Characterization of the bark storage protein gene (*JcBSP*) family in the perennial woody plant *Jatropha curcas* and the function of *JcBSP1* in *Arabidopsis thaliana*

Ming-Jun Zhang[1,2], Qiantang Fu[2], Mao-Sheng Chen[2], Huiying He[2], Mingyong Tang[2], Jun Ni[3], Yan-Bin Tao[2] and Zeng-Fu Xu[2,3]

[1] School of Life Sciences, University of Science and Technology of China, Hefei, Anhui, China
[2] CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, The Innovative Academy of Seed Design, Chinese Academy of Sciences, Menglun, Mengla, Yunnan, China
[3] State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Forestry, Guangxi University, Nanning, Guangxi, China

ABSTRACT

Background. Bark storage protein (BSP) plays an important role in seasonal nitrogen cycling in perennial deciduous trees. However, there is no report on the function of BSP in the perennial woody oil plant *Jatropha curcas*.

Methods. In this study, we identified six members of *JcBSP* gene family in *J. curcas* genome. The patterns, seasonal changes, and responses to nitrogen treatment in gene expression of *JcBSP*s were detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Overexpression of *JcBSP1* in transgenic *Arabidopsis thaliana* was driven by a constitutive cauliflower mosaic virus (CaMV) *35S* RNA promoter.

Results. *JcBSP* members were found to be expressed in various tissues, except seeds. The seasonal changes in the total protein concentration and *JcBSP1* expression in the stems of *J. curcas* were positively correlated, as both increased in autumn and winter and decreased in spring and summer. In addition, the *JcBSP1* expression in *J. curcas* seedlings treated with different concentrations of an NH_4NO_3 solution was positively correlated with the NH_4NO_3 concentration and application duration. Furthermore, *JcBSP1* overexpression in *Arabidopsis* resulted in a phenotype of enlarged rosette leaves, flowers, and seeds, and significantly increased the seed weight and yield in transgenic plants.

Subjects Molecular Biology, Plant Science, Forestry

Keywords *Jatropha curcas*, *JcBSP* gene family, Seasonal nitrogen cycling, Nitrogen induction, Overexpression, *Arabidopsis thaliana*

Submitted 10 November 2021
Accepted 24 January 2022
Published 8 February 2022

Corresponding authors
Yan-Bin Tao, taoyanbin@xtbg.ac.cn
Zeng-Fu Xu, zfxu@gxu.edu.cn

Academic editor
Wei Ma

Additional Information and Declarations can be found on page 15

DOI **10.7717/peerj.12938**

INTRODUCTION

Seasonal nitrogen cycling (SNC) is important in deciduous perennials to ensure the sufficient use of nitrogen resources. It is also a decisive factor for plant fitness in perennial (*May & Killingbeck, 1992*). The process of SNC involves the degradation of proteins when

How to cite this article Zhang M-J, Fu Q, Chen M-S, He H, Tang M, Ni J, Tao Y-B, Xu Z-F. 2022. Characterization of the bark storage protein gene (*JcBSP*) family in the perennial woody plant *Jatropha curcas* and the function of *JcBSP1* in *Arabidopsis thaliana*. *PeerJ* **10**:e12938
http://doi.org/10.7717/peerj.12938

leaves shed in autumn, the transportation of the released amino acids to the perennial tissues (bark and wood) to synthesize storage proteins, and then used for the growth of new stems and leaves in spring (*Babst & Coleman, 2018*; *Wildhagen et al., 2010*). When the deciduous perennial trees overwinter, nitrogen is transported from senescing leaves to perennial tissues for storage (*Ryan & Bormann, 1982*). For example, before poplar leaf senescence, protein is hydrolyzed and transported to stems and roots in the form of amino acids, which results in approximately 90% of the nitrogen being removed from the leaves (*Chapin & Kedrowski, 1983*; *Pregitzer et al., 1990*). Through longitudinal section observation of *Populus* stems in winter and summer, it has been found that the phloem parenchyma cells and xylem ray cells contained only a large central vacuole in summer, while the central vacuole in these cells was replaced by many small protein storage vacuoles in winter (*Clausen & Apel, 1991*; *Cleve & Apel, 1993*; *Cleve, Clausen & Sauter, 1988*; *Cooke & Weih, 2005*; *Sauter, Cleve & Wellenkamp, 1989*; *Sauter & Cleve, 1992*; *Wetzel, Demmers & Greenwood, 1989a*). This protein is a kind of vegetative storage protein (VSP) and is a designated bark storage protein (BSP) (*Cooke & Weih, 2005*). It is an important form of nitrogen storage for perennial woody plants in winter.

Previous studies have shown that the poplar *BSP* is composed of a multigene family, including three subfamilies: *BSP*, *wound-inducible 4* (*WIN4*), and *poplar nitrogen-inducible 288* (*PNI288*) (*Coleman, Chen & Fuchigami, 1992*; *Wildhagen et al., 2010*). All three subfamily genes can respond to nitrogen induction (*Coleman, Baíiados & Chen, 1994*; *Lawrence et al., 2001*; *Lawrence et al., 1997*), but only *BSP* has been found to exhibit consistent seasonal expression changes with the total protein concentration in bark. The expression of *BSP* was increased in winter, whereas the expression of *WIN4* and *PNI288* increased only in spring (*Wildhagen et al., 2010*), and *BSP* has been reported to respond to short light duration and low temperature induction (*Black et al., 2001*; *Cleve & Apel, 1993*; *Coleman et al., 1991*; *Coleman et al., 1993*; *Lawrence et al., 2001*; *Wildhagen, Bilela & Rennenberg, 2013*). Therefore, only *BSP* is directly related to nitrogen storage during plant dormancy in winter among these three subfamilies.

Jatropha curcas is a perennial woody oil plant of the Euphorbiaceae family. It has received widespread attention because its seed oil is recognized as a promising feedstock for biodiesel production (*Divakara et al., 2010*; *Kamel et al., 2018*; *Makkar & Becker, 2009*; *Mazumdar et al., 2018*; *Mohibbeazam, Waris & Nahar, 2005*; *Pandeya et al. 2012*; *Pramanik, 2003*; *Vaknin et al., 2018*; *Yi et al., 2014*). Although *J. curcas* grows in tropical and subtropical regions, it is also a deciduous tree. Adult *J. curcas* begins to defoliate in autumn and stays dormant in winter until the next spring, when it enters the growing season. In this study, to identify *J. curcas BSP* (*JcBSP*) genes that may be involved in seasonal nitrogen cycling, we examined the expression of *JcBSP* family members in response to seasonal changes and nitrogen induction and found that the expression of *JcBSP1* was positively correlated with the total protein concentration in the stems during seasonal changes and with the exogenous nitrogen application. To further determine the roles of *JcBSP1* in plant growth and development, we characterized phenotypic changes in transgenic *Arabidopsis thaliana* overexpressing *JcBSP1* and found that transgenic plants exhibited phenotypes of enlarged

rosette leaves, flowers, and seeds. These findings laid the foundation for further research on the function of *BSP* genes in the plant growth and development.

MATERIALS & METHODS

Plant materials and nitrogen treatment

Four-year-old adult *J. curcas* were grown in the experimental field of Xishuangbanna Tropical Botanical Garden (21°54′N, 101°46′E; 580 m in altitude) in Yunnan Province. Wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and the transgenic lines were grown in an environmentally controlled room at 22 °C under a 16-h light/8-h dark photoperiod. Wild-type *J. curcas* seeds were sown in sand that had been washed several times with distilled water and grown at 30 °C under a 12-h light/12-h dark photoperiod. Then, two-month-old *J. curcas* seedlings were randomly divided into three groups, with 12 plants per group. The three groups were treated with different concentrations of NH_4NO_3 solution (0, 5, and 50 mM). The seedlings were watered every week with 100 mL of a NH_4NO_3 solution per cup of seedlings.

Sequence analysis

We used BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) to analyze the cDNA sequence, CDS and amino acid sequence of *JcBSP* gene family members in the NCBI database. The conserved domains of deduced protein sequences were analyzed by the NCBI Conserved Domain Database (NCBI-CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The alignment of amino acid sequences was performed using DNAMAN software (version 6, Lynnon Biosoft Corporation, Canada, https://www.lynnon.com/dnaman.html).

