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Arabidopsis RING E3 ubiquitin ligase AtATL80 is negatively involved in phosphate mobilization and cold stress response in sufficient phosphate growth conditions☆

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ABSTRACT

Phosphate (Pi) remobilization in plants is critical to continuous growth and development. AtATL80 is a plasma membrane (PM)-localized RING E3 ubiquitin (Ub) ligase that belongs to the *Arabidopsis Tóxicos en Levadura* (ATL) family. *AtATL80* was upregulated by long-term low Pi (0–0.02 mM KH_2PO_4) conditions in *Arabidopsis* seedlings. *AtATL80*-overexpressing transgenic *Arabidopsis* plants (*35S:AtATL80-sGFP*) displayed increased phosphorus (P) accumulation in the shoots and lower biomass, as well as reduced P-utilization efficiency (PUE) under high Pi (1 mM KH_2PO_4) conditions compared to wild-type plants. The loss-of-function *atatl80* mutant line exhibited opposite phenotypic traits. The *atatl80* mutant line bolted earlier than wild-type plants, whereas *AtATL80*-overexpressors bloomed significantly later and produced lower seed yields than wild-type plants under high Pi conditions. Thus, AtATL80 is negatively correlated not only with P content and PUE, but also with biomass and seed yield in *Arabidopsis*. In addition, *AtATL80*-overexpressors were significantly more sensitive to cold stress than wild-type plants, while the *atatl80* mutant line exhibited an increased tolerance to cold stress. Taken together, our results suggest that AtATL80, a PM-localized ATL-type RING E3 Ub ligase, participates in the Pi mobilization and cold stress response as a negative factor in *Arabidopsis*.

1. Introduction

Phosphorus (P) is an essential macronutrient required for the biogenesis of biomembranes and nucleic acids. P is also a critical component of metabolic processes, such as the regulation of enzymatic activities and signal transduction [1]. P is acquired by plants in the form of phosphate (Pi) via Pi transporters in the roots. Pi transporters regulate the remobilization of Pi from source organs to sink organs, thereby sustaining growth under Pi-limited conditions [2]. About 78% of stored Pi was demonstrated to be redistributed from older leaves to sink organs in *Arabidopsis thaliana* [3]. Thus, Pi transporters play major roles in both initial Pi uptake and its mobilization within plants [2]. In *Arabidopsis*, members of the PHOSPHATE TRANSPORTER 1 (PHT1) family are localized to the plasma membrane (PM) [4]. There are nine such PHT1 proteins, eight of which are transcriptionally induced by Pi starvation [5,6]. Of these, PHT1;1, PHT1;4, PHT1;8, and PHT1;9 were each reported to play a role in Pi acquisition in roots under Pi-sufficient conditions [7,8]. PHT1;5 was shown to function in the remobilization of Pi between source and sink organs [9]. The cellular roles of the rest of the PHT1s remain unknown.

AtATL80 belongs to the *Arabidopsis Tóxicos en Levadura* (ATL) family, which is composed of 80 members in *Arabidopsis* [10]. The ATL family all contains the RING motif, and therefore is likely a RING-type E3 ligase [11]. The ATL proteins have four additional characteristic motifs; a trans-membrane domain, a basic amino-acid-rich region, a conserved GLD tri-peptide sequence, and a C-terminal diverse region. The cellular functions of basic amino-acid-rich region and GLD tri-peptide sequence remain unknown. It was proposed that the C-terminal diverse region mediates protein–protein interaction, and therefore may be the substrate binding region [12]. Currently, the physiological roles of only a few ATL family members are known. ATL31 regulates the ratio of carbohydrate to nitrogen via the Ub-26S proteasome-mediated degradation

Abbreviations: ATL, *Arabidopsis Tóxicos en Levadura*; P, phosphorus; Pi, phosphate; PM, plasma membrane; PSR, Pi starvation-response; PUE, phosphorus utilization efficiency; RING, really interesting new gene.

☆ The nucleotide sequence data reported here has been deposited in the GenBank database under accession number NM_101935.2 (*AtATL80*).

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of 14-3-3 proteins [13]. ATL78 is a negative regulator of the cold stress response and a positive regulator of drought stress response in *Arabidopsis* [14]. Both ATLs are localized to the PM [13,14].

In this report, a possible cellular role of AtATL80 was investigated in terms of Pi remobilization. An RT-PCR assay indicated that *AtATL80* is up-regulated by Pi starvation in *Arabidopsis* seedlings. AtATL80 is localized at the PM. Phenotypic analyses of a loss-of-function mutant (*atatl80*) and *AtATL80*-overexpressing transgenic lines (*35S:AtATL80-sGFP*) suggested that AtATL80 is involved in Pi mobilization as a negative regulator. Our data further suggest that AtATL80 is a negative factor of the cold stress tolerance response in *Arabidopsis*.

2. Materials and methods

2.1. Plant materials and growth conditions

The *atatl80* T-DNA insertion mutant (SALK_046204) was obtained from the Arabidopsis Biological Resource Center (ABRC). Sterilized *Arabidopsis* seeds were sown with various concentrations (0, 0.02, 0.5, or 1 mM) of KH_2PO_4 as described previously [15] and grown for 5–10 days. *Arabidopsis* seedlings were transferred to modified MS medium [16] supplemented with 1% sucrose and 1.5% agar, with high Pi (1 mM KH_2PO_4) or low Pi (50 μM KH_2PO_4), and grown for additional 2 weeks. The total dry weight, P content, and P utilization efficiency (PUE) were determined. Mature *Arabidopsis* plants were irrigated with modified half-strength Hoagland solution supplemented with 250 μM KH_2PO_4, as described by Nagarajan et al. [9], and flowering time and seed yield were monitored.

