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Cloning and characterization of a SnRK2 gene from *Jatropha curcas* L.

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ABSTRACT. Although the SnRK2 class of Ser/Thr protein kinases is critical for signal transduction and abiotic stress resistance in plants, there have been no studies to examine SnRK2 in *Jatropha curcas* L. In the present study, JcSnRK2 was cloned from *J. curcas* using the rapid amplification of cDNA end technique and characterized. The JcSnRK2 genomic sequence is 2578 base pairs (bp), includes 10 exons and 9 introns, and the 1017-bp open reading frame encodes 338 amino acids. JcSnRK2 was transcribed in all examined tissues, with the highest transcription rate observed in the roots, followed by the leaves and stalks; the lowest rate was observed in flowers and seeds. JcSnRK2 expression increased following abscisic acid treatment, salinity, and drought stress. During a 48-h stress period, the expression of JcSnRK2 showed 2 peaks and periodic up- and downregulation. JcSnRK2 was rapidly activated within 1 h under salt and drought stress, but not under cold stress. Because of the gene sequence and expression similarity of JcSnRK2 to AtSnRK2.8, primarily in the roots, an eukaryotic expression vector containing the JcSnRK2 gene (pBI121-JcSnRK2) was constructed and introduced to the *Arabidopsis* AtSnRK2.8 mutant *snf2.8*. JcSnRK2-overexpressing plants exhibited higher salt and drought tolerance, further demonstrating the function of JcSnRK2 in the osmotic stress response. *J. curcas* is highly resistant to extreme salt and drought conditions and JcSnRK2 was found to be activated under

these conditions. Thus, JcSnRK2 is potential candidate for improving crop tolerance to salt and drought stress.

Key words: JcSnRK2; *Jatropha curcas* L.; Salt tolerance; Drought resistance; Transgenic; Abscisic acid

INTRODUCTION

Osmotic stress during plant development can severely reduce crop yields and quality (Xiong and Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Plants have developed sophisticated defense mechanisms against adverse external stress conditions. For example, reversible phosphorylation/dephosphorylation of proteins is important for signal transduction (Boudsocq and Laurière, 2005).

Sucrose non-fermenting 1-related protein kinase (SnRK) Ser/Thr protein kinases participate in signal transduction and include 3 subfamilies: SnRK1, SnRK2, and SnRK3. SnRK1 primarily regulates the saccharometabolism pathway and has been implicated in pollen development and salt and disease resistance. SnRK2 and SnRK3 are plant-specific and primarily associated with stress resistance (Gancedo, 1998; Honigberg and Lee, 1998). Son of Sevenless 2 (SOS2) is the founding member of the *Arabidopsis thaliana* SnRK3 subfamily and participates in SOS signaling pathways. With SOS1 and SOS2, the cellular Na^+/H^+ ion transport pump enables the smooth discharge of excess-sodium-ion through a series of calcium-binding and gene-activation reactions, ensuring ionic balance and preventing salt-stress damage (Liu et al., 2000; Shi et al., 2000; Qiu et al., 2002).

SnRK2 is closely associated with plant stress responses. Some SnRK2 members are activated by abscisic acid (ABA) and participate in anti-salt and anti-drought stress reactions. SnRK2 members have relatively variable C-end structural domains and share 2 conserved amino acid regions in the activation loop (DFGYSKSSVLHSQPKSTVGTPAYIAPE) and ATP-binding domain (GXGXXGX). The conserved activation loop domain is the protein kinase phosphorylation target site; and the Ser and Thr residues are critical for SnRK2 activity (Belin et al., 2006). The ATP-binding domain is further divided into domains I (SnRK box) and II (ABA box). Domain I is relatively well-conserved among SnRK2 members, while domain II varies greatly among SnRK subfamilies, particularly in its acidic patch. SnRK2 II and SnRK2 III contain several aspartic acid residues, while SnRK2 I contains abundant glutamic acid (Hardie, 1999; Halford et al., 2000). ABA-box-containing SnRK2 II and SnRK2 III members are grouped with SnRK2a, and SnRK2 I members with SnRK2b. Based on this grouping, SnRK2 II and SnRK2 III can be activated by ABA and show different degrees of response. Accordingly, these 2 classes may share functions, such as involvement in ABA regulation pathways and hyperosmotic stress responses (Boudsocq et al., 2004; Yoshida et al., 2006).

SnRK2 Ser/Thr protein kinases are plant-specific and participate in the hyperosmotic stress response and ABA signaling. The first identified SnRK2 gene, PKABA1, was found in a cDNA library from ABA-treated wheat (*Triticum aestivum*) and is upregulated by ABA and dehydration (Anderberg and Walker-Simmons, 1992; Holappa and Walker-Simmons, 1995). The 10 SnRK2 proteins identified in *Arabidopsis* (SnRK2.1-SnRK2.10) comprise 3 subclasses: I (SnRK2.1, SnRK2.4, SnRK2.5, SnRK2.9, and SnRK2.10), II (SnRK2.2, SnRK2.3, and SnRK2.6), and III (SnRK2.7 and SnRK2.8). Except for SnRK2.9, these kinases are hyperosmotic stress-activated. Subclass II and III members are also activated by ABA and play important

roles in drought stress signaling. However, there are no SnRK2 protein kinases activated by cold stress (Hrabak et al., 2003). SnRK2.8 overexpression in *Arabidopsis* significantly improves drought tolerance (Umezawa et al., 2004; Shin et al., 2007). *Arabidopsis* double mutants with disrupted SnRK2.2 and SnRK2.3 exhibited high ABA sensitivity as well as severely repressed seed germination and root development (Fujii and Zhu, 2009; Fujita et al., 2009).

Structural and biochemical analyses revealed that the SnRK2 molecular architecture includes catalytic and C-terminal regulatory domains; the latter is essential for kinase activity and the ABA response (Yunta et al., 2011). Although all SAPK can be activated by NaCl, only SAPK8, SAPK9, and SAPK10 are activated by ABA (Kobayashi et al., 2004). OsSAPK4 overexpression significantly enhanced salt tolerance in transgenic rice (Diédhiou et al., 2008). Additionally, TaSnRK2.4 from wheat and ZmSAPK8 from corn were recently reported to enhance multi-stress and salt tolerance, respectively, in transgenic *Arabidopsis* (Mao et al., 2010; Ying et al., 2011).

Jatropha curcas L., a perennial plant of the Euphorbiaceae family with a relatively short growth cycle, has recently attracted attention as a potential biodiesel source. *J. curcas* can accumulate seed oil content to approximately 40% and strongly resists severe drought, salinity, temperature stress, and nutrient deficiency (Gao et al., 2012). *J. curcas* can be used as a model for basic research of ligneous plants; furthermore, studying its anti-adversity characteristics may contribute to the identification of superior varieties. Because of this species short research history, limited information is available regarding how and why *J. curcas* survives in severe environments. Although SnRK2 has been thoroughly studied in *Arabidopsis* and rice, its function in *J. curcas* remains unknown.

In a preliminary analysis, we found that JcSnRK2 is the only SnRK2 gene present in *J. curcas*. In this study, we cloned JcSnRK2 cDNA and genomic DNA fragments from *J. curcas* leaves, performed bioinformatic analysis, and studied the expression patterns of JcSnRK2. JcSnRK2 overexpression in the *snf2.8 Arabidopsis* mutant produced transgenic plants with significantly increased water deficit and salt stress resistance. These findings demonstrate that JcSnRK2 responds to abiotic stress and is important in the osmotic stress response. Further studies are necessary to determine the exact mechanisms involved in this response.