Phylogenetic analysis

To examine the phylogenetic relationships of the BSP homologues from different species, we retrieved the deduced protein sequences from the NCBI database (https://www.ncbi.nlm.nih.gov/) and selected the sequences from species belonging to Euphorbiaceae and *Populus* with the highest sequence similarity to JcBSPs. Sequences of PtdPNI288 and PtdWIN4 were derived from the reference literature (*Cooke & Weih, 2005*; *Wildhagen et al., 2010*). A phylogenetic tree was built in the MEGA program (version 7.0, https://megasoftware.net/) using the neighbor-joining method with 1000 bootstrap replicates.

RNA extraction

The samples for RNA extraction included different tissues (roots, stems, shoot apices, young leaves, mature leaves, male flowers, female flowers, fruits and seeds) of four-year-old adult *J. curcas*, stems collected from October 2019 to August 2020, two-month-old *J. curcas* seedlings treated with different concentrations of NH_4NO_3 solution for 0, 2, 4, 6, and 8 weeks, and leaves of one-month-old WT and transgenic *Arabidopsis*. These samples were quickly frozen in liquid nitrogen and stored at −80 °C. Total RNA was extracted using the silica adsorption method (*Ding et al., 2008*) , and the concentration and purity of RNA were detected by spectrophotometry and agarose gel electrophoresis, respectively.

qRT-PCR analysis

Reverse transcription of total RNA was performed using the PrimeScript® RT reagent kit with gDNA Eraser (Takara, Dalian, China). qRT-PCR was performed on the Roche 480 real-time PCR detection system using LightCycler® 480 SYBR Green I Master Mix (Roche Diagnostics, Indianapolis, IN, USA). The qRT-PCR reactions were performed under the following conditions: 5 min at 95 °C for the initial denaturation, followed by 42 cycles of 10 s at 95 °C, 20 s at 57 °C, and 20 s at 72 °C for the PCR amplification, and 1 cycle of 30 s at 95 °C, 30 s at 65 °C and 0.06 °C/s heating up to 95 °C for the melting curve. Data was analyzed using the $2^{-\Delta\Delta CT}$ method as described by *Livak & Schmittgen (2001)*. All expression data obtained in the qRT-PCR assay were normalized to the expression of *JcActin1* (*Zhang et al., 2013*) and *AtActin2*. The primers used for qRT-PCR are listed in Table S1.

Protein extraction

Stem samples of four-year-old adult *J. curcas* collected from October 2019 to August 2020 were also used for total protein extraction. The bark + phloem and xylem + pith dissected from stems were ground into powder mixtures. Fifty milligrams of powder were homogenized in 300 μl of protein extract buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol). The mixture was incubated at 4 °C for 30 min and then centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was collected and analyzed by a Bradford protein assay kit (BL524A, GUANGKE Technology Company, Kunming).

Correlation analysis

Correlation analysis between the total protein concentration and *JcBSP1* expression was performed by the method of Spearman's rank correlation using R package ggpubr (version 0.4.0, https://cran.r-project.org/).

Construction of the *JcBSP1* overexpression vector and *Arabidopsis* transformation

The primers XD423 (CGAGCTCATGGCTATGGCGACGGTGAT) and XD424 (CGGATCCTCACTCTTCATCATAACGGA) carrying *Sac*I and *Bam*HI restriction sites, respectively, were used to clone the full-length *JcBSP1* CDS. Then, the PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). To generate the *35S:JcBSP1* overexpression vector, *Sac*I and *Bam*HI were used to digest the plant transformation vector pOCA30 (*Chen & Chen, 2002*) and the pGEM-T Easy vector containing the *JcBSP1* sequence, respectively, and then, the resulting fragments were ligated by using T4 DNA Ligase (Promega). The generated *35S:JcBSP1* plasmid was transferred to *Agrobacterium tumefaciens* EHA105. Transformation of *Arabidopsis* was performed using the floral dip method (*Clough & Bent, 1998*).

Table 1 Sequence information for members of the *JcBSP* family in *J. curcas*.

Gene name	GenBank accession number	cDNA (bp)	CDS (bp)	Number of amino acids (aa)
JcBSP1	XM_012218517	1,248	972	323
JcBSP2	XM_012214829	1,144	1,017	338
JcBSP3	XM_012218526	959	843	280
JcBSP4	XM_012218520	1,038	702	233
JcBSP5	XM_012222025	1,274	1,062	353
JcBSP6	XM_012222124	1,174	1,041	346

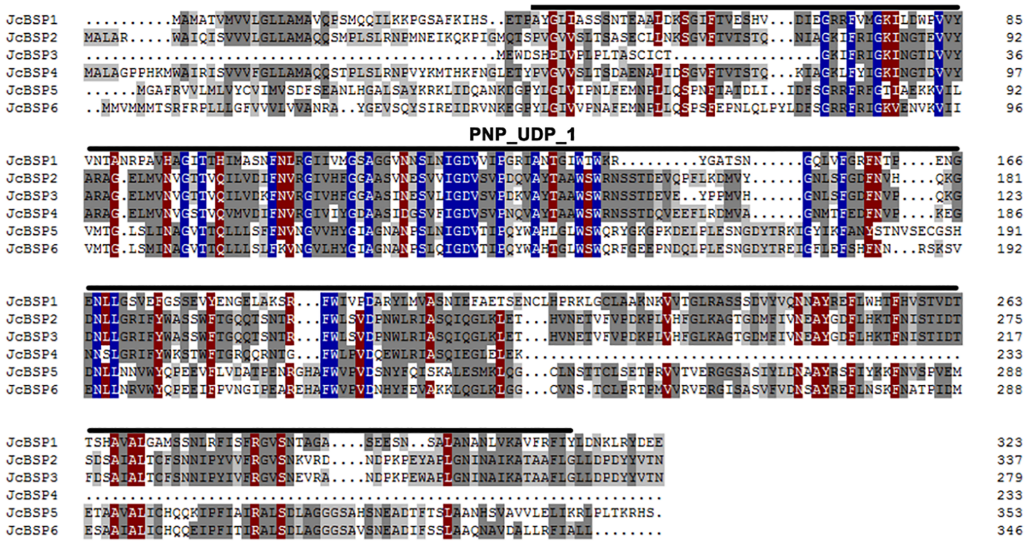

Figure 1 **Protein sequence alignment of *JcBSP* family members of *J. curcas*.** Identically conserved amino acid sequences are shown with a dark blue background, and partially conserved amino acid sequences are shown with grey and brown backgrounds; the conserved PNP_UDP_1 domain of JcBSP is indicated with overlining.

Full-size DOI: 10.7717/peerj.12938/fig-1

RESULTS

Identification of the members of the *JcBSP* gene family

We found six members of the *JcBSP* gene family in *J. curcas* from the NCBI database using BLAST and designated them as *JcBSP1*, *JcBSP2*, *JcBSP3*, *JcBSP4*, *JcBSP5*, and *JcBSP6* (Table 1). All the *JcBSP* gene family members contain a conserved domain, purine nucleoside phosphorylase_uridine phosphorylase_1 (PNP_UDP_1), which is a signature of the phosphorylase superfamily (Fig. 1).

To investigate the evolutionary relationships among *BSP* homologous genes, we performed phylogenetic analysis of genes from *J. curcas* and other species. The phylogenetic tree showed that JcBSP1, JcBSP5, and JcBSP6 were closely related to the BSP homologues from Euphorbiaceous species, and JcBSP2, JcBSP3, and JcBSP4 were closely related to the BSP homologues from poplar (Fig. 2).

