

# Genetic Dissection of Drought Resistance of Common Bean at the Seedling Stage by Genome-wide Association Study

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## Research Article

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# Abstract

A variety of adverse conditions, including drought stress, severely affect common bean production. Molecular breeding for drought resistance has been proposed as an effective and practical way to improve the drought resistance of common bean. A genome-wide association analysis was conducted to identify drought-related loci based on survival rates at the seedling stage using a natural population consisting of 400 common bean accessions and 3832340 SNPs. The coefficient of variation ranged from 40.90% to 56.22% for survival rates in three independent experiments. A total of 12 associated loci containing 89 significant SNPs were identified for survival rates at the seedling stage. Four loci overlapped in the region of the QTLs reported to be associated with drought resistance. According to the expression profiles, gene annotations and references of the functions of homologous genes in *Arabidopsis*, 39 genes were considered potential candidate genes selected from 199 genes annotated within all associated loci. A stable locus (Locus\_10) was identified on chromosome 11, which contained LEA, aquaporin, and proline-rich protein genes. We further confirmed the drought-related function of an aquaporin (*PvXIP1;2*) located at Locus\_10 by expression pattern analysis, phenotypic analysis of *PvXIP1;2*-overexpressing *Arabidopsis* and *Agrobacterium rhizogenes*-mediated hairy root transformation systems, indicating that the association results can facilitate the efficient identification of genes related to drought resistance. These loci and their candidate genes provide a foundation for crop improvement via breeding for drought resistance in common bean.

## Key Message

A whole-genome resequencing-derived SNP dataset used for genome-wide association analysis revealed 12 loci significantly associated with drought stress based on survival rate after drought stress at seedling stage. We further confirmed the drought-related function of an aquaporin (*PvXIP1;2*) located at Locus\_10.

## Introduction

Common bean is the most important food legume for direct human consumption and provides proteins, vitamins, minerals and income to many people in Africa and Latin America (Beebe et al., 2013; Broughton et al., 2003). The growth, development, and production of common bean require sufficient water. However, climate change is leading us towards a hotter, drier world (Gupta et al., 2020). It is generally considered that common beans have higher water requirements during the growth stage and lower resistance to drought stress than other food legumes, such as chickpea, cowpea or peanut (Broughton et al., 2003). However, approximately 60% of common bean-producing areas around the globe suffer from different degrees of drought stress (Asfaw et al., 2013; Funk et al., 2008; Muñoz et al., 2006). In China, common bean is mainly produced on a small scale and is generally cultivated on relatively infertile land, which is susceptible to abiotic and biotic stresses. Under such conditions, due to the lack of irrigation equipment, common bean production depends on natural precipitation, so drought is the main reason for bean yield losses. Climate models predict that the main production area for common beans in China will become successively drier in the future (Piao et al., 2010). In addition, agricultural water

demand accounts for more than two-thirds of global water consumption, which has increased sharply in recent decades and will further increase in the future (Wada et al., 2011; Fang et al., 2015). It is estimated that the world's population will increase from 7.5 billion presently to 9.7 to 10 billion by 2050, at which time an additional 1 million hectares of arable land will be needed to ensure food security, so the agricultural water demand will accordingly be doubled. However, climate change could give rise to a 50 percent drop in the availability of fresh water (Gupta et al., 2020). Molecular breeding for drought resistance has been proposed to be an effective and practical way to ensure sustained bean productivity (Langridge and Reynolds, 2015; Qian et al., 2016) but requires a better understanding of the genetic architecture of drought resistance. Therefore, the dissection of the genetic basis of drought resistance is indispensable for the improvement of common bean.

Studies on the drought resistance of common bean have been performed for a long time, and many drought-resistant varieties have been selected for crop improvement of common bean (Acosta-Gallegos et al., 1995; Beebe et al., 2008; Singh, 1995; Singh et al., 2001). However, limited drought resistance-related quantitative trait loci (QTLs) have been identified in common bean using different populations and molecular markers. In the absence of an effective linkage map, Schneider et al. (1997) reported nine random amplified polymorphic DNA (RAPD) markers associated with drought resistance, although they were not anchored linkage groups (LGs). A linkage map was generated, which uses an intragene pool RIL population derived from 'BAT477' and 'DOR364' and integrates 186 amplified fragment length polymorphisms (AFLPs), RAPD and simple sequence repeats (SSRs) to map drought resistance-related QTLs based on yield traits (Asfaw et al., 2012; Blair et al. 2012) and root traits (Asfaw and Blair 2012) at the maturity stage in different locations. A total of 5 yield QTLs and 9 root QTLs were found to be associated with drought resistance (Asfaw and Blair 2012; Blair et al. 2012). With the technological development of resequencing and gene microarrays, single nucleotide polymorphisms (SNPs) have been applied to construct linkage maps to dissect the genetic basis of drought resistance in common bean based on yield traits, phenological traits and biomass traits (Mukeshimana et al., 2014; Villordo-Pineda et al., 2015; Trapp et al., 2015; Briñez et al., 2017). Mukeshimana et al. (2014) constructed a linkage map of the RIL population derived from 'SEA5' and 'CAL96' using 2122 SNP markers for the identification of QTLs associated with agronomic traits under drought stress. A total of 14 drought resistance-related QTLs were consistently identified in different environments. More recently, genome-wide association studies (GWASs) have rapidly become a powerful tool for dissecting candidate regions associated with complex quantitative traits and have been successfully applied for identifying drought-related genes in many crop species including maize, rice and wheat (Wang et al., 2016; Guo et al., 2018; Li et al., 2019). In common bean, Hoyos-Villegas et al. (2017) performed a GWAS using 96 common bean accessions and 3968 SNPs for the identification of drought tolerance-related loci based on yield traits, and eight drought-related SNPs were detected. However, all these drought-related studies mentioned above in common bean were conducted at the late growth stage, and the genetic basis of drought resistance at the early stage remains to be elucidated.

Drought can occur during the whole period or at specific stages of crop development, including the seed germination, seedling growth, flowering and grain filling stages. There are differences in drought

resistance mechanisms in different growth stages (Levitt, 1972). In the common bean production area of China, drought stress often occurs at the early growth stage of common bean, which directly affects the rate of germination and seedling growth vigour of common bean and even results in the death of seedlings, severely restricting yield increases (Li et al., 2013). Therefore, identifying stable drought-related QTLs or alleles at early growth stages is also essential for crop improvement in common bean. Wu et al. (2021a, b) conducted a GWAS using 438 common bean accessions to identify drought-related SNPs associated with root and germination traits at the bud stage. A series of candidate SNPs were detected, which greatly enriched the genetic information of drought resistance and root traits at the bud stage, and a set of candidate genes were identified, including transcription factors and protein kinases (Wu et al., 2021a, b). However, genetic information associated with drought resistance of common bean at the seedling stage is lacking. Different characteristics were used to evaluate drought resistance in different growth stages; for instance, yield and phenological traits were used at the late growth stage (Blair et al., 2012; Mukeshimana et al., 2014), and the relative germination rate and germination index were used at the bud stage (Tan et al., 2017). In the seedling stage, the survival rate of seedlings after repeated drought is frequently used to evaluate the drought resistance of different genotypes (Wang et al., 2007; Li et al., 2015; Wang et al., 2015). Qin's research group evaluated the tolerance of maize to severe drought stress at the seedling stage and then performed GWAS based on 368 maize inbred lines and 560000 SNPs to identify a set of drought resistance-related genes (Liu et al., 2013; Mao et al., 2015; Wang et al., 2016). Few studies have dissected the genetic basis of drought resistance in common bean at the seedling stage.

In this study, a natural population consisting of 400 common bean accessions was used to evaluate drought resistance at the seedling stage, and the survival rate after repeated drought was measured. Combining 3832340 SNPs, a GWAS was performed to identify candidate regions associated with drought resistance. Our objective is (a) to identify SNPs associated with drought resistance at the seedling stage, (b) to identify potential drought-related genes within candidate regions and (c) to perform preliminary functional validation of potential candidate genes.

## **Materials And Methods**

### **Population materials and genotypic data used for GWAS**

A total of 400 common bean accessions representing broad genetic diversity, including 329 from China and 71 from the world, were used for the GWAS (Table S1). This panel is derived from a whole-genome resequencing panel of 683 accessions as previously described (Wu et al., 2020).

A set of SNPs generated from the whole-genome resequencing project contained 4811097 SNPs (Wu et al., 2020). For association analysis, the SNP dataset was further filtered based on the exclusion of data with a missing rate  $\geq 20\%$  and minor allele frequency  $< 0.05$  to obtain 3832340 qualified SNPs.

