chromosome location, conserved motifs, conserved domains, expression patterns, and interaction proteins. Moreover, transgene lines overexpression of candidate walnut PP2A genes were obtained to confirm the roles in response to osmotic stress. The results of the current study revealed that walnut PP2A genes play positive roles in osmotic stress response by interacting with JrVIP1 protein to control ROS scavenging in a Ca- and ABA-dependent manner.

Materials and methods

Plant materials and treatments

Three-year-old 'Xiangling' walnut (a variety widely planted in China) grafting seedlings were planted in flowerpots and grown in a greenhouse at the College of Forestry, Northwest Agriculture and Forestry University (China) with a 14/10 h photoperiod and relative humidity 70% \pm 5% under 22 \pm 2 °C and used as the materials for the stress treatments. The soil and growth environment as well as the genetic background are consistent. Considering that osmotic stress always resulted from drought, and saline-alkali conditions and PP2A genes probably relate to ABA signaling, the plants were treated with 20% (w/v) PEG₆₀₀₀, 300 mmol/L NaCl, 20 mmol/L CaCl₂ and 30 μ mol/L ABA by watering the roots, respectively. Normally watered plants were used as controls. After treatment for 5 d,

the leaves were collected and stored at –80 °C for RNA isolation. All treatments were replicated three times and each replicate included five seedlings.

Identification of PP2A members from walnut transcriptomes

The walnut leaf transcriptomes under stresses of drought, salt, and ABA (same as above treatments) were sequenced and a preliminary sequence set was obtained. Then firstly 'protein phosphatase' was subjected to search the transcriptomes' database to obtain PP2A family candidate sequences, which were further separately blasted (BLAST: Basic Local Alignment Search Tool (nih.gov)) to judge what homologous proteins they belong to. Next, the open reading frame (ORF) of each potential walnut PP2A (marked as JrPP2A) was confirmed using ORF Finder (www.ncbi.nlm.nih.gov/orffinder). The conserved domains of JrPP2A proteins were confirmed based on the online tools including CD-Search (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Pfam (<http://pfam.janelia.org/>), and SMART (<http://smart.embl-heidelberg.de/>). The conservative motifs were found using MEME online tools (<https://meme-suite.org/meme/>) and Tbttools^[20] with the following parameters: the motif number was 20, any repetition with motif width 17~50. After these analyses, members of the JrPP2A family were confirmed. The amino acid number, molecular weight and theoretical isoelectric point (pI) of JrPP2A proteins were analyzed by Expasy (<https://web.expasy.org/protparam/>). To clarify the evolutionary relationship of JrPP2A proteins, a neighbor-joining phylogenetic tree with a bootstrap replicate value of 1,000 was constructed in MEGA7 using the JrPP2A proteins, 25 Arabidopsis PP2A proteins downloaded from TAIR and 63 *H. brasiliensis* PP2A proteins downloaded from NCBI (*Hevea brasiliensis* (ID 503)-Genome-NCBI (nih.gov)). The phylogenetic tree was modified using Evolview (www.evolgenius.info/evolview) and the JrPP2A members were grouped referring to the topology of the phylogenetic tree.

Chromosomal location, gene structure and promoter analysis of JrPP2As

The genomic DNA sequence of JrPP2As were confirmed according to the walnut (*Juglans microcarpa* \times *J. regia*) genome (www.ncbi.nlm.nih.gov/genome) and the chromosomal location information of JrPP2As was determined. The exon-intron structure of each JrPP2A was defined using Gene Structure Display Server 2.0 (GSDS 2.0: <http://gsds.gao-lab.org/>). An upstream 2,000 bp promoter sequence of each JrPP2A was obtained according to the genome, and the potential cis-acting regulatory elements of the promoters were predicted by PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>).

Expression analysis of JrPP2As under osmotic stress

The leaves of 3-year-old walnut plants treated separately by PEG₆₀₀₀, NaCl, CaCl₂ and ABA for 5 d were sampled for RNA isolation. The total RNA of each sample was isolated using the cetyltrimethylammonium ammonium bromide (CTAB) method^[1, 5] and digested with DNase (Takara, Dalian, China) to ensure the RNA quality. The RNA concentration was tested using the Thermo Scientific™ NanoDrop™ One. 0.5 μ g RNA of each sample was reversely transcribed into cDNA using the Prime Script™ RT reagent Kit (CWBI, Beijing, China). The cDNA was diluted 10-fold with ddH₂O and used as the template of

real-time fluorescent quantitative PCR (qRT-PCR). The 20 μ L reaction solution contains 10 μ L SYBR Green Real-time PCR Master Mix (CW BIO), 0.5 μ M each forward and reverse primer, and 2 μ L cDNA template. StepOne™ Real-Time PCR System was adopted to perform the qRT-PCR. The amplification parameters: 94 °C/30 s, followed by 44 cycles of 94 °C/12 s, 60 °C/30 s, 72 °C/40 s, then 81 °C/1 s. The internal reference gene is *18S rRNA* (HE574850)^[21]. All related primers are listed in [Supplemental Table S1](#). The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method^[22].

Yeast two hybrid assay

Yeast two hybrid (Y2H) assay was used to clarify whether JrPP2A proteins could interact with basic leucine zipper protein (bZIP) JrVIP1. *JrVIP1* was cloned into pGBKT7 vector (marked as BD) to form the bait recombinant (BD-JrVIP1). Each *JrPP2A* was independently inserted into the pGADT7_Rec vector (marked as AD) to generate the prey recombinants (AD-JrPP2As). Meanwhile, *JrVIP1* was inserted into pGADT7_Rec to form AD-JrVIP1, while each *JrPP2A* gene was independently cloned into pGBKT7 to generate BD-JrPP2As. Then the interaction between BD-JrVIP1 and each AD-JrPP2A as well as AD-JrVIP1 and each BD-JrPP2A were confirmed in yeast Y2H by grown on the SD/-Ade/-His/-Leu/-Trp/X- α -Gal/Aureobasidin A (QDO/X/A) medium plates. The empty AD and BD functioned as interaction control^[1,5]. The related primers were listed in [Supplemental Table S2](#).

Qualitative and quantitative analysis of reactive oxygen species

To understand the osmotic stress response function, the *JrPP2A* genes were separately inserted into the pROKII vector to generate recombinant vectors 35S::*JrPP2A*s. Then each 35S::*JrPP2A* was transformed into *Arabidopsis* using the *Agrobacterium tumefaciens*-mediated method^[1,5]. The kanamycin-resistant transformed seedlings were further confirmed by PCR and qRT-PCR methods. The most overexpression lines were chosen for osmotic stress response analysis. The seeds of WT and *JrPP2A* transgenic lines were sown on 1/2MS (Murashige and Skoog) agar medium for 12 d, then the seedlings were transferred into soil to grow to one month old for treatments, including 10% PEG₆₀₀₀, 10% PEG₆₀₀₀ + 10 mol/L CaCl₂, 10% PEG₆₀₀₀ + 10 μ mol/L ABA, 100 mmol/L NaCl, 100 mmol/L NaCl + 10 mol/L CaCl₂, 100 mmol/L NaCl + 10 μ mol/L ABA. After 3 d of treatments, the leaves were harvested for reactive oxygen species (ROS) determination. The 3,3'-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining were applied to qualitative confirmation of the ROS generation. The H₂O₂ content was determined using the Hydrogen Peroxide Assay Kit (colorimetry, A064-1, NJC BIO, Nanjing, China). The total ROS content was tested using the chemiluminescence method according to the manufacturer's instructions of the Reactive Oxygen Species Assay Kit (E004, NJC BIO).

