FUNCTIONAL CHARACTERIZATION OF ZjPYL8 FROM SOUR JUJUBE: ENHANCING THE SENSITIVITY OF STOMATA AND ROOTS TO ABA IN Arabidopsis thaliana

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ABSTRACT

Abscisic acid (ABA) is a vital plant hormone that regulates plant growth, development, and stress response. The growth of Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. Chou, commonly known as Suanzao in Chinese, is significantly influenced by environmental factors, particularly drought and salt stresses. In this study, we aimed to isolate and characterize a putative ABA receptor named ZjPYL8 (Ziziphus jujube PYR1-LIKE 8) from sour jujube. By introducing ZjPYL8 into Arabidopsis thaliana (A. thaliana), we sought to investigate its impacts on ABA-responsive pathways and assess resulting phenotypic changes. Our results demonstrated that overexpression of ZjPYL8 in A. thaliana led to a significant reduction in stomatal aperture and root length under ABA treatment, while the wild type (WT) was relatively insensitive to ABA. Moreover, when subjected to salt treatment, ZjPYL8 transgenic plants exhibited shorter roots than WT. These findings suggest that the overexpression of ZjPYL8 in A. thaliana enhances salt stress tolerance and supports the hypothesis that ZjPYL8 serves as a putative ABA receptor in sour jujube, potentially enhancing the plant’s adaptability to drought and salt stresses. Notably, ZjPYL8 appears to mediate plant responses to ABA, resembling the behavior of most ABA receptors in A. thaliana, including stomatal closure and regulation of root length.

Key words: ZjPYL8, osmotic stress, salt stress, abscisic acid, sour jujube

INTRODUCTION

Sour jujube (Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. Chou) is an important fruit tree in China, traditionally used for medicinal purposes. It is mainly cultivated in dry, sunny mountainous regions [Ming et al. 1986, Liu et al. 1994, He et al. 2009, Wang et al. 2011]. Previous studies have demonstrated its ability to alleviate drought-induced damage by adjusting the activity of protective enzymes like SOD, CAT, and AXP, as well as modulating the levels of osmoregulatory substances [He et al. 2009]. Flavonoids, the main pharmacodynamics components in Semen Ziziphi Spinosa, play a pivotal role in regulating the osmotic balance in sour jujube under drought conditions.

The plant hormone abscisic acid (ABA) plays a crucial role in regulating plant growth, development, and stress adaptation [Cutler et al. 2010]. The
ABA-induced signaling pathway is a core pathway that controls responses to drought and salt stresses in plants [Zhu et al. 2002]. PYR1 (Pyrabactin resistance 1), PYL (PYR1-like), and RCAR (regulatory components of ABA receptor) families of soluble receptors are bound and activated by ABA [Ma et al. 2009, Park et al. 2009]. In the absence of ABA, clade A PP2Cs (Phytochrome-associated protein phosphatase type 2C) interact with SnRK2 (SNF1-related protein kinase 2) kinases, including SnRK2.2, SnRk2.3, and SnRK2.6 (OST1), through dephosphorylation [Soon et al. 2012]. When ABA levels increase, the PYR/PYL/RCAR receptors inhibit the phosphatase activity of PP2Cs, leading to the release and activation of SnRK2s through autophosphorylation [Belda-Palazon et al. 2018]. The downstream effectors are then activated through phosphorylation by SnRK2s [Fujii et al. 2009].

While there may be functional redundancies among PYLs (ABA receptors) in Arabidopsis thaliana (A. thaliana), each PYL exhibits unique expression patterns and biochemical characteristics [Lee et al. 2015]. For example, transgenic lines overexpressing AtPYL8 (Arabidopsis thaliana PYR1-LIKE 8) showed increased sensitivity to glucose during germination and seedling stages, whereas knockout of AtPYL8 led to ABA-insensitive recovery of lateral root growth under stress conditions [Zhao et al. 2014]. FsPYL8 (Fagus sylvatica PYR1-LIKE 8) was found to positively regulate the ABA signaling pathway in response to abiotic stress [Saavedra et al. 2010]. In addition, MYC2, a key transcription factor involved in jasmonic acid (JA) responses, interacted with PYL6, thereby linking the ABA and JA pathways [Aleman et al. 2016].