MATERIAL AND METHODS

Plant materials

Mature *J. curcas* seeds and flowers were harvested in Sanyang, China. After drying, the seeds were stored at room temperature in a dry environment and the flowers were immersed in a protective liquid and stored at -80°C. Young seedlings were germinated from seeds at 30°C under 24-h light conditions. Seeds of the *Arabidopsis* SnRK2.8 mutant *snf2.8* (Columbia ecotype) were obtained from the *Arabidopsis* Information Resource (TAIR, http://www.arabidopsis.org/index.jsp) and grown under the following conditions: 16-h light (24°C)/8-h dark (18°C), illumination intensity of 100-200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and 70% relative humidity.

Cloning of full-length JcSnRK2 cDNA and genomic DNA

Total RNA for cDNA synthesis and genomic DNA were extracted from the leaves of 1-month-old *J. curcas* seedlings using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany)

and the Plant Genomic DNA Kit (Tiangen, Beijing, China), respectively, according to the manufacturer instructions. First-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa, Shiga, Japan).

For cloning of SnRK2 or SnRK2-like genes from *J. curcas*, the primers P1 and P2 (Table 1) were designed based on the conserved amino acid sequences of SnRK2-like genes (from NCBI GenBank), and then used to amplify the conserved fragment in *J. curcas*. The following program was used for reverse transcription polymerase chain reaction (RT-PCR): 95°C for 3 min, followed by 35 cycles of amplification (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min) and extension at 72°C for 10 min. Next, 5' and 3' rapid amplification of cDNA ends were carried out based on the internal fragment using 3 universal primers [Oligo(dT)18, AP1, and AP2, Table 1] and 5 gene-specific primers (5PR, 5P1, 5P2, 3P1, and 3P2, Table 1). Full-length cDNA and genomic DNA were then amplified using 2 gene-specific primers (SP1 and SP2, Table 1). The PCR products obtained were subsequently purified using the Universal DNA Purification Kit (Tiangen), subcloned into the pMD19-T vector (TaKaRa), and sequenced (Beijing Genomics Institute, Beijing, China).

Table 1. Primers used for RT-PCR and Q-PCR.

Primer	Sequence (5'→3')	Description
P1	GAGATNATNAAYCATAGRTCATTGA	Degenerate primer, forward
P2	GATCYTCAAAWGGATAWGCDCCAA	Degenerate primer, reverse
Oligo(dT)$_{18}$	GCTGTCAACGATACGCTACGTAACG GCATGACAGTG(T)18	Universal adaptor primer, RT
AP1	GTCAACGATACGCTACGTAACG	Universal adaptor primer, outer
AP2	TACGTAACGGCATGACAGTG	Universal adaptor primer, nested
5PR	GTGTTCCCACTGTAGACTTAGGTTG	5'-RACE RT primer
5P1	TAGTTGTTGAAAGAAAAATCGTGCC	5'-RACE forward primer, outer
5P2	ACTATGGCTAAATGAGTTGGTGTGA	5'-RACE forward primer, nested
3P1	CTACAGTGGGAACACCAGCCTAT	3'-RACE forward primer, outer
3P2	AGATGTTTGGTCTTGTGGGGTTA	3'-RACE forward primer, nested
SP1	ATGGAGCGTTATGAGATATTGAGAG	Full-length cDNA and gene primer, forward
SP2	TCACAATGCACAAACAAAATCACCAC	Full-length cDNA and gene primer, reverse
QP1	TGAAGATGAGGCACGATT	Q-PCR gene-specific primer, forward
QP2	CCACTGTAGACTTAGGTTGA	Q-PCR gene-specific primer, reverse
JcActP1	TGGAGCAGAGAGATTCCGATG	Q-PCR reference gene primer, forward
JcActP2	CACCACTGAGCACAATGTTACC	Q-PCR reference gene primer, reverse
AREB1-F	CATACCAGCAATCGCAACAG	Q-PCR gene-specific primer, forward
AREB1-R	CTCCAAGTCCCACAAGACCA	Q-PCR gene-specific primer, reverse
LEA-F	TCCAACCCACTCACACCACCA	Q-PCR gene-specific primer, forward
LEA-R	CAGAAGCCAAACCCTCCTCA	Q-PCR gene-specific primer, reverse
RD29A-F	GCAATGAGCATGAGCAAGATC	Q-PCR gene-specific primer, forward
RD29A-R	TCGGAAGACACGACAGGAAAC	Q-PCR gene-specific primer, reverse
RD29B-F	TCTGACCACACCAAACCCATT	Q-PCR gene-specific primer, forward
RD29B-R	CCTCTCTTTTCGCTTCCCAG	Q-PCR gene-specific primer, reverse
At18sF	GAGTCATCAGCTCGCGTTGAC	Q-PCR reference gene primer, forward
At18sR	CTTCACCGGATCATTCAATCG	Q-PCR reference gene primer, reverse
Xba-F1	GCGTCTAGAATGGAGCGTTATGAGATATTGAGAG	Full-length cDNA primer (*Xba*I site), forward
Bam-R1	CGCGGATCCTCACAATGCACAAACAAAATCACCAC	Full-length cDNA primer (*Bam*HI site), reverse

Quantitative real-time PCR (qRT-PCR)

Organ-specific expression analysis of JcSnRK2 was performed in the roots, stalks, and leaves of 1-month-old *J. curcas* seedlings as well as *J. curcas* seeds and flowers. For stress response analysis, 1-month-old *J. curcas* seedlings cultured under non-stressed conditions were transferred to solutions containing 250 mM NaCl (salt stress), 1 mM ABA, or 20% PEG6000 (drought stress). To induce cold stress, the seedlings were maintained at 4°C for 0,

1, 3, 6, 12, 24, or 48 h. To assess the expression levels of the stress-response genes AREB1, LEA, RD29A, and RD29B in transgenic *Arabidopsis* plants, JK2 and *snf2.8* seedlings were subjected to drought stress for 4 h. Leaves from all treated seedlings were harvested separately at the indicated time points and then immediately frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the Plant Total RNA Extraction Kit (Tiangen) and first-strand cDNA was synthesized from the total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer instructions. qRT-PCR was carried out in the Bio-Rad iCycler MyiQ Real-Time PCR System using SsoFast™ EvaGreen® Supermix (Bio-Rad). The reaction procedures were as follows: denaturation at 95°C for 2 min, followed by 45 cycles of 95°C for 10 s, 60°C for 1 min, and a final melting curve profile (65°-95°C). Transcript levels were quantified using the comparative CT method. Each data point represents the average of 3 replicates, and the data were analyzed using the Bio-Rad iQ5 software.

Generation of transgenic plants

The open reading frame (ORF) of JcSnRK2 was amplified using the primers *Xba*F1 and *Bam*R1 and inserted into the pBI121 expression vector between the *Xba*I and *Bam*HI sites as a β-glucuronidase (GUS)-fused fragment driven by the CaMV35S promoter. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* LBA4404 and transformed into the *snf2.8 Arabidopsis* mutant via floral infiltration (Clough and Bent, 1998). Positive transgenic plants were confirmed through kanamycin spraying, GUS staining, and PCR analyses. After screening, the JK2 line, which presented 100% kanamycin-resistant T3 homozygous seeds, was used for further analysis. *snf2.8* mutant plants transfected with pBI121 were used as controls.