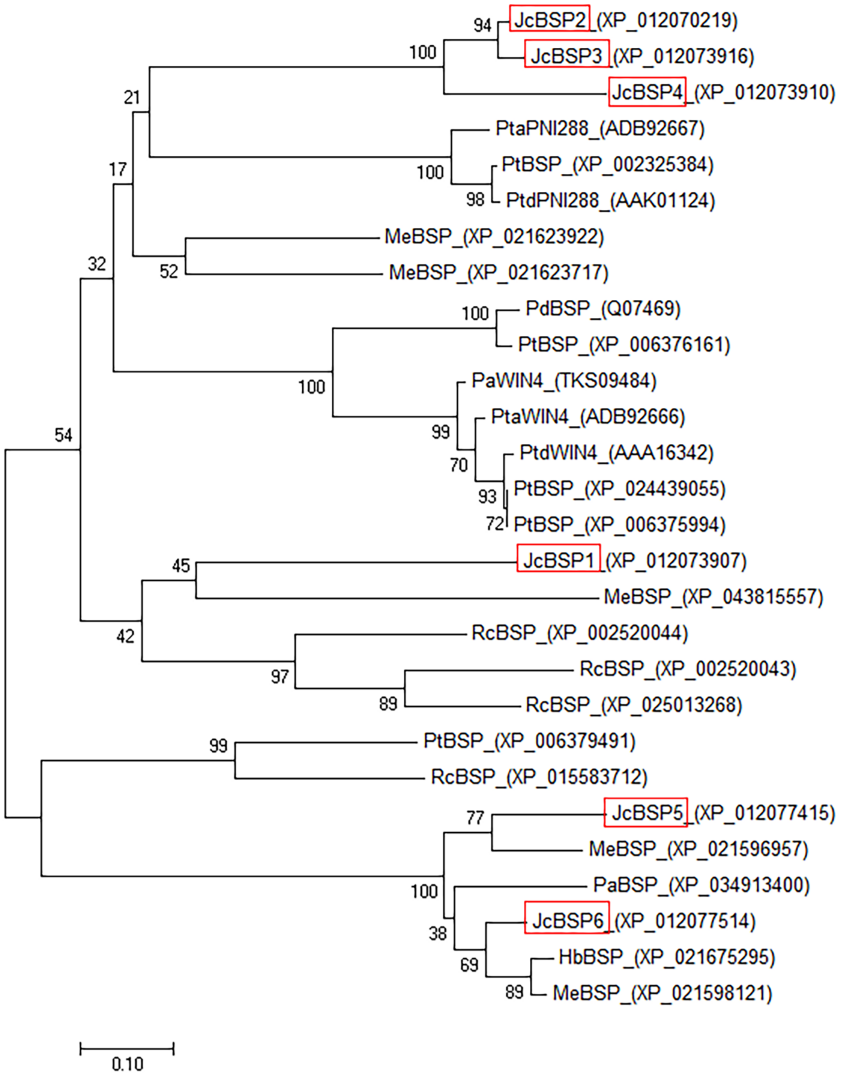

Figure 2 **Phylogenetic tree analysis of BSP homologues.** The homologues compared with *J. curcas* BSPs include *Ricinus communis* RcBSP; *Populus trichocarpa* PtBSP; *Populus deltoids* PdBSP; *Populus alba* PaBSP and PaWIN4; *Hevea brasiliensis* HbBSP; *Manihot esculenta* MeBSP; *P. trichocarpa* × *P. deltoids* PtdWIN4 and PtdPNI288; and *P. trichocarpa* × *P. alba* PtaWIN4 and PtaPNI288. The phylogenetic tree was constructed by the neighbor-joining method in MEGA 7.0 software; one thousand replicates were used for the bootstrap test; red frame: JcBSP family members.

Full-size DOI: 10.7717/peerj.12938/fig-2

The expression patterns of *JcBSP*s in *J. curcas*

To analyze the expression patterns of *JcBSP* family members, we used qRT-PCR to detect the expression levels of *JcBSP*s in roots, stems, shoot apices, young leaves, mature leaves, male flowers, female flowers, fruits, and seeds of adult *J. curcas*. The results showed that *JcBSP1* and *JcBSP2* exhibited similar expression patterns, in which both were highly expressed in shoot apices, stems, young leaves and female flowers; *JcBSP3* was mainly expressed in roots, stems, shoot apices, male flowers and fruits; the expression of *JcBSP4* was concentrated in reproductive tissues, with the highest expression level in female flowers; the expression of

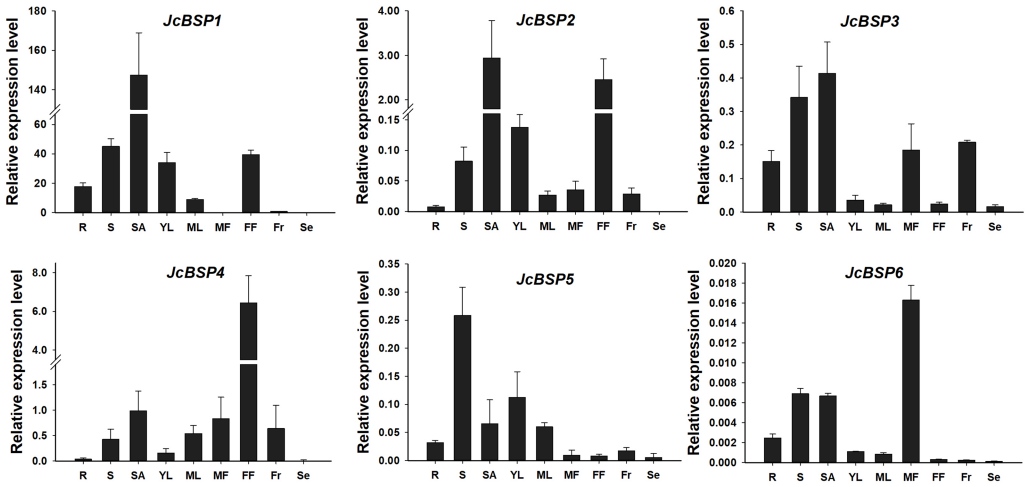

Figure 3 **Expression analysis of *JcBSP*s in various tissues of adult *J. curcas*.** The qRT-PCR results were obtained from three biological replicates and three technical replicates. The values were normalized to the expression of *JcActin1*. Error bars denote the standard deviation (SD) calculated from three biological replicates. R, roots; S, stems; SA, shoot apices; YL, young leaves; ML, mature leaves; MF, male flowers; FF, female flowers; Fr, fruits; Se, seeds.

Full-size DOI: 10.7717/peerj.12938/fig-3

JcBSP5 was concentrated in vegetative tissues, with the highest expression level in stems; and *JcBSP6* was remarkably expressed in male flowers (Fig. 3). Based on these results, we hypothesized that *JcBSP*s could play important roles in the growth and development of various organs, except seeds, in which all the members were barely expressed. This finding also indicates that JcBSPs may be vegetative storage proteins rather than seed storage proteins.

Seasonal changes in total protein concentrations and *JcBSP* expression in the stems of *J. curcas*

In perennial deciduous trees, most nitrogen resources in senescing leaves are transported to perennial tissues (bark and wood), and stored as proteins during autumn and winter; the next spring, these proteins are hydrolyzed to amino acids, which are transported from perennial tissues to growing tissues (*Chapin & Kedrowski, 1983*; *Cooke & Weih, 2005*; *Sauter, Cleve & Wellenkamp, 1989*). Therefore, we investigated whether the total protein concentration in *J. curcas* stems was relevant to seasons.

From October 2019 to August 2020, we sampled the stems of adult *J. curcas* in two parts (bark + phloem and xylem + pith), as shown in Fig. 4A, and examined the total protein concentrations of the samples. The results showed that the total protein concentrations in the bark + phloem and xylem + pith were approximately 10.5 mg/g FW in October; then, *J. curcas* entered the dormant stage in December, and the total protein concentrations reached a peak in the bark + phloem (12.3 mg/g FW) and xylem + pith (16.4 mg/g FW). When *J. curcas* began to enter the growing season in March, the total protein concentrations decreased sharply to 7 mg/g FW in the bark + phloem, which further decreased to 5.5 mg/g FW in August. The total protein concentration in the xylem + pith showed a similar trend

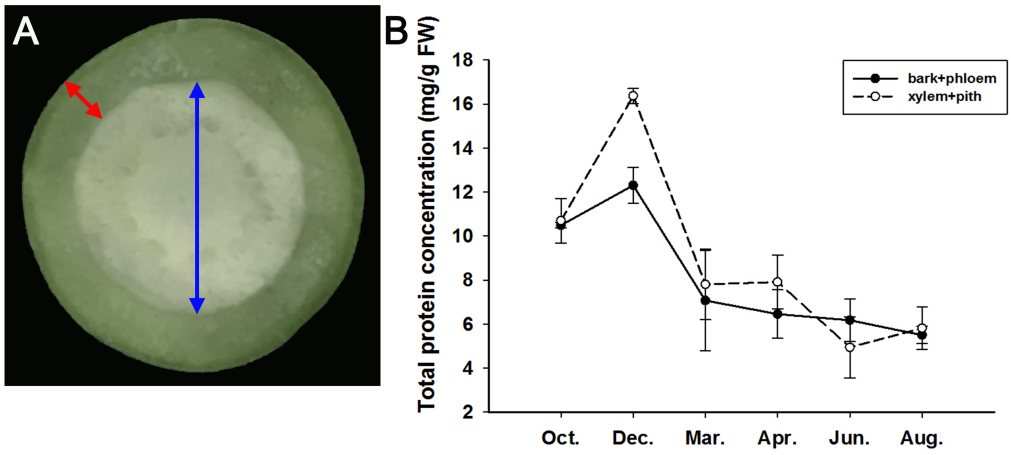

Figure 4 **Seasonal changes in total protein concentrations in the stems of adult *J. curcas*.** (A) Cross section of stems. The red arrow shows the bark and phloem, and the blue arrow shows the xylem and pith. (B) Total protein concentrations of *J. curcas* stems. The results were obtained from three biological replicates. Error bars denote the SD calculated from three biological replicates.

