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Auxin Influx Carrier AUX1 Confers Acid Resistance for Arabidopsis Root Elongation Through the Regulation of Plasma Membrane H^+-ATPase

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The plant plasma membrane (PM) H^+-ATPase regulates pH homeostasis and cell elongation in roots through the formation of an electrochemical H^+ gradient across the PM and a decrease in apoplastic pH; however, the detailed signaling for the regulation of PM H^+-ATPases remains unclear. Here, we show that an auxin influx carrier, AUXIN RESISTANT1 (AUX1), is required for the maintenance of PM H^+-ATPase activity and proper root elongation. We isolated a *low pH-hypersensitive 1* (*loph1*) mutant by a genetic screen of *Arabidopsis thaliana* on low pH agar plates. The *loph1* mutant is a loss-of-function mutant of the *AUX1* gene and exhibits a root growth retardation restricted to the low pH condition. The ATP hydrolysis and H^+ extrusion activities of the PM H^+-ATPase were reduced in *loph1* roots. Furthermore, the phosphorylation of the penultimate threonine of the PM H^+-ATPase was reduced in *loph1* roots under both normal and low pH conditions without reduction of the amount of PM H^+-ATPase. Expression of the *DR5:GUS* reporter gene and auxin-responsive genes suggested that endogenous auxin levels were lower in *loph1* roots than in the wild type. The *aux1-7* mutant roots also exhibited root growth retardation in the low pH condition like the *loph1* roots. These results indicate that AUX1 positively regulates the PM H^+-ATPase activity through maintenance of the auxin accumulation in root tips, and this process may serve to maintain root elongation especially under low pH conditions.

Keywords: *Arabidopsis thaliana* • Auxin • AUX1 • Phosphorylation • PM H^+-ATPase • Root.

Abbreviations: AHA, Arabidopsis plasma membrane H^+-ATPase; AUX1, AUXIN RESISTANT1; EMS, ethylmethane sulfonate; GUS, β-glucuronidase; PP2C-D, D-clade type 2C protein phosphatase; PM, plasma membrane; qRT-PCR, quantitative real-time PCR; RT–PCR, reverse transcription–PCR; *SAUR*, *SMALL AUXIN UP-RNA*; SSLP, simple sequence length polymorphism; WT, wild type.

Introduction

The plant plasma membrane (PM) H^+-ATPase actively transports protons (H^+) out of cells across the PM and the resulting electrochemical gradient provides a driving force for the uptake of numerous nutrients including K^+, NO^{3-}, SO_4^{2-}, PO_4^{3-}, amino acids, peptides and sucrose through corresponding secondary transporters (Sze et al. 1999, Palmgren 2001, Sondergaard et al. 2004). The uptake of osmotic solutes by PM H^+-ATPases in concert with coupled secondary transporters enhances water uptake as a consequence of the resultant water potential gradient leading to cell turgor maintenance and plant cell expansion. Thus, the PM H^+-ATPase is essential for elongation growth in hypocotyls (Haruta and Sussman 2012, Takahashi et al. 2012, Spartz et al. 2014) and roots (Haruta et al. 2014). In addition, plant cells maintain pH homeostasis under various pH conditions and the PM H^+-ATPase is involved in the regulation of pH homeostasis (Young et al. 1998, Palmgren 2001).

The PM H^+-ATPase, which is a member of the P-type ATPase super family, consists of a single polypeptide with 10 transmembrane domains and a C-terminal cytoplasmic domain which serves as an autoinhibitory domain (Palmgren 2001). The catalytic activity of the PM H^+-ATPase is regulated mainly by phosphorylation of the penultimate threonine residue (Thr947 in Arabidopsis H^+-ATPase2, AHA2) in the C-terminus and subsequent interaction of the 14-3-3 protein with the phosphorylated C-terminus (Sondergaard et al. 2004, Duby and Boutry 2009). The 14-3-3 protein interaction permits release of the catalytic domain from the autoinhibitory domain, leading to activation of the PM H^+-ATPase (Palmgren 2001). Many physiological signals including blue light in guard cells (Kinoshita and Shimazaki 1999, Ueno et al. 2005), white light in photosynthetic cells (Okumura et al. 2012a, Okumura et al. 2012b, Okumura et al. 2016), NaCl stress (Kerkeb et al. 2002), acid stress (Bobik et al. 2010) and the fungal toxin fusicoccin induce phosphorylation of the penultimate threonine of PM H^+-ATPase (Fuglsang et al. 1999, Svennelid et al. 1999). In addition to the external environmental stimuli, endogenous signals, such as sucrose as a photosynthetic product and the phytohormones auxin and gibberellin, also phosphorylate the threonine of the PM H^+-ATPase (Niittylä et al. 2007, Chen et al. 2010, Takahashi et al. 2012, Spartz et al. 2014). On the other hand, another phytohormone, ABA, inhibits the phosphorylation (Zhang et al. 2004, Hayashi et al. 2011, Hayashi et al. 2014).