### **Phenotyping common bean drought resistance at the seedling stage**

To evaluate the drought resistance of common bean at the seedling stage, survival rate (SR) tests were performed in a greenhouse in Beijing, China, including three independent repeats in 2017 (from September to December) and 2018 (from March to June and from September to December). Each independent repeat contained two replicated assays. A completely random design was applied in this experiment. In each assay, a total of 40 plastic boxes (65 × 40 × 20 cm, length × width × depth) were used for planting, which were filled with 13 kg mixed soil containing uniform topsoil, vermiculite and nursery substrate (mass ratio, 4: 3: 3). Before sowing, each box was watered until the relative soil water content reached approximately 40%, as measured by SU-LB (Mengchuangweiye Technology Co., Ltd). Then, each box was divided into 12 rows (Fig. S1). The first and twelfth rows were designed as guard rows, so each box contained rows to accommodate 10 materials. All of the genotypes were randomly planted, and 12 plants of each genotype were grown per row in each assay before being covered with 2 kg of mixed soil. The relative soil water content was maintained until all genotypes developed trifoliolate leaves, and then the number of seedlings was recorded. Subsequently, water was withheld, and the relative soil water content was measured by SU-LB every day. Approximately 6 d after the relative soil water content reached approximately 0%, when all seedlings were severely wilted, each box was rewatered until the relative soil water content reached approximately 40%. Three days after rewatering, the number of viable plants of every accession was recorded. Plants with green leaves and vigorous stems were regarded as survivors. Then, water was withheld again, and each box was rewatered again approximately 6 d after the relative soil water content reached approximately 0%. Three days after rewatering, the number of viable plants of every accession was recorded again.

In each independent repeat, the seedling SR was calculated as follows:  $SR = (N_1/N \times 100\% + N_2/N \times 100\%) \times 2^{-1}$ , where SR represents the survival rate in an independent repeat;  $N_1$  represents the mean of the number of viable plants in two assays after the first drought treatment;  $N_2$  represents the mean of the number of viable plants in two assays after the second drought treatment;  $N$  represents the mean of the total number of seedlings in two assays. The three independent repeats were denoted as SR1, SR2, and SR3.

SR1, SR2, SR3 were used for statistical analysis. Descriptive statistical analysis and significance testing were performed using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA), and correlations among independent repeats were calculated using the function `cor.test()` in the `psych` package of R 3.5.1 based on Pearson's correlation coefficient (Best and Roberts, 1975).

## Genome-wide association study

To identify potential related SNPs, GWAS was performed using the compressed mixed linear models (CMLM) program of Tassel 5.0 (Bradbury et al., 2007). Principal components (PCs) of the association panel were calculated by PLINK 1.9 software based on all SNPs after filtration (Purcell et al., 2007). The first three PCs were used as the PC matrix for the associated method. The kinship matrix (K matrix) was calculated using Tassel 5.0. The *P value* threshold of the entire population was  $1 \times 10^{-5}$ . The

chromosome region with an associated SNP  $\pm$  LD decay distance was considered a candidate segment or associated locus (represented by prefix "Locus\_"), and the LD decay distance of common bean was 107 kb (Wu et al., 2020). The lead SNPs (SNPs with the lowest *P value*) for each locus were defined as candidate SNPs.

## Locus identification, candidate gene prediction and candidate sequence analysis

The physical region of drought resistance-related QTLs was determined by the physical position of the left border marker and the right border marker or 107 kb around each lead SNP, which were obtained by searching marker information in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). If the physical position of the candidate segment overlapped with the physical region of the reported drought-related QTL, this candidate segment was considered an overlapping locus.

According to their physical positions, we selected genes of all candidate segments from the gene structure annotation file (gff3 format) of common bean accession G19833 v1.0 (Schmutz et al. 2014). All selected genes were annotated using the gene function annotation file of common bean accession G19833 v1.0 (Schmutz et al. 2014). Genes that were stress-responsive transcription factors, such as a NAC, MYB, or bHLH transcription factors or whose homologous genes were involved in the drought response, osmotic response, and abscisic acid (ABA) response in *A. thaliana* in a previous study, were considered candidate genes. Combining the expression data changes of common bean seedlings after drought stress (Wu et al., 2014; Pereira et al., 2020), we selected candidate genes for further functional analysis. Aquaporin protein sequences were aligned by the Muscle program, and a Bio neighbour-joining tree based on distance with 1000 bootstrap replicates was constructed using MEGA X (Kumar et al., 2018).

## Plant materials and growth conditions for the functional investigation of candidate genes

The common bean cultivar YUEJINGDOU was used for the expression level assay. Seeds were germinated on soil in a greenhouse equipped with a supplemental lighting and cooling system. The environmental settings for common bean growth were 23°C at night and 25°C at daytime with a photoperiod of 16 h:8 h (day:night). Uniformly developed trifoliolate leaves were selected for drought treatment, and water was withheld from common bean seedlings grown in soil for the durations indicated. For mannitol, ABA, and NaCl treatments, seedlings were cultured by hydroponics until they developed trifoliolate leaves, then seedlings were transplanted in a solution containing 300 mM mannitol, 100  $\mu$ M ABA, or 150 mM NaCl for the durations indicated.

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the wild-type (WT) control in the present study, and all transgenic lines were generated in the background of Col-0. Seeds were sterilized with

chlorine, vernalized for 3 d at 4°C, and sown on half-strength Murashige and Skoog (MS) medium. Plants were grown in a growth chamber under continuous light (70  $\mu\text{mol m}^{-1} \text{s}^{-1}$ ) with a photoperiod of 16 h:8 h day:night at 22°C. For the root length assay with the young seedlings, four-day-old seedlings were transplanted to MS medium supplemented with 100, 200, 300 mM mannitol, 50, 100, 150  $\mu\text{M}$  ABA or 50, 100, 150 mM NaCl for 10 days. Then, photos were taken, root length was measured, and MS medium was used as a control. For drought stress resistance analysis, one-week-old seedlings were transplanted to pots filled with a mixture of soil and sand (1:1) for 3 additional weeks. Water was withheld from the treatment group for 13 days before rewatering, photos were taken, and the SR was recorded.

## Constructs and generation of transgenic *Arabidopsis thaliana*

Full-length cDNA of *PvXIP1;2* with *Hind*III and *Spe*I restriction sites was amplified with the primers *PvXIP1;2\_1F/1R* (Table S2). The amplified fragments were cloned into the modified pCAMBIA1300-GFP expression vector to generate the 35S::*PvXIP1;2*-GFP fusion vector. The fusion vector was transferred into *Agrobacterium tumefaciens* GV3101 and transformed into Col-0 by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected with MS medium containing 50 mg/L hygromycin and were identified by PCR amplification with the primers *PvXIP1;2\_2F/2R* (Table S2). T3 or T4 homozygous lines were used for further functional investigation.

## Subcellular localization

The pCAMBIA1300-*PvXIP1;2*-GFP fusion vector was used for *Agrobacterium*-mediated transient expression in *Nicotiana benthamiana* (Sheludko et al., 2007), and pCAMBIA1300-GFP was used as a control. Seedlings were cultivated in the dark for one day and then under normal conditions for one day after infiltration, and then fluorescence signals were captured using a confocal microscope (LSM880 ZEISS, Germany).

## RNA extraction and expression analysis

Total RNA was extracted from different organs of common bean seedlings under various treatments using the RNeasy Pure Plant Kit (TIANGEN, Beijing, China). First-strand cDNA was synthesized using the UEIris II RT-PCR system for First-Strand cDNA Synthesis Kit (BIORIGIN, Beijing, China) according to the manufacturer's instructions. Quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) was conducted on an ABI7500 system (Applied Biosystems, New York, USA) using the TransStart Top Green qPCR SuperMix Kit (TransGen, Beijing, China). The PCR procedure included preincubation at 95°C for 30 s, followed by 45 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 15 s and extension at 72°C for 34 s. The relative expression level was calculated and analysed using the  $2^{-\Delta\Delta\text{Ct}}$  method. *PvActin* was used as an internal control to normalize the transcriptional

abundance. The primer pairs *PvXIP1;2*\_QF/QR and *PvActin*\_QF/QR were used for RT-qPCR (Table S2). Experiments were carried out with three replications.

## Ion leakage, proline, and malondialdehyde measurements

Four-week-old *Arabidopsis thaliana* seedlings were well watered or not watered for 13 days, and then the leaves were sampled to measure the malondialdehyde (MDA) and proline contents as well as ion leakage (IL). The MDA content was measured using an MDA Assay Kit (Solarbio, Beijing, China) according to the manufacturer's instructions. The proline content was measured using a Micro Proline (Pro) Content Assay Kit (Solarbio, Beijing, China) according to the manufacturer's instructions. IL was measured according to a previously described method (Jiang and Zhang, 2001). Collected leaves were cut into well-proportioned strips and incubated in 10 ml distilled water at 25°C for 8 h. The initial conductivity (C1) was measured by a conductivity metre (SevenExcellence™, Switzerland). Then, the samples were boiled for 10 min. The conductivity (C2) was measured again when the samples cooled to 25°C. IL was calculated as follows:  $IL = C1/C2 \times 100\%$ .

