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# PLANT U-BOX PROTEIN 10 negatively regulates abscisic acid response in Arabidopsis

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**Abstract**

MYC2 is well known as a positive regulator for abscisic acid (ABA) signaling but whether PLANT U-BOX PROTEIN 10 (PUB10) is involved in ABA responses has not been reported. Here, we show that the E3 ubiquitin ligase PUB10 modulates ABA signaling in Arabidopsis. *PUB10ox* (*35S:PUB10-myc*) and *myc2* loss-of-function mutants were hyposensitive to ABA during germination, whereas *pub10* loss-of-function and *MYC2ox* (*35S:MYC2-GFP*) mutants were hypersensitive. In addition, *pub10* mutants showed hypersensitivity to high salt and osmotic stress during germination; by contrast, *PUB10ox* line displayed the opposite phenotype. ABA-induced expression of *KIN2* (*Cold- and ABA-Inducible Protein*), *RD22* (*Responsive to Dehydration 22*), *ANAC019* (*NAC Domain-Containing Protein 19*), and *ANAC055* (*NAC Domain-Containing Protein 55*) was enhanced in both *pub10* and *MYC2ox* plants. Taken together, *pub10* plants phenocopied *MYC2ox* plants, whereas *PUB10ox* plants phenocopied *myc2* in ABA response. Our results provide evidence that PUB10 negatively regulates ABA signaling in Arabidopsis.

**Keywords:** Abscisic acid, ANAC019, ANAC055, Arabidopsis, MYC2, PUB10

## Introduction

Abscisic acid (ABA) is a phytohormone present in all vascular plants and it participates in various developmental and physiological processes during the plant life cycle, including seed development, seed dormancy, germination, and abiotic stress responses [1]. The phosphorylation and dephosphorylation of protein are key post-translational modifications in ABA signal transduction [2]. In addition, regulation of protein stability via ubiquitination of key components of the ABA signaling pathways also plays an important role [3].

E3 ubiquitin ligases are responsible for the specificity of ubiquitination by recruiting appropriate target proteins [4]. Until now, only a limited number of Plant U-box (PUB) E3 ligases have been characterized as both positive and negative regulators of ABA signaling [5]. For instance, PUB12 and PUB13 were found to ubiquitinate ABI1 in the presence of both ABA and Pyrabactin Resistance 1 (PYR1) [6]. Another U-box E3 ligase, Carboxyl terminus of the Hsc70-Interacting Protein (CHIP), monoubiquitinated PP2A subunits and enhances their activities under stress conditions [7]. In addition, PUB9, PUB18, and PUB19 are involved in ABA signaling [8, 9].

MYC2 has been well characterized as a central transcriptional regulator in JA signaling [10, 11]. MYC2 was first isolated as a transcription factor that binds to the *RESPONSIVE DEHYDRATION 22* (*RD22*) promoter and subsequently characterized as a positive regulator of ABA signaling through genetic analysis [12, 13]. Hence, MYC2 seems to integrate at least two (ABA and JA) different signaling pathways to coordinate growth and development in Arabidopsis.

Crosstalk between ABA and JA signaling pathways has been poorly examined so far. Previously, we showed that MYC2 directly interacts with PUB10, and that PUB10 modulates JA signaling by destabilizing MYC2 protein [14]. However, how PUB10 regulates ABA responses is mostly unknown. Here, we showed that PUB10 is a negative regulator in ABA signaling.

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## Materials and methods

### Plant materials and growth conditions

*Arabidopsis thaliana* Columbia ecotype (Col-0), *myc2* (*jin1-9*, SALK_017005), *pub10* (SALK_017111), *MYC2ox*, and *PUB10ox* lines [14] were grown on 0.5% agar medium containing Murashige and Skoog (MS) salts, 1% sucrose, and 0.5 g/L MES hydrate at 22 °C under 16 h white fluorescent light (100 μmol $m^{-2}$ $s^{-1}$)/8 h dark. T-DNA insertion lines were obtained from the SALK collection [15].

### Germination test

Seeds were spread onto MS agar plates with or without 2 μM ABA, 150 mM NaCl, or 200 mM mannitol. After 4 days of stratification at 4 °C, seed germination was monitored from 0 to 7 days.