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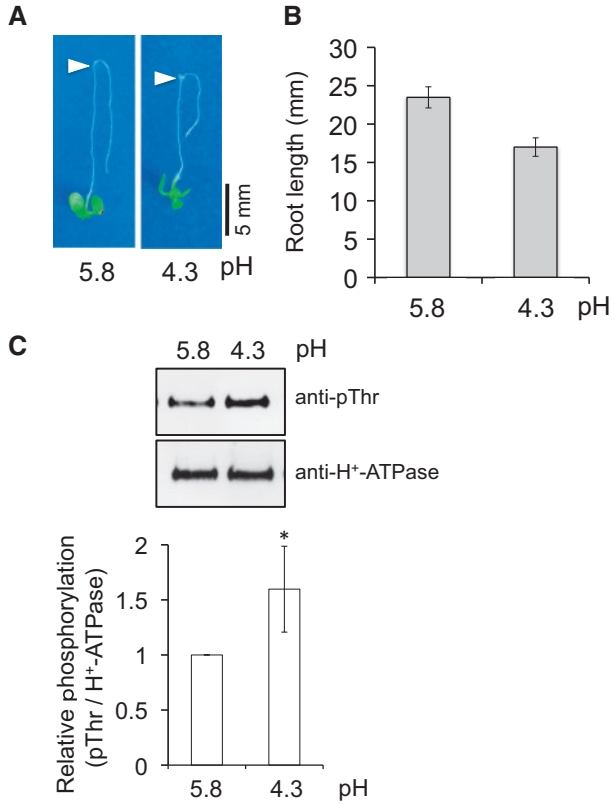

Fig. 1 Effects of low pH on root growth and phosphorylation of plasma membrane (PM) H^+-ATPase in Arabidopsis roots. (A and B) Wild-type (WT) Arabidopsis (Col-0) plants were grown on normal pH (pH 5.8) medium for 4 d and then transferred to normal (pH 5.8) or low (pH 4.3) pH media. The plants were grown in an inverted position for 3 d. (A) Photographs of the root-bending assay. Arrowheads indicate the positions of the root tips just after transfer. (B) Effect of acidic pH on root growth. Primary root lengths were measured using 7-day-old plants grown on normal and low pH media. Values represent the means of three independent experiments with SDs. Each measurement was performed using 25 roots in each treatment. (C) Phosphorylation status of the penultimate threonine (Thr) residue of the PM H^+-ATPase in roots grown in low pH medium. Three days after transfer, root tips were harvested and proteins were extracted. Immunoblots of phosphorylated PM H^+-ATPase and total PM H^+-ATPase were performed. The phosphorylation level of the PM H^+-ATPase in roots grown at pH 4.3 is presented as values relative to the phosphorylation level in roots grown at pH 5.8. Bars represent means with SDs ($n = 4$). An asterisk indicates a significant difference from pH 5.8 (Student's *t*-test; $^*P < 0.05$).

However, the underlying mechanism by which many stimuli as above phosphorylate and activate the PM H^+-ATPase is largely unknown.

Among the stimuli, acid stress induces phosphorylation of the penultimate threonine of the PM H^+-ATPase in *Nicotiana* suspension cells (Bobik et al. 2010), and constitutive activation of the PM H^+-ATPase by a molecular genetic approach facilitates acid resistance and growth in Arabidopsis seedlings under the low pH medium (Young et al. 1998). The results suggest that the PM H^+-ATPase is activated by acid stress and the enhanced activity may contribute to pH homeostasis and maintenance of growth under the stress condition. Thus, we considered that acid stress is a good stimulus to use to study regulation of the PM H^+-ATPase in Arabidopsis roots.