2.2. RT-PCR and real-time qRT-PCR analyses

The isolation of total RNA from various tissues in *Arabidopsis* was performed according to Ryu et al. [17]. The cDNA synthesis, RT-PCR, and real-time qRT-PCR analyses were conducted as described previously [17], using SYBR Premix Ex Taq II (Takara, Kyoto, Japan). qRT-PCR data were obtained using the IQ5 optical system software program (Bio-Rad, Hercules, CA, USA) and normalized to the *At4g26410* mRNA levels [9]. The primers used in this study are listed in Supplementary Table S1.

2.3. In vitro self-ubiquitination assay

The in vitro self-ubiquitination assay of the bacterially-expressed GST-ΔNAtATL80$^{60\text{-}197}$ and GST-ΔNAtATL80$^{60\text{-}197/C130S}$ recombinant proteins (500 ng) was conducted according to the established protocol [18]. Immuno-blot analysis was conducted using anti-GST and anti-Ub antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described by Kim and Kim [19].

2.4. Subcellular localization assay of AtATL80-sGFP in tobacco leaf epidermal cells

The GFP, AtATL80^{C130S}-GFP, ΔTM-AtATL80^{C130S}-sGFP, and PIP2-mCherry proteins were transiently expressed in three-week-old tobacco (*Nicotiana benthamiana*) leaves using the *Agrobacterium*-mediated infiltration method as described previously [20].

2.5. Generation of 35S:AtATL80-sGFP transgenic plants

35S:AtATL80-sGFP overexpressing transgenic plants were generated according to the method described previously [17]. Homozygous T3 lines were used in phenotypic analyses.

2.6. Measurement of total P content

Total P concentration was quantified using a modified version of the U.S. Environmental Protection Agency method 365.2, as recorded by Song et al. [21]. Total P content was measured using a spectrophotometer (model DU800; Beckman Coulter, Brea, CA, USA) at 650 nm, and expressed as total P/mg tissue dry weight.

2.7. Analysis of cold stress tolerance phenotypes

Light-grown three-week-old plants were subjected to a temperature drop (0 °C for 1 h → −4 °C for 3 h → 4 °C for 3 h) as described by Chinnusamy et al. [22] and then transferred to 22 °C. The survival of stress-treated plants was monitored after one week. The electrolyte leakage assay was conducted as described previously [14].

3. Results

3.1. AtATL80 is an Arabidopsis RING E3 Ub ligase that is localized to the plasma membrane

Publicly accessible in silico data (www.genevestigator.com) and *Arabidopsis* whole genome Affymetrix GeneChip (ATH1) analysis [15] showed that the *AtATL80* gene is induced by Pi starvation. The aim of this study was to explore the in vivo role of AtATL80.

The *AtATL80* gene encodes a 197-amino-acid protein ($M_r = 20.9$ kDa) (Fig. 1A). AtATL80 shares over 70% of its amino-acid sequence identity with other ATL RING E3 homologs from various plant species (Supplementary Fig. S1). Roles of these AtATL80 homologs are unknown. As with other ATL family members, AtATL80 contains a single C3H2C3-type RING motif, putative trans-membrane domain, basic amino-acid-rich region, end part of conserved GLD motif, and C-terminal diverged region (Fig. 1A and Supplementary Fig. S1).

When the *35S:AtATL80-sGFP* construct was transiently expressed in tobacco (*N. benthamiana*) leaf protoplasts, AtATL80-sGFP was barely detected, probably due to self-ubiquitination. In contrast, the AtATL80^{C130S}-sGFP protein, in which the conserved Cys residue in the RING motif was replaced by Ser, was predominantly detected in the PM. This localization pattern overlapped closely with the PM-localized PIP2. The ΔTM-AtATL80^{C130S}-sGFP protein, which lacks the trans-membrane domain, was observed in the cytosol (Fig. 1B).

The *GST-AtATL80* fusion construct was expressed in *Escherichia coli*. The full-length GST-AtATL80 was insoluble in bacterial cells, likely due to the presence of the hydrophobic N-terminal trans-membrane domain. The N-terminal transmembrane domain was deleted, after which the GST-ΔNAtATL80$^{60\text{-}197}$ mutant protein was successfully expressed as a soluble protein in bacterial cells. Fig. 1C revealed that the purified GST-ΔNAtATL80$^{60\text{-}197}$ protein displayed in vitro self-ubiquitination activity in the presence of Ub, ATP, E1, and E2, detected by both anti-Ub and anti-GST antibodies. In contrast, GST-ΔNAtATL80$^{60\text{-}197/C130S}$ showed a loss of self-ubiquitination activity. Taken together, these results suggest that AtATL80 is a PM-localized E3 Ub ligase in *Arabidopsis*.

3.2. The ATL80 gene is up-regulated during Pi starvation

RT-PCR analysis showed that *AtATL80* was expressed in all tissues examined, with its expression level being highest in mature leaves and stems (Supplementary Fig. S2).