## *Agrobacterium rhizogenes*-mediated transformation of seedlings and mannitol treatment

For the overexpression vector, full-length cDNA of *PvXIP1;2* with *NcoI* and *BstE* restriction sites was amplified with the primers *PvXIP1;2*\_3F/3R (Table S2) and then cloned into the pCAMBIA3301 vector. For the RNAi vector, a fragment with *NcoI* and *BstE* restriction sites was synthesized, including the sense and antisense fragments of *PvXIP1;2*, and cloned into the pCAMBIA3301 vector. The pCAMBIA3301 vector was used as control. The common bean cultivar YUEJINGDOU and the *A. rhizogenes* strain K599 were used for transformation. Transgenic common bean hairy root composite plants were generated by a previously described method (Estrada-Navarrete et al., 2007). When hairy roots developed, the seedlings with hairy roots were moved to water for 2 days for recovery. For measurement of the relative water content (RWC), the seedlings with uniform hairy roots were transferred to 200 mM mannitol or water for 24 h. All leaves were collected and weighed (M1), dried at 75°C for 2 days and weighed again (M2). The water content (WC) was calculated by the equation  $WC = M1 - M2$ , and the RWC was calculated by the equation  $RWC (\%) = (WC \text{ after mannitol treatment}) / (WC \text{ in water})$  (Wei et al., 2017 and 2019). For measurement of the relative root growth rate, the initial hairy root length (L1) was recorded, and then the seedlings were transferred to 200 mM mannitol or water for 3 days. The length (L2) was then remeasured, and the relative root growth rate was calculated as follows:  $\text{relative root growth rate } (\%) = (L2 - L1 \text{ at mannitol treatment}) / (L2 - L1 \text{ in water})$  (Wei et al., 2017).

## Results

# Phenotypic analysis of the survival rate of 400 common bean accessions

The seedling SR can reflect plant drought resistance mechanisms and cellular responses. It is less affected by environmental fluctuation, which helps to identify the underlying genetic determinants (Mao et al., 2015). Therefore, the drought resistance of 400 common bean accessions was assayed by calculating the SR after severe drought stress at the seedling stage. A large range of variation was detected, with the coefficient of variation (*CV*) varying from 40.90% for SR3 to 56.22% for SR2 (Table S3). The SR at different time points ranged from 0.00 to 100.00%, and the means were 50.81, 52.04, and 58.22, respectively (Table S3). All SR data showed a uniform distribution (Fig. S2). These results indicated that the drought resistance of the 400 common bean accessions was different at the seedling stage. Correlation analysis showed that pairwise positive correlations ( $p < 0.01$ ) were detected for SR1, SR2 and SR3 (Table S4), with correlation coefficients of 0.72, 0.56 and 0.54, respectively.

## Association analysis of drought resistance at the seedling stage

To identify drought resistance-related association loci, a reported genotypic dataset consisting of 3832340 SNPs was used for conducting GWAS for the SR. According to the definition described in the methods, a total of 12 associated loci containing 89 SNPs were identified (Fig. 1, Table S5), and 12 leak SNPs were detected, which were distributed on Chr. 2, 3, 5, 6, 7, 10, and 11 (Table S6). For SR1, 4 associated loci were detected, which were distributed on Chr. 6, 10, and 11 (Table S6). For SR2, 5 associated loci were detected, which were distributed on Chr. 5, 7, and 11 (Table S6). For SR3, 5 associated loci were detected, which were distributed on Chr. 2, 3, and 11 (Table S6). Among these associated loci, four loci collocated with QTLs previously reported to be related to drought resistance (Villordo-Pineda et al., 2015; Wu et al., 2021, Table S6). It is worth noting that Locus\_10, located on Chr. 11, was simultaneously associated with SR1, SR2 and SR3 and contained a significant SNP with the lowest *P value* of 4.36E-08. These results indicated that these associated loci were related to drought resistance.

## Prediction of candidate genes associated with drought resistance

Among all physical positions of the 12 associated loci, 199 genes were annotated (Table S7), including protein kinase genes, RING/U-box superfamily protein genes, MYB domain genes, and WRKY family transcription factors, which may be related to drought response (Fang et al., 2015; Mao et al., 2015; Li et al., 2019; Li et al., 2021). Combining the expression level changes after drought stress treatment (Wu et al., 2014; Pereira et al., 2020), 57 genes among these 199 genes were responsive to drought stress, of which 22 were upregulated and 35 were downregulated (Table S7). *Phvul.003G124000*, located at Locus\_3, is associated with SR3 and encodes a WRKY family transcription factor, and the expression of the gene was downregulated in common bean after drought treatment with a  $\text{Log}_2(\text{FC}) = -2.76$  (Table S7, Wu et al., 2014). We subsequently searched the NCBI database to determine the functions of these genes

in *A. thaliana*. Two genes, *Phvul.002G278200* and *Phvul.002G279300*, located at Locus\_1 and associated with SR3 (Fig. 1, Table S7) encode a protein kinase superfamily protein and calcium-dependent protein kinase 2, whose homologous genes in *Arabidopsis* are *AtSNRK2.4* and *AtCDPK2*, respectively. *AtSNRK2.4* is involved in root development and the response to osmotic stress (McLoughlin et al., 2012; Fujii et al., 2011), and *AtCDPK2* is involved in the ABA-activated signalling pathway (Zhu et al., 2007). A total of six genes were annotated in Locus\_2; among these genes, *Phvul.003G067700* encodes a homeobox-leucine zipper protein, whose homologous gene in *Arabidopsis* (*AtHB13*) is involved in drought resistance (Cabello et al., 2012), and *Phvul.003G067800* encodes MYB domain protein 88, whose homologous gene in *Arabidopsis* (*AtMYB88*) is also involved in drought resistance (Xie et al., 2010) (Fig. 1, Table S7). Another gene (*Phvul.007G220900*) located at Locus\_8 (Fig. 1, Table S7) encodes a RING/U-box superfamily protein whose homologous gene in *Arabidopsis* (*LOG2*) is involved in drought resistance (Kim et al., 2013). There were 39 candidate genes screened from all annotated genes (Table S8) according to the following rules: (1) it was a stress-responsive transcription factor, such as an NAC, MYB, or bHLH transcription factor; (2) its homologous genes in *A. thaliana* were identified in a previous study as being involved in the drought and ABA response and ABA signal transduction; and (3) its expression was upregulated or downregulated after drought treatment with  $|\text{Log}_2(\text{FC})| \geq 2$ . These results further showed that these significant SNPs were related to drought resistance, and potential genes associated with drought resistance were identified.

## A major locus on Chromosome No.11

A stable locus (Locus\_10) was detected at Chr. 11 and was simultaneously associated with SR1, SR2 and SR3 (Fig. 1). There are 41 genes contained at Locus\_10 (Fig. 2a-c). Combining the expression level changes after drought stress treatment (Wu et al., 2014; Pereira et al., 2020), among these 41 genes, 4 genes were upregulated and 9 genes were downregulated (Table S7, Fig. 2d). *Phvul.011G024800* encodes expansin-like A2, whose homologous gene in *Arabidopsis* (*AtEXLA2*) responds to ABA (Abuqamar et al., 2013), and the expression of the gene was downregulated in common bean after drought treatment (Table S7, Fig. 2b, d). *Phvul.011G025500* encodes rho guanyl-nucleotide exchange factor 1, whose homologous gene in *Arabidopsis* (*AtROPGEF1*) was involved in the ABA-activated signalling pathway and lateral root development (Li et al., 2016; Li et al., 2018), and the expression of the gene was downregulated in common bean after drought treatment (Table S7, Fig. 2b, d). *Phvul.011G025200* encodes a late embryogenesis abundant protein (LEA), *Phvul.011G025700* and *Phvul.011G025800* encode plasma membrane intrinsic proteins (*XIP1;1*, *XIP1;2*), and *Phvul.011G026700* encodes a proline-rich protein (*PRP2*) (Table S7, Fig. 2b). All these genes mentioned above were generally considered to be associated with drought resistance (Fang et al., 2015). Combining the expression level changes after drought stress treatment (Wu et al., 2014; Pereira et al., 2020), *Phvul.011G025200* and *Phvul.011G026700* were downregulated with  $\text{Log}_2(\text{FC})$  of -3.07 and -5.99, respectively, in common bean after drought treatment (Table S7). *Phvul.011G025800* was upregulated with a high  $\text{Log}_2(\text{FC})$  of 8.2 and was assumed to be the most promising candidate gene. *Phvul.011G025800* was located 74 kb upstream of the lead SNP (Chr11\_\_2209628,  $P = 4.36\text{E-}08$ ), which was annotated as aquaporin (named *PvXIP1;2*).

A phylogenetic tree based on XIPs from different species was constructed (Fig. S3a), indicated that the XIPs from legumes clustered together. The results of sequence alignment (Fig. S3b) showed that two highly conserved 'NPA signature motifs' were found in PvXIP1;2, but the first NPA motif was replaced with SPV, similar to that in soybean. The four residues forming the ar/R selectivity filter of PvXIP1;2 are valine from TMH2, isoleucine from TMH5, and alanine and arginine from R3 and R4, which are identical to those in soybean (Bienert et al., 2011, Fig. S3b). The results demonstrated that PvXIP1;2 is an aquaporin and belongs to the X-intrinsic protein (XIP) subfamily, which is absent in *Arabidopsis* (Reuscher et al., 2013; Ariani et al., 2015). Aquaporins are involved in almost every physiological process in plants and in the response to environmental stresses (Maurel et al., 2008; Li et al., 2014). Most aquaporins have been identified to be involved in resistance to drought, such as *MaPIP1;1* (Xu et al., 2014), *ScPIP1;1* (Wang et al., 2019), *RWC3* (Lian et al., 2004) and *TaAQP7* (Zhou et al., 2012). However, the function of *PvXIP1;2* remains to be elucidated. Therefore, *Phvul.011G025800* was assumed to be a promising candidate gene for further functional investigation.