The present investigation aimed to characterize the role of ZjPYL8, a PYL family member in sour jujube. Through the overexpression of ZjPYL8 in A. thaliana, we discovered that the transgenic lines exhibited heightened sensitivity to ABA during the seedling stage, implicating ZjPYL8 as an essential component in response to drought and salt stresses. These findings highlight the importance of studying the functionality of ZjPYL8 in understanding how sour jujube adapts to arid environments.

MATERIAL AND METHOD

Plant materials. Sterile seedlings of sour jujube were obtained using tissue culture techniques. The experimental group consisted of A. thaliana ecotype Colombia (Col-0), while Col-0 and the transgenic lines were cultured under conditions of 22°C, with a photoperiod of 16 hours light and 8 hours dark. Weekly watering was done using a nutrient solution.

Bioinformatical analysis. ZjPYL8 was isolated from the cDNA of Ziziphus jujuba using the A. thaliana, Z. jujuba Mill. var. jujube, Solanum lycopersicum L., and Glycine max (Linn.) Merr. sequences available on NCBI. Multiple sequences were compared using DNAMAN.

Generation of transgenic plants. ZjPYL8 was integrated into the pCAMBIA2300 vector. Inflorescences of A. thaliana with unopened flower buds were immersed in a transformation medium containing ZjPYL8 for 4–5 minutes. The transgenic lines were then cultured at 22°C with a photoperiod of 16 hours light and 8 hours dark. In the T3 generation, a homozygous hygromycin-resistant transgenic line was selected.

Physiological phenotypes of ZjPYL8 transgenic lines. The wild-type (WT) and transgenic lines were grown for three weeks, and the lower epidermal cells of leaves were soaked in a buffer solution (50 mM KCl, 0.1 mM CaCl2, 10 mM MES, pH 6.1). The change in pore size before and after ABA treatment was observed under a microscope. Three biological replicates were performed for each group, and statistical analysis was used to determine the differences. For A. thaliana, both Col-0 and ZjPYL8 transgenic lines were sown on 1/2 MS medium with varying concentrations of ABA (0, 0.5, or 1 μM). The germination rate was recorded daily after germination. Additionally, the WT and transgenic lines were vertically cultured in 1/2 MS medium containing 150 mM NaCl and 10 μM ABA for ten days, and measurements of root length and scanner photos were recorded.

Protein extraction and SDS-PAGE. Transgenic Arabidopsis lines overexpressing ZjPYL8 were selected when they reached 15 days old. These seedlings were immersed in plates containing ABA, https://czasopisma.up.lublin.pl/index.php/asphc

ZjPYL8 gene encodes a PYL8 in sour jujube. During the juvenile fruit stage of sour jujube, a highly expressed putative PYL8 gene was identified through screening a cDNA library. Subsequently, a full-length PYL-like gene, known as ZjPYL8, was isolated from the sour jujube cDNA using primers derived from PYL sequences in jujube, Arabidopsis, tomato, and soybean. Sequence analysis revealed that the ZjPYL8 open reading frame (ORF) is 585 bp in length, encoding 195 amino acids. Comparison of the DNA sequences showed an 82.05% sequence homology between ZjPYL8 and AtPYL8, GmPYL8 (Glycine max PYR1-LIKE 8), and SlPYL8 (Solanum lycopersicum PYR1-LIKE 8), indicating that ZjPYL8 belongs to the PYR/PYL/RCAR family (Fig. 1).

Transgenic arabidopsis lines overexpressed with ZjPYL8: phenotypic analysis. No significant differences were observed in the germination rate and root length between the ZjPYL8 transgenic lines and the wild-type (WT) plants (Fig. 2). These results suggest that the ZjPYL8 transgenic lines exhibit average growth under non-stressful conditions.