Arabidopsis seeds were sown on high Pi (1 mM KH_2PO_4), sufficient Pi (500 μM KH_2PO_4), low Pi (20 μM KH_2PO_4), or Pi-deprived (0 μM KH_2PO_4) substrate and grown for 5 and 10 days. *AtATL80*

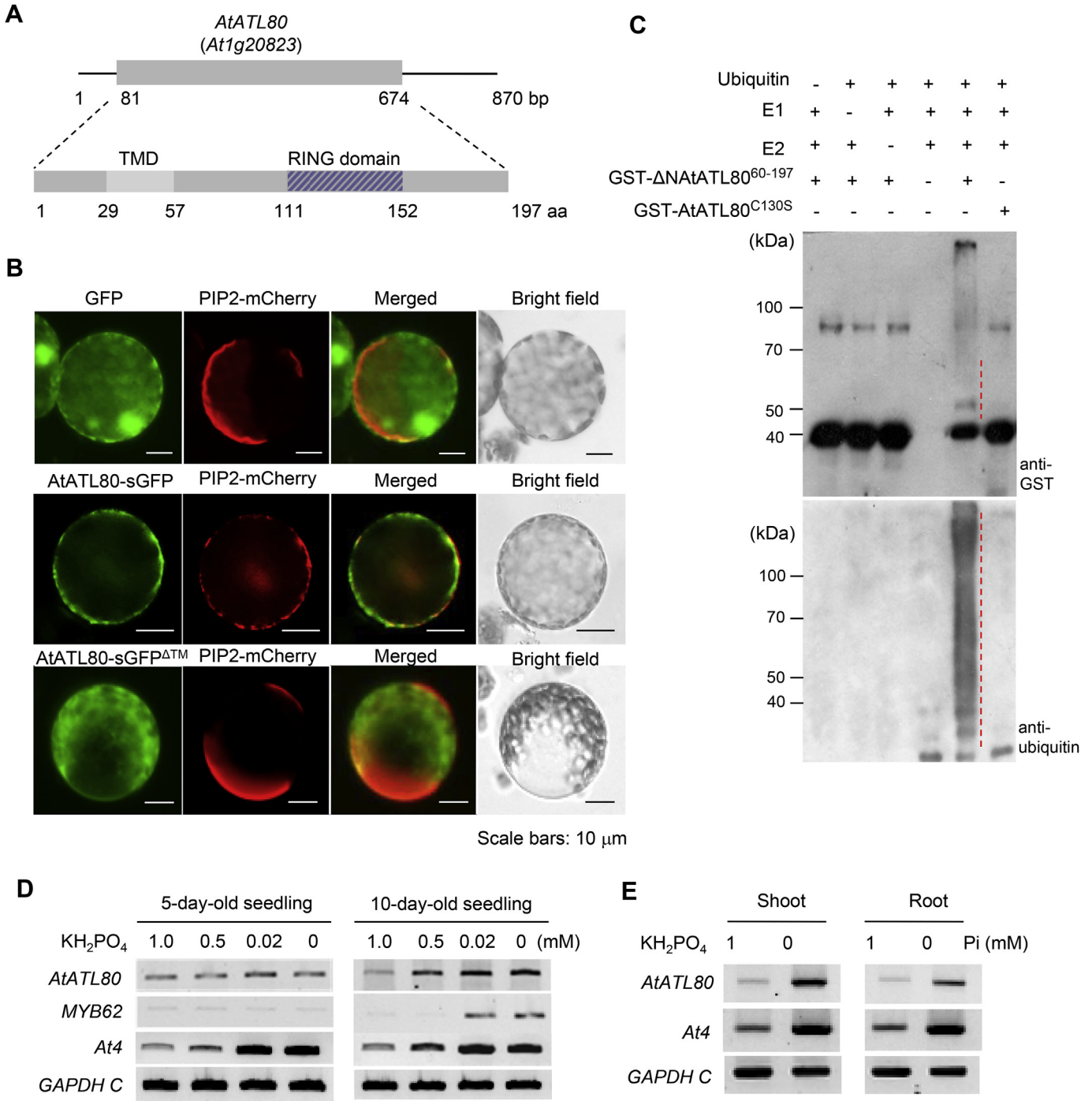

Fig. 1. Sequence, sub-cellular localization, in vitro Ub ligase analyses, and expression profiles of *Arabidopsis* RING E3 Ub ligase AtATL80. (A) Schematic representation of the *AtATL80* gene (Genbank accession No. NM_101935.2) and its predicted protein. There is no intron in the *AtATL80* gene. (B) Sub-cellular localization of the ATL80^{C130S}-GFP protein. GFP and PIP2-mCherry were used as specificity controls for cytosolic and PM-localized proteins, respectively. Scale bars = 10 μm. (C) In vitro self-ubiquitination assay of GST-ΔNAtATL80$^{60-197}$ and GST-ΔNAtATL80$^{60-197/C130S}$ fusion proteins (500 ng). Reaction products were analyzed by immuno-blotting with anti-GST antibody (upper panel) and anti-Ub antibody (lower panels). High-molecular-mass self-ubiquitinated ladders are indicated by vertical dashed lines. (D) Induction patterns of *AtATL80* under different Pi conditions. *GAPDH C* (glyceraldehyde-3-phosphate dehydrogenase C subunit) was used as a loading control. *At4* and *MYB62* were used as representative Pi starvation-response marker genes. (E) Induction of *AtATL80* in response to Pi-deprived (0 μM) conditions in shoot and root tissues of *Arabidopsis* seedlings.

was up-regulated by 500 μM Pi treatment, and attained a maximal induction level via 10 days of 0–20 μM Pi treatment, indicating that *AtATL80* was induced in response to long-term Pi starvation in *Arabidopsis* seedlings (Fig. 1D). The *At4* and *MYB62* genes were used as representative Pi starvation-response (PSR) marker genes [9,16]. The *At4* marker gene was strongly induced by a limited Pi supply (0–20 μM) for 5 days, while the induction of *MYB62* was detected after 10 days of 20 μM Pi treatment. The induction of *AtATL80* in response to low Pi condition was more evident in shoots than roots (Fig. 1E).