In this study, we isolated a *low pH-hypersensitive 1* (*loph1*) mutant from Arabidopsis using a root-bending assay. Root elongation in the *loph1* mutant was more severely suppressed than that in the wild type (WT) under low pH conditions. We show that *loph1* is a mutant allele of the auxin influx carrier *aux1* and that phosphorylation and activity of the PM H^+-ATPase in *aux1* roots were lower than those in WT roots. Our results provide new insight into the importance of auxin in root acid resistance through the maintenance of the PM H^+-ATPase activity in Arabidopsis.

Results

Involvement of PM H^+-ATPase in root elongation and increased PM H^+-ATPase phosphorylation under low pH conditions

The PM H^+-ATPase is involved in elongation growth and the regulation of pH homeostasis in Arabidopsis (Young et al. 1998, Palmgren 2001); therefore, we confirmed the effect of pH on elongation growth and PM H^+-ATPase activity in roots. We examined root growth using a root-bending assay (Wu and Zhu 1996) and determined the levels of PM H^+-ATPase activity and phosphorylation of the penultimate threonine of the PM H^+-ATPase in roots under normal and low pH conditions. As shown in **Fig. 1A, B** and **Supplementary Fig. S6**, root elongation was suppressed by 20% under low pH conditions (pH 4.3) compared with normal pH conditions (pH 5.8). Interestingly, the ATP hydrolysis activity of the PM H^+-ATPase (**Fig. 4B**) and the phosphorylation level of the penultimate threonine increased in roots under low pH conditions (**Fig. 1C**). These results suggested that the PM H^+-ATPase in roots is activated in response to low pH conditions. In addition, in the presence of PM H^+-ATPase inhibitors such as erythrosine B and vanadate, we observed strong suppression of root growth (**Supplementary Fig. S1**), suggesting that the PM H^+-ATPase plays an essential role in root growth.

Isolation of a low pH-hypersensitive mutant using a root-bending assay

To explore the activation mechanism of PM H^+-ATPase in roots in response to low pH conditions, we screened ethylmethane sulfonate (EMS)-treated Arabidopsis plants using a root-bending assay (Wu and Zhu 1996) on low pH agar plates (pH 4.3) and isolated a *loph1* mutant. Root elongation of the *loph1* mutant was suppressed markedly on pH 4.3 agar plates (**Fig. 2A**). The pH dependency of root elongation in the *loph1* mutant is shown in **Fig. 2B** and **Supplementary Fig. S6**. Elongation of *loph1* roots was suppressed significantly between pH 4.0 and 4.5, but was similar to that of the WT at pH 5.5 and 6.0. The results indicate that the *loph1* mutant was hypersensitive to low pH conditions during root elongation. We note that the *loph1* mutant also exhibited an agravitropic root phenotype (**Fig. 2A**). In addition, the cell length in the mature region of roots was almost same in both the WT and

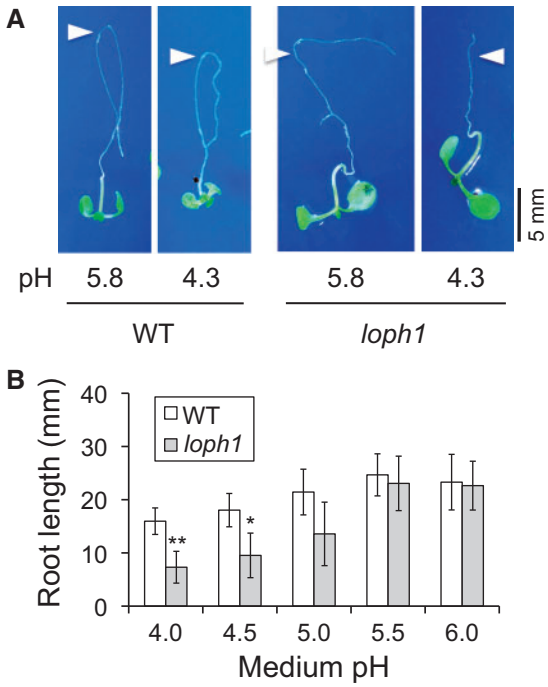

Fig. 2 Isolation of the *low pH-hypersensitive* (*loph*) *1* mutant. (A) Root-bending assay of WT and *loph1* roots. Arrowheads indicate the positions of the root tips just after transfer. (B) pH dependency of root elongation in the *loph1* mutant. Lengths of the primary roots were measured using 7-day-old plants grown under several pH conditions. Bars represent means with SDs ($n = 60$, pooled from triplicate experiments). Asterisks indicate significant differences between the WT and *loph1* (Student's *t*-test; $^{**}P < 0.01$ and $^{*}P < 0.05$).

loph1 under different pH conditions (**Supplementary Fig. S2**), suggesting that low pH (pH 4.3) may have an inhibitory effect on meristem activity in roots.