### Abscisic acid treatments

Ten-day-old Col-0, *pub10*, and *MYC2ox* Arabidopsis seedlings grown on MS agar plates were transferred to liquid MS medium containing 10 μM ABA. Treated seedlings were collected at the indicated time points for real-time RT-PCR analysis.

### Real-time RT-PCR analysis

Total RNA was extracted from Arabidopsis seedlings treated with ABA using an RNeasy Plant Mini Kit (Qiagen) including DNase I treatment. Reverse transcription was performed using 2 μg of each total RNA and oligo $(dT)_{20}$ primers by SuperScript III reverse transcriptase (Invitrogen). Real-time RT-PCR was performed using SYBR premix Ex Taq (Tli RNaseH plus) (TaKaRa) on the Bio-Rad CFX96 real-time system with gene-specific primers. Primer sequences are listed in Additional file 1: Table S1.

### Accession numbers

Sequence data can be found in the Arabidopsis Genome Initiative data library under the following accession numbers: ACT2 (AT3G18780), ANAC019 (AT1G52890), ANAC055 (AT3G15500), KIN2 (AT5G15970), MYC2 (AT1G32640), PUB10 (AT1G71020), RD22 (AT5G25610).

## Results and discussion

### PUB10 negatively regulates ABA response during seed germination

To examine PUB10 function in ABA responses, we analyzed the germination rates of *pub10*, *PUB10ox* (*35S:PUB10-myc*), *myc2* (*jin1-9*), and *MYC2ox* (*35S:MYC2-GFP*) seeds on MS agar plates containing 2 μM ABA. Germination rates of all genotypes in MS medium without ABA were almost the same as those of Col-0 (Fig. 1a). At 2 μM ABA, the germination rates of *pub10* and *MYC2ox* seeds were significantly reduced compared to Col-0 seeds (Fig. 1b), but germination of *PUB10ox* and *myc2* seeds showed a remarkable ABA-insensitive phenotype (Fig. 1b). These results indicate that the altered germination rates observed in *PUB10* and *MYC2* mutants are dependent on ABA sensitivity, and that PUB10 and MYC2 act as positive and negative ABA response regulators, respectively. These opposing ABA sensitivities between *PUB10* and *MYC2* mutants are consistent with our previous observation that MYC2 protein is destabilized by the E3 ubiquitin ligase PUB10 [14].