Measurement of root growth in transgenic plants

Following surface sterilization with 75% ethanol for 1 min and 5% NaClO for 5 min and several washes with sterilized water, JK2 and *snf2.8* seeds were placed on solid Murashige and Skoog (MS) medium (0.8% agar) for 1 week under the following growth conditions: 16-h light (24°C)/8-h dark (18°C), illumination intensity of 100-120 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and 70% relative humidity. The lengths of the seedling roots were measured after 1 week and root development was observed after 45 days of cultivation in soil.

Determination of seed germination rate

JK2 and *snf2.8* seeds were cultured on solid MS medium (0.8% agar) with 150 mM NaCl or 200 mM mannitol, respectively. A culture with no solution added was used as a control. Germination rates were evaluated based on the appearance of the first 2 leaves after 2 weeks.

Salt tolerance assay

Two-week-old aseptic seedlings showing similar growth were transferred to MS agar medium supplemented with different concentrations of NaCl (150, 200, or 250 mM) for 30 days. The number of seedlings with green leaves and the number with 4 leaves were counted. Additionally, 30- and 45-day-old *Arabidopsis* seedlings cultivated in soil were irrigated with a 450 mM NaCl solution from the bottom of the pot. Plants were observed at 2 weeks and 1

month after saline irrigation treatment.

Drought tolerance assay

Suitable aseptic seedlings, as described above, were selected and transferred to MS agar medium containing various concentrations of mannitol (200, 250, and 300 mM). Additionally, the water supply was withdrawn from *Arabidopsis* seedlings that had grown in soil for 30 or 45 days. Each plant was photographed after 1 month.

Determination of water loss rates

Fresh leaves of 30-day-old plants were weighed immediately after detachment to determine the fresh weight (FW). Subsequently, the leaves were maintained at 24°C under dark conditions at a relative humidity between 45-50% until the designated time point and then re-weighed to obtain the designated time weight (DTW). The leaves were completely dried in an oven at 85°C for 24 h to determine the dry weight (DW). Water loss rate (WLR) was calculated according to the following formula: WLR = (DTW-DW) / (FW-DW) x 100%.

RESULTS

JcSnRK2 cloning and sequence analysis

Full-length cDNA of JcSnRK2 exhibits an ORF of 1017 nucleotides (**Supplementary material**), encoding 338 amino acids. This sequence was nearly identical to the SnRK homolog of *Medicago truncatula* (XM_003615109) (88%) and SnRK2.7 of *Arabidopsis* (NM_120165) (85%). It also shared some similarities with *Oryza sativa* SAPK1 (AB125302.1), *Solanum lycopersicum* SRK2C (NM_001247424.1) and *Zea mays* SnRK2.1 (NM_001154177), showing 78, 78, and 77% similarity with these sequences, respectively. The results are shown in Figure 1.

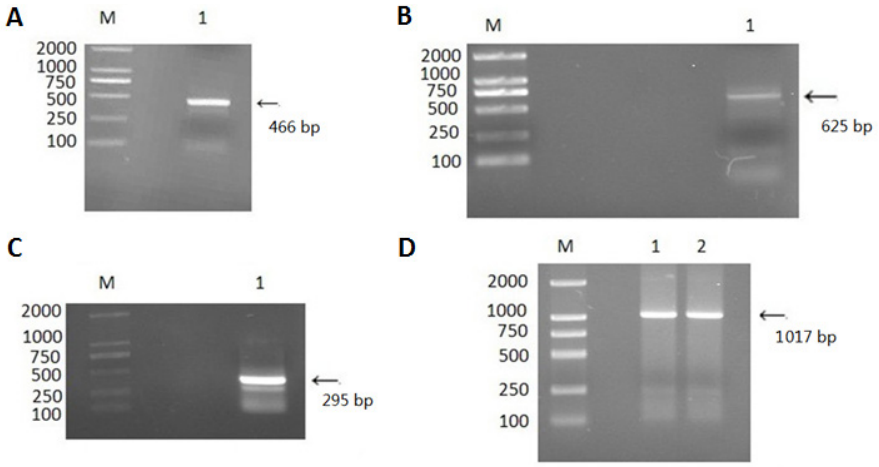

Figure 1. Gel electrophoresis of RT-PCR products. **A.** PCR products of the amplified conserved fragment. **B.-D.** PCR products from 3' RACE, 5' RACE, and full-length cDNA amplification, respectively. *Lane M* = 2000-bp DNA marker. *Lanes 1-2* = PCR products.

To analyze the homology between the JcSnRK2 gene and the 10 well-studied SnRK2 genes with similar functions in *Arabidopsis*, a phylogenetic tree was constructed based on their amino acid sequences. The results showed that JcSnRK2 was the most similar to AtSnRK2.7 and AtSnRK2.8 (Figure 2A). The JcSnRK2 protein contained both conserved domains found in the SnRK2 family, i.e., the ATP-binding domain (GXGXXGX, near the N-terminus) and the activation loop (DFGYSKSSVLHSQPKSTVGTPAYIAPE), which is a target of protein kinase phosphorylation (Figure 2B). Domain I (SnRK box) and domain II (ABA box) in the