## Expression patterns of *PvXIP1;2*

To investigate the expression of *PvXIP1;2* in different organs of common bean, total RNA was extracted from roots, leaves, trifoliates and stems for quantitative real-time RT-qPCR analysis. The results showed that *PvXIP1;2* was expressed in various tissues of common bean (Fig. 3a).

The expression file data indicated that *PvXIP1;2* transcript levels were strongly induced by drought stress (Wu et al., 2014; Pereira et al., 2020). To verify the expression data, we performed quantitative real-time RT-qPCR using RNA isolated from drought-treated common bean, and the results confirmed the expression data (Fig. 4a). Furthermore, various stress treatments were applied to common bean with trifoliates, including salt, mannitol and ABA treatments, to determine the transcriptional response of *PvXIP1;2* to abiotic stress. The results demonstrated that the expression of *PvXIP1;2* was induced in leaves and roots after salinity stress, simulated drought and ABA treatments (Fig. 4b-c). These results suggested that the *PvXIP1;2* transcript level was affected by various stress treatments.

To determine the subcellular localization of the PvXIP1;2 protein, its ORF was introduced into the pCAMBIA1300-GFP vector upstream of the *GFP* gene to create a *PvXIP1;2-GFP* fusion construct. Then, we transformed the *PvXIP1;2-GFP* fusion construct into *Nicotiana benthamiana* by injection. A strong fluorescent signal derived from GFP alone was observed in the cytoplasm and nuclei (Fig. 3b), whereas transformed cells carrying *PvXIP1;2-GFP* showed a strong green fluorescence signal in the plasma membrane, indicating the plasma membrane localization of *PvXIP1;2*.

## Overexpression of *PvXIP1;2* increases the drought resistance of *Arabidopsis*

To further understand the function of *PvXIP1;2*, we constructed a *35S::PvXIP1;2* vector. After floral-dip transformation of *Arabidopsis*, three homozygous lines (L6, L8 and L10) were selected from the T4 generation for further functional investigation (Fig. 5a). To investigate the drought resistance of transgenic *Arabidopsis* overexpressing *PvXIP1;2*, WT and transgenic *Arabidopsis* were grown for 3 weeks in soils before water was withheld for 13 d and then rewatered. Most transgenic plants remained turgid, and their leaves remained green, whereas the WT plants wilted, and their leaves became yellow (Fig. 5b). When data from three different experiments were analysed, approximately 80% of the transgenic plants survived stress after rewatering, which was approximately four times higher than that of WT plants (Fig. 5c). Osmotic adjustment and antioxidant defence systems are the main pathways of drought tolerance-associated mechanisms (Fang et al., 2015). Therefore, the MDA and proline contents and IL were measured in WT and transgenic plants under drought stress and well-watered environments to identify the function of *PvXIP1;2* in these physiological processes. MDA, which causes oxidative injury to the cytomembrane, accumulates in leaves when plants experience stress. No evident difference was observed in the MDA content of WT and transgenic plants under well-watered conditions, whereas a significantly lower MDA content was observed in transgenic plants than in the WT under drought treatment (Fig. 5d). Consistent with these results, a lower IL was observed in transgenic plants than in the WT under drought treatment, whereas there was no significant difference in WT and transgenic plants under well-watered conditions (Fig. 5e), suggesting that the more severe membrane damage after water was withheld in the WT could at least partly be attributed to the inability of these plants to efficiently eliminate MDA due to the disruption of antioxidant defence systems under severe drought stress. In addition, proline, which is an essential osmotic substance, accumulated more in transgenic plants than in WT plants under drought stress conditions, while no significant difference was observed in WT and transgenic plants (Fig. 5f). These results indicated that heterologously overexpressing *PvXIP1;2* in *Arabidopsis* improved resistance to drought stress due to reduced lipid peroxidation and membrane injury and increased accumulation of osmotic substances.

## ***35S::PvXIP1;2* transgenic *Arabidopsis* is resistant to osmotic, salt and ABA stresses**

*PvXIP1;2* is a mannitol-, salt- and ABA-induced gene (Fig. 4b-c), indicating that *PvXIP1;2* might also be necessary for plant responses to osmotic, salt and ABA stresses. To further identify the results, four-day-old WT and transgenic seedlings were transformed on MS medium containing different concentrations of mannitol, NaCl and ABA for 10 days. There was no significant difference in WT and transgenic plants (Fig. 6a) on MS medium without treatments. The addition of mannitol, NaCl and ABA significantly inhibited root development, and the degree of inhibition increased with increasing concentrations of mannitol, NaCl and ABA (Fig. 6b-f). The root development of WT plants was more inhibited than that of transgenic plants in all treatments except 150 mM NaCl (Fig. 6b-f). These results demonstrated that the overexpression of *PvXIP1;2* improved the resistance of *Arabidopsis* to osmotic, salt and ABA stresses.

# ***PvXIP1;2* improves stress resistance in transgenic common bean hairy roots**

An *Agrobacterium rhizogenes*-mediated hairy root transformation system was used to investigate the function of *PvXIP1;2* in common bean under osmotic stress conditions. The K599 strain harbouring the *PvXIP1;2*-overexpression (*PvXIP1;2*-OE) and *PvXIP1;2*-RNA interference (*PvXIP1;2*-RNAi) constructs was injected into the hypocotyls of common bean seedlings for the generation of transgenic hairy roots. The transgenic hairy roots grew out of the injection sites approximately 10 days later. *PvXIP1;2* expression in *PvXIP1;2*-OE roots was 4 times higher than that in control hairy roots, whereas that in *PvXIP1;2*-RNAi roots was only half of that in the control (Fig. 7a). The original roots were removed before the plants with hairy roots were subjected to 200 mM mannitol to simulate osmotic stress. After keeping in mannitol for 24 h, the leaves of the plants with *PvXIP1;2*-OE roots performed better than the control leaves; however, the leaves from the plants with *PvXIP1;2*-RNAi roots were more wilted than the control leaves (Fig. 7b-c). The results of the RWC showed that seedlings with *PvXIP1;2*-OE roots had a higher water content than the control seedlings, whereas seedlings with *PvXIP1;2*-RNAi roots had a lower water content (Fig. 7d). After keeping in mannitol for 72 h, the relative hairy root growth rates were measured and compared. The results demonstrated that the relative growth rate of *PvXIP1;2*-OE hairy roots was significantly higher, whereas this parameter in *PvXIP1;2*-RNAi roots was significantly lower than that of control roots (Fig. 7e-f). These results indicated that *PvXIP1;2* confers resistance to drought in common bean and further demonstrated that these loci detected in this study were related to drought resistance.

## **Discussion**

To the best of our knowledge, this is the first attempt to conduct a genome-wide association study to identify drought resistance-related QTLs in common bean at the seedling stage. In the present study, the largest SNP dataset consisting of 4.8 M SNPs, which was generated by resequencing a panel of 683 common bean accessions collected from China and elsewhere, including landrace and breeding lines representing both Andean and Mesoamerican gene pools (Wu et al., 2020), was used for conducting the GWAS. A total of 400 common bean accessions derived from the resequencing population by random sampling were used to evaluate drought resistance at the seedling stage. Both the number of individuals and the density of markers are relatively large in genome-wide association analysis of common bean. In the present study, 12 association loci containing 89 significant SNPs were identified to be associated with SR. Four loci overlapped with reported drought-related QTLs. One locus located on Chr. 11 was stably detected in three independent repeats (Table S6). Seven genes whose homologous genes in *Arabidopsis* were involved in the response to drought or ABA were found to be located in these loci (Table S7), suggesting the excellent reliability of the genetic loci of drought resistance identified in this study. More importantly, a case study of *PvXIP1;2* demonstrated that the candidate loci identified in this study are highly valuable for further identification of the causal genes

associated with drought resistance, and some of these loci may be used for genetic improvement of drought resistance in common bean at the seedling stage.

Drought resistance studies in common bean have mainly focused on the late developmental stage, and a few drought-related QTLs have been identified using different molecular markers (Schneider et al., 1997; Asfaw et al., 2012; Asfaw and Blair, 2012; Blair et al., 2012; Mukeshimana et al., 2014; Villordo-Pineda et al., 2015; Trapp et al., 2015; Briñez et al., 2017; Hoyos-Villegas et al., 2017; Berny-Mier et al., 2019). Limited studies have reported drought-related QTLs in common at the early growth stage. Wu et al. (2021) evaluated the drought resistance of a common bean natural-variation population at the bud stage based on nine root traits and conducted a GWAS to identify drought-related loci. A set of loci and candidate genes were found to be associated with drought resistance at the bud stage. Among these loci, three loci associated with roots were collocated with Locus\_1, Locus\_7 and Locus\_8 identified in the present study, indicating that good root development after germination was related to the drought resistance of common bean at the seedling stage. In addition, a SNP associated with days to flowering and the reproductive period was collocated with Locus\_12 identified in this study (Villordo-Pineda et al., 2015;). The overlapping loci were detected by different mapping populations at different growth stages, indicating the possibility of finding common regulatory genes for the whole growth period.