3.3. Dry weight, total P content, and P utilization efficiency were altered in atatl80 mutant and 35S:AtATL80-sGFP transgenic plants in the presence of high Pi

Expression of *AtATL80* is modulated by different concentrations of Pi (Fig. 1D and E). Therefore, it was hypothesized that AtATL80 plays a role in Pi metabolism. Wild-type, *atatl80* T-DNA knock-out mutant (*atatl80*) (Supplementary Fig. S3A and B), and *AtATL80-sGFP*-overexpressing transgenic (*35S:AtATL80-sGFP*) (Supplementary Fig. S3C) *Arabidopsis* plants were grown in high Pi (1 mM) or low Pi (50 μM) conditions for additional two weeks (Fig. 2A). Under high Pi (1 mM) conditions, the dry weight of the three-week-old wild-type plants was 21.7 ± 0.8 mg/5 seedlings, while that of *atatl80* mutant was 26.9 ± 1.0 mg/5 seedlings (Fig. 2B). This suggests that loss of *AtATL80* resulted in a slight increase in dry weight under high Pi conditions. In contrast, the dry weight of *35S:AtATL80-sGFP* transgenic plants was markedly reduced to 10.5 ± 0.6–14.8 ± 0.5 mg/5 seedlings under the same conditions. Thus, AtATL80 appeared to have a negative impact on seedling growth under high Pi conditions.

Total P content per mg of dry weight was also affected under high Pi conditions. Fig. 2C revealed that the P content of the wild-type shoot tissues was 366 ± 6.0 μmol/mg DW with 1 mM Pi supply. The mutation of *AtATL80* reduced the P content to 316 ± 14.0 μmol/mg DW, while the overexpression of *AtATL80* increased P content to 500 ± 20.0–538 ± 14.0 μmol/mg DW. These results indicate that loss and overexpression of *AtATL80* resulted in the decrease and increase, respectively, of P content in shoot tissues under high Pi conditions.

P utilization efficiency (PUE) is defined as the amount of biomass produced per unit of total P accumulated in the shoots [21]. When compared with the PUE ($2.9 \pm 0.1 \times 10^{-2}$ g DW/mg P) of the wild-type plants, that of the *atatl80* mutant increased by approximately 17% ($3.4 \pm 0.2 \times 10^{-2}$ g DW/mg P), whereas the PUE of *35S:AtATL80-sGFP* transgenic plants decreased by approximately 35% ($1.9 \pm 0.1 \times 10^{-2}$ g DW/mg P) to 28% ($2.1 \pm 0.1 \times 10^{-2}$ g DW/mg P) (Fig. 2D). Taken together, our phenotypic analyses showed that the overexpression of *AtATL80* caused P to accumulate in shoot tissues, and thereby decrease seedling growth and PUE. This raised the possibility that AtATL80 may be negatively involved in Pi distribution in shoot tissues under high Pi conditions.

However, the phenotypes of wild-type, *atatl80* mutant, and *AtATL80*-overexpressors were similar under low Pi (50 μM) conditions (Supplementary Fig. S4A–D). In addition, the differences in root phenotypes were undetected under both high Pi (1 mM) and low Pi (50 μM) conditions (Fig. 2A and Supplementary Fig. S4A).

3.4. Overexpression of AtATL80 resulted in the delayed flowering time and decrease in seed yield in sufficient Pi conditions

Under the sufficient Pi growth condition (250 μM KH_2PO_4 in soil), wild-type *Arabidopsis* bolted approximately 28 days after germination, while the *atatl80* mutant bolted 25–26 days after germination (Fig. 3A). *AtATL80*-overexpressors displayed a late flowering phenotype and bolted 31–33 days after germination. At 32 days after germination, the inflorescence lengths of wild-type, *atatl80* mutant, and *AtATL80* overexpressors were 8.0 ± 0.7 cm, 13.0 ± 0.5 cm, and 0.3 ± 0.1 (line #1) – 1.8 ± 0.3 (line #2) cm, respectively (Fig. 3B). These results indicated that the loss of *AtATL80* resulted in earlier flowering. In contrast, the overexpression of *AtATL80* delayed flowering significantly under Pi sufficient conditions.

Additionally, the effects of the mutation and overexpression of *AtATL80* on seed yield were monitored. As shown in Fig. 3C, seed yield was not statistically different between wild type (54.8 ± 1.5 mg/plant) and *atatl80* mutant (56.8 ± 1.1 mg/plant) plants. However, the seed yield of *35S:AtATL80-sGFP* transgenic plants was approximately 54–79% (11.5 ± 1.1 – 25.1 ± 1.2 mg/plant) lower than that of wild type plants (Fig. 3C), indicating that constitutive expression of *AtATL80* resulted in the reduction of seed yield under Pi sufficient conditions.