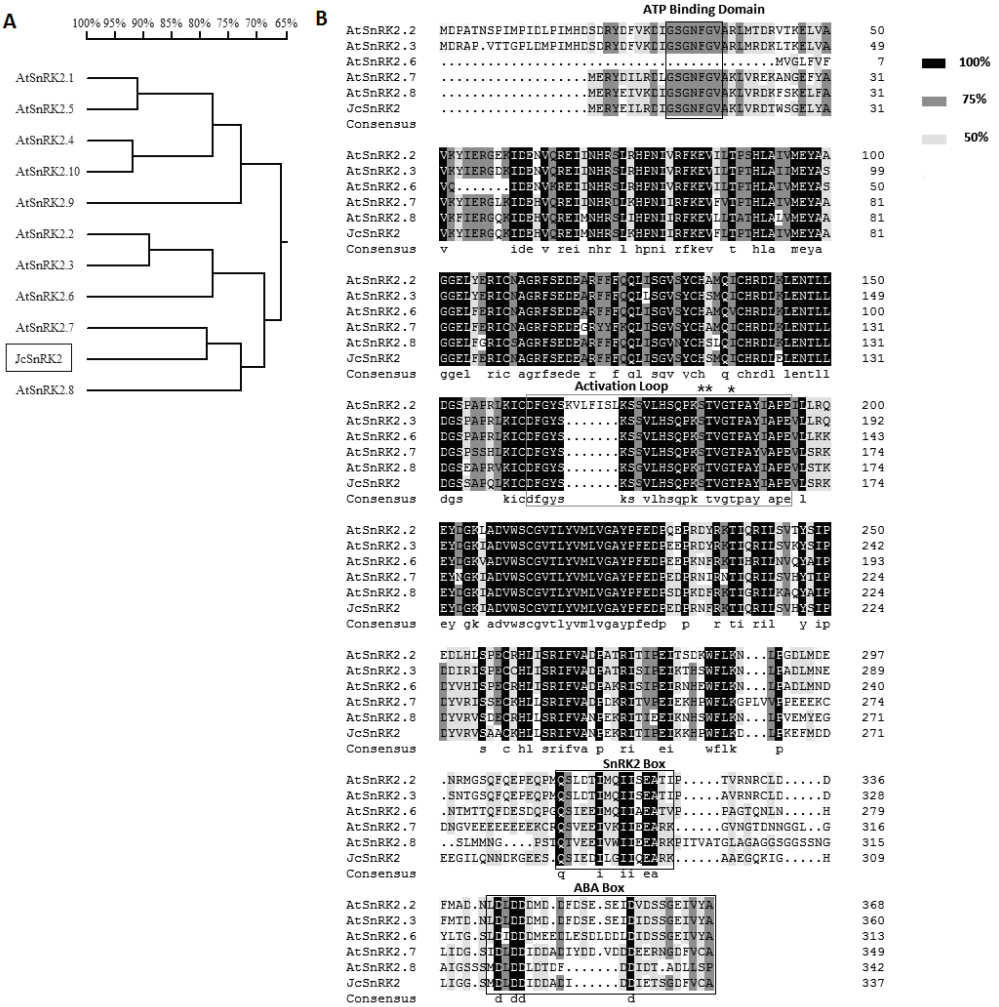

Figure 2. Sequence analysis of JcSnRK2 using DNAMAN. **A.** Phylogenetic relationships between JcSnRK2 and the 10 members of the SnRK2 family in *Arabidopsis*. The GenBank ID numbers were as follows: AtSnRK2.1 (At5g08590), AtSnRK2.2 (At3g50500), AtSnRK2.3 (At5g66880), AtSnRK2.4 (At1g10940), AtSnRK2.5 (At5g63650), AtSnRK2.6 (At4g33950), AtSnRK2.7 (At4g40010), AtSnRK2.8 (At1g78290), AtSnRK2.9 (At2g23030), and AtSnRK2.10 (At1g60940). A higher percentage indicates a closer genetic relationship. **B.** Amino acid sequence alignment of JcSnRK2 with the 5 AtSnRK2a family members. The ATP-binding domain, activation loop, SnRK2 box and ABA box are framed. Potential phosphorylated residues are indicated by asterisks. The black, dark gray, and light gray shaded areas indicate 100, 75, and 50% conservation among the 6 kinases, respectively.

C-terminal structural domain of the JcSnRK2 amino acid sequence were relatively conserved. These findings indicate that the cloned JcSnRK2 protein possessed the typical characteristics of the SnRK2 family based on structural analysis and that JcSnRK2 is activated by ABA.

Organization of the JcSnRK2 gene

To examine the intron-exon organization of JcSnRK2, PCR was performed using DNA as the template and the SP1 and SP2 gene-specific primers (Table 1). PCR amplification produced a 2578 base pair (bp) genomic fragment, which included a 1017-bp coding sequence interrupted by 1561 bp of introns. Comparison of the JcSnRK2 genomic and cDNA sequences indicated that 10 exons and 9 introns were present. These exons were 120, 76, 102, 52, 92, 94, 104, 101, 18, and 218 bp long, while the introns were 227, 526, 128, 291, 89, 109, 97, 94, and 40 bp in length (Figure 3). All of introns exhibited typical structural characteristics of plant introns, showing high A+T content (**Supplementary material**).

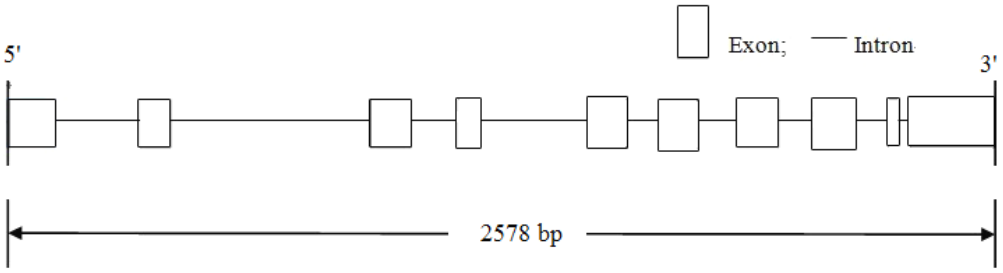

Figure 3. Diagram of the JcSnRK2 gene. The boxes and lines indicate exons and introns, respectively.

Pattern of JcSnRK2 expression

JcSnRK2 mRNA transcript levels were examined by qRT-PCR to assess the spatial and temporal expression pattern of JcSnRK2 in specific organs and tissues. JcSnRK2 was transcribed in all examined tissues but was most highly expressed in the roots, followed by the leaves and stalks. The lowest expression levels were observed in flowers and seeds (Figure 4A). The higher expression levels of JcSnRK2 observed in seedling roots suggest that this protein may function as a fundamental signaling molecule related to soil water and nutrient status.

Because SnRK2 genes are known to be involved in various stress responses, we subjected 1-month-old *J. curcas* seedlings to different stresses, as described in the Material and Methods section. The leaves of all seedlings were analyzed by qRT-PCR. As shown in Figure 4B, JcSnRK2 transcript levels increased 1 h after ABA treatment and then decreased after 3 h. Thereafter, the transcript levels gradually increased, until the highest level was reached after 24 h. Finally, expression decreased at 48 h. Under NaCl stress, JcSnRK2 transcript levels in the leaves increased significantly after 1 h of treatment and decreased after 3 h. The levels remained low until increasing again at 24 h, followed by a decrease at 48 h (Figure 4C). In PEG-treated leaves, a significant increase was observed after 1 h of treatment, followed by another increase at 12 h (Figure 4D). No regular pattern was detected under cold stress (Figure 4E). These results demonstrate that JcSnRK2 is activated by ABA, salt stress, and drought stress, showing

a double-peaked expression pattern, whereas it does not appear to be involved in cold stress. A rapid and significant JcSnRK2 reaction was detected following NaCl and PEG treatment.

