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# Loss of *Ribosomal Protein L24A* (*RPL24A*) suppresses proline accumulation of *Arabidopsis thaliana ring zinc finger 1* (*atrzf1*) mutant in response to osmotic stress

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ABSTRACT

Proline (Pro) metabolism in plants is involved in various cellular processes mediated during abiotic stress. However, the Pro-regulatory mechanisms are unclear. We used a suppressor mutation technique to isolate novel genes involved in the regulation of Pro metabolism in *Arabidopsis*. Using *atrzf1* as a parental plant for T-DNA tagging mutagenesis, we identified a suppressor mutant, termed *proline content alterative 21* (*pca21*), that displayed reduced Pro contents compared with the *atrzf1* under osmotic stress conditions. Genomic Thermal Asymmetric Interlaced (TAIL)-PCR revealed *pca21* harbored an inserted T-DNA in the region of *At2g36620* that encodes Ribosomal Protein L24A. In general, the *pca21* mutant partially suppressed the insensitivity of *atrzf1* to osmotic stress and abscisic acid during seed germination and early seedling stage. Additionally, the *pca21* mutant had increased MDA content and lower expression of several Pro biosynthesis-related genes than the *atrzf1* mutant during drought condition. These results suggest that *pca21* acts as partial suppressor of *atrzf1* in the osmotic stress response through the Pro-mediated pathway.



## 1. Introduction

Plants subjected to drought stress accumulate osmolytes, such as proline, glucose, betaine, fructans, pinitol, and inositol, in the cytosol of their cells [1]. Proline (Pro) production is an important potential response to drought and can have many other protective functions. These include protecting membrane structure [2], regulating cellular redox potential [3], scavenging reactive oxygen species [4], and modulating antioxidant free radical contents [5]. Furthermore, Pro may function in the metabolic signaling of sugar status [6].

Ribosomal Proteins (RPs) reportedly help stabilize the ribosomal complex, mediate polypeptide synthesis, and have extra-ribosomal functions like regulating plant morphogenetic and environmental stress responses. These extra-ribosomal functions include regulation of cell differentiation, RNA chaperone activity, cell development, and pathogen resistance [7–9]. Transcript levels of *RP* genes are modulated differentially by abiotic stresses. The expression of *RPL13A*, *RPL30*, *RPS14* and *RPS16* genes is regulated by abscisic acid (ABA) and cytokinin [10]. The overexpression of eggplant *RPL13* in potato enhanced resistance to fungal pathogens [11]. Cold stress induces transcription of *RPS13*, *RPS6* and *RPL37* in soybeans [12]. In *Arabidopsis*, *RPL10A*, *RPL10B* and *RPL10C* genes are involved in plant development and protein translation under ultraviolet B stress [13]. The collective evidence implies that *RP* genes associate with plant environmental stress response through a crosstalk of multiple stress-regulated genes.

We have previously shown that mutations in the *AtRZF1* gene, which encodes E3 ubiquitin ligase, have less sensitive to dehydration, but *AtRZF1*-overexpressing plants were hyper-sensitive due to their reduced Pro contents [14]. To investigate the function of AtRZF1 in drought-induced Pro accumulation, genetic screening for suppressors of *atrzf1* was conducted by inserting a T-DNA tag. The *atrzf1* suppressed lines displayed altered Pro contents compared

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with the parent line in drought stress condition. The mutants were termed *proline content alternative* (*pca*) mutants.

In this study, we isolated an *atrzf1* background mutant, *pca21*, which displayed sensitivity to ABA and dehydration stress. The T-DNA insertion in *pca21* was located on the fifth exon region of the *At2g36620* gene. Data demonstrated that PCA21 influences the drought-related physiological parameters and molecular components during dehydration stress.

## 2. Materials and methods

### 2.1. Plant materials, growth conditions, and stress induction

*Arabidopsis* (Col-0) plants were grown in growth chambers under intense light (120 μmol $m^{-2}$ $s^{-1}$) at 22 °C, 60% relative humidity, for 16-h. Ten-day-old *Arabidopsis* seedlings were treated with 400 mM mannitol or 100 μM ABA. Samples were obtained 0, 3, and 6 h after treatment, quickly frozen in liquid nitrogen and stored at −80 °C.

### 2.2. Assay of Pro and malondialdehyde (MDA) content

Pro contents were measured as previously described [15]. Leaves (about 100 mg) extracted in 1 mL of 3% sulfosalicylic acid were reacted with 100 μL of ninhydrin reagent. The reaction mixture was extracted with 200 μL of toluene and vortexed. Absorbance of the toluene layer at 520 nm was determined in a UV/VIS spectrophotometer (JASCO, Tokyo, Japan). The Pro concentration was extrapolated from a standard curve.