Determination of calcium content in transgenic Arabidopsis plants

The leaves of WT and *JrPP2A* transgenic lines were ground and dried into a powder, independently. Then each sample powder was placed into a centrifuge tube, in which 1.9 mL of 1% HCl was added and mixed well with a vortex shaker. The mixture was centrifuged (15,000 r/min) after digested at 37 °C for 48 h. 1.5 mL of the supernatant was taken to measure the

concentration of Ca²⁺ using a TAS-990 atomic absorption spectrometer at 422.7 nm.

Pull-down assay

The CDSs of *JrPP2As* and *JrVIP1* were independently cloned into the vectors of pET30a and pGEX4T-1 and then transformed into Rosetta for expression of JrPP2A-His and GST-JrVIP1 proteins by using 0.1 mM IPTG (isopropyl-b-thiogalactopyranoside). Soluble GST or GST-JrVIP1 fusion proteins were extracted and immobilized using a glutathione HiCap matrix (Qiagen). JrPP2A-His was incubated with immobilized GST or GST-JrVIP1, and the interaction was checked by western blotting analysis. The related primers were included in [Supplemental Table S2](#).

Statistical analysis

All the data were organized and analyzed using Excel 2023 and SPSS (Chicago, Illinois, USA). The sample variability was described by standard deviation (S.D.) from three repeated assays. The differences between WT and *JrPP2A* transgenic lines were evaluated using Tukey's multiple comparison test ($p < 0.05$).

Results

Sequence characteristics and nomination of walnut PP2A proteins

A total of 18 putative *JrPP2A* genes were screened from walnut transcriptome, among these 18, three lacked PP2A catalytic domain. Therefore, 15 genes in *J. regia* were identified as PP2A family members in the current study. To better describe these 15 *JrPP2A* genes, their positions on the walnut chromosomes were analyzed, and found that these 15 walnut PP2A genes were unevenly distributed on 10 chromosomes: Four were distributed on the 15th chromosome, which has the maximum number of *JrPP2As*, followed by the 01st and 16th chromosome, those have two *JrPP2As*, accordingly; The chromosomes of 02nd, 03rd, 05th, 09th, 10th and 12th, each covers only one *JrPP2A* gene. In addition, there was no *JrPP2A* member presented on the chromosomes of the 04th, 06th, 07th, 11th, 13th and 14th. Considering the conciseness of the description, the *JrPP2A* genes were named *JrPP2A01* to *JrPP2A15* according to their orders in the chromosomes ([Fig. 1](#), [Table 1](#)). The ORFs of the 15 *JrPP2As* were between 651 bp (*JrPP2A12*) and 1,764 bp (*JrPP2A10*), consisting of 216~587 amino acids. The molecular weight of the proteins ranged from 24.15 kDa (*JrPP2A12*) to 65.61 kDa (*JrPP2A10*), and the pI ranged from 4.80 (*JrPP2A02*) to 8.37 (*JrPP2A01*) ([Table 1](#)).

Classification of JrPP2A proteins according to phylogenesis and gene structure

Classification may have a potential relationship with gene function. Therefore, the 15 *JrPP2A* proteins were classified according to the widely accepted approaches mainly including evolutionary relationship and gene structure. To investigate the genetic relationship, total 25 *Arabidopsis* PP2As (three A subunits-*AtPP2AA*, nine B' subunits-*AtPP2AB'*, six B'' subunits-*AtPP2AB''*, two B55 subunits-*AtPP2AB55*, and five C subunits-*AtPP2AC*), 36 rubber tree PP2As (four A subunits-*HbPP2AA*, 14 B' subunits-*HbPP2AB'*, six B'' subunits-*HbPP2AB''*, four B55 subunits-*HbPP2AB55*, and eight C subunits-*HbPP2AC*) as well as the 15 walnut PP2As were aligned to construct a phylogenetic tree

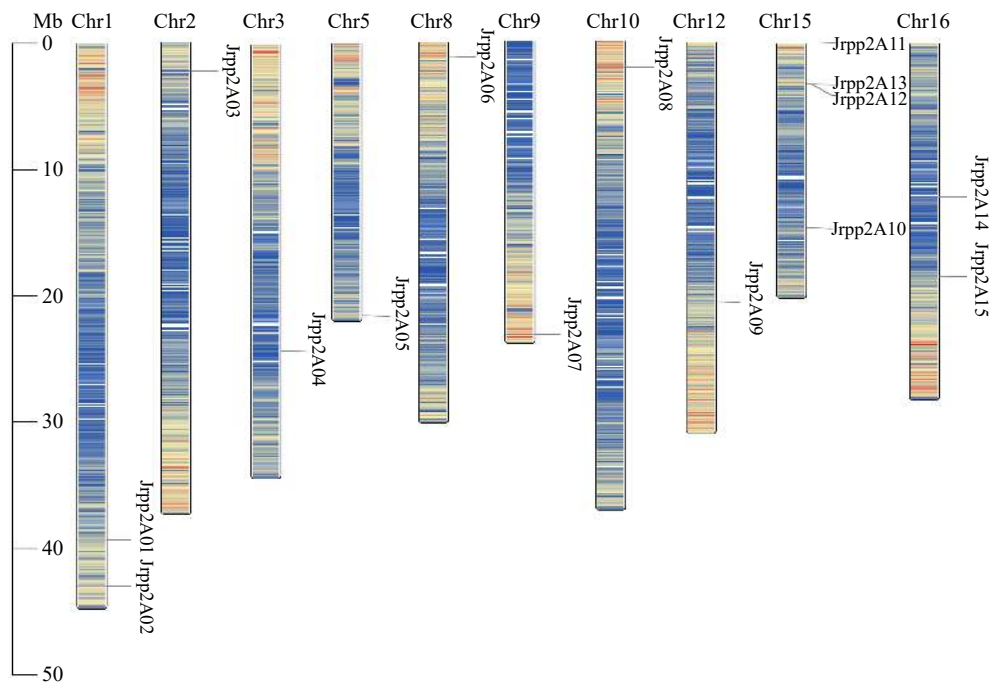


Fig. 1 Distribution of the *JrPP2As* on chromosomes of the *J. regia* genome. The chromosome number is shown on the top side of each chromosome.

Table 1. Sequence characteristics of 15 *JrPP2As*.

Gene names	Type	Transcriptome No.	Gene Bank accession No.	Gene ID	Chromosome	ORF length (bp)	Number of amino acids	Molecular weight (kDa)	pI
JrPP2A01	B'	comp30409_c0	XM_018967286.2	LOC108992675	ch1	1,452	483	55.13	8.37
JrPP2A02	B''	comp28147_c0	XM_018971752.2	LOC108996025	ch1	1,254	417	49.44	4.8
JrPP2A03	B55	comp26329_c0	XM_018982199.2	LOC109003870	ch2	1,506	501	56.72	6
JrPP2A04	B'	comp20037_c0	XM_018993415.2	LOC109011996	ch3	1,503	500	57.52	6.24
JrPP2A05	C	comp26715_c0	XM_018952439.2	LOC108981323	ch5	681	226	25.7	4.94
JrPP2A06	B'	comp24655_c0	XM_018979195.2	LOC109001785	ch8	1,503	500	56.94	7.61
JrPP2A07	C	comp32898_c0	XM_018969187.2	LOC108994090	ch9	918	305	34.78	5.24
JrPP2A08	B'	comp25187_c0	XM_018958784.2	LOC108986228	ch10	1,575	524	59.87	7.97
JrPP2A09	B''	comp9850_c0	XM_018985083.2	LOC109005962	ch12	1,431	476	54.74	4.93
JrPP2A10	A	comp28348_c0	XM_035685846.1	LOC109013629	ch15	1,764	587	65.61	4.88
JrPP2A11	B55	comp28413_c1	XM_018989098.2	LOC109008856	ch15	918	305	34.06	5.16
JrPP2A12	B55	comp28413_c1	XM_018989097.2	LOC109008856	ch15	651	216	24.15	6.21
JrPP2A13	C	comp27670_c1	XM_018980847.2	LOC109002920	ch15	912	303	34.84	4.88
JrPP2A14	C	comp23892_c0	XM_018963562.2	LOC108989812	ch16	921	306	35.01	4.83
JrPP2A15	B'	comp13497_c0	XM_018951013.2	LOC108980163	ch16	1,530	509	58.52	7.18