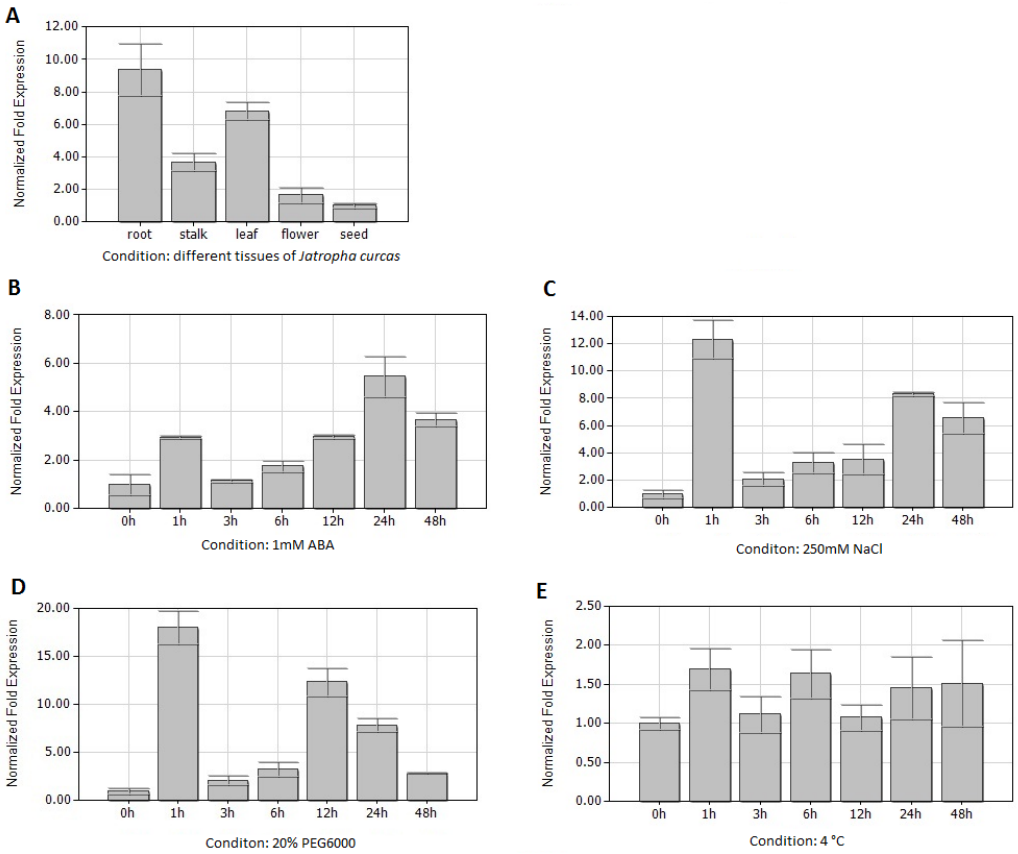

Figure 4. Pattern of JcSnRK2 expression in *Jatropha curcas* detected via quantitative real-time PCR. **A.** Pattern of JcSnRK2 expression in various tissues. Flowers and seeds were collected in Hainan Province, China. Roots, stalks, and leaves were obtained from 1-month-old *J. curcas* seedlings. **B.-E.** Pattern of JcSnRK2 expression under 1 mM ABA treatment, salt treatment (250 mM NaCl), drought stress (20% PEG6000), and cold stress (4°C). The primers QP1 and QP2 were used to detect JcSnRK2 transcript levels. The *J. curcas* actin gene was used as an internal control for all analyses and amplified using the primers JcAct1 and JcAct2.

JK2 plants show stronger potential in preliminary stages of development

In addition to kanamycin screening, transgenic plants were confirmed by GUS staining (Figure 5) and PCR amplification of both JcSnRK2 DNA and cDNA fragments isolated from JK2 seedlings.

Additional experiments were performed to assess the impact of JcSnRK2 expression in transgenic *Arabidopsis*. After culturing in MS medium for 1 week, the JK2 roots had grown to an average length of 1.7 cm, which was 0.4 cm longer than those of *snf2.8* plants (Figure 6A and B). After 45 days under natural conditions, the JK2 plants possessed more developed

root systems with more lateral roots (Figure 6C). These results indicate that the expression of JcSnRK2 stimulated root growth in *Arabidopsis snf2.8* mutants.

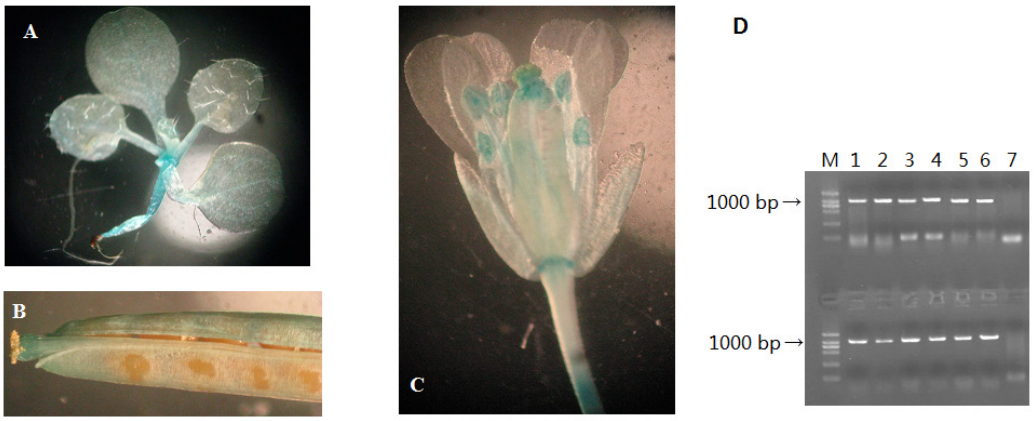

Figure 5. Confirmation of JK2 transgenic plants. **A.-C.** GUS staining of the seedlings, pods, and flowers of JK2 transgenic plants, respectively. **D.** Gel electrophoresis of PCR products. The top row shows PCR products amplified from cDNA using the SP1 and SP2 primers, while the bottom row shows PCR products from amplified DNA. *Lane M* = DNA marker 2000. *Lanes 1-6* = 6 individual JcSnRK2 transgenic lines. *Lane 7* = *snf2.8* (transformed with pBI121 as a control).

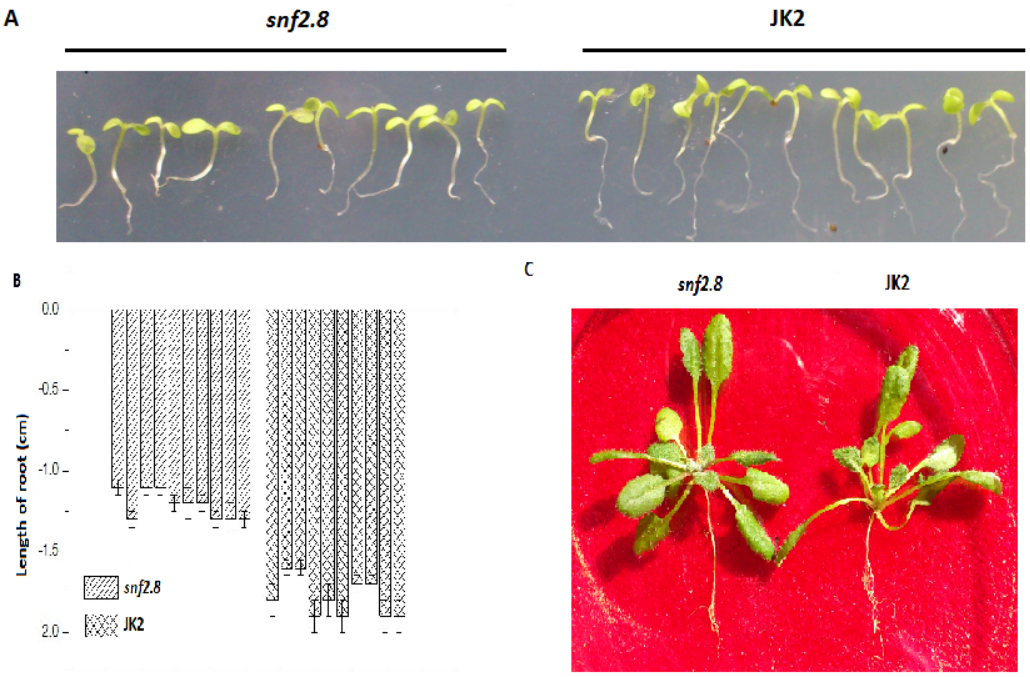

Figure 6. Comparison of root growth between *snf2.8* and JK2 plants. **A.** and **B.** Comparison of primary root lengths. Seeds sterilized were cultured in solid MS medium for 1 week, at which point the root lengths were measured. **C.** Comparison of root system development after 45 days of cultivation.