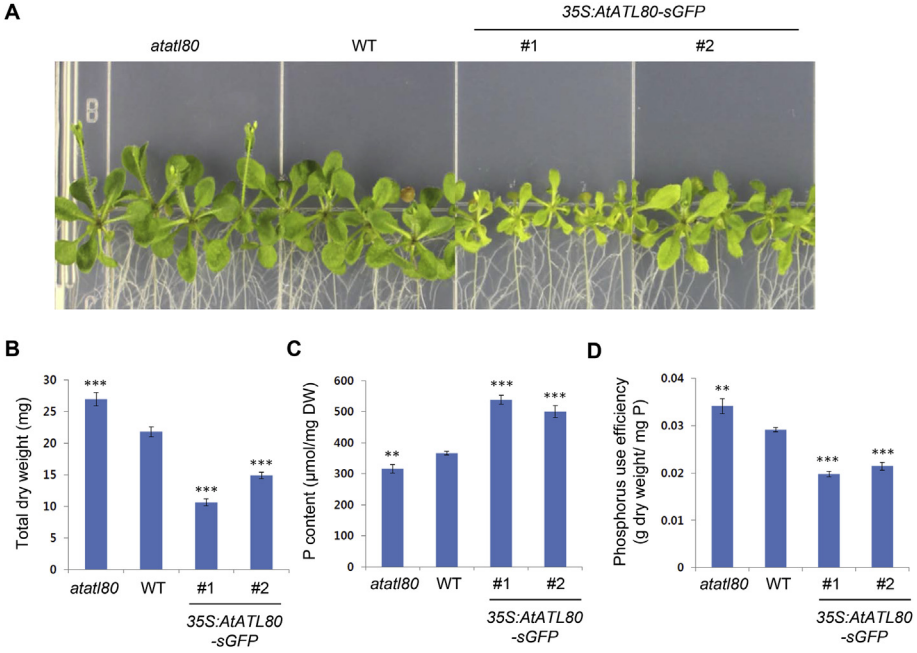

Fig. 2. Dry weight, total P content in the shoots, and PUE of wild-type (WT), *atatl80* mutant, and *35S:AtATL80-sGFP* transgenic (lines #1 and #2) plants grown with high Pi (1 mM KH_2PO_4). (A) Overall morphology of three-week-old *Arabidopsis* plants. (B–D) Dry weight (B), total P content in shoot tissues (C), and PUE (g dry weight mg^{-1} P) (D) of *Arabidopsis* plants (n = 12 replicates of 5 seedlings each). Error bars indicate ±SE. Double (**) and triple asterisks (***) indicate the statistical significance as determined by Student's *t* test at $P < 0.01$ and $P < 0.001$, respectively.

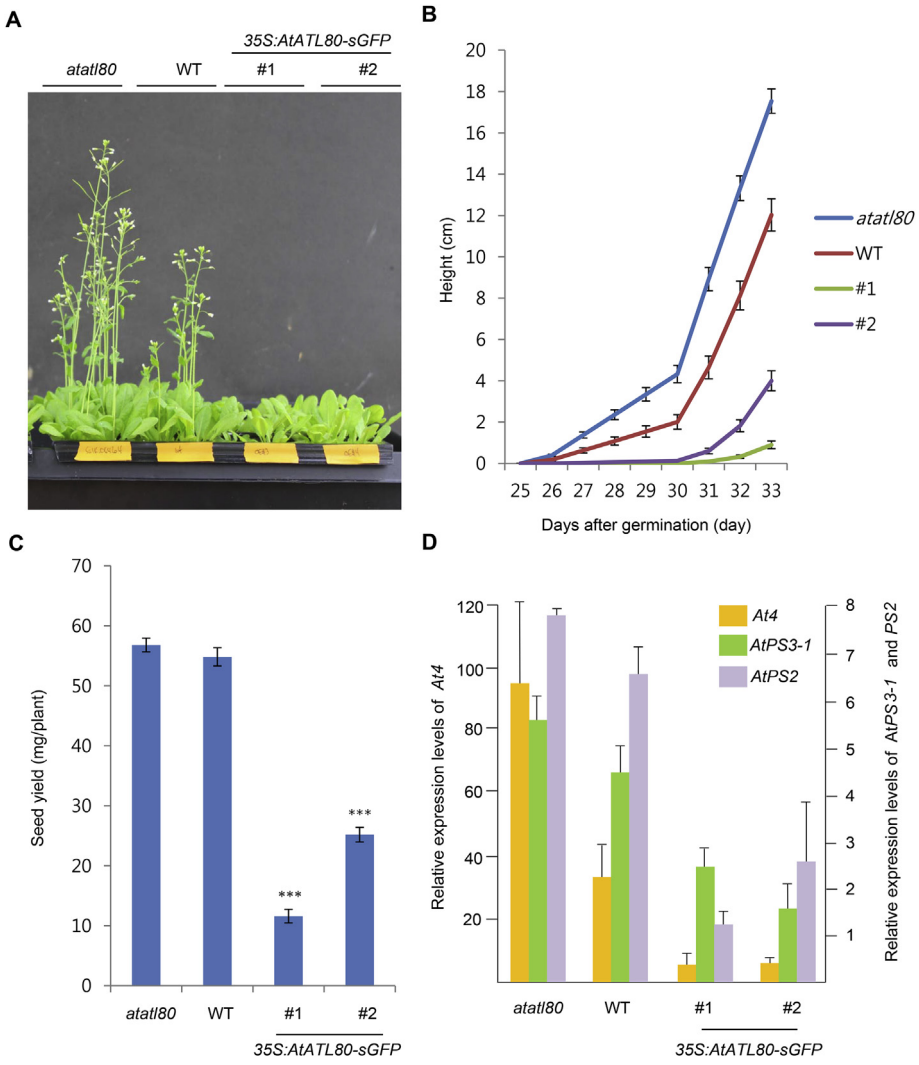

Fig. 3. Phenotypic analysis of mature wild-type (WT), *atatl80* mutant, and *35S:AtATL80-sGFP* transgenic plants fertilized with 250 μM KH_2PO_4. (A) Growth morphology of *Arabidopsis* plants 30 days after germination. (B) Growth patterns of inflorescence stems of *Arabidopsis* plants 25–33 days after germination. Error bars represent ±SE (n = 60). (C) Seed yields (mg seeds per plant). Error bars represent ±SE (n = 60). Triple asterisks (***) indicate significant differences at $P < 0.001$ (*t*-test). (D) Negative regulation of *At4*, *AtPS3-1*, and *PS2* by AtATL80. Relative expression levels of *At4*, *AtPS3-1*, and *PS2* were determined by real-time qRT-PCR. *At4g26410* was used as an internal control.