Full-size DOI: 10.7717/peerj.12938/fig-4

(Fig. 4B). The total protein concentration in the *J. curcas* stem exhibited a seasonal change, which accumulated in autumn and winter and decreased in spring and summer. This result indicates that the total protein in the stems is a form of nitrogen storage during the overwintering period of *J. curcas*, and this protein is reallocated during the growing seasons.

Based on the above results, we examined the seasonal course of *JcBSP* expression in the same samples mentioned above (Fig. 4A) to investigate whether the expression of *JcBSP*s shows the same seasonal changes as the total protein concentration. The results showed that the expression of *JcBSP* family members in stems exhibited different patterns over the seasonal course (Fig. 5). From autumn to winter, the expression of *JcBSP1* in the two parts of the stems increased rapidly, with a higher level in xylem + pith, and then decreased sharply in spring and remained low until August. The seasonal changes in *JcBSP1* expression in the two parts of the stem were entirely consistent with those of the total protein concentration. However, the seasonal expression patterns of *JcBSP2*, *JcBSP4* and *JcBSP5* in stems were inconsistent with those of the total protein concentration. In addition, only in the xylem + pith did the expression of *JcBSP3* and *JcBSP6* show the same seasonal changes as that of the total protein concentration, but their expression levels were much lower than that of *JcBSP1*. Therefore, we analyzed the correlation between seasonal changes in the total protein concentration and *JcBSP1* expression. It turned out that there were significant correlations between them in the bark + phloem ($r = 0.63$, $P < 0.01$) and the xylem + pith ($r = 0.67$, $P < 0.01$) (Fig. 6). These results suggest that *JcBSP1* might play an important role in seasonal nitrogen cycling.

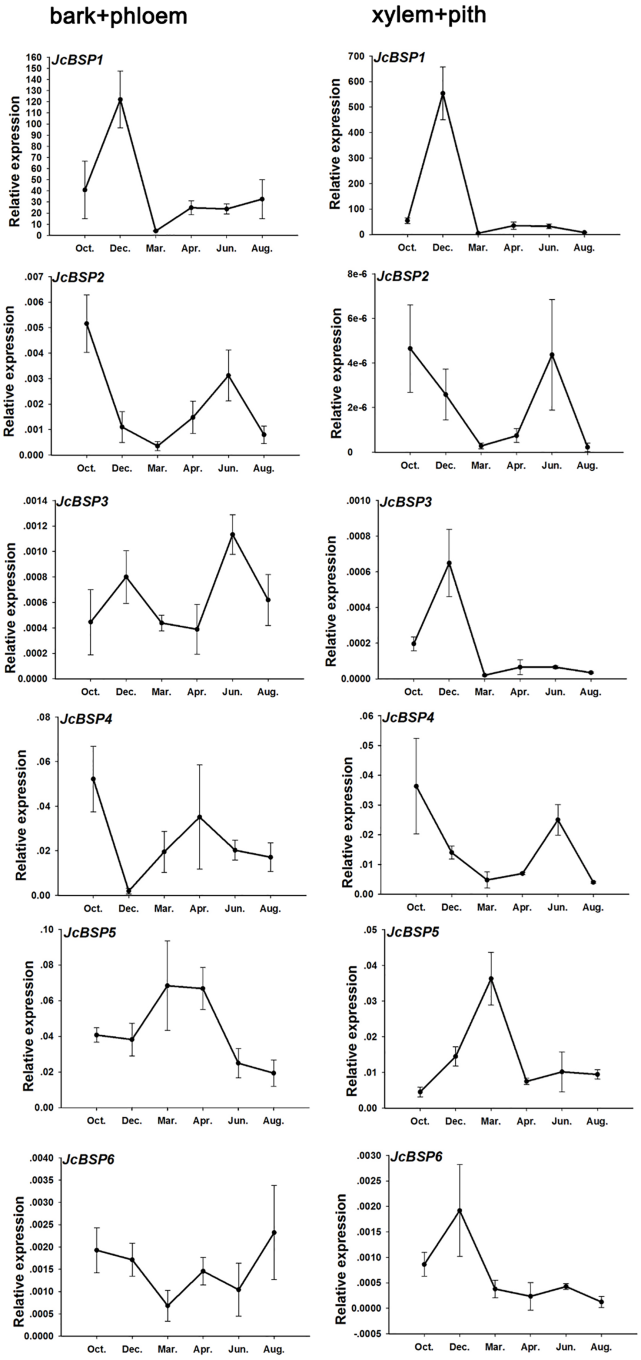

Figure 5 **Seasonal changes in *JcBSP* expression in the two parts of *J. curcas* stems.** The qRT-PCR results were obtained from three biological replicates and three technical replicates. The values were normalized to the expression of *JcActin1*. Error bars denote the SD calculated from three biological replicates.
Full-size DOI: 10.7717/peerj.12938/fig-5

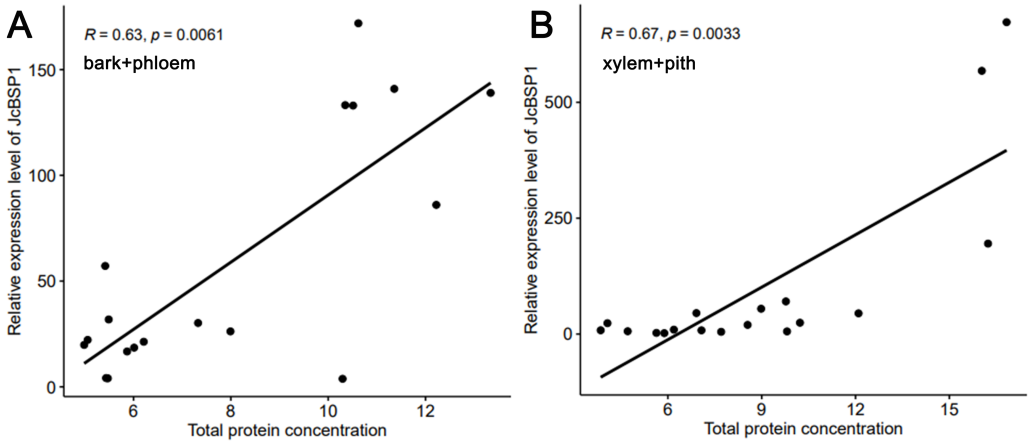

Figure 6 **Correlation analysis between the seasonal changes in total protein concentration and *JcBSP1* expression in the bark + phloem (A) and the xylem + pith (B).**

Full-size DOI: 10.7717/peerj.12938/fig-6

JcBSP expression in response to nitrogen

To further verify the correlation between *JcBSP*s and seasonal nitrogen cycling, we investigated the response of these genes to nitrogen induction. We applied a 0, 5, and 50 mM NH_4NO_3 solution to two-month-old *J. curcas* seedlings. After 8 weeks of treatment, we found that the group treated with the 5 mM NH_4NO_3 solution grew better than the other two groups (Fig. 7A). This result indicated that nitrogen supply in a certain concentration range could effectively promote the growth of *J. curcas*.

We collected the leaves of *J. curcas* seedlings treated with different concentrations of NH_4NO_3 for 0, 2, 4, 6, and 8 weeks to detect changes in *JcBSP* family member expression. The results showed that the expression of *JcBSP1* increased obviously along with the increased NH_4NO_3 concentration and application duration; *JcBSP2* expression increased remarkably with the 5 mM NH_4NO_3 treatment for 2–8 weeks and with 50 mM NH_4NO_3 treatments for 8 weeks; *JcBSP4* expression was induced obviously with only the 50 mM NH_4NO_3 treatment for 4 weeks; however, the expression of *JcBSP3*, *JcBSP5* and *JcBSP6* was not induced by NH_4NO_3 treatment (Fig. 7B). The results indicated that *JcBSP1* and *JcBSP2* expression is responsive to nitrogen induction. In particular, *JcBSP1* expression was positively correlated with the nitrogen concentration and application duration. Combined with the seasonal changes in *JcBSP1* expression in stems, we further concluded that JcBSP1 might be a form of nitrogen storage in the seasonal nitrogen cycling in *J. curcas*.