using NJ method. As shown in Fig. 2, these PP2A proteins were divided into five clusters according to the branch of the evolutionary tree and subunits covered in *Arabidopsis* and rubber PP2As. The B' cluster covered most PP2A proteins, including five *JrPP2As* (*JrPP2A01*, *JrPP2A04*, *JrPP2A06*, *JrPP2A08*, *JrPP2A15*), nine *AtPP2As* and fourteen *HbPP2As*. Group A had the least number of PP2As, containing one *JrPP2A* (*JrPP2A10*), three *AtPP2As* and four *HbPP2As*. Subfamily B'' covered 2 *JrPP2A* proteins (*JrPP202*, *JrPP209*), while subclass B55 included three *JrPP2As* (*JrPP203*, *JrPP211*, *JrPP212*). The other four *JrPP2As* (*JrPP2A05*, *JrPP2A07*, *JrPP2A13*, *JrPP2A14*) were clustered in C subgroup (Fig. 2).

Considering that the exon-intron structure can provide prominent evidence supporting the phylogenetic relationships of a gene family, the diversity of the intron-exon construction

leads to the gene structure being varied. Thus, a structural analysis was performed using these 15 CDSs and genomic sequences of *JrPP2A* genes in the GSDS online. As shown in Fig. 3a, the intron numbers of the 15 *JrPP2A* genes varied widely among the five subfamilies. In detail, the A and B' sub-family *JrPP2A* genes contained 12 and 11 introns, accordingly. The intron numbers of C subclass *JrPP2A* genes ranged from 6 (*JrPP2A14*) to 10 (*JrPP2A13*). The B55-like *JrPP2A* genes possessed the most introns (14), while the B' sub-group *JrPP2A* genes had the fewest introns (only 1~2). The performance of the introns validated that the structure of exon-intron was similar in the same subfamily, despite some differences in the length of exons. The showing exon-intron structure was consistent with the evolutionary relationship.

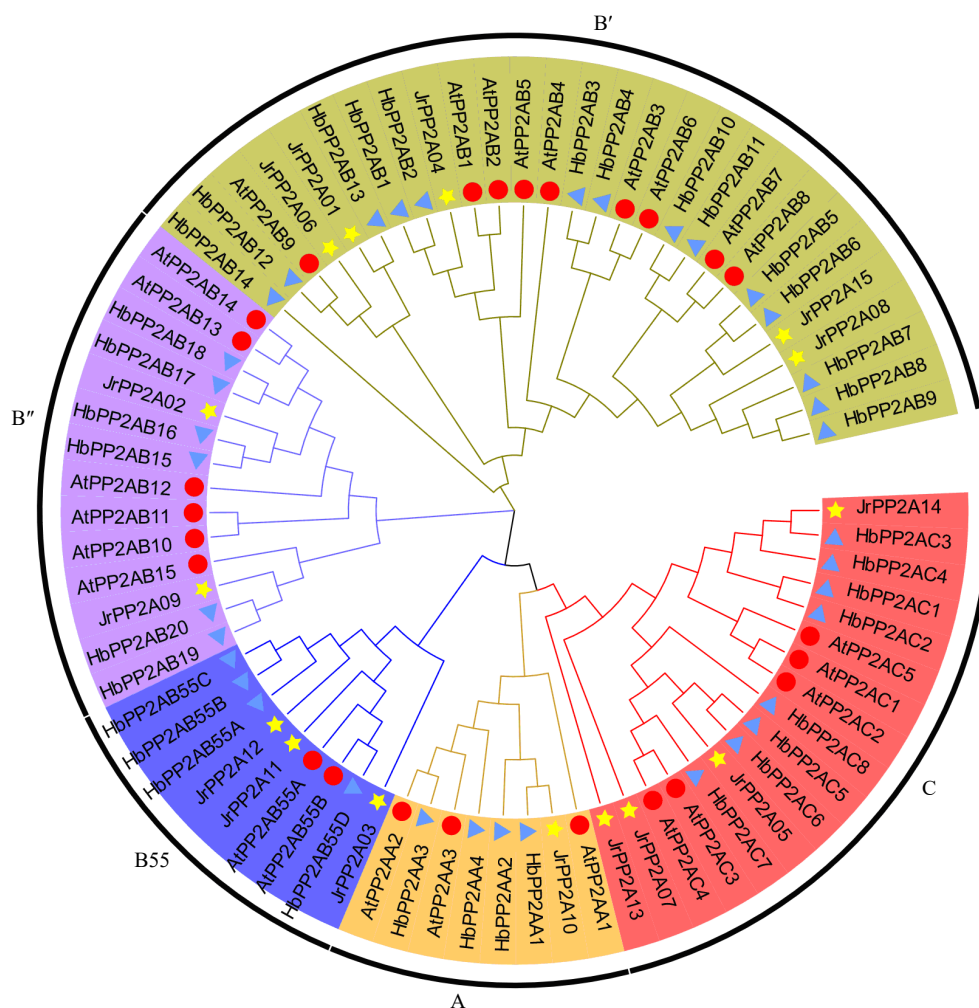


Fig. 2 Phylogenetic relationship of PP2A proteins from *J. regia*, *A. thaliana* and *H. brasiliensis*. A, C, B55, B', B'' means five sub-family of PP2As, respectively, which are displayed in different colors. A total of 15 walnut PP2As are represented by yellow five-pointed stars, 25 Arabidopsis PP2As are represented by red circles, 36 *H. brasiliensis* PP2As are represented by blue triangles.

Conserved motifs and domains of walnut PP2A proteins

A total of 20 conserved motifs were identified from the 15 walnut PP2A proteins using an online MEME tool and the basic information (width and best possible match sequence) was downloaded and displayed in Table 2. Each motif included 17–50 amino acids. The proteins categorized within the same group shared a similar motif composition, which further verified the group definitions. As shown in Fig. 3b, some unique motifs could be detected only in specific subgroups. In detail, motif 19, motif 10, motif 7, motif 9, motif 2, motif 1, motif 3, motif 4 and motif 6 was detected in B' subunit JrPP2A proteins; Motif 14, motif 5, motif 11, motif 1 and motif 8 was found in all C subunit JrPP2As. Motif 16, motif 7, motif 6 and motif 9 was contained in A subunit of JrPP2As; Motif 20, motif 6, motif 15, motif 18 and motif 17 was located in subgroup B55 JrPP2As; while subgroup B'' JrPP2A proteins only contained motif18, motif2 and motif7.

The conserved domains of JrPP2A proteins further confirmed the sub-classifications that were presented by the motifs. As shown in Fig. 3c, the different subunits of JrPP2As were obviously varied, the A sub-family members covered the 'HEAT

superfamily' domain, the B sub-family members covered the 'B56 superfamily' domain, the C sub-family members covered the 'MPP superfamily' domain, the B55 sub-family members covered 'CDC55 superfamily' domain, the B' sub-family members covered 'PPP2R superfamily' domain.