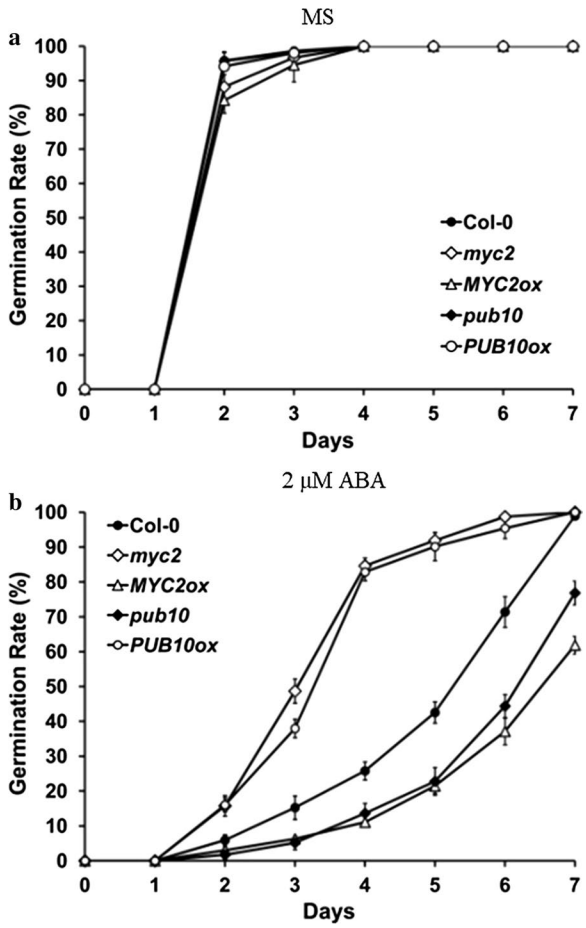


**Fig. 1** ABA affects *myc2, MYC2ox, pub10, and PUB10ox* mutant germination. Col-0, *myc2*, *MYC2ox*, *pub10*, and *PUB10ox* seed germination rates were measured on MS medium containing 0 (**a**) or 2 μM (**b**) ABA. The percentage of seeds showing 1 mm radical length was scored. Symbols represent average values ± SD (n = 3). Col-0 (filled circle), *myc2* (empty diamond), *MYC2ox* (empty triangle), *pub10* (filled diamond), and *PUB10ox* (empty circle)

### PUB10 positively regulates salt and osmotic stress tolerance during seed germination

ABA plays a key role in abiotic stress responses including drought, salt, and osmotic stress [1]. To evaluate the

effect of PUB10 on salt and osmotic stress responses, we analyzed the seed germination rates of *pub10* and *PUB10ox* on MS agar plates containing 150 mM NaCl or 200 mM mannitol. The germination rate of *pub10* seeds was significantly decreased in high salt medium compared to Col-0 seeds, but *PUB10ox* seeds showed an increased germination rate compared to Col-0 seeds (Fig. 2a). The germination rates of *pub10* and *PUB10ox* seeds in mannitol-containing medium were similar to those in high salt medium. *PUB10ox* and *pub10* seeds showed tolerant and hypersensitive phenotypes to osmotic stress, respectively (Fig. 2b). Growth inhibition of *pub10* mutant seedlings was stronger than that of Col-0 seedlings (Fig. 2c). Taken together, these results indicated that PUB10 acts as a positive regulator for salt and osmotic stress tolerance.

### PUB10 negatively regulates ABA-responsive gene expression

To investigate how PUB10 regulates gene expression in response to ABA, we performed a time-course analysis for ABA-responsive gene expression in *pub10* and *MYC2ox* plants after ABA treatment. To determine the role of PUB10 in the MYC2-dependent ABA signaling pathway, we monitored the expression of *KIN2*, *RD22*, *ANAC019*, and *ANAC055* genes previously reported as ABA-responsive genes regulated by MYC2 [12, 15, 16]. *KIN2* and *RD22* expression were enhanced more than twofold in *pub10* mutants compared to Col-0 6 h after ABA treatment (Fig. 3a, b). A previous report showed that transcription levels of *KIN2* and *RD22* were enhanced in *MYC2ox* and reduced in *myc2* after ABA treatment [12]. Therefore, enhanced expression of *KIN2* and *RD22* in *pub10* could be caused by increased MYC2 protein level. Transcription levels of two NAC transcription factors, *ANAC019 and ANAC055*, were induced by ABA, dehydration, and salt, and are also known as direct MYC2 targets [15–18]. *ANAC019* and *ANAC015* expression were higher in *pub10* and *MYC2ox* plants than those in Col-0 plant at 3 to 6 h after ABA treatment (Fig. 3c, d). Similar expression patterns in *pub10* and *MYC2ox* after ABA treatment support the notion that PUB10 negatively regulates target gene expression by destabilizing MYC2 protein. Overall, our results indicate that PUB10 negatively regulates ABA or salt responses by modulating MYC2 protein levels.

Although MYC2 has been first identified as a positive regulator of ABA responses [11, 12], MYC2 function in ABA signaling is almost unknown. Rather, MYC2 has been intensively studied as a key regulator in the JA signaling pathway [10]. MYC2 directly binds to the promoters of two NAC transcription factors, ANAC019 and ANAC055 and activates their transcription levels. These