MDA content was determined by the thiobarbituric acid (TBA) reaction as described previously [16]. Approximately 200 mg of leaves were homogenized with 2 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 10 min. Two milliliters of supernatant was mixed with 0.6% TBA, incubated in hot water (100 °C) for 15 min, cooled immediately on ice, and centrifuged at 6000 rpm for 10 min. Absorbance at 532, 450 and 600 nm was determined, and MDA concentration was estimated by subtracting the non-specific absorption at 450 and 600 nm from the absorption at 532 nm.

### 2.3. Assay of the T-DNA insertion region by TAIL-PCR

Genomic DNA was isolated from *pca21* mutant and Thermal Asymmetric Interlaced (TAIL)-PCR was performed using arbitrary degenerate (AD) and T-DNA left border (LB) end primers (Supplementary Table S1). Purified PCR products following tertiary TAIL-PCR were cloned into the pGEM T-easy vector (Promega, Madison, WI) for DNA sequence analysis, and the flanking sequences obtained were used to perform to a BLAST search using the NCBI program (http://www.ncbi.nlm.nih.gov). Finally, specific primers were designed (Supplementary Table S1) and used in combination with the LB primer to amplify specific fragments that were sequenced to confirm the T-DNA insertion site.

### 2.4. Statistical analysis

Statistical analysis was used Excel and SPSS software (Ver. 23, IBM, New York, USA). Analysis of variance was used to compare the statistical differences by Student's *t*-test, at a significant level of $0.01 < P < 0.05$ or $P < 0.01$.

## 3. Results and discussion

### 3.1. Isolation of pca21 mutant

To find novel genes related to Pro metabolism, we recently created a T-DNA tagging population using the *atrzf1* mutant as the parental plant [17]. Since the *atrzf1* mutant accumulates Pro under drought conditions, the T-DNA-tagged pool was screened for reduced Pro contents to dehydration stress compared with the *atrzf1*. We identified a partial suppressor mutant, termed *proline content alterative 21* (*pca21*) that displayed reduced Pro contents compared with the *atrzf1* under water deficit conditions. The Pro level of *pca21* was slightly higher than that of the wild type (WT) plant (Fig. 1A). The results indicate that the *PCA21* can regulate the Pro production of the *atrzf1* mutant in response to drought conditions.

### 3.2. Response of the pca21 mutant to osmotic stress and ABA

To investigate whether *pca21* mutant is involved in osmotic stress and ABA responses, we examined the rate of seed germination and cotyledon greening in WT, *atrzf1*, and *pca21* plants in the presence of mannitol or ABA. In Murashige and Skoog (MS) medium [18], there was no difference in seed germination and seedling growth among the plants (Supplementary Fig. S1A). The germination rate was similar between *pca21* and *atrzf1* mutants, except at 2 days after sowing of seeds on MS plates containing 400 mM mannitol. The *pca21* and *atrzf1* plants displayed significantly higher seed germination compared with the WT at 3 and 4 days during osmotic stress condition (Supplementary Fig. S1B). In addition, the germination rate of the *pca21* mutant was markedly more affected than the *atrzf1* mutant by 1 μM ABA treatment, while the germination rate of *pca21* was similar with WT plants (Supplementary Fig. S1C). These results suggest that the *pca21* mutant is a suppressor of *atrzf1* against the ABA response rather than the osmotic stress during seed germination.

Comparison of the *pca21* mutant with the *atrzf1* revealed fewer green cotyledons after seeds were sown on MS plates containing 350 or 400 mM mannitol, or 0.5 or 1 μM ABA at the indicated times, whereas the rate of cotyledon greening in the *pca21* mutant was markedly higher compared with WT plants (Fig. 1B and C and Supplementary Fig. S1A). These data indicate that PCA21 partially suppresses AtRZF1 in response to dehydration and ABA during early seedling development.

### 3.3. Expression analysis of the Pro biosynthesis-related genes

To analyze the expression levels of Pro biosynthesis genes related to dehydration stress, we examined two genes induced by ABA, high salt and osmotic stresses: *Delta 1-pyrroline-5-carboxylate synthase 1* (*P5CS1*) and *Delta 1-pyrroline-5-carboxylate reductase* (*P5CR*) [19,20]. The transcript levels of Pro biosynthesis genes were significantly induced more in *atrzf1* than WT after mannitol treatment. The *pca21* mutant was induced slightly less than *atrzf1*, but slightly higher than WT (Fig. 2A and B). This indicates that PCA21 can mediate the expression of AtRZF1-regulated Pro biosynthesis genes during osmotic stress. AtMYB2 transcription factor is involved in the up-regulation of *P5CS1* expression [21]. As shown in Fig. 2C, dehydration-induced expression of *AtMYB2* was slightly increased in *atrzf1* plants than in WT and *pca21* plants. These molecular changes suggest that the Pro content of the *pca21* mutant was reduced more than *atrzf1*.