The *cis*-acting regulatory elements in JrPP2As' promoters

To analyze the potential transcriptional regulation of JrPP2A genes in abiotic stress response, putative *cis*-acting elements in JrPP2A promoters were identified. As a result, a total of 55 *cis*-acting elements in the promoters were identified and the JrPP2A genes in the same subfamily possessed similar *cis*-elements. These elements belonged to four types: hormone responsiveness, light responsiveness, abiotic stress response, plant growth, and development (Supplemental Table S3). The elements in 'abiotic stress response' and 'light-responsive' were abundant with 15 and 22 hits (Supplemental Tables S4 & S5), accordingly. The elements Box 4 and G box (light responsive related), MYC (drought and ABA responsiveness related), and MYB (drought-inducible) could be found in most JrPP2As' promoters. In addition, 70% of the JrPP2A genes covered ABA-responsive element (ABRE), suggesting that JrPP2As may be

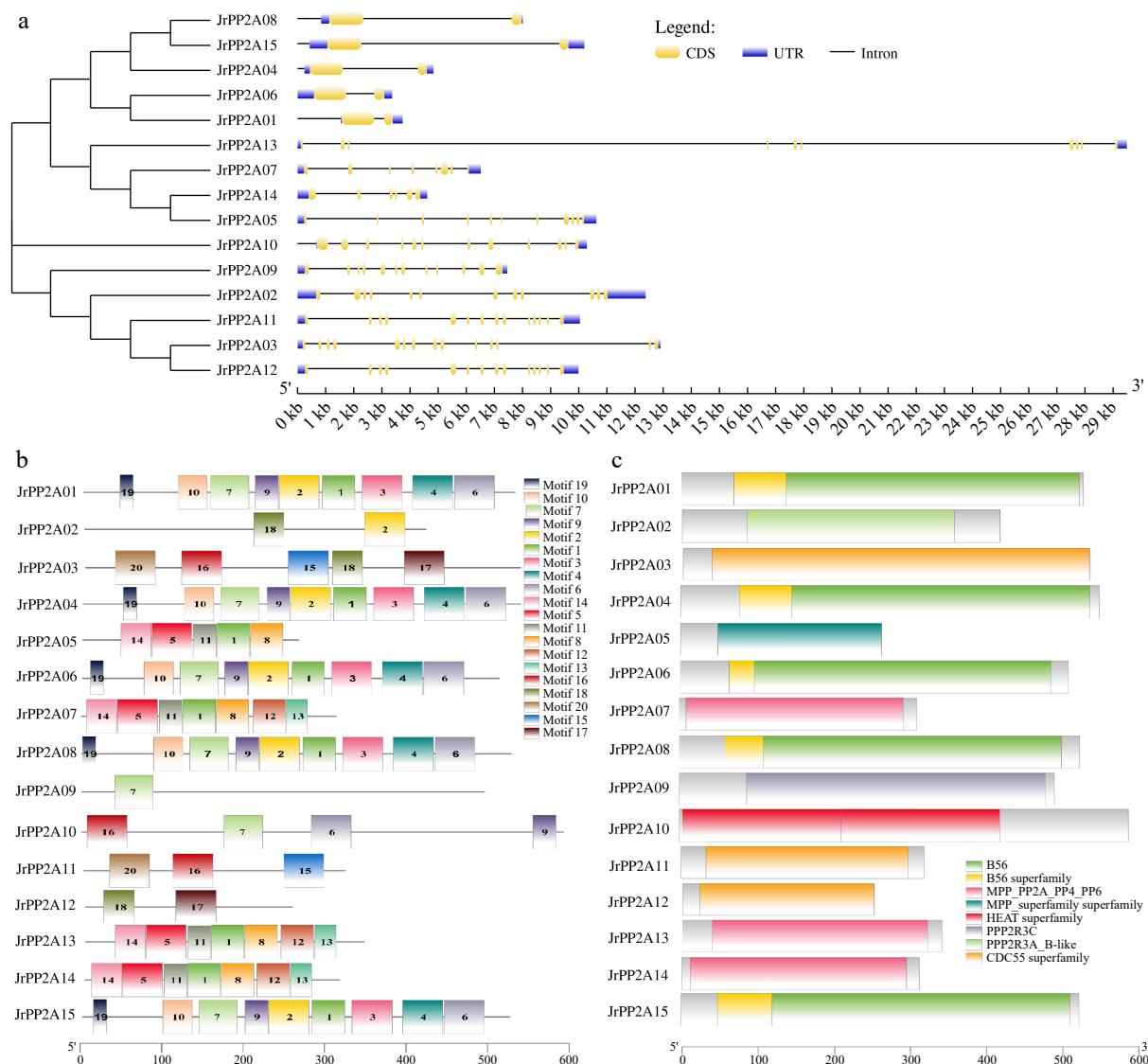


Fig. 3 Gene structure of walnut *PP2As*. (a) Exon-intron distribution map. The vertical phylogenetic tree and gene structure of *JrPP2As* was constructed by GSDS online software. Yellow boxes indicate exons; blue boxes indicate upstream or downstream; black lines indicate introns. (b) Conserved motif analysis: 20 separate patterns were identified with the MEME suite and each pattern was depicted with different colors. (c) Distribution of conserved domains.

associated with the regulation of the ABA pathway. Meanwhile, some elements such as CAT-box and O2 were predicted to be involved in plant growth and development (Supplemental Tables S3–S5). These results implied that the walnut *PP2A* genes may play abundant functions in plant growth, development and response to abiotic stresses.

Expression patterns of *JrPP2A* genes under osmotic stresses and ABA

To validate the possible functions of *JrPP2A* genes in osmotic stress response and whether involving in ABA signaling, the transcript abundance of 15 selected *JrPP2A* genes were analyzed under drought (PEG₆₀₀₀), salt (NaCl), calcium (CaCl₂) and ABA treatment.

Under PEG₆₀₀₀ treatment

The response of *JrPP2A* genes to drought stress was different. *JrPP2A15* was up-regulated to the highest level while *JrPP2A01* was the lowest one. *JrPP2A15* was induced to 1.12~27.86-fold

of other genes. *JrPP2A14* was also up-regulated to exceed 4.00 and ranked just after *JrPP2A15*. The expression value of *JrPP2A12*, *JrPP2A04*, and *JrPP2A03* exceeded 3.00, while the transcription level of *JrPP2A02*, *JrPP2A05*, and *JrPP2A10* was 1.54~1.68. The other five (*JrPP2A06*, *JrPP2A08*, *JrPP2A09*, *JrPP2A11*, *JrPP2A13*) genes differed not obviously with a level between 2.26 and 2.85 (Fig. 4a).

Response to NaCl stress

Most of the *JrPP2A* genes except *JrPP2A02* were induced by NaCl with varied levels. The expression level of *JrPP2A14* was the maximum while not significantly different from the second gene *JrPP2A04*. The transcription values of *JrPP2A12*, *JrPP2A03*, *JrPP2A08*, and *JrPP2A15* were similar and ranged from 2.74 to 2.42. The expression levels of *JrPP2A05*, *JrPP2A07*, *JrPP2A10*, *JrPP2A11*, and *JrPP2A13* were changed not significantly, their values were 2.01~2.22. The expression value of *JrPP2A09* (1.32) was significantly lower than the above 10 up-regulated genes

Table 2. Motif sequences identified by the MEME tool.