Although JK2 and *snf2.8* germinated at similar rates under non-stress conditions in MS medium, their germination rates differed under osmotic stress in MS medium supplemented with 150 mM NaCl or 200 mM mannitol. The percentages of germination observed for JK2 and *snf2.8* seeds were 87.64 and 79.56%, respectively, under salt stress and 84.61 and 70.56%, respectively, under drought stress (Figure 7). Osmotic stress inhibited seed germination to different degrees; however, this effect was decreased in JK2 seeds, indicating that JcSnRK2 expression can enhance salt and drought tolerance during the germination phase. These findings indicate that JK2 plants display faster root growth and a higher seed germination capacity, which may confer a stronger potential during the preliminary stages of development.

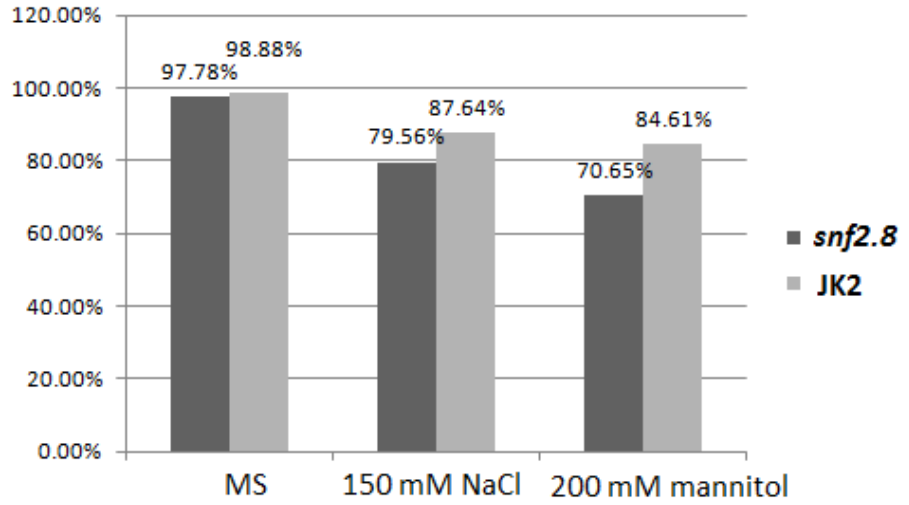

Figure 7. Germination rates of *snf2.8* and JK2 seeds under different conditions. MS is the control, and NaCl and mannitol were used to induce salt and drought stress, respectively.

JcSnRK2 overexpression confers salt tolerance

To determine whether JcSnRK2 overexpression enhanced the salt tolerance of *snf2.8 Arabidopsis* mutants, the ability to maintain green leaves and the appearance of 4 leaves were assessed as measures of tolerance and viability under salt stress. Both the growth and development of *snf2.8* were greatly reduced by salt stress. As shown in Table 2 and Figure 8, only 7.25% of the *snf2.8* plantlets remained green under 150 mM NaCl treatment, while 0% remained green when the NaCl concentration was increased to 200 or 250 mM. Percentages of plants with 4 leaves were 27.54, 17.14, and 6.25% under treatment with 150, 200, or 250 mM NaCl, respectively. In contrast, JcSnRK2 overexpression enhanced salt tolerance: 57.39% of the plantlets maintained their green color and 80% developed 4 leaves under 150 mM NaCl treatment. These values were 8-fold and 3-fold higher than the percentages of *snf2.8* plants showing green color and 4 leaves, respectively. Although the growth and development of JK2 plants was inhibited by higher salt concentrations, the effect was weaker. When the NaCl concentration was increased to 200 mM, the green color and 4-leaf rates were nearly 2-fold higher in JK2 than *snf2.8* plants. Moreover, the green color rate was 15.63% in JK2 plants, in comparison to 0% in *snf2.8* plants.

Under the most severe salt stress, however, both the JK2 and *snf2.8* plants were completely white. In addition to seedling analysis, 30- and 45-day-old *Arabidopsis* seedlings cultivated in soil were irrigated with a 450 mM NaCl solution from the bottom of the pot, with observations performed at 2 weeks and 1 month. We found that JK2 plants exhibited increased salt tolerance based on the presence of slightly curled and wilted leaves (Figure 9).

Table 2. Statistics for the green color and 4-leaf rates under salt stress.

Type	Factor assessed	NaCl concentration (mM)		
		150	200	250
snf2.8	Maintenance of green plantlets	7.25%	0%	0%
	Appearance of 4 leaves	27.54%	17.14%	6.25%
JK2	Maintenance of green plantlets	57.39%	15.63%	0%
	Appearance of 4 leaves	80%	46.88%	14.44%

$P < 0.05$.

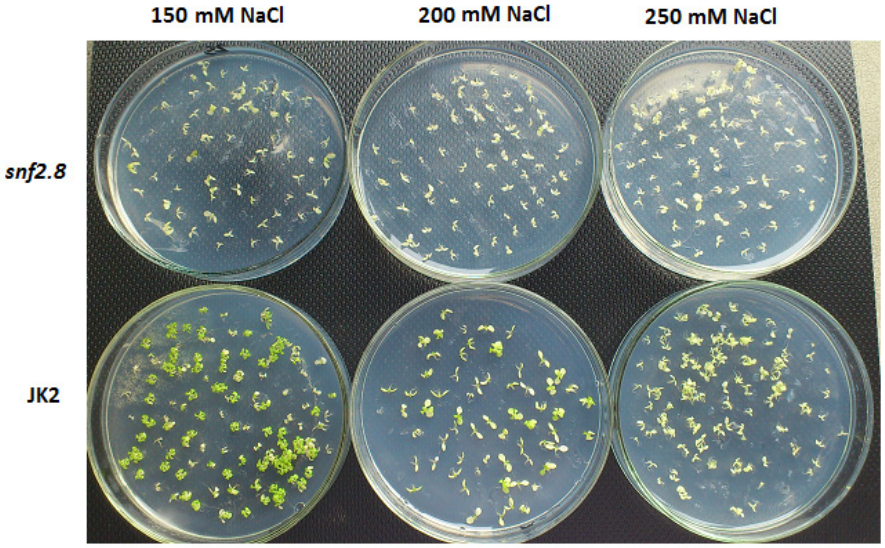

Figure 8. Green color and 4-leaf rates under salt stress. Seedlings were cultured on MS agar supplemented with different concentrations of NaCl (150, 200, and 250 mM) for 30 days.

Overexpression of JcSnRK2 enhances drought resistance in *Arabidopsis*

To assess the response of JcSnRK2-overexpressing plants to drought stress, aseptic seedlings were treated with 200, 250, or 300 mM mannitol for 1 month. Both the JK2 and *snf2.8* plants exhibited more tightly curled leaves and obvious growth inhibition under higher drought stress conditions, but the effect was much less severe in JK2 than in *snf2.8* plants. Specifically, following treatment with 200 or 250 mM mannitol, the 3rd and 4th leaves of *snf2.8* plants were more curled, while JK2 plants maintained relatively normal growth. Under treatment with 300 mM mannitol, all *snf2.8* rosette leaves were severely curled, while JK2 plants displayed similar signs of water deficiency, as observed in *snf2.8* plants under 250 mM mannitol (Figure 10). After mature plants had been cut off from the water supply for 1 month,