Predicting candidate genes around lead SNPs within LD decay distances could be an effective method for identifying causal genes (Mao et al., 2015; Wu et al., 2020). However, despite the relatively low LD decay of 107 kb in common bean (Wu et al., 2020), one association locus in this study contained more than ten genes on average. A total of 199 genes were annotated among all associated loci (Table S7), so it is rather difficult to pinpoint the causal genes for these loci. The combined analysis of the expression profiles, gene annotations and references of the functions of homologous genes in *Arabidopsis* is a feasible and efficient way to narrow down the candidate genes. Among these genes, seven were reported to be involved in osmotic development, root development, the ABA-activated signalling pathway and drought resistance and were located at Locus\_1, Locus\_7, Locus\_8 and Locus\_10 (Table S7). Locus\_1 identified in this study was also associated with root traits (Table S6, Wu et al., 2021). The gene *Phvul.002G278200*, which encodes a protein kinase superfamily protein, was located at Locus\_1, and its homologues in *Arabidopsis* (*AtSNRK2.4*) were identified to be involved in root development and osmotic stress (McLoughlin et al., 2012; Fujii et al., 2011). Locus\_8 identified in this study was also associated with root traits (Table S6, Wu et al., 2021) and contained the drought-related gene *Phvul.007G220900*, which encodes a RING/U-box superfamily protein and was reported to enhance resistance to drought in *Arabidopsis* (*LOG2*) (Kim et al., 2013). These results further demonstrated that these loci identified in the study were related to drought resistance and that the candidate genes are worth further functional study. In addition to the seven genes reported in *Arabidopsis*, many unreported loci and genes for drought resistance were detected in our association analysis. There were 39 candidate genes screened from all annotated genes (Table S8) according to their expression profiles, gene annotations and references to the functions of homologous genes in *Arabidopsis*. For example, *Phvul.011G026700* encodes proline-rich protein 2 and was downregulated after drought treatment with  $\text{Log}_2(\text{FC})$  of 5.99, and *Phvul.011G025800*

encodes aquaporins and was upregulated after drought treatment with a  $\text{Log}_2(\text{FC})$  of 8.2, indicating that these are promising causal genes for further functional investigation. Nevertheless, the functions of these genes in response to drought stress need to be confirmed by molecular experiments.

Aquaporins are channel proteins that are responsible for water transport during seed germination, cell elongation, stomatal movements and abiotic stress responses (Maurel et al., 1997 and 2008). Some members of the aquaporin family were identified to enhance resistance to drought, such as *MaPIP1* (Xu et al., 2014) and *ScPIP1* (Wang et al., 2019). These aquaporins were induced by NaCl and water deficiency treatment, increased primary root elongation, reduced membrane injury and accumulated osmotic substances under drought stress. *PvXIP1;2* is an aquaporin and belongs to the X-intrinsic protein (XIP) subfamily, which is absent in *Arabidopsis* (Reuscher et al., 2013; Ariani et al., 2015). In the present study, we confirmed the function of *PvXIP1;2* in the response to drought, salt, osmotic and ABA stresses by expression level comparison and phenotypic analysis of *Arabidopsis* overexpression and *Agrobacterium rhizogenes*-mediated hairy root transformation systems. Overexpression lines of *PvXIP1;2* showed significant changes in the root growth rate, membrane injury and osmotic substances compared to the control plants. *PvXIP1;2* played a positive role in mediating drought, salt, osmotic and ABA stresses. These results are consistent with previous studies demonstrating that overexpression of AQP genes confers abiotic stress resistance to transgenic plants (Gao et al., 2010; Sade et al., 2010; Zhou et al., 2012; Xu et al., 2014; Wang et al., 2019). The same conclusion was observed in common bean using a hairy root transformation system, further indicating the function of *PvXIP1;2* in enhancing resistance to drought.

In conclusion, our study provides relatively rich genetic information on drought resistance in common bean at the seedling stage. A panel of 400 common bean accessions was employed to perform a GWAS for drought resistance at the seedling stage, and 4 of 12 candidate loci identified by GWAS overlapped with reported drought-related QTLs. A stable locus containing promising causal genes was identified in three independent experiments. A set of genes were identified as prospective candidate genes involved in drought response at the seedling stage. Importantly, a case study of *PvXIP1;2* indicated the feasibility of mining candidate genes by GWAS. The associated loci and causal genes identified in this study provide insights into the genetic basis of plant mechanisms in response to drought and an important foundation for the genetic improvement of drought resistance in common bean at the seedling stage in the future.

## Abbreviations

GWASs: Genome-wide association studies

IL: ion leakage

MDA: malondialdehyde

QTLs: Quantitative trait loci

RWC: Relative water content

SNPs: Single nucleotide polymorphisms

SR: Survival rate

WT: Wild-type

## Declarations

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## Conflicts of interest/Competing interests

The authors declare no conflict of interest

## Availability of data and material

The data supporting the findings of this study are available from the corresponding author (Jing Wu) upon request.

## Author contributions

L. W and J. W designed the project. L. W conducted the main experiments. Y. C., LF. W and S. W participated in data analysis. L. W., S. W and J. W wrote the manuscript.