A mutation in *AUX1* is responsible for the *loph1* phenotype

Backcrosses of *loph1* with the WT showed that *loph1* was a recessive mutant. We performed map-based cloning of the *LOPH1* locus to identify the gene responsible for the *loph1* phenotype. The *LOPH1* locus showed strong linkage to the simple sequence length polymorphism (SSLP) marker nga168 on chromosome 2 (see the Materials and Methods). We then searched The Arabidopsis Information Resource (TAIR) database and found that an auxin influx carrier *AUXIN RESISTANT1* (*AUX1*, At2g38120) is located close to nga168. In addition, *aux1* mutants exhibit an agravitropic root phenotype (Bennett et al. 1996). We sequenced the *AUX1* gene from *loph1* genomic DNA and identified a single nucleotide substitution from G to C in the end of the second intron of the *AUX1* gene (**Fig. 3A**). This mutation was located at the splicing recognition site of the second intron and produced multiple mis-spliced products of *AUX1* mRNA (**Fig. 3B**, arrowheads). Transformation of the WT *AUX1* gene under its own promoter into the *loph1* mutant complemented both the low pH hypersensitivity during root growth and agravitropic root phenotypes (**Fig. 3C**). Furthermore, a known loss-of-function mutant of *AUX1*, *aux1-7* (Swarup et al. 2004), also exhibited low pH hypersensitivity during root growth and agravitropic root phenotypes (**Supplementary Figs. S3, S6**). These results demonstrated that the *loph1* phenotypes were due to a mutation in the *AUX1* gene.

AUX1 is required for the PM H$^+$-ATPase activity in roots

To explore the role of AUX1 in the regulation of PM H$^+$-ATPase activity, we examined ATP hydrolysis activity, H$^+$ extrusion activity and phosphorylation levels of the PM H$^+$-ATPase in WT and *loph1* roots under normal (pH 5.8) and low (pH 4.3) pH conditions (**Fig. 4**). Both the ATP hydrolysis activity (**Fig. 4B**) and the phosphorylation level of PM H$^+$-ATPase (**Fig. 4A**) increased in WT roots in response to low pH conditions. In the *loph1* mutant, the responsiveness of PM H$^+$-ATPase activity and phosphorylation to low pH remained; however, the levels were lower than those in the WT. H$^+$ extrusion activity and the phosphorylation level of PM H$^+$-ATPase in *loph1* roots were also lower than in the WT (**Fig. 4C**). However, these were probably not due to the expression levels of PM H$^+$-ATPases in roots because expression of the major H$^+$-ATPase isoforms, *AHA1* and *AHA2*, and PM H$^+$-ATPase protein concentrations did not differ significantly between *loph1* and the WT (**Supplementary Fig. S4**; **Fig. 4A, C**, lower panels). In addition, our results indicated that low pH had no effect on *AHA1* and *AHA2* expression levels in either WT or *loph1* roots. Taken together, these results suggested that AUX1 plays an important role in maintaining proper PM H$^+$-ATPase activity in roots.

The *aux1* mutant affects auxin homeostasis and the expression levels of auxin-inducible genes in roots