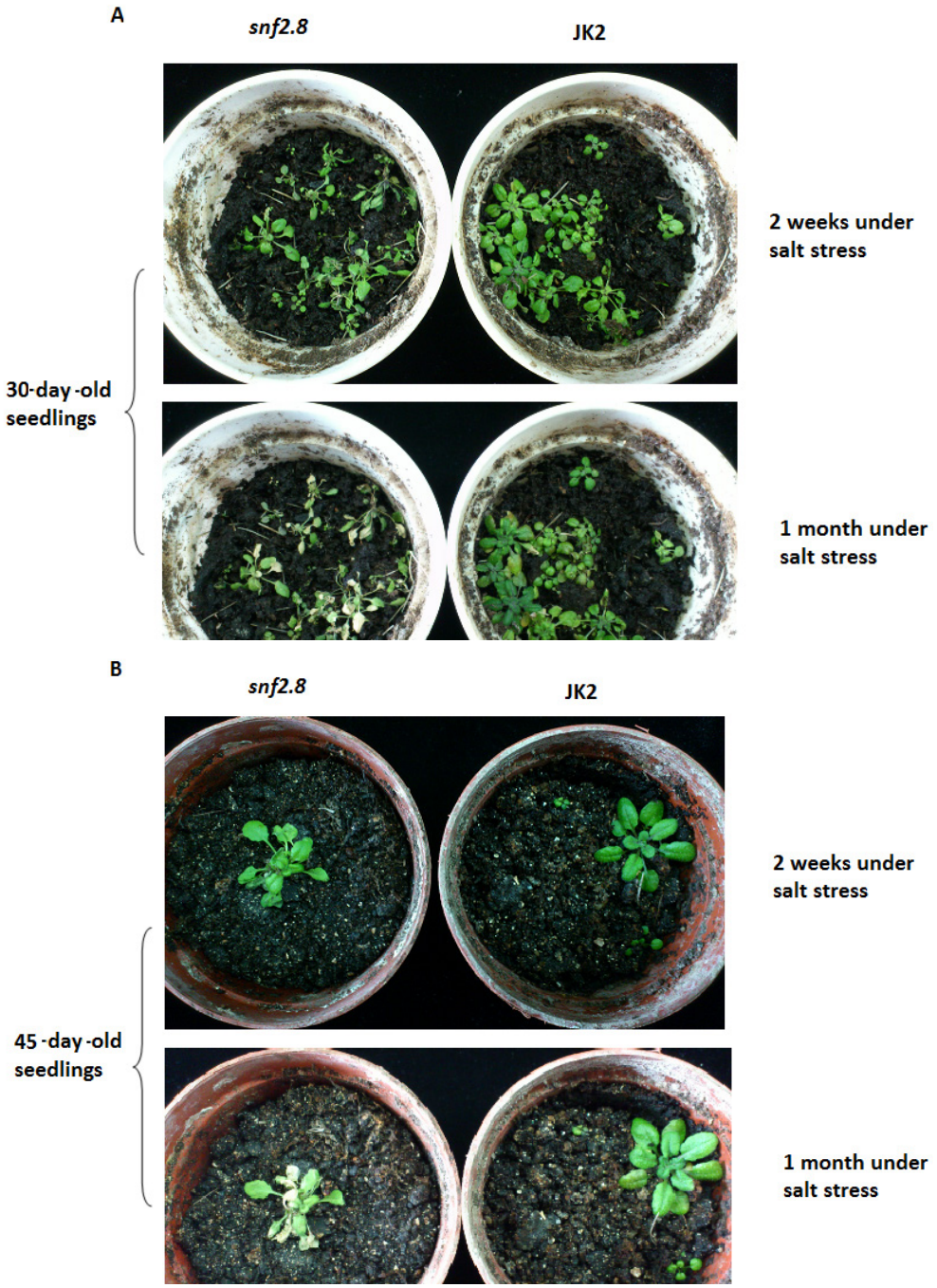

Figure 9. Performance of *snf2.8* and transgenic plants overexpressing JcSnRK2 (JK2) under salt stress. **A.** and **B.** Growth of 30- and 45-day-old plants, respectively, under salt stress (irrigation with 450 mM NaCl) after 2 weeks and 1 month.

the *snf2.8* leaves were severely curled and wilted, whereas the JK2 leaves showed a less significant effect (Figure 11). The lower water loss rate observed in excised leaves may have accounted for the stronger drought resistance of JK2 plants (Figure 12). Finally, stress response genes, including AREB1, LEA, RD29A, and RD29B, were upregulated to a greater extent in JcSnRK2-overexpressing plants under drought stress for 4 h, further supporting that JcSnRK2 improves tolerance to water loss (Figure 13).

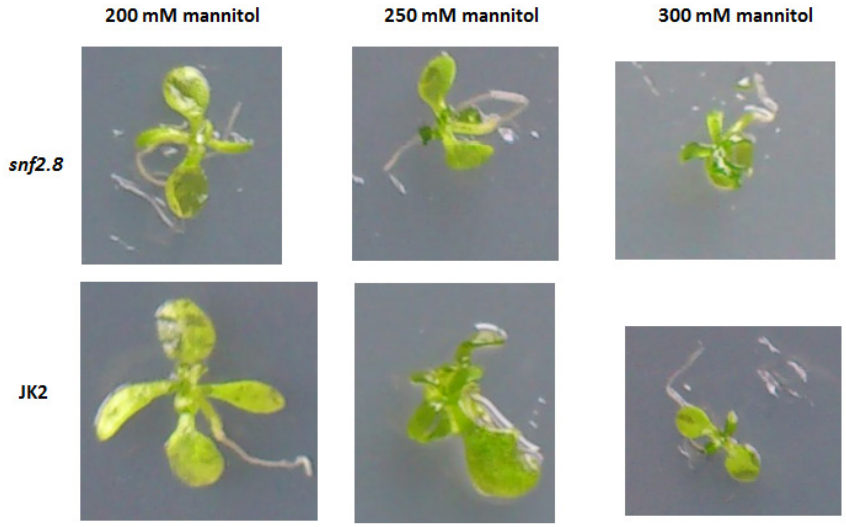

Figure 10. Performance of *snf2.8* and JK2 plants under mannitol stress. Seedlings were cultured on MS agar medium supplemented with different concentrations of mannitol (200, 250, and 300 mM) for 30 days.

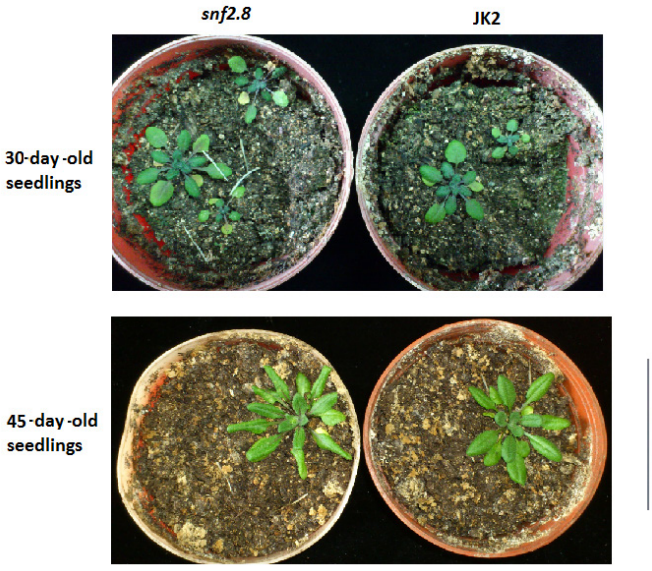

Figure 11. Growth of *snf2.8* and JK2 plants under drought stress. Mature 30- and 45-day-old plants were cut off from the water supply for 1 month and then photographed.