Overexpression of ZjPYL8 in A. thaliana reduces germination rate and root length under ABA treatment. The function of ZjPYL8 in response to ABA

MG132 + ABA, and distilled water as control. Samples were taken at 0, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min. The seedlings were carefully dried and transferred into labeled sterilization centrifuge tubes, then rapidly frozen in liquid nitrogen for 5 minutes. Afterward, the seedlings were ground into a dry powder and mixed with 400 μL of 2× protein buffer per 250 μL sample. The mixture was then incubated in a water bath at 80°C for 10 minutes and subsequently centrifuged at 12,000 rpm for 5 minutes. The resulting protein was stored at –20°C. Protein samples were subjected to protein electrophoresis at 150 V for 1 hour. After staining and destaining, the target bands were observed and photographed.

RESULTS

Fig. 1. Alignment of the DNA sequences of ZjPYL8, AtPYL8, GmPYL8, and SIPYL8 using DNAMAN software (Lynnon Biosoft, USA)
Fig. 2. Results of germination and root tests on ZjPYL8 heterogeneous transgenic lines. (A) germination examinations of 10-day-old WT and genetic transformation lines; (B) statistical analysis of germination tests; (C) root length tests of 2-year-old WT and transgenic lines; (D) statistical analysis of root length tests. Bar = 2 cm

treatment was evaluated using WT and ZjPYL8-OE lines maintained on 1/2 MS medium supplemented with ABA. The germination rate, green cotyledon occurrence rate, and root length were measured under normal and ABA conditions. Our findings showed no significant difference between the WT and transgenic lines in ideal conditions. However, when exposed to ABA, the ZjPYL8-OE lines exhibited lower germination, green cotyledon occurrence, and root length than WT (Fig. 3A–D). These results indicate that overexpression of ZjPYL8 increased sensitivity to ABA, highlighting its role in seed germination and seedling establishment.

**Overexpression of ZjPYL8 in A. thaliana regulates ABA-induced stomatal closure.** To investigate the involvement of ZjPYL8 in regulating stomatal aperture, we conducted ABA treatment on both the WT and ZjPYL8-OE lines. The overexpression transgenic lines showed significantly lower stomatal aperture than the WT after being treated with 10 μM ABA for 1 and 2 hours (Fig. 4). These results demonstrated that overexpression of ZjPYL8 increased sensitivity to exogenous ABA. Under osmotic stress, plants restrict water loss by narrowing the stomatal opening of guard cells. Therefore, these findings suggest that ZjPYL8 may play a crucial role in sour jujube’s adaptation to drought stress.

**Overexpression of ZjPYL8 in A. thaliana improves the sensibility of the seedling to salt stress.** No significant differences were observed between the WT and ZjPYL8-OE lines under optimal conditions. However, upon exposure to NaCl, the overexpression of ZjPYL8 resulted in a lower germination rate, green cotyledon occurrence rate, and root length than the WT (Fig. 5A–D). These findings indicate an increase in salt sensitivity due to ZjPYL8 overexpression.
Fig. 3. Germination and root length assays of ZjPYL8-OE lines treated with ABA. (A) germination tests of 10-day-old WT and transgenic lines with ABA treatment; (B) statistical analysis of germination tests is shown in image; (C) root length testing of 2-week-old WT and ZjPYL8-OE lines treated with ABA; (D) statistical analysis of root length tests. *P < 0.05, **P < 0.01, t-test, n = 90. Bar = 2 cm