Overexpression of *J. curcas JcBSP1* in *Arabidopsis* led to enlarged rosette leaves, flowers, and seeds

Next, we investigated the function of *JcBSP1* in plant growth and development in transgenic *Arabidopsis*. Twenty-two independent *35S:JcBSP1* transgenic *Arabidopsis* lines were generated (Fig. 8A). *JcBSP1* expression in seven transgenic lines showing similar phenotypes was analyzed, and most of transgenic lines yielded abundant transgene expression (Fig. S1). We investigated in further detail the phenotypes of two independent transgenic lines, L4

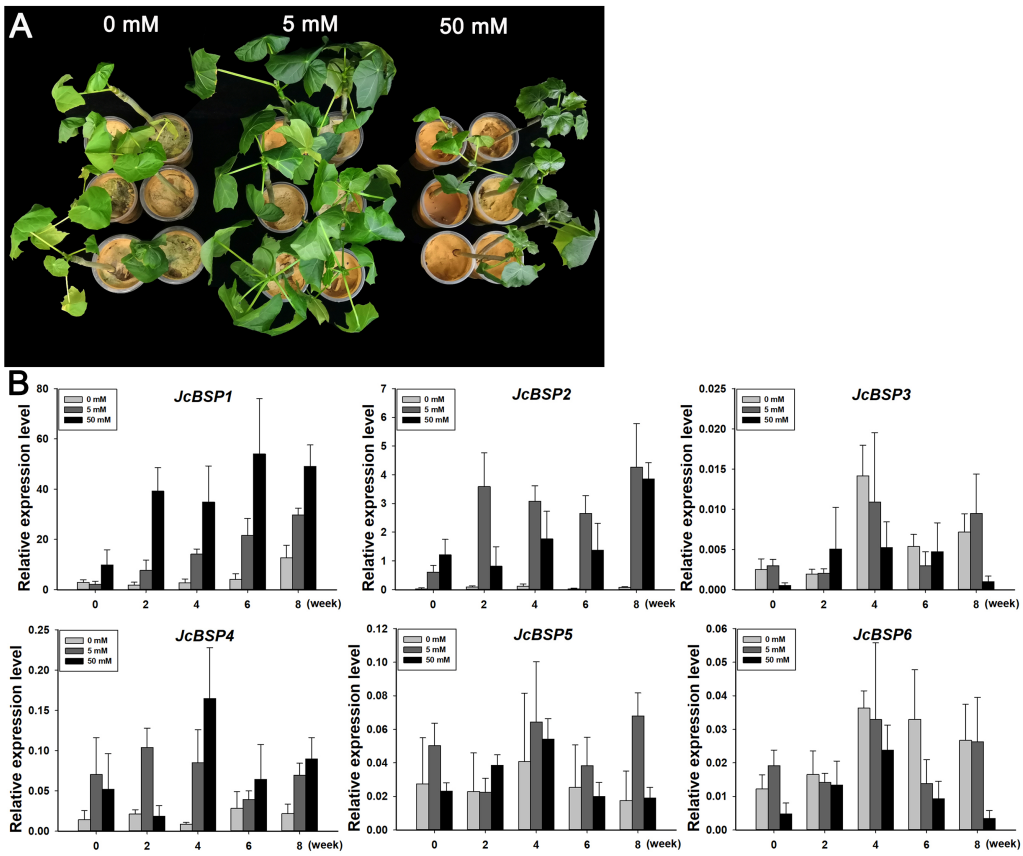

Figure 7 Changes in *JcBSP* expression in the leaves of two-month-old *J. curcas* seedlings treated with NH_4NO_3. (A) *J. curcas* seedlings treated with different concentrations of NH_4NO_3 for 8 weeks. (B)*JcBSP* expression in response to NH_4NO_3 treatment. The qRT-PCR results were obtained from three biological replicates and three technical replicates. The values were normalized to the expression of *JcActin1*. Error bars denote the SD calculated from three biological replicates.

Full-size DOI: 10.7717/peerj.12938/fig-7

and L10, which exhibited high and intermediate expression levels of *JcBSP1*, respectively (Fig. 8B).

During plant growth and development, we found that *35S:JcBSP1* transgenic *Arabidopsis* produced larger rosette leaves and flowers than the wild-type (WT) plants (Figs. 8C and 8D). As shown in Figs. 8E and 8F, the rosette leaf lengths and widths were all significantly increased in transgenic lines. And larger seeds and significantly increased hundred-seed weights were found in transgenic plants (Figs. 9A and 9B). Consequently, the seed yields in transgenic lines were significantly higher than that of the WT (Fig. 9C). These results indicated that *JcBSP1* was able to affect plant growth and development.

DISCUSSION

BSP, a kind of VSP, is a main nutrient storage protein in perennial woody plants and a form of nitrogen storage in vegetative tissues (*Cooke & Weih, 2005*; *Staswick, 1994*). It is different from seed storage protein, which accumulates during seed maturation and

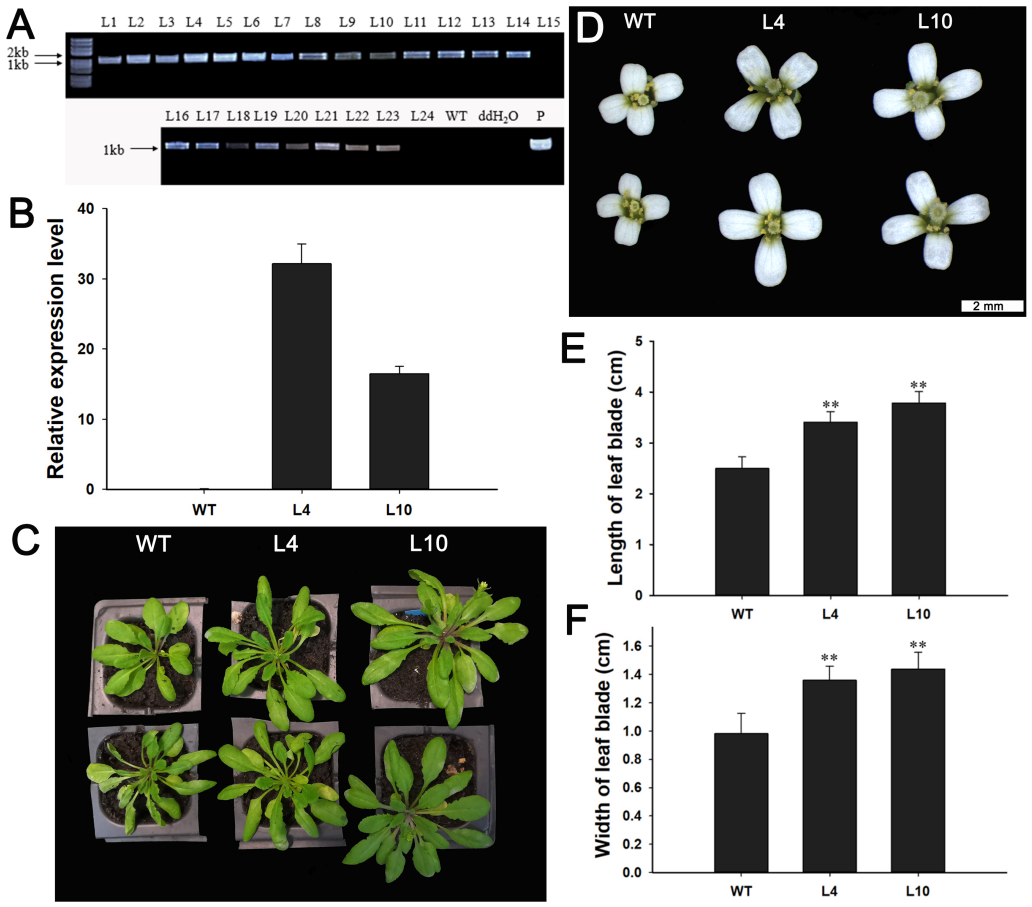

Figure 8 **Phenotypic changes in *35S:JcBSP1* transgenic *Arabidopsis*.** (A) PCR identification of *35S:JcBSP1* transgenic *Arabidopsis*. WT, wild-type negative control; ddH_2O, ddH_2O negative control; P, positive control. (B) qRT-PCR analysis of *JcBSP1* expression in WT and transgenic *Arabidopsis* (L4 and L10). The qRT-PCR results were obtained from three biological replicates and three technical replicates. The values were normalized to the expression of *AtActin2*. Error bars denote the SD calculated from three biological replicates. (C and D) Rosette leaves and flowers from WT and transgenic *Arabidopsis*. (E and F) Leaf length and width of WT and transgenic *Arabidopsis*. The values are presented as the means ± standard deviations ($n = 8$). Student's *t*-test was used for significance analysis: $^{**}P \leq 0.01$.