Motif	Width	Motif consensus
1	41	TIVYGFYDETERHNGIAELLEIFGSIIDGFALPLKEEHKIF
2	50	KVAKRYIDHSFVLRLDLFDSEDPREYELKTLHRIYKGFMVHR PFIRK
3	50	HKPKSIGLYHQQLSYCITQFVEKDPKLADTVIRGLLKYPVPTNS QKEVMF
4	50	PAEFQRCMVPLFRQIGCCNLSSHQVAERALFLWNNDHIVNLI AQNRNVI
5	50	NVQPVKSPVTICGDIHQGFHDLIELFRIGGNCPDTNYLFMGDY VDRGYYS
6	50	PIIFPALEKNARSHWNQAVQNLTLNVRKIFSEMDPELFEECQR QFQDEA
7	48	DIKRQTLIELVDFVASGSGKFTETAQEMIKMVSVNLFRLVPPK REN
8	41	CLHGLSPSIETLDNIRVIDRIQEVPHGPMCDLLWSDPDD
9	29	EPSFDPAPWPHLQJYVELLLRFVSSSETDA
10	37	VEALPAFKDVPNSEKQNLFISKLNLCVDFDFDPTK
11	29	ETFTLLALKVRYPDRTJLRGNHESRQI
12	41	WGVSPRGAGYLFGGDVVSQFNHTNNLDLICRAHQVMEGYK
13	27	WFQDKGIVTVWASAPNYCYRCGNVAAIL
14	38	SHADLDRQIEQLKECKPLPEAEVKVLCDAKEILVEES
15	50	AHAHDFNINSINSNSDGETFISADDLRLNWLNEISNQCFNIID MKPANM
16	50	YKTEFQSHEPEFDYLSLEIEEKINKIRWCQTQNGALLSSNDK TIKFW
17	50	MDSGPVATFKVHENLRPKLCELYENDSIFDKFECCJSGDGJHF ATGSYSN
18	38	EVITSAEFHPIHCNLLAYSSSRGFIRLIDMRCSALCDQ
19	17	TMIKQILSKLPRKPSKS
20	50	PLEWKFSQVQGERPAGEEVQEVDIISAIEFDKSGDHLAVGDRG GRVVJFE

while higher than the other three (*JrPP2A01*, *JrPP2A02*, and *JrPP2A06*). Meanwhile, *JrPP2A01* and *JrPP2A06* displayed similar expression levels (Fig. 4b).

Under CaCl_2 treatment

Most *JrPP2A* genes excluding *JrPP2A01* were induced by CaCl_2 with a large range (0.55~5.39). The expressions of *JrPP2A02*, *JrPP2A05*, *JrPP2A06*, and *JrPP2A13* were at a level less than 1.00. *JrPP2A09*, *JrPP2A07*, *JrPP2A08*, and *JrPP2A10* were up-regulated to 2.06~2.62, in which *JrPP2A08* was significantly higher than *JrPP2A07* and *JrPP2A10*. *JrPP2A03* and *JrPP2A15* were similar with the expression values 3.01 and 3.28, accordingly. Meanwhile, the difference between *JrPP2A03* and *JrPP2A08* was not significant. The other four, *JrPP2A04*, *JrPP2A11*, *JrPP2A12*, and *JrPP2A14*, was showed most obviously transcription and exceed 4.00 (Fig. 4c).

Under ABA treatment

All *JrPP2A* genes were induced by ABA. *JrPP2A04* was transcribed highest and significantly differed from all others. The expression levels of *JrPP2A14* and *JrPP2A12* also exceeded 4.00 and ranked second and third, accordingly, with a non-significant difference. *JrPP2A15*, *JrPP2A11* and *JrPP2A03* were up-regulated to a level ranging from 3 to 4. The expression values of *JrPP2A09* and *JrPP2A05* were 2.22~2.75 with significant difference. *JrPP2A01* and *JrPP2A06* were transcribed with a level lower than 1.00. The other five genes (*JrPP2A02*, *JrPP2A07*, *JrPP2A08*, *JrPP2A10* and *JrPP2A13*) were expressed in the level between 1.16~1.82 (Fig. 4d).

Overexpression of *JrPP2As* promote osmotic stress tolerance involving in Ca and ABA signal

To confirm the roles of *JrPP2As* in osmotic stress response, three members, *JrPP2A07*, *JrPP2A9*, and *JrPP2A14*, were chosen

according to classification and expression under PEG_{6000} , NaCl, CaCl_2 , and ABA treatments. *JrPP2A07*, *JrPP2A9*, and *JrPP2A14* were separately overexpressed in *A. thaliana*. The transgenic lines with the highest expression value (160.89-, 112.20-, 101.83-fold of WT, accordingly) were selected for analysis (Supplemental Fig. S1a). The results showed that the 42-d old of WT and transgenic seedlings were grown normally without ROS generation under control condition, however, when exposed to PEG_{6000} and NaCl stress, NBT staining (represent O_2^-) of WT was deeper than that of transgenic lines (Fig. 5a). Quantitative determination of H_2O_2 also revealed a similar pattern as NBT staining (Supplemental Fig. S2a). The total ROS content of WT was 1.49~1.68-fold and 1.37~1.48-fold of *JrPP2A07*, *JrPP2A9*, and *JrPP2A14* transgenic lines under PEG_{6000} and NaCl stress, accordingly (Fig. 5b). Moreover, the cell damage reflected by electrolyte leakage (EL) rate was similar to ROS accumulation. The EL rate of WT was 1.53~1.91-fold and 1.57~1.73-fold of *JrPP2A07*, *JrPP2A9*, and *JrPP2A14* transgenic lines under PEG_{6000} and NaCl stress, accordingly (Supplemental Fig. S1b). However, the changes of catalase (CAT) activity were opposite to that of ROS content and EL rate (Supplemental Fig. S1c). These results suggested that *JrPP2A07*, *JrPP2A9*, *JrPP2A14* could positively improve plant drought and salt inducing osmotic stress tolerance.

Considering the up-regulating expression by CaCl_2 and ABA, we want to know whether the *JrPP2A* genes are associated with Ca and ABA signaling when responding to osmotic stress. Therefore, the 42-day old seedlings were also treated simultaneously with PEG_{6000} and CaCl_2 , PEG_{6000} and ABA, NaCl and CaCl_2 , NaCl and ABA. Interestingly, we observed that compared to pure PEG_{6000} and NaCl treatments, the addition of CaCl_2 and ABA resulted in changes in resistance physiological indicators of *JrPP2As*' transgenic plants, suggesting further improvement in osmotic stress resistance (Fig. 5a & b, Supplemental Fig. S1b & S1c). The NBT staining, H_2O_2 content and total ROS accumulation under PEG_{6000} + CaCl_2 and PEG_{6000} + ABA was less than under PEG_{6000} , under NaCl + CaCl_2 and NaCl + ABA was less than under NaCl, accordingly (Fig. 5a & b, Supplemental Fig. S2a). The cell damage was alleviated after adding CaCl_2 and ABA. The EL rate of transgenic lines under NaCl + ABA was 52.56%~84.00% of that under NaCl (Supplemental Fig. S1b), while the CAT activity was increased by adding CaCl_2 and ABA (Supplemental Fig. S1c). Moreover, the Ca uptake in *JrPP2A* transgenic plant cells was improved compared to WT. Under control conditions, the Ca content in transgenic plants' cells was 1.06~1.13-fold of WT. When exposed to PEG_{6000} and NaCl, the degree of Ca increases in transgenic plants was also higher than that of WT. The Ca content of WT and transgenic lines under PEG_{6000} stress were 1.18~1.38-fold of themselves under control. Adding CaCl_2 and ABA significantly improved the Ca accumulation compared to a single treatment of PEG_{6000} and NaCl (Fig. 5c), further confirmation of the relationship of Ca with osmotic stress in *JrPP2A* genes' response pathway.