Fig. 4. ABA-induced stomatal closure in the WT and ZjPYL8-OE lines. (A and B) Microscopic images of stomatal apertures in both WT and ZjPYL8-OE lines following treatment with 10 μM ABA. White light (150 μmol m⁻² s⁻¹) was used to clarify epidermal peels with closed stomata for 2 hours, followed by 1 and 2 hours of treatment with 10 μM ABA. (C) Quantitative analysis of stomatal aperture measurements. The error bars indicate the means and standard errors of three biological replicates (t-test, n = 150, ***P < 0.001). Bar = 5 μm (A, B)
ABA may stabilize ZjPYL8 in A. thaliana. Our results revealed a significant increase in ZjPYL8 expression 10 minutes after ABA treatment, with peak expression observed at 30 minutes (Fig. 6A). Since the 35S promoter drove ZjPYL8, we did not anticipate any effect of ABA treatment on its expression. To validate these results, we performed qPCR analysis and found no significant change in ZjPYL8 expression levels following treatment with 10 μM ABA (Fig. 6C). Additionally, our findings demonstrated that MG132 had no significant impact on the expression of ZjPYL8 transcripts (Fig. 6B, C). These findings suggest that ZjPYL8 may be involved in ABA-mediated physiological responses in sour jujube, although further investigation is necessary to comprehend the underlying mechanisms fully.

DISCUSSION

Plants often encounter salt and osmotic stress and have developed intricate mechanisms to mitigate their detrimental effects. As a resilient plant, Sour jujube exhibits characteristics of drought tolerance, salt resistance, and alkali resistance. However, the underlying resistance mechanisms of sour jujube remain poorly understood. The ABA signal transduction pathway is activated by ABA, which plays a crucial role in reducing the negative impacts of stress in plant cells. Among the ABA receptor family, PYL8 is a member known to participate in plant responses to ABA [Belda-Palazon et al. 2018]. Furthermore, PYL8 has been extensively documented to regulate plant responses to high glucose, leaf

Fig. 6. Protein abundance of ZjPYL8 under ABA and MG132 treatments. (A) The protein levels in 14-day-old ZjPYL8-OE lines following treatment with 10 µM ABA at intervals of 0, 10, 20, 30, and 40 minutes. (B) Protein abundance in the ZjPYL8-OE line, aged 14 days, after a 3-hour exposure to 10 M MG132 in 1/2 MS liquid culture, and subsequently treated with 10 µM ABA for 0, 10, 20, 30, and 40 minutes. A band marked with an asterisk indicates a non-specific protein. (C) The quantification of ZjPYL8 transcript levels using q-PCR. The experiment was repeated at least three times, and equivalent findings were achieved each time.

senescence, drought stress, stomatal movement, and root development in A. thaliana, exhibiting unique regulatory properties [Lim et al. 2013, Xing et al. 2016, Zhao et al. 2016, Qi et al. 2020b]. While screening our laboratory’s previous transcriptome data, a PYL8-like gene highly expressed under dehydration was discovered in sour jujube. We subsequently cloned the PYL8-like gene and compared it to the PYL8 sequences found in Arabidopsis, soybean, and tomato. The sequence exhibited 82.05% homology, confirming that the gene we obtained corresponds to PYL8 in sour jujube, and we named it ZjPYL8 (Fig. 1). By analyzing the expression level of ZjPYL8 in A. thaliana under ABA treatment, we observed a significant increase in ZjPYL8 protein abundance in the transgenic lines (Fig. 6A). However, since the 3SS promoter controls ZjPYL8 in the transgenic lines, its expression level should not be significantly altered under different physiological conditions. Our hypothesis was supported by qPCR data (Fig. 6C), which showed no significant changes in ZjPYL8 expression levels. Interestingly, when MG132 was added, the protein abundance of ZjPYL8 in the transgenic lines remained consistently high, both before and after ABA treatment (Fig. 6B). These findings suggest that auxin may overcome the phenotype of the ABA core signaling pathway by inhibiting plant root growth during ABA treatment [Deak and Malamy 2005]. Our research indicates that the degradation mechanism of ZjPYL8 could promote plant root development under normal conditions and enhance plant adaptability to adverse environmental conditions. Our findings demonstrated that overexpression of ZjPYL8 conferred a resistant phenotype under de-
Fig. 7. The activation of ZjPYL8 and ZjPYR1 by ABA improves plant resistance to drought environments by down-regulating the stomatal aperture, root length, and germination rate.