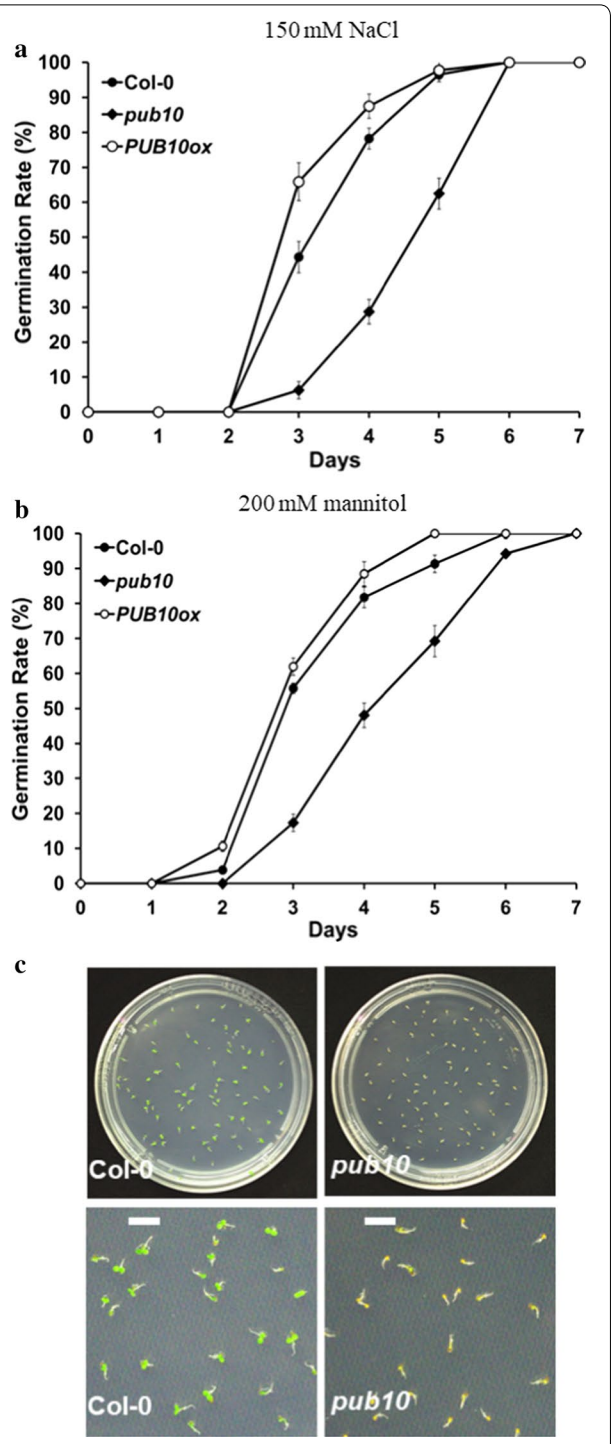


**Fig. 2** *pub10* and *PUB10ox* germination in the presence of high salt and osmotic stress. Col-0, *pub10*, and *PUB10ox* seed germination rate on MS medium containing 150 mM NaCl (**a**) and 200 mM mannitol (**b**). Early post-germinative growth of Col-0 and *pub10* mutants 5 days after germination on 150 mM NaCl medium (**c**). The percentage of seeds showing 1 mm radical length was scored after 4 days of stratification at 4 °C. Seed germination was monitored from 0 to 7 days. Symbols represent average values ± SD (n = 3). Col-0 (filled circle), *pub10* (empty triangle), and *PUB10ox* (empty circle). Scale bar = 10 mm