We next examined the expression of Pi starvation-response (PSR) marker genes. Real-time qRT-PCR results showed that the transcript levels of *At4*, a member of the *At4/IPS1* gene family [23], were higher in the *atatl80* mutant and lower in *35S:AtATL80-sGFP* transgenic plants, relative to the wild-type (Fig. 3D). The transcript levels of *AtPS3-1*, a member of the glycerol-3-phosphate permease family [16], and *AtPS2*, a member of a pyrophosphatase family [24], were also higher in the *atatl80* mutant and lower in *35S:AtATL80-sGFP* lines (Fig. 3D). These results indicate that the PSR genes are negatively regulated by AtATL80 in *Arabidopsis*.

Overall, the mutation of *AtATL80* produced an earlier-flowering phenotype, whereas overexpression of *AtATL80* resulted in a delayed flowering time and decrease in seed yield under high Pi conditions. Thus, it appears that AtATL80 acts as a negative factor in flowering and seed yield in *Arabidopsis*. In addition, these phenotypic properties were likely associated with the altered Pi concentrations evidenced by the changes in the PSR gene activities.

3.5. *AtATL80 is negatively involved in cold stress tolerance response*

Decreasing Pi concentrations were reported to increase sucrose synthesis and photosynthetic activity, and thus trigger acclimatization in response to cold stress [25]. The aforementioned results indicate that the loss and overexpression of *AtATL80* resulted in the decrease and increase, respectively, of P content in shoot tissues (Fig. 2). *Arabidopsis* plants grown with 250 μM KH_2PO_4 were subjected to cold stress (0 °C for 1 h → −4 °C for 3 h → 4 °C for 3 h) (Fig. 4A). The survival rates of cold stressed plants were monitored one week after transfer from low-temperature to normal (22 °C) condition. The survival rate of wild-type plants was 57.7 ± 3.2%, while 75.5 ± 2.0% of *atatl80* mutants survived (Fig. 4B). This indicates that *atatl80* mutants are more tolerant to cold stress than wild type plants. In contrast, *AtATL80*-overexpressors showed markedly reduced survival rates [24.7 ± 2.3% for line #1 and 23.1 ± 1.9 for line #2] compared to wild-type plants. Electrolyte leakage was measured from leaves of cold-treated three-week-old plants. Consistent with the results from whole plants, leaves from

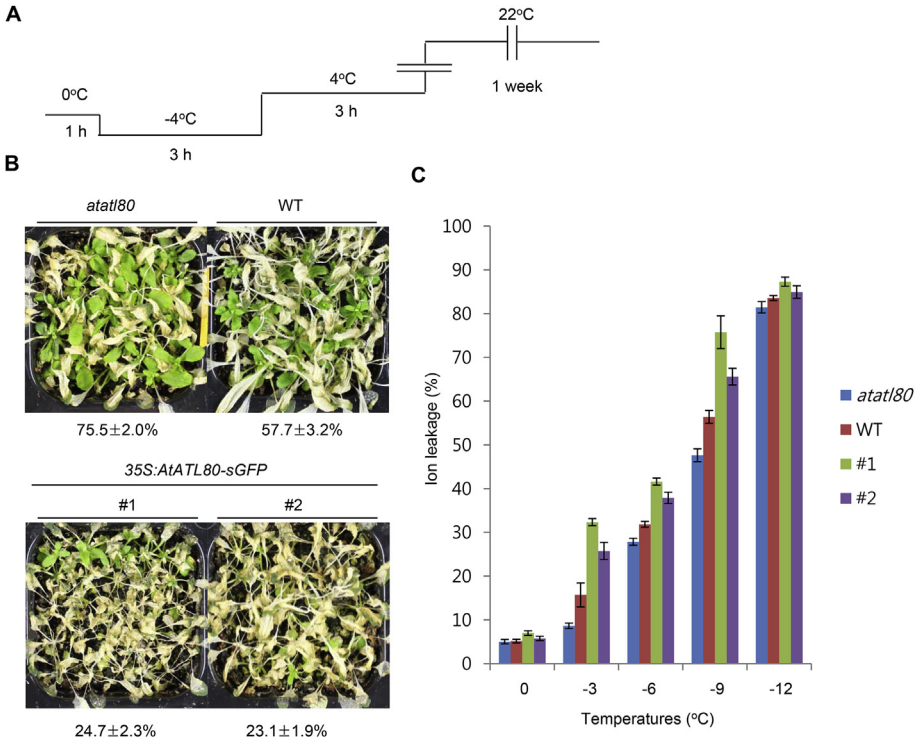

Fig. 4. Overexpression of *AtATL80* resulted in decreased tolerance to cold stress. (A) Schematic representation of the low-temperature treatment (0 °C for 1 h → −4 °C for 3 h → 4 °C for 3 h) under sufficient Pi conditions (250 μM Pi). (B) Survival rates of wild-type (WT), *atatl80* mutant, and *35S:AtATL80-sGFP* transgenic plants after cold stress. Results are expressed as means ± SE from four independent experiments. More than 25 plants were used in each experiment. (C). Electrolyte leakage analysis. The degree of ion leakage of cold stress-treated (−3, −6, −9, and −12 °C) leaves was monitored. Results are expressed as means ± SE from three independent experiments.

transgenic lines #1 and #2 had higher rates of electrolyte leakage than wild-type leaves in response to low temperature (Fig. 4C). In contrast, *atatl80* mutant leaves displayed lower electrolyte leakage relative to wild-type leaves. Collectively, these results suggest that AtATL80 is negatively involved in the cold stress tolerance response.