AUX1 has been shown to act as an auxin influx carrier (Yang et al. 2006); therefore, we expected auxin homeostasis in *loph1* roots to differ from that in WT roots. To test this hypothesis, we examined auxin homeostasis in roots using the auxin-responsive reporter gene construct *DR5:GUS* (Ulmasov et al. 1997) in which expression of the β-glucuronidase (GUS) gene was driven by the auxin-responsive promoter, *DR5*. In WT roots, GUS staining was observed in the root tips and stele. GUS staining in the stele of the *loph1* mutant was lower than that in the WT under normal pH conditions (pH 5.8); however, low pH conditions (pH 4.3) enhanced the GUS staining in the stele, suggesting that low pH conditions increased the auxin concentration in both WT and *loph1* roots. In the *loph1* mutant, the responsiveness of GUS staining to low pH remained, but the levels of GUS staining were lower than those in the WT (**Fig. 5A**). In addition, the expression levels of the known auxin-responsive genes *SMALL AUXIN UP-RNA 9* (*SAUR9*), *SAUR19* (Hagen and Guilfoyle 2002, Spartz et al. 2014) and *IAA1* (Abel et al. 1995) in the *loph1* roots were lower than those in the WT under the normal pH condition (**Fig. 5B**). These results suggested that AUX1 is required for auxin homeostasis in roots and that the endogenous auxin accumulation in the *loph1* mutant is lower than that in the WT.

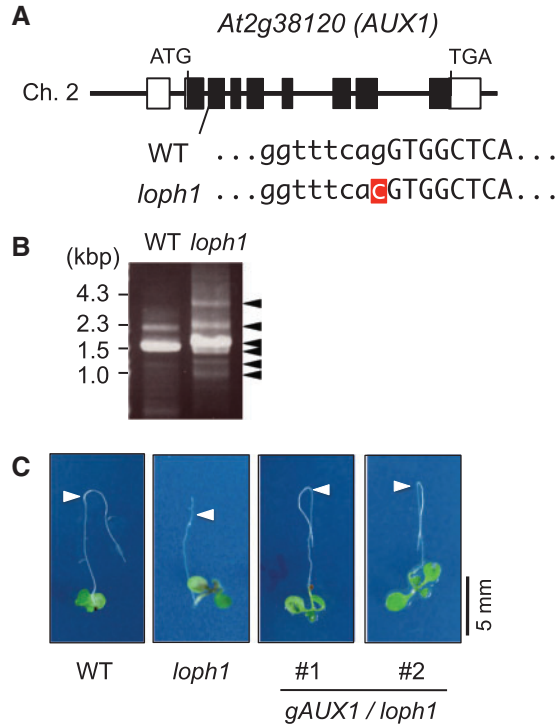

Fig. 3 Identification of the gene responsible for the *loph1* phenotype. (A) Genomic structure of the *AUX1* gene on chromosome 2. Boxes and bold lines indicate exons and introns, respectively. The coding region sequence is indicated by black boxes. The *loph1* mutant contains a G-to-C nucleotide substitution in the end of the second intron. (B) *AUX1* mRNA expression determined by RT—PCR analysis of 7-day-old WT and *loph1* roots. Arrowheads indicate *AUX1* cDNAs amplified from the *loph1* roots. (C) Functional complementation of the *loph1* mutant by the WT genomic *AUX1* gene. The root-bending assay was performed as in **Fig. 1A** using WT and *loph1* roots and the roots of *loph1* mutants transformed with a WT genomic *AUX1* gene (g*AUX1*/*loph1* #1 and #2).

Discussion

Isolation of low pH-hypersensitive mutant *loph1* revealed the importance of auxin in the acid resistance in Arabidopsis roots

To clarify the mechanism that regulates PM H^+-ATPase activity, we focused on an Arabidopsis root response to the acid stimulus in this study. Because the PM H^+-ATPase activity is required for root elongation (**Supplementary Fig. S1**) and low pH conditions activate the PM H^+-ATPase through phosphorylation of the penultimate threonine (Bobik et al. 2010; **Figs. 1C, 4A, B**), we screened the low pH-hypersensitive mutant *loph1* that showed root growth retardation only under low pH conditions (**Fig. 2**). The *LOPH1* gene was the *AUX1* gene (**Fig. 3**) which encodes an auxin influx carrier in the root apex (Bennet et al. 1996, Swarup et al. 2004). In addition, AUX1 plays a key role in the regulation of auxin homeostasis (Swarup and Péret 2012; **Fig. 5**). From the multiple phenotypic analyses with respects to the H^+-ATPase (**Figs. 2, 4**), we concluded that auxin accumulates in root tips in response to acid pH via AUX1, and the enhanced accumulation in turn activates the PM H^+-ATPase activity through phosphorylation of the penultimate threonine of the PM H^+-ATPase in root tips.