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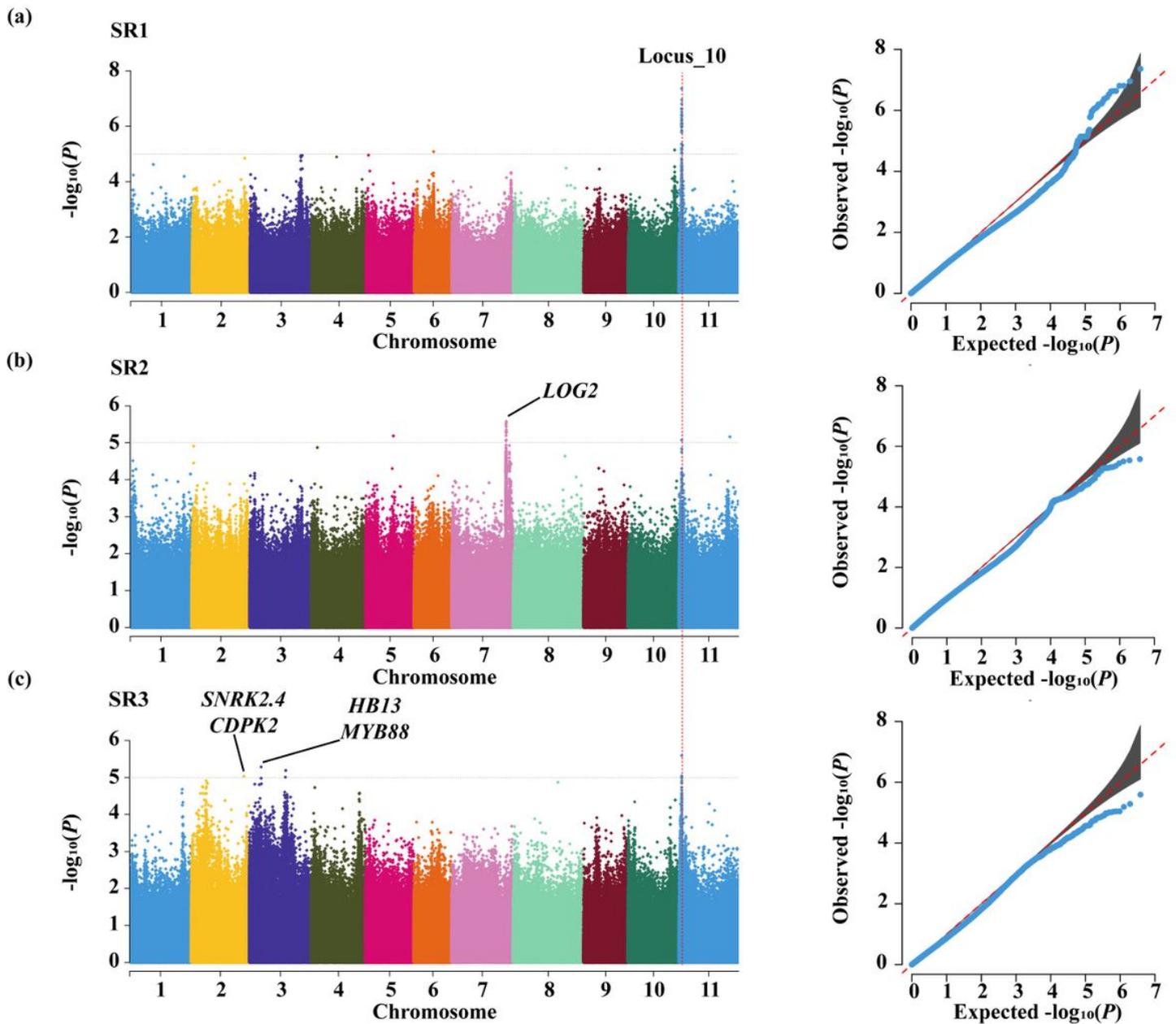
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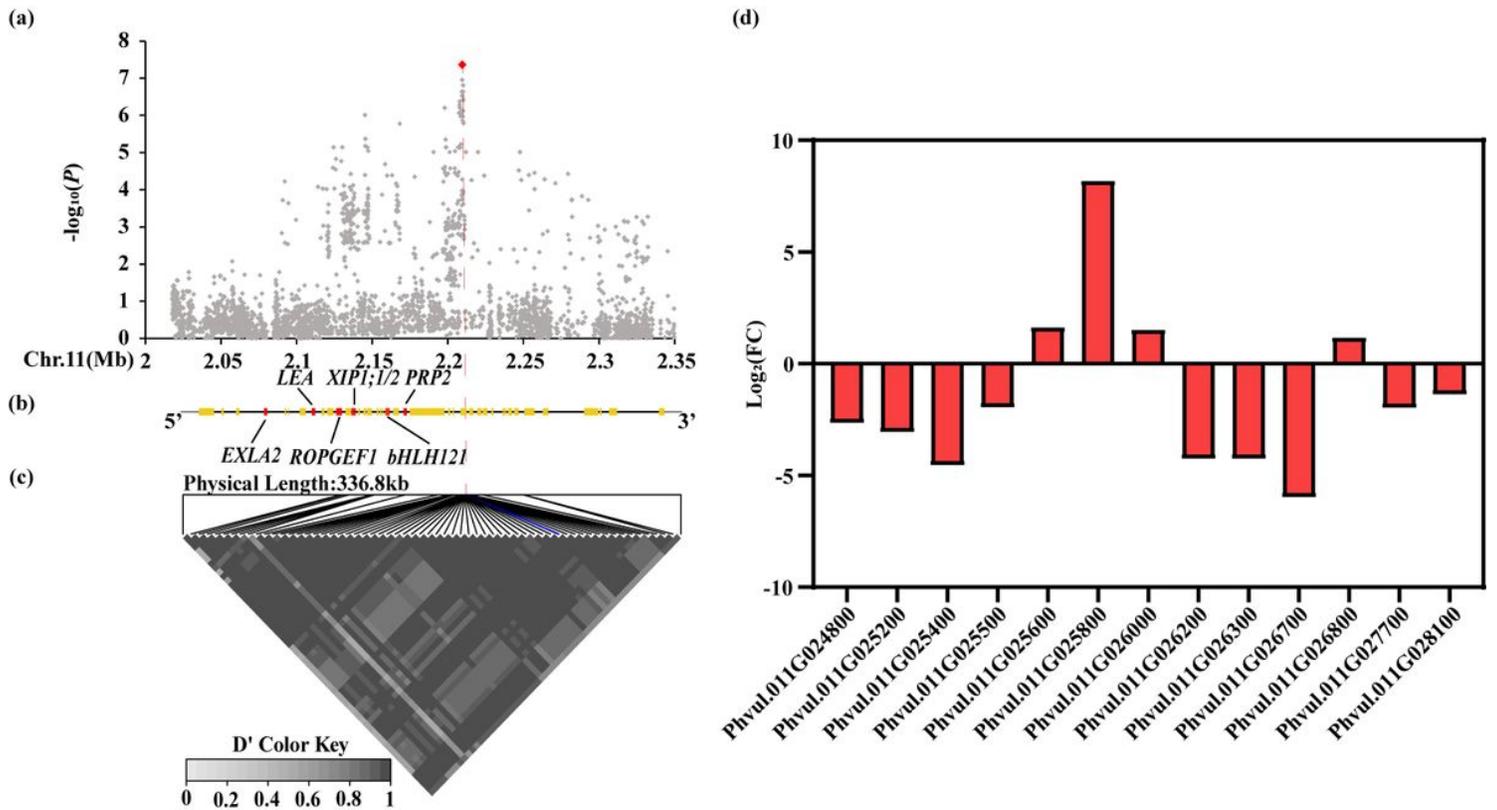
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## Figures



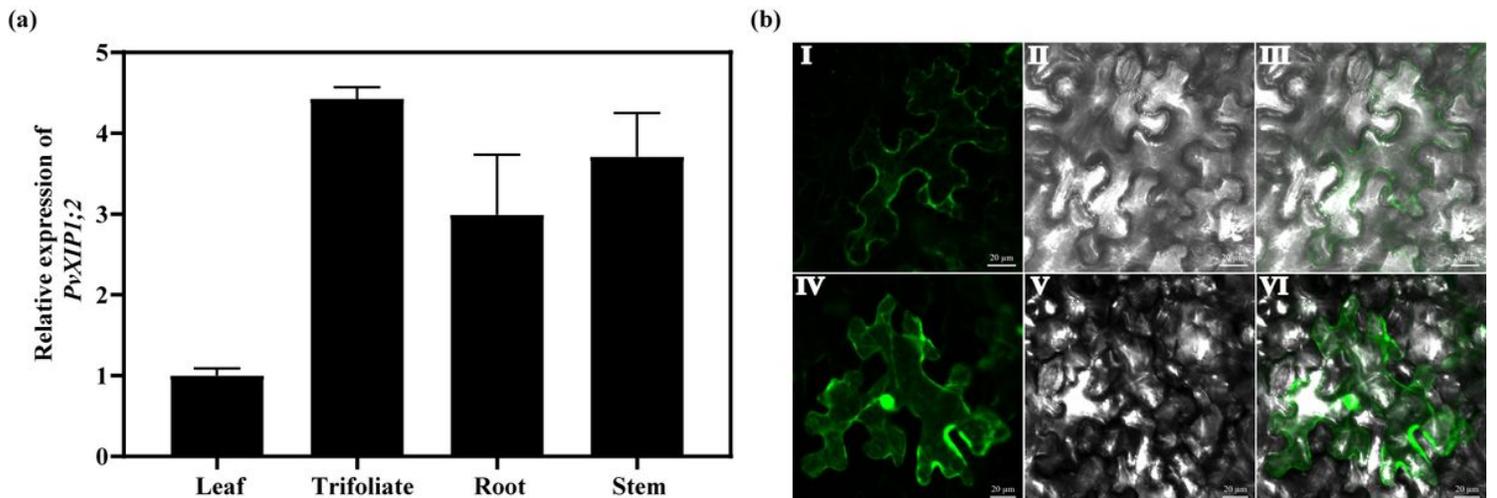
**Figure 1**

Genome-wide association results for the SR in three independent experiments. Manhattan plots (left) and quantile-quantile plots (right) are presented for (a) SR1, (b) SR2 and (c) SR3. For the Manhattan plots,  $-\log_{10}P$  from a genome-wide scan was plotted against the position of the SNPs on each of 12 chromosomes, and the horizontal grey dashed line indicates the significance threshold ( $P = 1 \times 10^{-5}$ ). For the quantile-quantiles plots, the horizontal axis indicates the expected  $-\log_{10}P$ , and the vertical axis indicates the observed  $-\log_{10}P$ . The names of reported drought- or ABA-related genes are indicated near the association signals. The vertical red dashed line indicates the stable loci detected in three independent experiments.