4. Discussion

AtATL80 is a PM-localized RING E3 Ub ligase (Fig. 1A–C). The *AtATL80* gene was upregulated under low Pi conditions (Fig. 1D–E). These *AtATL80* expression profiles are reminiscent of those of Pi remobilization transporter genes, such as *PHT1;5*, *PHT2;1*, and *GmPT1* [9,21,26]. Overexpression of *AtATL80* caused P accumulation in shoot tissues, lower biomass, and reduced PUE under high Pi (1 mM) conditions compared to wild-type plants (Fig. 2). The loss-of-function mutation of *AtATL80* produced opposite phenotypes. Thus, these phenotypic properties adversely resembled those of Pi-remobilizing *PHT1;5* transgenic lines. *PHT1;5*-overexpressors possessed a larger biomass and decline in total P content in shoots when grown in high Pi media relative to wild-type plants, whereas the *pht1;5* mutant exhibited the opposite phenotypes [9]. The PSR genes, including *At4*, *AtPS3-1*, and *AtPS2*, were upregulated in *atatl80* mutant and down-regulated in *AtATL80*-overexpressing plants (Fig. 3D). These results are in agreement with the results indicating that *atatl80* mutant and *AtATL80*-overexpresors contained lower and higher Pi content, respectively, in the shoots (Fig. 2C).

The *atatl80* mutant line bolts earlier than wild-type plants, whereas *AtATL80*-overexpressors possess late flowering phenotypes (Fig. 3A and B). In addition, the degree of *AtATL80* gene activity is negatively linked to seed yield (Fig. 3C). Thus, AtATL80 is negatively correlated not only with P content, but also with flowering time and seed yield in *Arabidopsis*. These results are consistent with the previous study that showed that *PHT1;5*-overexpressing plants exhibited premature senescence along with an increase in Pi remobilization [9]. In light of these results, we speculated that AtATL80 may be involved in Pi mobilization within shoot tissues as a negative regulator.

In addition, the overexpression of *AtATL80* resulted in markedly reduced tolerance to cold stress (Fig. 4). A relationship between Pi signaling and cold stress response was proposed; cellular Pi concentrations influence cold acclimatization by affecting photosynthetic carbon metabolism [25]. Lower Pi concentrations increased sucrose phosphate synthase protein amount and sustained photosynthetic gene activities, which, in turn, positively affected acclimatization to cold stress [25]. Overexpression of OsSPX1, which is involved in Pi homeostasis in rice shoots, resulted in the decreased leaf Pi content and improved cold tolerance in tobacco and *Arabidopsis* plants [27]. Because P content in *AtATL80*-overexpressors was significantly higher than in wild-type plants (Fig. 2C), the cold stress-sensitive phenotype of *35S:AtATL80* plants may be associated with decreased sucrose synthesis and photosynthetic activity. Thus, our results suggest a possible functional link between Pi mobilization and the cold stress tolerance response.

Overall, our data suggest that AtATL80, an *Arabidopsis* PM-localized RING E3 Ub-ligase, participates in Pi mobilization and cold stress response as a negative factor. The cellular mechanism by which AtATL80 is involved in the Pi mobilization and cold response is currently unknown. The root-specific Pi transporter PHT1s (PHT1;1, PHT1;2, PHT1;3, and PHT1;4) are ubiquitinated by RING E3 Ub ligase NLA, which indicates that PM-localized Pi transporters are regulated by the ubiquitin system [28]. These results raised the possibility that AtATL80 may be involved in the ubiquitination of

PM-localized proteins, including Pi transporters. Thus, additional experiments are needed to identify the target protein(s) of AtATL80, and to decipher the links between AtATL80 and Pi mobilization.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.015.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.06.015.