Full-size DOI: 10.7717/peerj.12938/fig-8

provides a nitrogen source for embryo development (*Autran, Halford & Shewry, 2001*; *Gacek, Bartkowiak-Broda & Batley, 2018*; *Kawakatsu et al., 2010*). In *Populus*, *BSP* has been found to be highly expressed in the bark, dormant cambium and bud (*Coleman, Baíiados & Chen, 1994*; *Cooke & Weih, 2005*) , and the *bspA* promoter has been shown to be predominantly active in bark (*Zhu & Coleman, 2001*). In this study, we identified six members of the *JcBSP* gene family in *J. curcas*, and none of them were expressed in seeds (Fig. 3), indicating that JcBSP might be a nutrient storage protein rather than seed storage protein. In addition, *JcBSP1*, *JcBSP2* and *JcBSP4* were highly expressed in female flowers, and *JcBSP3* and *JcBSP6* were relatively highly expressed in male flowers, suggesting that they may be involved in the development of female and male flowers, respectively.

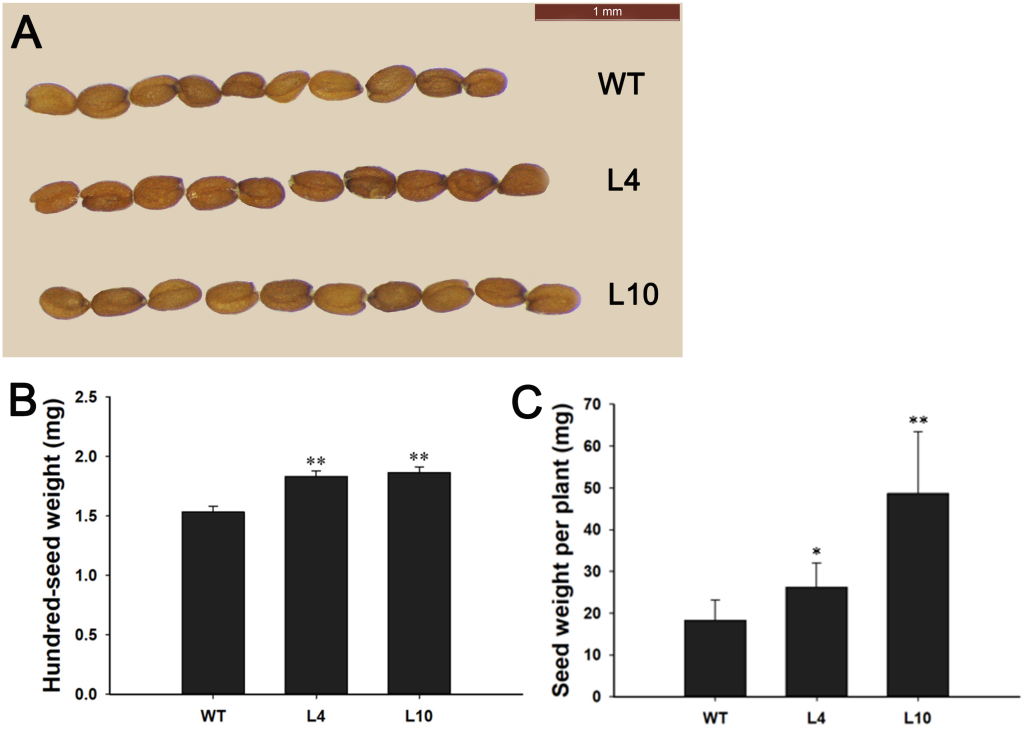

Figure 9 Overexpression of *JcBSP1* increased the size, weight, and yield of seeds in transgenic *Arabidopsis*. Seed size (A), hundred-seed weight (B) ($n = 3$) and seed yield per plant (C) ($n = 6$) were analyzed in WT and transgenic *Arabidopsis* lines L4 and L10. The values are presented as the means ± standard deviations. Student's *t*-test was used for significance analysis: $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

Full-size DOI: 10.7717/peerj.12938/fig-9

In perennial woody plants, BSP plays an important role in seasonal nitrogen cycling (*Wetzel, Demmers & Greenwood, 1989b*; *Wetzel & Greenwood, 1989*; *Wildhagen et al., 2010*). During autumn and winter, nitrogen-rich amino acids are transported from senescing leaves to perennial tissues and subsequently used to synthesize proteins for nitrogen storage (*Geßler, Kopriva & Rennenberg, 2004*; *Hörtensteiner & Feller, 2002*). BSP is the main form of nitrogen storage in trees during the dormant period, which accumulates in autumn and winter (*Cooke & Weih, 2005*; *Wetzel, Demmers & Greenwood, 1989b*). In this study, we found that the seasonal changes in *JcBSP1* expression in stems were consistent with those of the total protein concentration, as both increased in autumn and winter and then decreased in spring and summer (Figs. 4B and 5). And there is a significant correlation between seasonal changes in the total protein concentration and *JcBSP1* expression (Fig. 6), suggesting that JcBSP1 might be the main protein stored in the stem of *J. curcas* during overwintering. Moreover, the expression of *JcBSP1* was positively correlated with the nitrogen concentration and application duration (Fig. 7B). Therefore, we hypothesized that JcBSP1 might play an important role in the seasonal nitrogen cycling of *J. curcas*, acting as a form of nitrogen storage in the stems during overwintering. In addition, this study was conducted in the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, which is located in a tropical region of China. According to rainfall, there are

two seasons, a rainy season from May to October and a dry season from November to April of the following year, in Xishuangbanna area. The dry season is further divided into the foggy-cool season from November to February of the following year and the dry-hot season from March to April. Although the foggy-cool season has little precipitation, there is a large amount of dense fog from night to noon, which has a certain compensation effect on the water demand of plants in dry season; the dry-hot season has a dry climate, low precipitation and large daily temperature differences (*Zhao et al., 2009*). As shown in Figs. 4B and 5, both of the total protein concentration and *JcBSP1* expression in the stems were decreased to a very low level at the beginning of the dry-hot season. Hence, further studies are required to link the seasonal changes in total protein concentration and *JcBSP1* expression to possible drought-related protein mobilization.

It is well known that nitrogen is an important nutrient for plant growth and development. *Lemaitre et al. (2008)* showed that when *Arabidopsis* grew under low nitrogen conditions, rosette biomass and seed yield were limited. Storage proteins are considered as nitrogen source that are utilized for plant growth (*Sözen, 2004*; *Titus & Kang, 1982*). In this study, we found that overexpression of *JcBSP1* could promote the growth and development of rosette leaves, flowers, and seeds in transgenic *Arabidopsis* (Figs. 8 and 9). This finding further indicates the JcBSP1 might be a form of nitrogen storage in plants, serving as a nutrient provider. Similarly, overexpression of a storage protein gene *AmA1* in potato could increase the growth and production of tubers (*Chakraborty, Chakraborty & Datta, 2000*). By analyzing cell architectures, the cell areas in cortex, perimedullary and pith regions of the tuber were found to be increased, which indicated the AmA1 storage protein in potato tuber was correlated with cell growth (*Agrawal et al., 2013*). In cabbage, when the nitrogen supply can't meet the need of plant growth, the leaf cells became smaller while the number of cell layers remained unchanged (*Kano et al., 2007*). It turns out that both endogenous and exogenous nitrogen sources could affect cell growth. Accordingly, overexpression of *JcBSP1* in transgenic *Arabidopsis* may also promote the cell growth, resulting in enhanced plant growth and production. Furthermore, it is worthy to mention here that although the expression level of *JcBSP1* in the transgenic line L4 was higher than that in L10 (Fig. 8B), the leaves and seeds in L4 were relatively smaller than those in L10 (Figs. 8C, 8E, 8F; 9). We hypothesized that this phenotype might be caused by the excessively high *JcBSP1* transgene expression, which might lead to excess JcBSP1 protein storage and subsequently excess nitrogen accumulation in L4 plants. Previous study showed that under the excess nitrogen conditions, both cell number and size were found to be reduced in leaves (*MacAdam, Volenec & Nelson, 1988*). In addition, about half of the Rubisco are inactive or only half of the catalytic sites are functional, which certainly leads to a decrease in photosynthetic efficiency and therefore a retardation in plant growth (*Chapin, Schulze & Mooney, 1990*; *Cheng & Fuchigami, 2000*; *Millard, 1988*). As shown in Fig. 7A, the excessive nitrogen supply does have a certain negative impact on *J. curcas* growth. Consistently, *Barbosa et al. (2010)* also found that when the adding nitrogen concentration was below 40 mM, it stimulated *Arabidopsis* root growth, while the concentration was higher than 40 mM, root elongation was inhibited.