It should be noted that the auxin efflux carrier, PIN2, is required for the adaptation of Arabidopsis roots to alkaline stress by modulating proton secretion probably via PIN2-trasported auxin (Xu et al. 2012). Since auxin accumulation in root tips was also enhanced in response to alkaline conditions, Arabidopsis roots may use a similar system to overcome pH stress around the rhizosphere.

PM H^+-ATPase activity is required for root elongation under low pH conditions

We found that both ATP hydrolysis activity and the phosphorylation level of the penultimate threonine of the PM H^+-ATPase increased in response to low pH conditions in WT roots (**Figs 1C, 4B**). In contrast, root elongation was suppressed slightly by 20% under low pH conditions compared with normal pH conditions (**Fig. 1A, B; Supplementary Fig. S6**). These results suggest that the enhanced activity of PM H^+-ATPase under the low pH condition is required to sustain pH homeostasis for root elongation because a more severe pH gradient should be formed across the PM under low pH conditions than under normal pH conditions. Indeed, root elongation in the *loph1* mutant, which exhibits lower PM H^+-ATPase activity than the WT, was suppressed markedly under low pH conditions (**Fig. 2**). However, ATP hydrolysis activity of the PM H^+-ATPase in the *loph1* root was increased to approximately 2.2 nmol phosphate (Pi) h^{-1} μg $protein^{-1}$ in response to low pH conditions (**Fig. 4B**), which is a value similar to that of the WT under normal pH conditions. These results suggest that root elongation under low pH conditions may require PM H^+-ATPase activity >2.2 nmol Pi h^{-1} μg $protein^{-1}$ under our experimental conditions. Further investigation is required to determine the accurate threshold of PM H^+-ATPase activity for root elongation under low pH conditions.

We also note that transformation of *AHA2 P68S*, which is a constitutive active PM H^+-ATPase (Merlot et al. 2007), into the *loph1* mutant partially restored root elongation under low pH conditions (**Supplementary Fig. S5**), indicating again that PM H^+-ATPase activity is required for root elongation under low pH conditions. However, full elongation in the transgenic roots may need factors other than the PM H^+-ATPase activity that are expressed or activated by auxin under low pH conditions.

Endogenous auxin positively regulates PM H^+-ATPase activity in roots

Our analysis indicated that low pH conditions increased endogenous auxin accumulation in WT root tips, and that the auxin level in *loph1* roots is lower than that in WT roots under both normal and low pH conditions (**Fig. 5A**). Corresponding to the results, both the activity and the phosphorylation of the penultimate threonine in the PM H^+-ATPase were lower in *loph1* roots than in WT roots under normal and low pH conditions (**Fig. 4**). These results show the positive correlation between the endogenous auxin level and the PM H^+-ATPase