References

[1] H. Rouached, A.B. Arpat, Y. Poirier, Regulation of phosphate starvation responses in plants: signaling players and cross-talks, Mol. Plant 3 (2010) 288–299.
[2] T.J. Chiou, S.I. Lin, Signaling network in sensing phosphate availability in plants, Annu. Rev. Plant Biol. 62 (2011) 185–206.
[3] E. Himmelblau, R.M. Amasino, Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence, J. Plant Physiol. 158 (2001) 1317–1323.
[4] U.S. Muchhal, J.M. Pardo, K.G. Raghothama, Phosphate transporters from the higher plant *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 10519–10523.
[5] A.S. Karthikeyan, D.K. Varadarajan, U.T. Mukatira, M.P. D'Urzo, B. Damsz, K.G.H. Raghothama, Regulated expression of Arabidopsis phosphate transporters, Plant Physiol. 130 (2002) 221–233.
[6] S.R. Mudge, A.L. Rae, E. Diatloff, F.W. Smith, Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*, Plant J. 31 (2002) 341–353.
[7] H. Shin, H.S. Shin, G.R. Dewbre, M.J. Harrison, Phosphate transport in *Arabidopsis*: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments, Plant J. 39 (2004) 629–642.
[8] E. Remy, T.R. Cabrito, R.A. Batista, M.C. Teixeira, I. Sá-Correia, P. Duque, The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation, New. Phytol. 195 (2012) 356–371.
[9] V.K. Nagarajan, A. Jain, M.D. Poling, A.J. Lewis, K.G. Raghothama, A.P. Smith, Arabidopsis Pht1;5 mobilizes phosphate between source and sink organs and influences the interaction between phosphate homeostasis and ethylene signaling, Plant Physiol. 156 (2011) 1149–1163.
[10] L. Aguilar-Henonin, J. Bravo, P. Guzmán, Genetic interactions of a putative *Arabidopsis thaliana* ubiquitin-ligase with components of the *Saccharomyces cerevisiae* ubiquitination machinery, Curr. Genet. 50 (2006) 257–268.
[11] E. Kraft, S.L. Stone, L. Ma, N. Su, Y. Gao, O.S. Lau, X.W. Deng, J. Callis, Genome analysis and functional characterization of the E2 and RING type E3 ligase ubiquitination enzymes of *Arabidopsis*, Plant Physiol. 139 (2005) 1597–1611.
[12] V. Aguilar-Hernández, L. Aguilar-Henonin, P. Guzmán, Diversity in the architecture of ATLs, a family of plant ubiquitin-ligases, leads to recognition and targeting of substrates in different cellular environments, PLoS One 6 (2011) e23934.
[13] T. Sato, S. Maekawa, S. Yasuda, Y. Domeki, K. Sueyoshi, M. Fujiwara, Y. Fukao, D.B. Goto, J. Yamaguchi, Identification of 14-3-3 proteins as a target of ATL31 ubiquitin ligase, a regulator of the C/N response in Arabidopsis, Plant J. 68 (2011) 137–146.
[14] S.J. Kim, W.T. Kim, Suppression of Arabidopsis RING E3 ubiquitin ligase AtATL78 increases tolerance to cold stress and decreases tolerance to drought stress, FEBS Lett. 587 (2013) 2584–2590.
[15] J. Misson, K.G. Raghothama, A. Jain, J. Jouhet, M.A. Block, et al., A genome-wide transcriptional analysis using *Arabidopsis thaliana* Affymetrix gene chips determined plant responses to phosphate deprivation, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 11934–11939.
[16] B.N. Devaiah, R. Madhuvanthi, A.S. Karthikeyan, K.G. Raghothama, Phosphate starvation responses and gibberellic acid biosynthesis are regulated by the MYB62 transcription factor in *Arabidopsis*, Mol. Plant 2 (2009) 43–58.
[17] M.Y. Ryu, S.K. Cho, W.T. Kim, The Arabidopsis C3H2C3-type RING E3 ubiquitin ligase AtAIRP1 is a positive regulator of an ABA-dependent response to drought stress, Plant Physiol. 154 (2010) 1983–1997.
[18] S.K. Cho, M.Y. Ryu, C. Song, J.M. Kwak, W.T. Kim, Arabidopsis PUB22 and PUB23 are homologous U-Box E3 ubiquitin ligases that play combinatory roles in response to drought stress, Plant Cell. 20 (2008) 1899–1914.
[19] J.H. Kim, W.T. Kim, The Arabidopsis RING E3 ubiquitin ligase AtAIRP3/LOG2 participates in positive regulation of high-salt and drought stress responses, Plant Physiol. 162 (2013) 1733–1749.
[20] S.J. Kim, M.Y. Ryu, W.T. Kim, Suppression of Arabidopsis RING-DUF1117 E3 ubiquitin ligases, AtRDUF1 and AtRDUF2, reduces tolerance to ABA-mediated drought stress, Biochem. Biophys. Res. Commun. 420 (2012) 141–147.
[21] H. Song, Z. Yin, M. Chao, L. Ning, D. Zhang, D. Yu, Functional properties and expression quantitative trait loci for phosphate transporter GmPT1 in soybean, Plant Cell. Environ. 37 (2014) 462–472.
[22] V. Chinnusamy, M. Ohta, S. Kanrar, B.H. Lee, X. Hong, M. Agarwal, J.K. Zhu, ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*, Genes. Dev. 17 (2003) 1043–1054.
[23] H. Shin, H.S. Shin, R. Chen, M.J. Harrison, Loss of At4 function impacts phosphate distribution between the roots and the shoots during phosphate starvation, Plant J. 45 (2006) 712–726.
[24] N.N. Chandrika, K. Sundaravelpandian, S.M. Yu, W. Schmidt, ALFIN-LIKE 6 is involved in root hair elongation during phosphate deficiency in Arabidopsis, New. Phytol. 198 (2013) 709–720.
[25] V. Hurry, A. Strand, R. Furbank, M. Stitt, The role of inorganic phosphate in the development of freezing tolerance and the acclimatization of photosynthesis to low temperature is revealed by the *pho* mutants of *Arabidopsis thaliana*, Plant J. 24 (2000) 383–396.
[26] W.K. Versaw, M.J. Harrison, A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses, Plant Cell. 14 (2002) 1751–1766.
[27] L. Zhao, F. Liu, W. Xu, C. Di, S. Zhou, Y. Xue, J. Yu, Z. Su, Increased expression of OsSPX1 enhances cold/subfreezing tolerance in tobacco and *Arabidopsis thaliana*, Plant Biotechnol. J. 7 (2009) 550–561.
[28] T.K. Huang, C.L. Han, S.I. Lin, Y.J. Chen, Y.C. Tsai, et al., Identification of downstream components of ubiquitin-conjugating enzyme PHOSPHATE2 by quantitative membrane proteomics in Arabidopsis roots, Plant Cell. 25 (2013) 4044–4060.