In addition, VSPs may also play a role in plant defense. In *Arabidopsis*, *AtVSP1* and *AtVSP2* have been shown to enhance plant resistance to diseases and insects (*Berger, Mitchell-Oldsb & Stotz, 2002*; *Ellis & Turner, 2001*; *Liu et al., 2005*). Furthermore, *AtVSP1* and *AtVSP2* have been found to be highly expressed in flowers (*Utsugi et al., 1998*), which implies a mechanism used by *Arabidopsis* to protect reproductive structures (*Liu et al., 2005*). Interestingly, most *JcBSP*s were also highly expressed in female or male flowers (Fig. 3). Thus, in addition to being a provider of nitrogen resources, *JcBSP*s may also play other roles in plant growth and development, which requires further study.

CONCLUSIONS

In this study, six members of the *JcBSP* gene family were identified in *J. curcas*, which were expressed in various tissues, except seeds. Among these members, only the expression of *JcBSP1* was positively correlated with the total protein concentration in the stems during seasonal changes and with the exogenous nitrogen application. We thus supposed that JcBSP1 could play an important role in seasonal nitrogen cycling as a form of nitrogen storage. By the function analysis of *JcBSP1* in transgenic *Arabidopsis*, we found that *JcBSP1* was able to enhance the plant growth and production. This suggests that *JcBSP1* could be useful in crop breeding.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Central Laboratory of the Xishuangbanna Tropical Botanical Garden for providing the research facilities.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by funding from the National Natural Science Foundation of China (No. 31771605) and the Chinese Academy of Sciences (No. kfj-brsn-2018-6-008). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
The National Natural Science Foundation of China: No. 31771605.
The Chinese Academy of Sciences: No. kfj-brsn-2018-6-008.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Ming-Jun Zhang performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

- Qiantang Fu, Huiying He and Mingyong Tang performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Mao-Sheng Chen analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Jun Ni performed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Yan-Bin Tao and Zeng-Fu Xu conceived and designed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The qRT-PCR primers, *JcBSP1* expression in wild-type (WT) and transgenic *Arabidopsis* lines and raw measurements are available in the Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12938#supplemental-information.

REFERENCES

Agrawal L, Narula K, Basu S, Shekhar S, Ghosh S, Datta A, Chakraborty N, Chakraborty S. 2013. Comparative proteomics reveals a role for seed storage protein AmA1 in cellular growth, development, and nutrient accumulation. *Journal of Proteome Research* **12**:4904–4930 DOI 10.1021/pr4007987.

Autran JC, Halford NG, Shewry PR. 2001. The biochemistry and molecular biology of seed storage proteins. In: Lea PD, Morot-Gaudry JF, eds. *The assimilation of nitrogen by plants*. Berlin: Springer-Verlag, 295–341 DOI 10.1007/978-3-662-04064-5_12.

Babst BA, Coleman GD. 2018. Seasonal nitrogen cycling in temperate trees: transport and regulatory mechanisms are key missing links. *Plant Science* **270**:268–277 DOI 10.1016/j.plantsci.2018.02.021.

Barbosa JM, Singh NK, Cherry JH, Locy RD. 2010. Nitrate uptake and utilization is modulated by exogenous γ-aminobutyric acid in *Arabidopsis thaliana* seedlings. *Plant Physiology and Biochemistry* **48**:443–450 DOI 10.1016/j.plaphy.2010.01.020.

Berger S, Mitchell-Oldsb T, Stotz HU. 2002. Local and differential control of vegetative storage protein expression in response to herbivore damage in *Arabidopsis thaliana*. *Physiologia Plantarum* **114**:85–91 DOI 10.1046/j.0031-9317.2001.1140112.x.

Black BL, Parmentier-Line CM, Fuchigami LH, Coleman GD. 2001. Ecotypic and genetic variation in poplar bark storage protein gene expression and accumulation. *Tree Physiology* **21**:1289–1297 DOI 10.1093/treephys/21.17.1289.

Chakraborty S, Chakraborty N, Datta A. 2000. Increased nutritive value of transgenic potato by expressing a nonallergenic seed albumin gene from *Amaranthus hypochondriacus*. *Proceedings of the National Academy of Sciences of the United States of America* **97**:3724–3729 DOI 10.1073/pnas.050012697.

Chapin FS, Kedrowski RA. 1983. Seasonal changes in nitrogen and phosphorus fractions and autumn retranslocation in evergreen and deciduous taiga trees. *Ecology* **64**:376–391 DOI 10.2307/1937083.

Chapin FS, Schulze ED, Mooney HA. 1990. The ecology and economics of storage in plants. *Annual Review of Ecology and Systematics* **21**:423–447 DOI 10.1146/annurev.ecolsys.21.1.423.

Chen CH, Chen ZX. 2002. Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. *Plant Physiology* **129**:706–716 DOI 10.1104/pp.001057.

Cheng LL, Fuchigami LH. 2000. Rubisco activation state decreases with increasing nitrogen content in apple leaves. *Journal of Experimental Botany* **51**:1687–1694 DOI 10.1093/jexbot/51.352.1949-a.

Clausen S, Apel K. 1991. Seasonal changes in the concentration of the major storage protein and its mRNA in xylem ray cells of poplar trees. *Plant Molecular Biology* **17**:669–678 DOI 10.1007/BF00037052.

Cleve BV, Apel K. 1993. Induction by nitrogen and low temperature of storage-protein synthesis in poplar trees exposed to long days. *Planta* **189**:157–160 DOI 10.1007/BF00201357.

Cleve BV, Clausen S, Sauter JJ. 1988. Immunochemical localization of a storage protein in poplar wood. *Plant Physiology* **133**:371–374 DOI 10.1016/S0176-1617(88)80219-0.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**:735–743 DOI 10.1046/j.1365-313x.1998.00343.x.

Coleman GD, Baíiados MP, Chen THH. 1994. Poplar bark storage protein and a related wound-induced gene are differentially induced by nitrogen. *Plant Physiology* **106**:211–215 DOI 10.1104/pp.106.1.211.

Coleman GD, Chen THH, Ernst SG, Fuchigami L. 1991. Photoperiod control of poplar bark storage protein accumulation. *Plant Physiology* **96**:686–692 DOI 10.1104/pp.96.3.686.

Coleman GD, Chen THH, Fuchigami LH. 1992. Complementary DNA cloning of poplar bark storage protein and control of its expression by photoperiod. *Plant Physiology* **98**:687–693 DOI 10.1104/pp.98.2.687.

Coleman GD, Englert JM, Chen THH, Fuchigami LH. 1993. Physiological and environmental requirements for poplar (*Populus deltoides*) bark storage protein degradation. *Plant Physiology* **102**:53–59 DOI 10.1104/pp.102.1.53.

Cooke JEK, Weih M. 2005. Nitrogen storage and seasonal nitrogen cycling in *Populus*: bridging molecular physiology and ecophysiology. *New Phytologist* **167**:19–30 DOI 10.1111/j.1469-8137.2005.01451.x.

Ding LW, Sun QY, Wang ZY, Sun YB, Xu ZF. 2008. Using silica particles to isolate total RNA from plant tissues recalcitrant to extraction in guanidine thiocyanate. *Analytical Biochemistry* **374**:426–428 DOI 10.1016/j.ab.2007.11.030.

Divakara BN, Upadhyaya HD, Wani SP, Gowda CLL. 2010. Biology and genetic improvement of *Jatropha curcas* L.: a review. *Applied Energy* **87**:732–742 DOI 10.1016/j.apenergy.2009.07.013.

Ellis C, Turner JG. 2001. The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. *The Plant Cell* **13**:1025–1033 DOI 10.2307/3871361.

Gacek K, Bartkowiak-Broda I, Batley J. 2018. Genetic and molecular regulation of seed storage proteins (SSPs) to improve protein nutritional value of oilseed rape (*Brassica napus* L.) seeds. *Frontiers in Plant Science* **9**:890 DOI 10.3389/fpls.2018.00890.

Geßler A, Kopriva S, Rennenberg H. 2004. Regulation of nitrate uptake at the whole-tree level: interaction between nitrogen compounds, cytokinins and carbon metabolism. *Tree Physiology* **24**:1313–1321 DOI 10.1093/treephys/24.12.1313.

Hörtensteiner S, Feller U. 2002. Nitrogen metabolism and remobilization during senescence. *Journal of Experimental Botany* **53**:927–937 DOI 10.1093/jexbot/53.370.927.