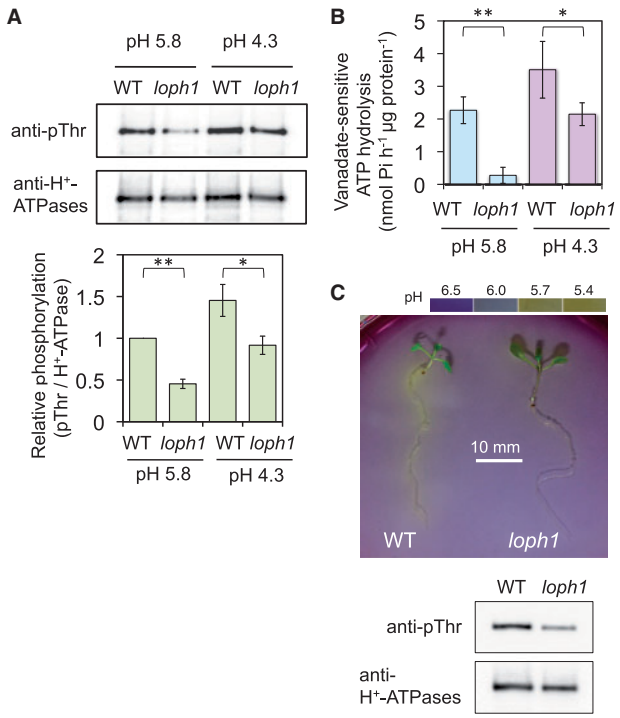

Fig. 4 Effect of the *loph1* mutation on PM H$^+$-ATPase activity in roots. (A) Phosphorylation status of the penultimate threonine residue of the PM H$^+$-ATPase in WT and *loph1* mutant roots. Immunoblots of phosphorylated PM H$^+$-ATPase and total PM H$^+$-ATPase were performed as described in **Fig. 1C**. Phosphorylation levels of the PM H$^+$-ATPase are presented as values relative to the phosphorylation level in WT plants grown at normal pH. Bars represent means with SDs (n = 3). Asterisks indicate significant differences between the WT and *loph1* (Student's t-test; $^{**}P < 0.01$ and $^{*}P < 0.05$). (B) ATP hydrolysis activity of the PM H$^+$-ATPase in WT and *loph1* roots. Crude extract was prepared from the root tips of plants grown as described in **Fig. 1B**, and vanadate-sensitive ATP hydrolysis activity was measured by determining Pi release from ATP. Values represent means of four independent experiments with SDs. Asterisks indicate significant differences between the WT and *loph1* (Student's t-test; $^{**}P < 0.01$ and $^{*}P < 0.05$). (C) Monitoring of media acidification around seedlings using a pH indicator dye. One-week-old WT and *loph1* seedlings were transferred onto solid medium containing 0.02% (w/v) bromocresol purple and 0.8% (w/v) agar adjusted to pH 6.5. After incubation for 15 h, the plates were photographed. Experiments repeated on three occasions gave similar results. Phosphorylation status of the penultimate threonine residue of the PM H$^+$-ATPase in WT and *loph1* mutant roots grown under the same conditions as the media pH monitoring. Immunoblots of phosphorylated PM H$^+$-ATPase and total PM H$^+$-ATPase were performed as in **Fig. 1C.** Experiments repeated on two occasions gave similar results.

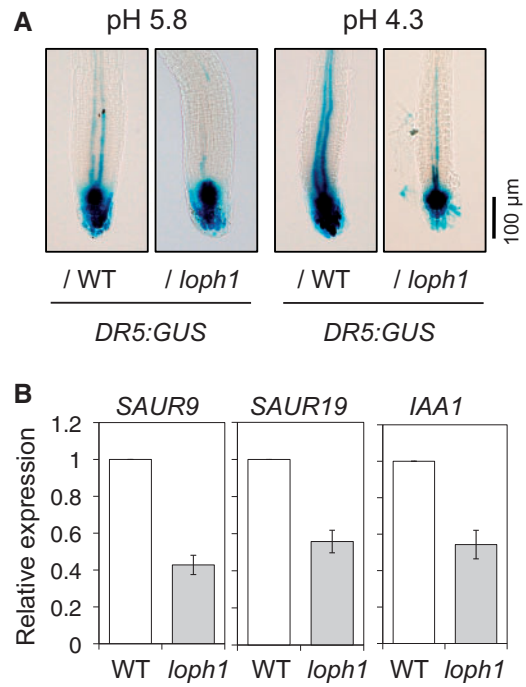

Fig. 5 Analyses of auxin homeostasis and auxin-responsive gene expression in *loph1* roots. (A) Auxin levels in WT and *loph1* root tips. Accumulation of auxin in root cells was detected by GUS staining in roots of transgenic plants expressing the *GUS* gene under control of the auxin-responsive promoter, *DR5*. Plants were grown as described in **Fig. 1A** and used for GUS staining. (B) Transcript levels of auxin-responsive genes in WT and *loph1* root tips. mRNAs were extracted from 5 mm segments containing the root tips of 2-week-old seedlings grown on normal medium (pH 5.8). Relative expression levels were determined by qRT-PCR analysis. Values show the means of three biological replicates with SDs.

activity in roots. Auxin has long been implicated in the activation of the PM H$^+$-ATPase (Cleland 1972). Our recent investigation indicated that auxin induces activation of the PM H$^+$-ATPase through phosphorylation of the penultimate threonine and promotes hypocotyl elongation within several minutes in Arabidopsis (Takahashi et al. 2012). Thus, it is likely that endogenous auxin acts as an important physiological signal and promotes elongation growth through activation of the PM H$^+$-ATPase also in roots. It should be noted that the increase of endogenous auxin was not associated with proton excretion and the phosphorylation level of the PM H$^+$-ATPase in roots from Brachypodium (Pacheco-Villalobos et al. 2016), suggesting that the decrease of the endogenous auxin level may affect the PM H$^+$-ATPase activity and phosphorylation level of the PM H$^+$-ATPase in roots. Further investigations will be needed to clarify the relationship between the endogenous auxin level and the PM H$^+$-ATPase activity in roots.