Interactions between JrVIP1 protein and JrPP2As

JrVIP1 is a basic leucine zipper protein (bZIP) and homologous to the VirE2-INTERACTING PROTEIN1 (VIP1) from *A. thaliana* (Supplemental Fig. S3), whose dephosphorylation can be mediated by PP2A in osmotic and other stresses^[11, 23]. In our previous study, to analyze the relationship between walnut response to drought and demethylation, we constructed BD-JrVIP1, which was used to screen the possible interacting

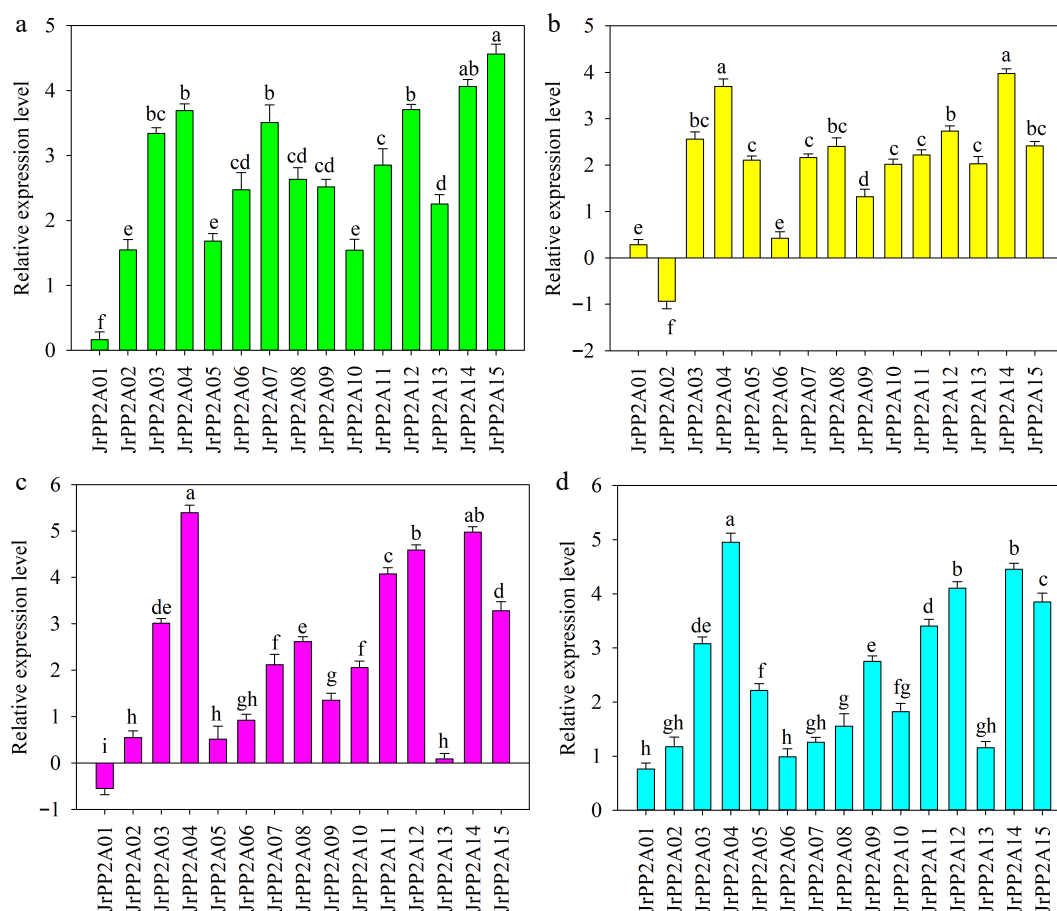


Fig. 4 QRT-PCR analysis of the expression of *JrPP2A* genes under drought, NaCl, CaCl₂ and ABA treatments. The relative expression level is expressed as relative to the internal reference gene and at 0 h. Error bars represent the SD (n = 3). Lowercase letters indicate significant differences among different *JrPP2A* genes under each treatment according to the Student's t-test ($p < 0.05$). (a) PEG₆₀₀₀ stress. (b) NaCl stress. (c) CaCl₂ treatment. (d) ABA treatment.

proteins from the walnut yeast two hybrid library, and found that there were many *JrPP2A* members. To verify whether the biological function of the *JrPP2A* family members was associated with *JrVIP1*, in this study, AD-*JrPP2As* were constructed and submitted to the Y2H system to confirm the interaction with BD-*JrVIP1*. The results showed that *JrPP2A02*, *JrPP2A05*, *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* could interact with *JrVIP1*, respectively (Fig. 6a). The interactions were also tested by AD-*JrVIP1* and BD-*JrPP2As* (Fig. 6b). Moreover, *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* were selected to further verify their interactions with *JrVIP1* by an *in vitro* pull-down assay (Fig. 6c). These data confirmed that *JrVIP1* could interact with *JrPP2A07*, *JrPP2A09*, and *JrPP2A14*. Since *VIP1* homologous were believed to be a vital protein for phosphorylation or dephosphorylation in stress response^[11, 23], we are more convinced that the *JrPP2A* genes are related to stress response involving in phosphorylation.

Discussion

The plant *PP2A* gene has been regarded as important members in response to various external stimulus^[24, 25]. To provide scientific guidance in the cultivation and management of walnut trees and then to guarantee the yield and quality of walnuts under adverse conditions, the molecular mechanism of adaptation to adversity is urgently to be revealed. Many

environmental factors may lead to osmotic stress, so the regulation of osmotic stress response is particularly noteworthy. Therefore, the walnut transcriptomes under different abiotic stresses were sequenced to establish a basic database for identifying key genes that adapt or respond to environmental stresses. In the current study, 15 *JrPP2A* genes with prominent expression levels that can respond to various stressors were identified from the transcriptome data mentioned above. This quantity, 15 *JrPP2A* genes, is less than the number of members of the *PP2A* family in other plants such as *Arabidopsis* and rubber trees. The possible reason is that the *JrPP2A* family genes selected in this study are shared genes screened from transcriptomes under various stresses, and mainly transcriptionally expressed genes that respond to osmotic stress. It cannot be guaranteed that all members can be induced to express under stress, nor can these genes be screened in the transcriptome database. Walnuts are likely to have other *PP2As* that may not be clear about their response to osmotic stress. In terms of evolutionary relationship, the 15 *JrPP2A* proteins shared a high similarity with the *PP2As* from other species, such as *A. thaliana*^[11, 23], *O. sativa*^[26], and *H. brasiliensis*^[13] and grouped into five subfamilies (Fig. 1, Table 1). The ORF length, pl, amino acid number, and molecular weight of *JrPP2As* were similar to those from *Arabidopsis* and *H. brasiliensis*^[11,13,27], confirming that these 15 *JrPP2As* belong to *PP2A* protein