Kamel DA, Farag HA, Amin NK, Zatout AA, Ali RM. 2018. Smart utilization of jatropha (*Jatropha curcas* Linnaeus) seeds for biodiesel production: optimization and mechanism. *Industrial Crops and Products* **111**:407–413 DOI 10.1016/j.indcrop.2017.10.029.

Kano Y, Nakagawa H, Sekine M, Goto H, Sugiura A. 2007. Effect of nitrogen fertilizer on cell size and sugar accumulation in the leaves of cabbage (*Brassica oleracea* L.). *HortScience* **42**:1490–1492 DOI 10.1590/S0102-05362007000400022.

Kawakatsu T, Hirose S, Yasuda H, Takaiwa F. 2010. Reducing rice seed storage protein accumulation leads to changes in nutrient quality and storage organelle formation. *Plant Physiology* **154**:1842–1854 DOI 10.1104/pp.110.164343.

Lawrence SD, Cooke JEK, Greenwood JS, Korhnak TE, Davis JM. 2001. Vegetative storage protein expression during terminal bud formation in poplar. *Canadian Journal of Forest Research* **31**:1098–1103 DOI 10.1139/cjfr-31-6-1098.

Lawrence SD, Greenwood JS, Korhnak TE, Davis JM. 1997. A vegetative storage protein homolog is expressed in the growing shoot apex of hybrid poplar. *Planta* **203**:237–244 DOI 10.1007/s004250050187.

Lemaitre T, Gaufichon L, Boutet-Mercey S, Christ A, Masclaux-Daubresse C. 2008. Enzymatic and metabolic diagnostic of nitrogen deficiency in *Arabidopsis thaliana* Wassileskija accession. *Plant & Cell Physiology* **49**:1056–1065 DOI 10.1093/pcp/pcn081.

Liu YL, Ahn JE, Datta S, Salzman RA, Moon J, Huyghues-Despointes B, Pittendrigh B, Murdock LL, Koiwa H, Zhu-Salzman K. 2005. Arabidopsis vegetative storage protein is an anti-insect acid phosphatase. *Plant Physiology* **139**:1545–1556 DOI 10.1104/pp.105.066837.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**:402–408 DOI 10.1006/meth.2001.1262.

MacAdam JW, Volenec JJ, Nelson CJ. 1988. Effects of nitrogen on mesophyll cell division and epidermal cell elongation in tall fescue leaf blades. *Plant Physiology* **89**:549–556 DOI 10.1104/pp.89.2.549.

Makkar HPS, Becker K. 2009. *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of Lipid Science and Technology* **111**:773–787 DOI 10.1002/ejlt.200800244.

May JD, Killingbeck KT. 1992. Effects of preventing nutrient resorption on plant fitness and foliar nutrient dynamics. *Ecology* **73**:1868–1878 DOI 10.2307/1940038.

Mazumdar P, Singh P, Babu S, Siva R, Harikrishna JA. 2018. An update on biological advancement of *Jatropha curcas* L.: New insight and challenges. *Renewable and Sustainable Energy Reviews* **91**:903–917 DOI 10.1016/j.rser.2018.04.082.

Millard P. 1988. The accumulation and storage of nitrogen by herbaceous plants. *Plant, Cell and Environment* **11**:1–8 DOI 10.1111/j.1365-3040.1988.tb01769.x.

Mohibbeazam M, Waris A, Nahar N. 2005. Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India. *Biomass and Bioenergy* **29**:293–302 DOI 10.1016/j.biombioe.2005.05.001.

Pandeya VC, Singh K, Singh JS, Kumar A, Singh B, Singh RP. 2012. *Jatropha curcas*: a potential biofuel plant for sustainable environmental development. *Renewable and Sustainable Energy Reviews* **16**:2870–2883 DOI 10.1016/j.rser.2012.02.004.

Pramanik K. 2003. Properties and use of *Jatropha curcas* oil and diesel fuel blends in compression ignition engine. *Renewable Energy* **28**:239–248 DOI 10.1016/S0960-1481(02)00027-7.

Pregitzer KS, Dickmann DI, Hendrick R, Nguyen PV. 1990. Whole-tree carbon and nitrogen partitioning in young hybrid poplars. *Tree Physiology* **7**:79–93 DOI 10.1093/treephys/7.1-2-3-4.79.

Ryan DF, Bormann FH. 1982. Nutrient resorption in northern hardwood forests. *BioScience* **32**:29–32 DOI 10.2307/1308751.

Sauter BJJ, Cleve BV, Wellenkamp S. 1989. Ultrastructural and biochemical results on the localization and distribution of storage proteins in a poplar tree and in twigs of other tree species. *Holzforschung* **43**:1–6 DOI 10.1515/hfsg.1989.43.1.1.

Sauter JJ, Cleve BV. 1992. Seasonal variation of amino acids in the xylem sap of Populus x canadensis and its relation to protein body mobilization. *Tree* **7**:26–32 DOI 10.1007/BF00225228.

Sözen E. 2004. Vegetative storage proteins in plants. *Anadolu University Journal of Science and Technology* **5**:1–7.

Staswick PE. 1994. Storage proteins of vegetative plant tissues. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**:303–322 DOI 10.1146/annurev.pp.45.060194.001511.

Titus JS, Kang SM. 1982. Nitrogen metabolism, translocation, and recycling in apple trees. *Horticultural Reviews* **4**:204–246 DOI 10.1002/9781118060773.ch7.

Utsugi S, Sakamoto W, Murata M, Motoyoshi F. 1998. *Arabidopsis thaliana* vegetative storage protein (VSP) genes: gene organization and tissue-specific expression. *Plant Molecular Biology* **38**:565–576 DOI 10.1023/A:1006072014605.

Vaknin Y, Yermiyahu U, Bar-Tal A, Samocha Y. 2018. Global maximization of *Jatropha* oil production under semi-arid conditions by balancing vegetative growth with reproductive capacity. *GCB Bioenergy* **10**:382–392 DOI 10.1111/gcbb.12497.

Wetzel S, Demmers C, Greenwood JS. 1989a. Spherical organelles, analogous to seed protein bodies, fluctuate seasonally in parenchymatous cells of hardwoods. *Canadian Journal of Botany* **67**:3439–3445 DOI 10.1139/b89-420.

Wetzel S, Demmers C, Greenwood JS. 1989b. Seasonally fluctuating bark proteins are a potential form of nitrogen storage in three temperate hardwoods. *Planta* **178**:275–281 DOI 10.1007/BF00391854.

Wetzel S, Greenwood JS. 1989. Proteins as a potential nitrogen storage compound in bark and leaves of several softwoods. *Trees* **3**:149–153 DOI 10.1007/BF00226650.

Wildhagen H, Bilela S, Rennenberg H. 2013. Low temperatures counteract short-day induced nitrogen storage, but not accumulation of bark storage protein transcripts in bark of grey poplar (*Populus x canescens*) trees. *Plant Biology* **15**:44–56 DOI 10.1111/j.1438-8677.2012.00687.x.

Wildhagen H, Dürr J, Ehlting B, Rennenberg H. 2010. Seasonal nitrogen cycling in the bark of field-grown grey poplar is correlated with meteorological factors and gene expression of bark storage proteins. *Tree Physiology* **30**:1096–1110 DOI 10.1093/treephys/tpq018.

Yi CX, Reddy C, Varghese K, Bui TNH, Zhang SL, Kallath M, Kunjachen B, Ramachandran S, Hong Y. 2014. A new *Jatropha curcas* variety (JO S2) with improved seed productivity. *Sustainability* **6**:4355–4368 DOI 10.3390/su6074355.

Zhang L, He LL, Fu QT, Xu ZF. 2013. Selection of reliable reference genes for gene expression studies in the biofuel plant *Jatropha curcas* using real-time quantitative PCR. *International Journal of Molecular Sciences* **14**:24338–24354 DOI 10.3390/ijms141224338.

Zhao JB, Zhang YP, Song FQ, Xu ZF, Xiao YL. 2009. A comparison of the phenological characteristics of introduced plant species in the Xishuangbanna Tropical Botanical Garden. *Chinese Bulletin of Botany* **44**:464–472 DOI 10.3969/j.issn.1674-3466.2009.04.008.

Zhu BL, Coleman GD. 2001. The poplar bark storage protein gene (*Bspa*) promoter is responsive to photoperiod and nitrogen in transgenic poplar and active in floral tissues, immature seeds and germinating seeds of transgenic tobacco. *Plant Molecular Biology* **46**:383–394 DOI 10.1023/A:1010600504740.