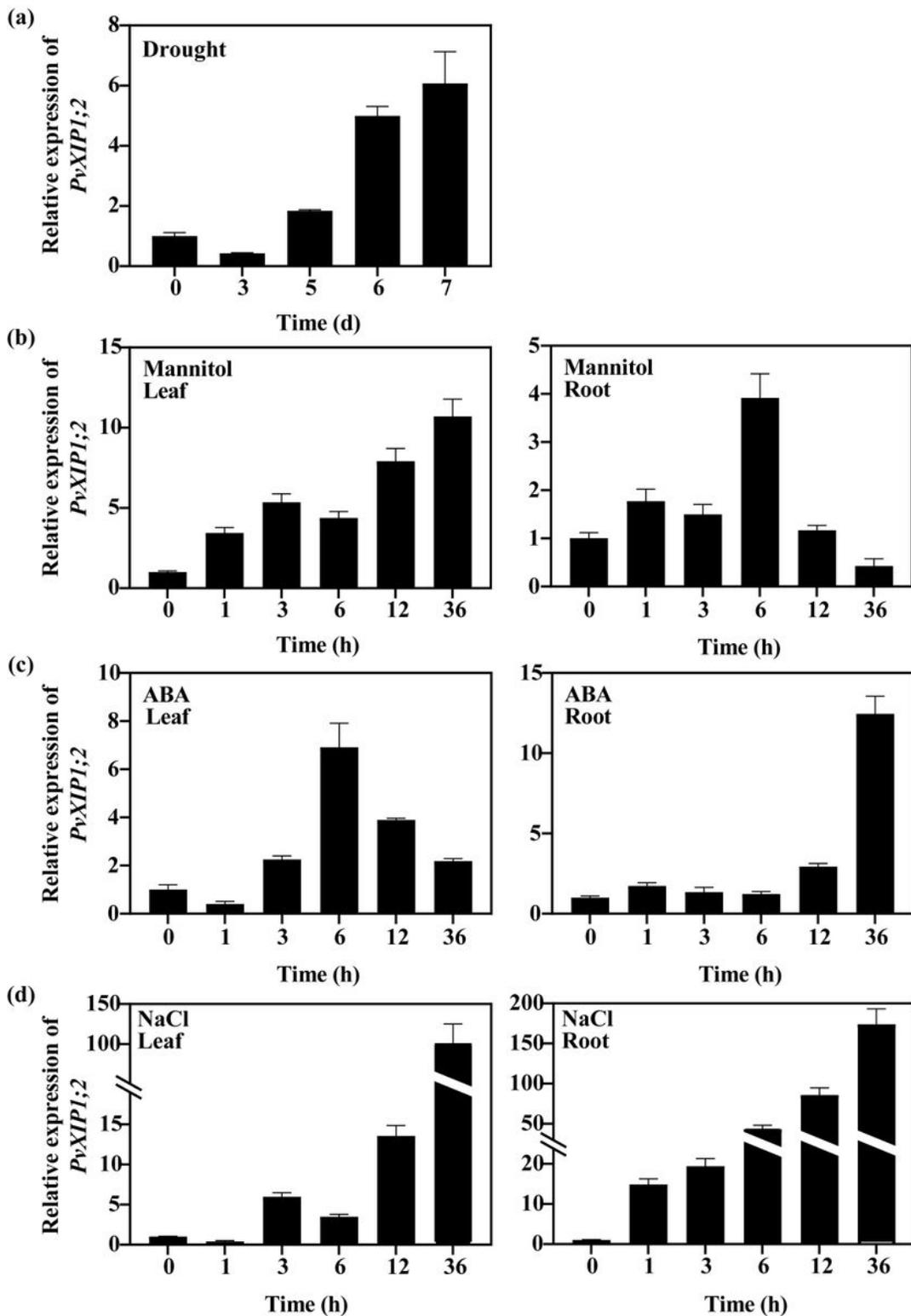
**Figure 2**

Genomic location of Locus\_10 detected in three independent experiments and LD block analysis. (a) Local Manhattan plot of Locus\_10 associated with SR1. The red point indicates the lead SNP, and the red dotted line indicates the position of the SNP. (b) Genes within this region are indicated, and the names of some potential drought-related genes are indicated. (c) Linkage disequilibrium heatmap surrounding Locus\_10 on chromosome 11. The darkness of the colour of each box corresponds to the  $D'$  value according to the legend, and the blue line indicates the lead SNP (Chr11\_\_2209628). (d) Transcript-level difference in candidate genes after drought treatment measured by comparisons of the  $\log_2(\text{fold change})$  of FPKM values within Locus\_10.



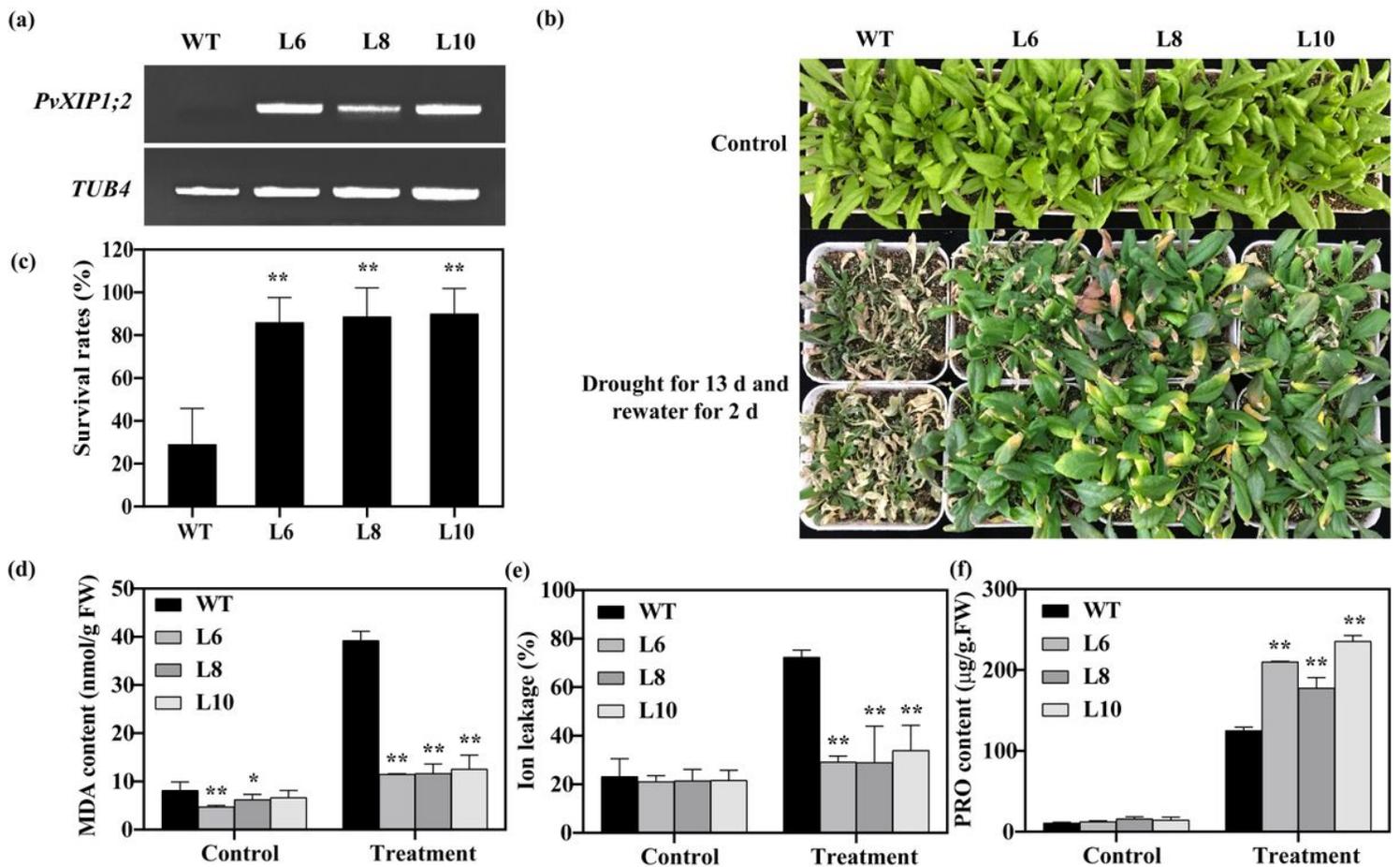
**Figure 3**

Expression patterns of PvXIP1;2. (a) Detection of PvXIP1;2 transcript accumulation in different tissues of common bean seedlings. Total RNA was isolated from various tissues of seedlings with trifoliolate leaves. Real-time reverse transcription-polymerase chain reaction (RT-qPCR) quantifications were normalized to the expression of PvActin. Values are means ( $\pm$  SE) of three replications. (b) Subcellular localization of PvXIP1;2. The PvXIP1;2-GFP fusion construct was expressed in epidermal cells of *Nicotiana benthamiana*, which were observed with a confocal microscope. Subcellular localization of the green fluorescent protein PvXIP1;2-GFP (I) and GFP alone (IV) indicated that PvXIP1;2 was located in the plasma membrane. (III) and (VI) show the combined bright field images for the morphology of the cells (II, V) and green fluorescence.