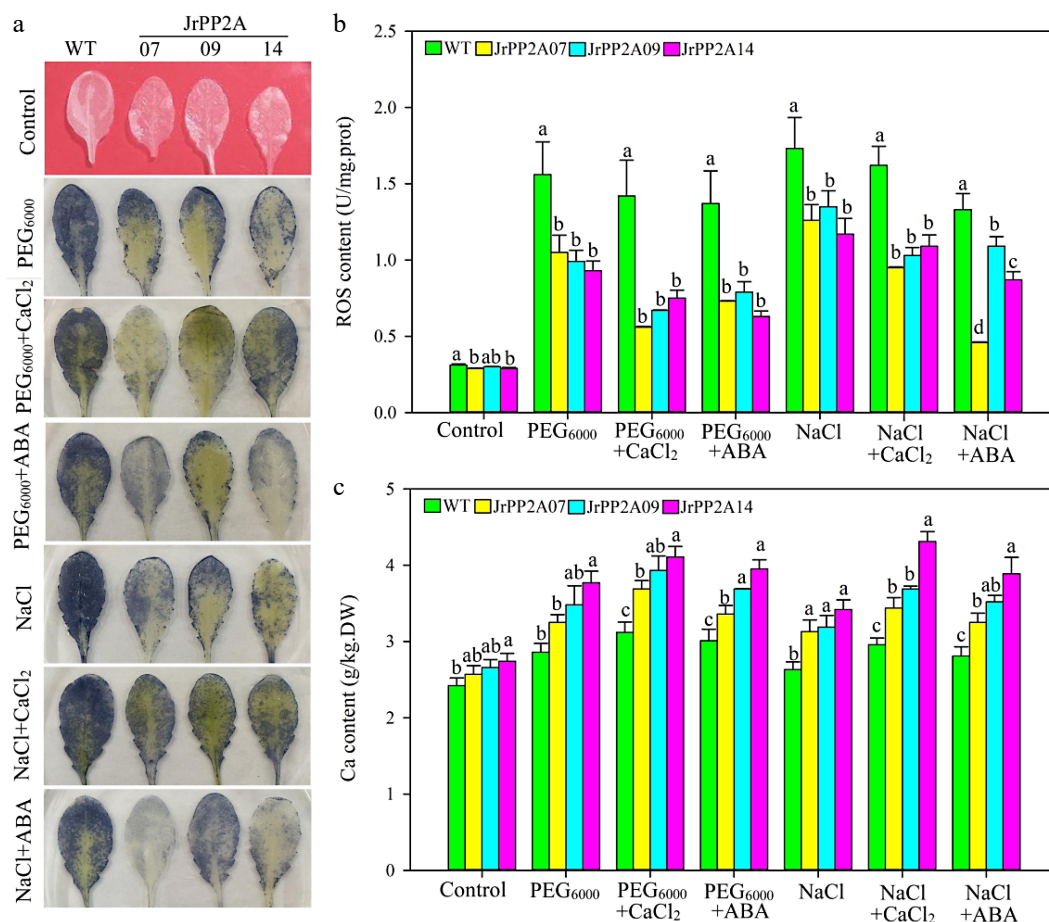


Fig. 5 Osmotic stress response function of *JrPP2As*. WT, wild type. *JrPP2A07*, *JrPP2A09*, *JrPP2A14*, the transgenic lines overexpression of *JrPP2A07*, *JrPP2A09*, *JrPP2A14*. Forty two-day old seedlings were treated with PEG₆₀₀₀, PEG₆₀₀₀ + CaCl₂, PEG₆₀₀₀ + ABA, NaCl, NaCl + CaCl₂, NaCl + ABA for 5 d. Control was normally watered. The significant differences among WT, *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* were marked with lowercase ($p < 0.05$). (a) NBT staining. (b) Total ROS content. (c) Total Ca content.

family.

To understand the biological functions of *PP2A* family members, originally, both intron/exon organizations and protein motif patterns were analyzed, and found that *JrPP2As* displayed diverse gene and protein structures. The intron/exon structure is an important pathway for gene functional evolution and the main reason for functional differences with its homologous proteins^[28]. Despite the conserved distribution pattern of exons in subgroups B and C, many other subgroups displayed diversity in exon structure and number, which was consistent with the protein pattern (Fig. 3). Most of B' subunit type *JrPP2As* contained motifs of 19, 10, 7, 9, 2, 1, 3, 4, and 6, which were related to B56 domain^[7, 13, 24]; while *JrPP2As* were related to MPP domain^[7, 24] (Fig. 3c). Because different motifs and the number of motifs were related to functions^[25–27], our results suggested that *JrPP2As* have potential abundant roles in walnut. Next, given that plant promoter *cis*-acting elements perform essential roles in regulating gene expression and may imply metabolic pathways and stress response^[29,30], the *cis*-acting elements in *JrPP2As* promoters were detected. We screened abundant *cis*-elements related to drought and salt stress response (Supplemental Tables S3–S5), such as MYC elements, W-box, MBS elements, MYB recognition site, TCA-element. The results preliminarily suggested that these *JrPP2As* may be involved in stress responses such as drought and salt

regulated by the upstream promoter elements.

Consequently, the response patterns of 15 *JrPP2A* genes exposed to PEG₆₀₀₀ and NaCl were determined and found that most of the *JrPP2As* were significantly induced by PEG₆₀₀₀ and NaCl. The transcription activity of genes in response to different stresses may effectively predict their potential functions. *Arabidopsis PP2AC5* was reported to be induced by drought and salt stresses, when it was overexpressed, the transgenic *Arabidopsis* was verified to improve the drought and salt stress tolerance^[31]. Compared with the loss-of-function mutant *pp2a-c5-1*, *PP2AC5* overexpression lines were conferred with better root and shoot growth under salt treatments^[32]. The drought and salt inducible *GmPP2A-B''71* could also enhance plant tolerance to drought and salt stresses via overexpression in soybean^[33]. A novel Ca²⁺-binding protein, named AtCP1 (AtPP2A-B''43), can be up-regulated by NaCl treatment, was also believed to positive on salt stress response^[34]. The mRNA levels of *Solanum tuberosum PP2Ac1*, *PP2Ac2a*, *PP2Ac2b* and *PP2Ac3* in leaves were up-regulated by salt stress, suggesting that the subunits might have vital roles in response to drought and salt stress^[35]. These reports and the inducible expression of *JrPP2As* let us believe that *JrPP2A* genes are likely to play roles in drought and salt stress response. From the expression level, *JrPP2A04*, *JrPP2A14* and *JrPP2A15* were the top three while *JrPP2A01* and *JrPP2A02* were the bottom two those may