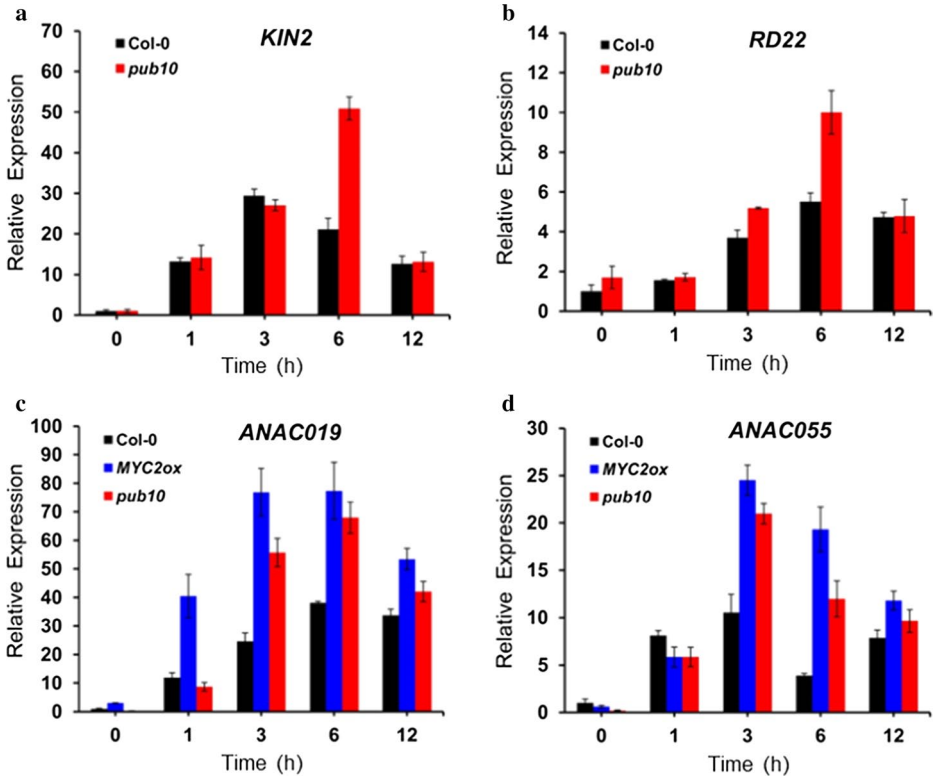


**Fig. 3** ABA-responsive gene expression levels in *pub10* and *MYC2ox* plants after ABA treatment. Time course expression levels of *KIN2* (**a**) and *RD22* (**b**) after ABA treatment in Col-0 and *pub10* plants. Time course expression levels of *ANAC019* (**c**) and *ANAC055* (**d**) after ABA treatment in Col-0, *MYC2ox*, and *pub10* plants. **a**–**d** transcript levels were normalized to *ACT2* expression levels, and bars represent average ± SD (n = 3 independent seedling pools)

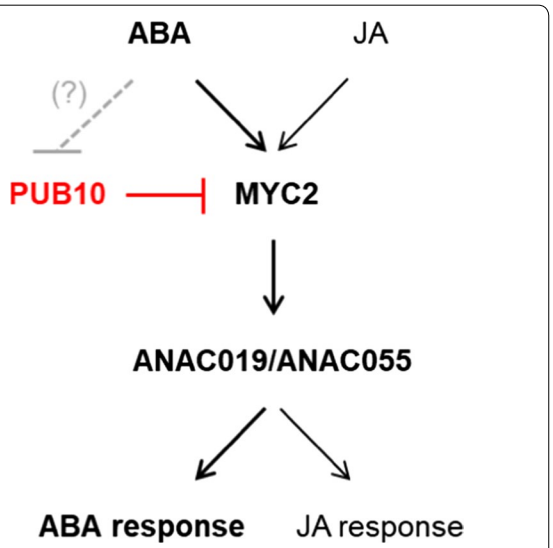


**Fig. 4** Proposed model of the role of PUB10 in ABA signaling pathway. MYC2 is a positive regulator for ABA responses and is activated by ABA. MYC2 directly activates two NAC transcription factor (TF) genes, *ANAC019* and *ANAC055*, and these NAC TFs enhance downstream ABA-responsive gene transcription. PUB10 negatively regulates ABA responses by destabilizing MYC2 protein. PUB10 protein level or activity might be modulated after ABA treatment

two NAC factors enhance the downstream transcription JA- and ABA-responsive genes, and also play key roles in the crosstalk between JA and ABA signaling (Fig. 4) [10, 15]. We suggest that PUB10 acts as a negative regulator of ABA signaling through MYC2; further study on the regulation of PUB10 protein by ABA is necessary to understand how PUB10 participates in the fine tuning of ABA signaling and JA crosstalk. Our results provide new insights for increasing ABA-mediated abiotic stress tolerance in plants.

## Additional file

**Additional file 1: Table S1.** Primers used in this study.

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**Authors' contributions**
CJ, and NHC conceived the experiments. JSS, PZ, and CJ conducted the experiments. JSS, CJ, and NHC analyzed the results. JSS, CJ, and NHC wrote the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

**Competing interests**
The authors declare that they have no competing interests.

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