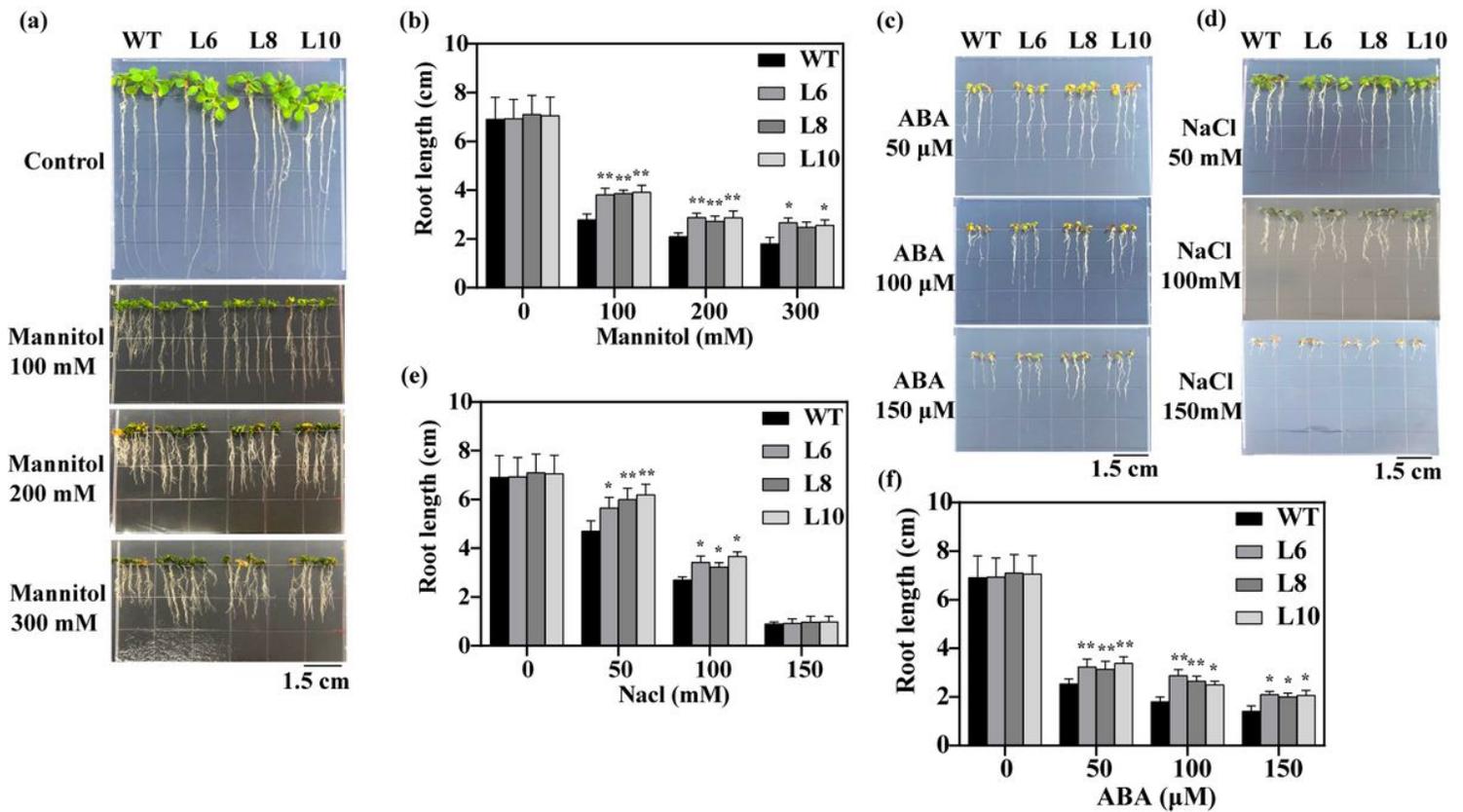
**Figure 4**

Regulation of *PvXIP1;2* transcripts by drought, mannitol, NaCl and ABA treatments. Assay of the accumulation of the *PvXIP1;2* transcript in common bean seedlings in response to drought (a), mannitol (b), ABA (c) and NaCl (d) treatments by real-time reverse transcription-polymerase chain reaction (RT-qPCR). The expression levels were normalized to that of *PvActin*. Values are means ( $\pm$  SE) of three replications.



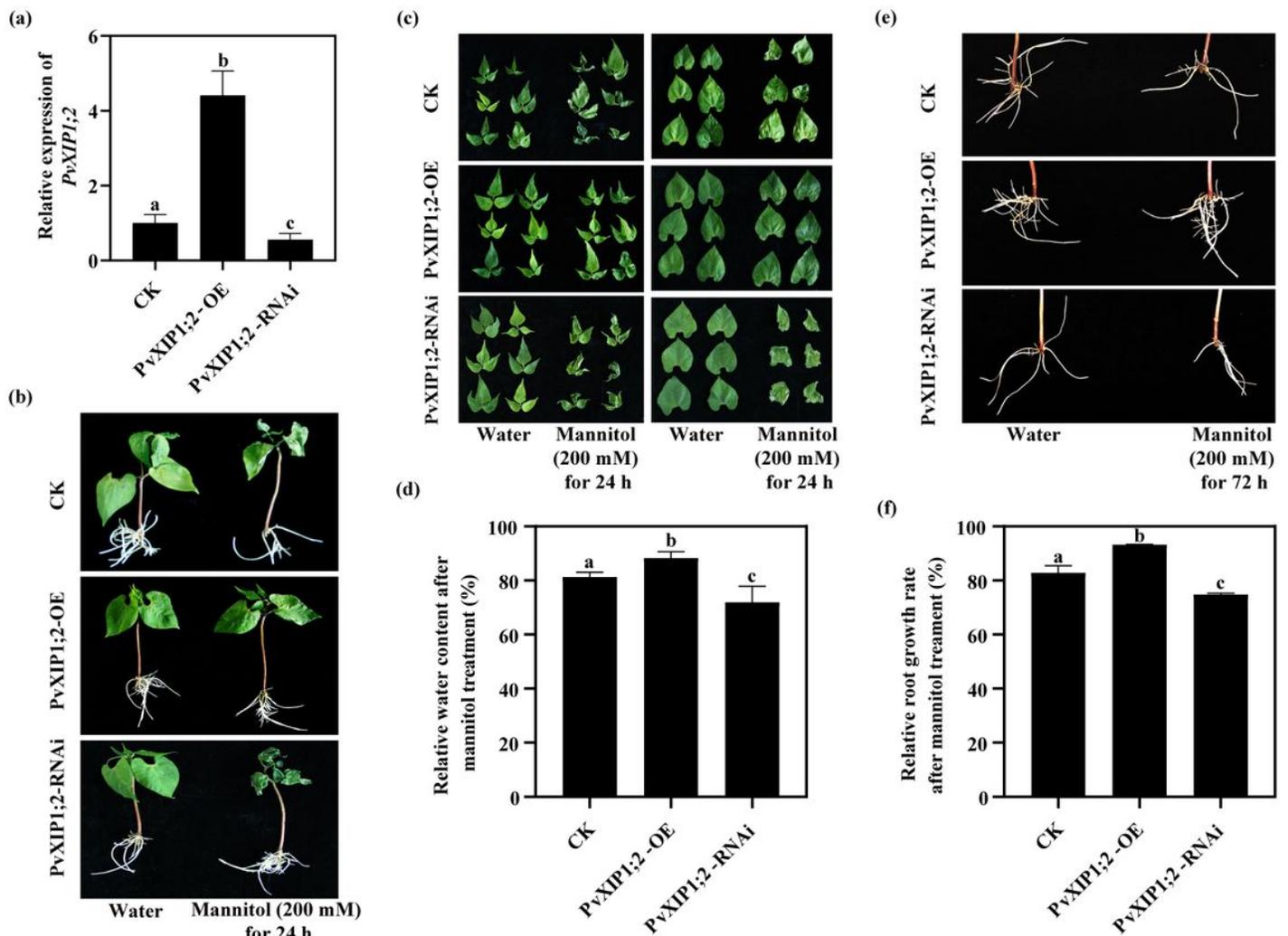
**Figure 5**

Overexpression of *PvXIP1;2* enhances drought resistance in *Arabidopsis thaliana*. (a) Detection of *PvXIP1;2* in cDNA of transgenic plants. *Tub4* was used as a control and was amplified by the primers *AtTub4\_QF/QR* (Table S2). (b) Drought resistance of 35::*PvXIP1;2* transgenic plants (L6, L8 and L10). The photographs show representative seedlings. (c) The SR of WT and transgenic plants after watering was withheld for 13 d and rewatered for 2 days, when the difference in the SR of WT and transgenic plants was greatest. Average SRs and standard errors were calculated on the basis of data from three replicated experiments. Thirty-six plants were tested in each experiment. (d) Malonaldehyde (MDA) content, (e) ion leakage (IL) and (f) proline content of the leaves of WT and transgenic plants were measured under well-watered and drought stress conditions. Values are means ( $\pm$  SE) of three replications. Thirty leaves were sampled to measure IL in each experiment. \* indicates statistically significant differences at the level of  $P < 0.05$ , \*\* indicates statistically significant differences at the level of  $P < 0.01$ , Student's t test, two-tailed.



**Figure 6**

Osmotic, salt and ABA responses of transgenic plants overexpressing PvXIP1;2 during the post-germination growth stage. (a) Osmotic effects, (c) ABA effects and (d) salt effects on the growth of germinated WT and transgenic seedlings. The photographs show representative seedlings. Root elongation of WT and transgenic plants was measured in response to mannitol (b), NaCl (e) and ABA (f). Values are the means ( $\pm$  SE) of three replications, and ten plants were tested in each experiment. \* indicates statistically significant differences at the level of  $P < 0.05$ , \*\* indicates statistically significant differences at the level of  $P < 0.01$ , Student's t test, two-tailed.



**Figure 7**

Differences in common bean seedlings with *PvXIP1;2*-OE and *PvXIP1;2*-RNAi transgenic hairy roots in response to mannitol treatment. (a) Expression levels of *PvXIP1;2* in transgenic hairy roots. Values are means ( $\pm$  SE) of three replications. (b) Performance of common bean plants with hairy roots in response to mannitol treatment. (c) Performance of common bean trifoliolate leaves (left) and primary leaves (right) after mannitol treatment. (d) All leaves were cut to measure the relative water content (RWC). Values are the means ( $\pm$  SE) of three replications, and ten plants were tested in each experiment. (e) Performance of common bean hairy roots after mannitol treatment. (f) The relative root growth rate was measured after mannitol treatment. Values are the means ( $\pm$  SE) of three replications, and ten plants were tested in each experiment. The different letters indicate statistically significant differences at the level of  $P < 0.05$ , Student's *t* test, two-tailed.

## Supplementary Files

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