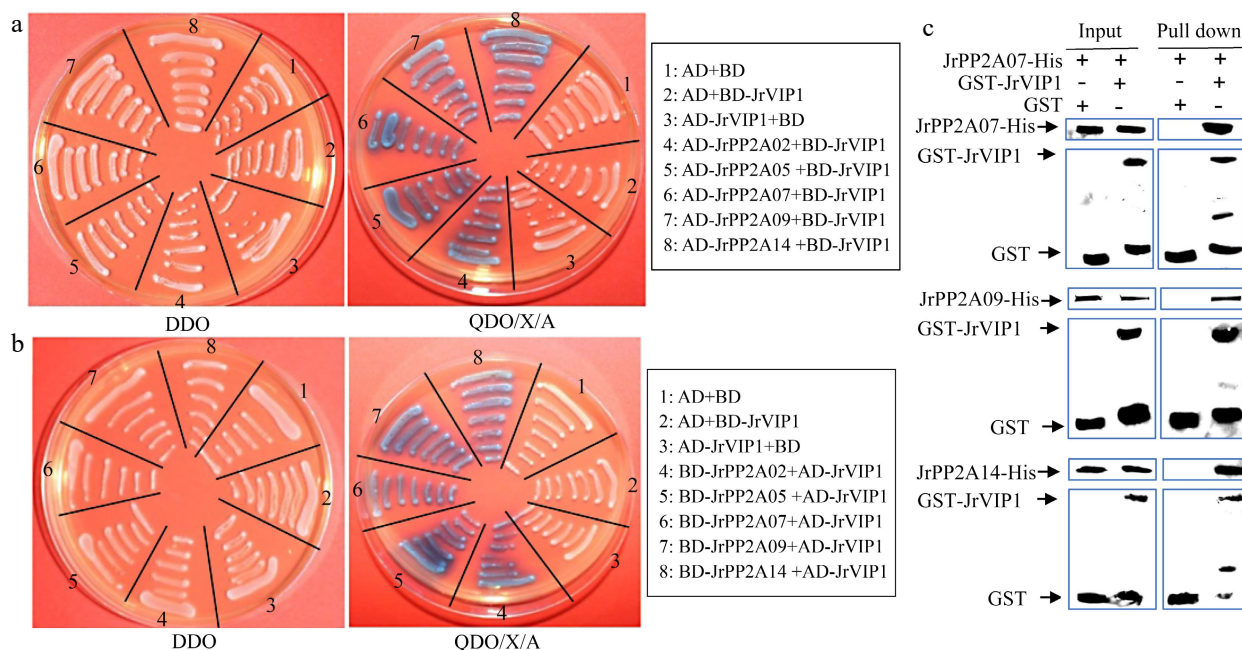


Fig. 6 Interaction analysis of JrVIP1 and JrPP2A proteins using yeast two-hybrid (Y2H) and *in vitro* pull-down assay. AD+BD, AD+BD-JrVIP1, AD-JrVIP1+BD, negative control. The DDO plate was used as positive control for growth. (a) JrVIP1 was used as the bait. (b) JrVIP1 was used as the prey. (c) An *in vitro* pull-down assay demonstrates the interaction between JrVIP1 with JrPP2A07, JrPP2A09, JrPP2A14. JrPP2A-His protein was incubated with immobilized GST or GST-JrVIP1 protein, and immunoprecipitated fractions were detected by anti-His antibody. The assay was performed three times with the same result.

deserve further attention (Fig. 4a–c).

To confirm the functions of *JrPP2A* genes in drought and salt-inducing osmotic stress response, *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* were independently overexpressed in *A. thaliana* and found that the transgenic plants showed less ROS accumulation, and lighter cell damage while higher antioxidant enzyme activity than those of WT under PEG₆₀₀₀ and NaCl stress (Supplemental Figs S1 & S2a, Fig. 5), determining that *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* are positive genes in walnut osmotic stress tolerance. Additionally, the process that plant PP2A responding to osmotic stress is implicated in ABA signaling^[36]. In this study, the transcription of *JrPP2As* under ABA treatment was tested and showed that B subunit *JrPP2A* genes were induced obviously by ABA, especially *JrPP2A04*. In other species, such as wheat, *TaPP2AB-α*, a novel B subunit of PP2A, was induced by the response to NaCl, PEG₆₀₀₀, cold, and ABA at the transcriptional level^[37]. Transgenic Arabidopsis overexpression of *TaPP2AB-α* displayed more lateral roots under mannitol or NaCl treatment^[37]. These results indicated that *TaPP2AB-α* could promote plant lateral root growth under osmotic conditions^[37]. TIP41, an interactor of PP2A present in *Arabidopsis*, was induced by long-term NaCl, polyethylene glycol and ABA treatments, proving that TIP41 mediates the participation of PP2A in ABA-mediated mechanisms^[38]. The mRNA levels of *A. thaliana* PP2AA3 in roots and shoots were up and down-regulated by drought and ABA treatments, suggesting that the subunits might have vital roles in response to drought and ABA treatments^[39]. Therefore, we believed that *JrPP2As* in response to osmotic stress involving in ABA signaling.

Under external stimulation, Arabidopsis PP2A protein stimulates the Ca accumulation or transport within cells and in turn activates B'' and C class PP2A proteins^[11, 23]. To investigate whether

the *JrPP2A* family genes are involved in calcium signaling in osmotic stress response, we also invested the transcription activity of the *JrPP2A* genes under the condition of adding exogenous CaCl₂. We were surprised to find that members of *JrPP2A* genes were significantly induced by CdCl₂ (Fig. 4d). Overexpression of *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* in Arabidopsis promoted the Ca accumulation within plant cells under PEG₆₀₀₀ and NaCl stress. Moreover, under CaCl₂ mediation conditions, the ROS accumulation and cell damage were reduced, while antioxidant protection was enhanced, indicating the effective improvement of the plant osmotic stress tolerance with increasing Ca uptake (Supplemental Figs S1 & S2a, Fig. 5). These performances confirmed the positive mediation of Ca in *JrPP2As*' abiotic stress response. The activation of B'' and C subunits of PP2A proteins mediates the dephosphorylation of VIP1^[11, 23]. Hypo-osmotic stress regulates the dephosphorylation and nuclear-localization of VIP1. When cells confronted with mechanical stress, VIP1 was transiently accumulated in the nucleus, where it controls the expression of the target genes and inhibits mechanical stress-induced root waving^[11]. In this process, Ca signal was accompanied to play necessary roles^[23]. These reports told that the interaction between PP2A and VIP is an important pathway for osmotic stress response. In walnut tree, we also discovered that JrVIP1, a homology of Arabidopsis VIP1, could interact with B'' subunit type JrPP2A02 and JrPP2A09 as well as C subunit type JrPP2A05, JrPP2A07 and JrPP2A14 (Fig. 6). Moreover, *JrVIP1* was also up-regulated by osmotic stress (Supplemental Fig. S2b & S2c). Therefore, we believe that the *JrPP2A* genes also mediate VIP1 dephosphorylation in response to osmotic stress and are involved in Ca signaling. Meanwhile, an osmotic stress responsive pathway mediated by JrVIP1 and JrPP2As was summarized

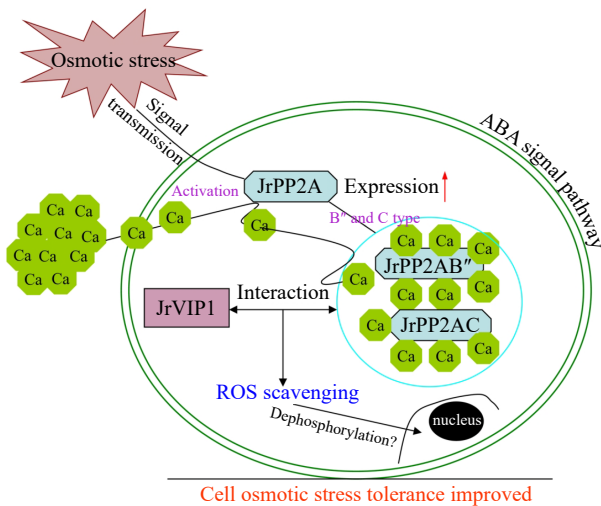


Fig. 7 The *JrVIP1* and *JrPP2As* mediated osmotic stress responsive pathway in walnut trees.

as Fig. 7.

Conclusions

In this study, we identified the *PP2A* family genes from the walnut transcriptome and a total of 15 *JrPP2As* were screened to be unevenly distributed on 10 of the walnut chromosomes. The *JrPP2As* genes were grouped into five subfamilies and members of the same subfamily shared similar gene structures and conserved protein motifs. Promoter element compositions imply that *JrPP2As* may be involved in hormone, light, growth, development, and abiotic stress responses. Most of the *JrPP2A* genes exhibited various expression levels to drought and salt-inducing osmotic stress, among which *JrPP2A07*, *JrPP2A09*, and *JrPP2A14* were improved to play positive roles in osmotic stress response involving Ca and ABA signaling. Moreover, B" and C types of *JrPP2A* proteins mediate the dephosphorylation of *JrVIP1* in osmotic stress response. Our findings generate novel insights into *PP2A* family genes in walnut and lay a foundation for further understanding their biological functions.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: He Y, Yang G; performing experiments: He Y, Chen S, Li C, Yang S; data analysis: Li C, Yang S, Sun Z, Hou S; draft manuscript preparation: He Y, Yang G; data analysis checking and manuscript revision: Yang G, Xu Z. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All the data were presented in the main manuscript and additional supporting files. The *Arabidopsis* and *H. brasiliensis* related datasets generated and/or analyzed during the current study are available in the TAIR database (www.arabidopsis.org) and NCBI (*Hevea brasiliensis* (ID 503)-Genome-NCBI (nih.gov)).

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Conflict of interest

The authors declare that they have no conflict of interest.

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