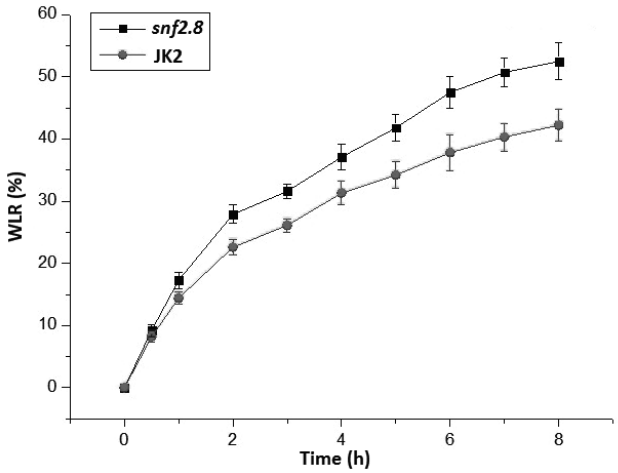

Figure 12. Comparison of water loss rates for detached rosette leaves of *snf2.8* and JK2 plants.

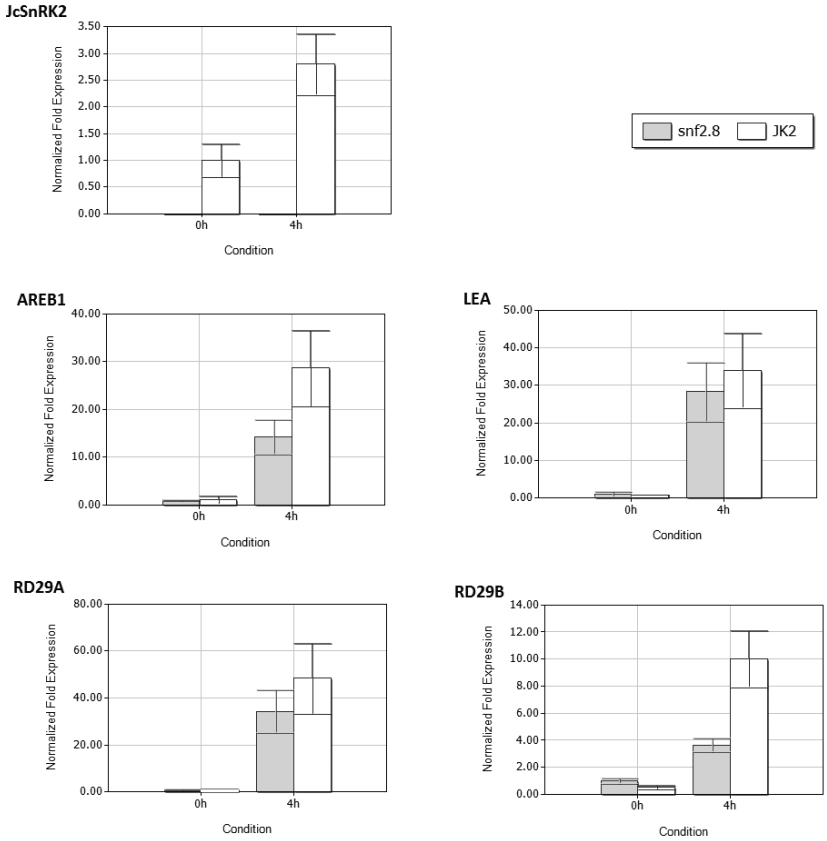

Figure 13. Expression patterns of several stress response genes in *snf2.8* and JK2 determined by quantitative real-time PCR. The *Arabidopsis* 18s gene was used as an internal control for all analyses and was amplified using the primers At18sF and At18sR.

DISCUSSION

Members of the SnRK2 family of plant-specific protein kinases have been identified in various species, such as *Arabidopsis*, *O. sativa*, *Z. mays*, and *T. aestivum*. However, no SnRK2 member has been previously reported in *J. curcas*. In this study, we characterized the expression profile of an SnRK2 gene from *J. curcas* and found that it possesses the typical features of the SnRK2 subfamily.

Sequence analysis revealed that JcSnRK2 contained the SnRK2 box, the 2 conserved domains of the SnRK2 family (ATP-binding domain and the activation loop), and the ABA box. Through site-directed mutagenesis and serial deletions, Belin et al. (2006) previously demonstrated that serine/threonine residues in the activation loop and serine in the SnRK2-specific box are critical for the activity of recombinant open stomata 1 kinase in *Arabidopsis* guard cells. The ABA box is typically found in ABA-activated SnRK2s. JcSnRK2 was classified as a SnRK2a because its amino acid sequence contained an acidic patch with a high Asp (D) content located near the C-terminus. The SnRK2a proteins of *Arabidopsis*, including AtSnRK2.2, AtSnRK2.3, AtSnRK2.6, AtSnRK2.7, and AtSnRK2.8, are known to be activated by ABA and hyperosmotic stress (Hrabak et al., 2003). Based on our results, JcSnRK2 exhibits the same features.

JcSnRK2 was transcribed in all examined tissues and showed the highest level of expression in seedling roots, similarly to the tissue expression pattern of AtSnRK2.8. This finding suggests that JcSnRK2 acts as a fundamental signaling molecule related to soil water and/or nutrient status. As observed for other salt-activated SnRK2s, JcSnRK2 was immediately activated by NaCl and upregulated by ABA under drought conditions. This response activity was similar to that of AtSnRK2.8, further suggesting that JcSnRK2 contributes to osmotic stress tolerance (Mizoguchi et al., 2010).

For functional analysis, the gene for JcSnRK2 was overexpressed in *snf2.8* (*Arabidopsis* SnRK2.8 mutant) plants because of its close genetic relationship and similar expression patterns to the 2 homologs. JcSnRK2 overexpression enhanced both the salt and drought resistance of *snf2.8*. Thus, JcSnRK2 activity may compensate for the deficiencies caused by disrupted AtSnRK2.8 function by regulating stress response genes, a function typically performed by AtSnRK2a (Hrabak et al., 2003).

The planting and cultivation of *J. curcas* in salinized and desertified lands to address ecological problems and to achieve economic returns represents a major arena for researching this species. As a ligneous species, *J. curcas* offers the advantages of short growth and blossom cycles. Thus, basic research employing *J. curcas* as a model plant species is partly aimed at achieving a better understanding of ligneous plants. Additionally, research focusing on anti-adversity factors in this species, such as JcSnRK2, may provide a foundation for the selection of saline- and drought-tolerant *J. curcas* varieties. Such varieties may ultimately lead to expansion of the cultivation acreage of *J. curcas*, decreased management costs, and sustainable farming systems, thereby benefiting the exploitation and application of *J. curcas*.

There were some limitations to our study. First, we did not identify the JcSnRK2 promoter sequence, making it difficult to precisely determine the functions of JcSnRK2. Second, we did not analyze non-mutated wild-type *Arabidopsis* or other SnRK2a mutants. Future studies will be necessary to address these issues.

In conclusion, salt and drought stress are the main causes of growth retardation and developmental deficiencies in plants, particularly fragile plants. Accordingly, overcoming abi-

otic damage and increasing crop yields and quality are important applications of research findings regarding stress-related genes. *J. curcas* is highly resistant to extreme salt and drought conditions, and JcSnRK2 is activated under these conditions. Thus, JcSnRK2 represents a potential candidate for genetically improving salt and drought tolerance in this crop.

Supplementary material

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