hydration conditions, highlighting the crucial role of the putative PYL8 receptor in seedling growth and establishment. High levels of ABA have been shown to inhibit stomatal movement and root growth [Gonzalez-Guzman et al. 2012, Xing et al. 2016]. In our study, we observed that overexpression of ZjPYL8 increased the sensitivity of plant stomata to ABA (Fig. 4). The transgenic plants exhibited significantly smaller stomatal apertures under ABA treatment than WT plants. However, there was no noticeable difference in stomatal aperture between WT and transgenic plants under white light irradiation. These results suggest that ZjPYL8 can effectively reduce transpiration rates in plants to prevent water loss during drought conditions. Unlike AtPYL8, ZjPYL8 does not appear to impact the stomatal aperture under steady-state conditions, indicating that ZjPYL8 may possess a unique regulatory mechanism in plant stomata (Fig. 4). The transgenic lines exhibited shorter root length phenotypes under ABA treatment and salt stress than WT plants (Figs 3, 5). This observation is consistent with the inhibitory effects of high concentrations of ABA on root growth, as well as the detrimental impact of high salt concentrations on root growth [Jones et al. 1920]. ABA regulates the expression of salt stress-responsive genes through transcription factors such as ABF2/AREB1 (abscisic acid-responsive elements-binding factor.
2/ABA-response element binding factor 1), ABF3, and ABF4/AREB2, which amplify the plant’s response to salt stress [Fernando et al. 2016]. Our results indicate that overexpression of ZjPYL8 in plants suppresses root growth under ABA treatment and salt stress. Osmotic stress and salt stress inhibit primary and lateral root growth partially in an ABA-dependent manner [LaRosa et al. 1987]. These findings suggest that ZjPYL8 functions as a putative ABA receptor in sour jujube, potentially revealing a drought resistance mechanism in this plant species.

The PYR/PYL family consists of multiple members, and the sensitivity of each receptor to ABA varies in plants [Yan et al. 2014, Zhao et al. 2014]. In a previous study, we successfully cloned and confirmed the presence of an ABA receptor named ZjPYR1 (Ziziphus jujube Pyrabactin resistance 1) in sour jujube [Qi et al. 2020a]. Unlike ZjPYL8, the response of ZjPYR1 to ABA was observed after 1 hour of ABA treatment. By comparing the expression patterns of ZjPYL8 and ZjPYR1 in transgenic lines, we observed a clear cooperative relationship between the two receptors in response to ABA treatment. In sour jujube, ZjPYL8 is responsible for the early response to ABA, while ZjPYR1 is responsible for the later response. Based on previous research findings and the results presented here, it can be inferred that ZjPYL8 and ZjPYR1 may work together to enhance the plant’s resistance to drought conditions by regulating stomatal aperture, root length, and germination rate (Fig. 7).

CONCLUSIONS

This study focused on cloning a putative ABA receptor known as ZjPYL8. To examine the impact of ZjPYL8 overexpression on plant responses to ABA, we introduced it into A. thaliana. When subjected to ABA treatment, the overexpression of ZjPYL8 in A. thaliana led to a significant decrease in stomatal aperture size and root length, highlighting its ABA-responsive nature. In contrast, the WT plants exhibited less sensitivity to ABA. Notably, ZjPYL8 transgenic plants displayed shorter root lengths under salt treatment than WT plants. Based on the above findings, we speculate that the overexpression of ZjPYL8 in A. thaliana can remarkably enhance plant resistance to various stressors and imply its potential role as an ABA receptor in sour jujube, enabling plants to better adapt to drought and salt stresses. Similar to other ABA receptors in A. thaliana, ZjPYL8 is involved in mediating plant responses, such as stomatal closure and root length, in the presence of ABA.

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