A recent study demonstrated that the phosphorylated threonine of PM H$^+$-ATPases is directly dephosphorylated by D-clade type 2C protein phosphatases (PP2C-Ds), and in auxin-mediated hypocotyl elongation, PP2C-Ds are inhibited by SMALL AUXIN UP RNA (SAUR) proteins that were up-regulated by auxin (Spartz et al. 2014). As shown in **Fig. 5B**, expression of the PP2C-D inhibitors, *SAUR9* and *SAUR19*, was suppressed markedly in the *loph1* roots. PP2C-D activity is probably enhanced by decreases in *SAUR9* and *SAUR19* expression, which is responsible for the decreased activity and phosphorylation of the penultimate threonine of PM H$^+$-ATPase in the mutant roots. Similarly, low pH-induced activation and phosphorylation of the PM H$^+$-ATPase in WT roots may be mediated by up-regulation of *SAUR* genes by the enhancement of auxin accumulation in the low pH

condition. Further comprehensive expression analysis in *loph1* roots will be needed to clarify AUX1-dependent regulation of the PM H^+-ATPase in root tips.

Generally, auxin is believed to be synthesized in the apical parts of plants, and the concentration of active auxin in cells is regulated by its biosynthesis, conjugation/deconjugation and polar transport (Ljung 2013). The directional polar transport of auxin, which is known to play an important role in plant development, is mediated by the auxin efflux carriers PIN-FORMED (PIN) and P-GLYCOPROTEIN (PGP) and by the auxin influx carriers AUX1 and LIKE-AUX1 (LAX). In Arabidopsis, AUX1 and LAX proteins are produced by a small family of genes, which encode multimembrane-spanning transmembrane proteins (Swarup and Peret 2012). Among the auxin carriers, AUX1 is localized in the collumella, stele and in lateral root cap tissues of roots, and plays an important role in auxin homeostasis through the regulation of auxin distribution in root tissues in co-operation with PINs and PGPs (Swarup et al. 2001, Swarup and Peret 2012). Our results suggested that the low pH condition induced endogenous auxin accumulation in WT root tips via AUX1 (**Fig. 5A**). Since AUX1 is a co-transporter of auxin and H^+ that takes up auxin using the H^+ gradient across the PM (Yang et al. 2006), the low pH condition might enhance the auxin uptake by AUX1. In addition, the H^+ gradient is maintained by the action of PM H^+-ATPase, because auxin uptake may be regulated by the PM H^+-ATPase activity. Thus, the PM H^+-ATPase may affect cell elongation through auxin distribution and uptake. However, a major problem remains to be answered. AUX1 is known to facilitate the transport of the dissociated form of IAA^-; therefore, it is difficult to explain the increase of auxin amount/transport based on the form of IAA under the low pH conditions where IAAH is enriched (Yang et al. 2006, Swarup and Pret 2012). How does AUX1 enhance the auxin accumulation under the low pH condition? Further analyses including modifications of AUX1 and auxin molecules will be needed to answer this question.

In this report, we characterized a novel mechanism that regulates the PM H^+-ATPase in Arabidopsis roots by analyzing a newly isolated low pH-hypersensitive mutant, *loph1*, which is a loss-of-function mutant of the auxin influx carrier AUX1. Our results increase our understanding of auxin-mediated regulation of PM H^+-ATPase activity during root elongation in response to pH conditions.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col) and a corresponding mutant *glabra1-1* (*gl1*) were used as controls in this study. The *gl1* background was used as the parental strain for generating EMS-mutagenized seeds. The *aux1-7* mutant was obtained from the Arabidopsis Biological Resource Center (ABRC, https://abrc.osu.edu/). Plants were grown on agar plates under fluorescent light (50 μmol m^{-2} s^{-1}) with a 16 h light/8 h dark cycle at 24 °C. Agar plates contained half-strength Murashige and Skoog salts and 1% (w/v) agar, pH 5.8 or pH 4.3.

Immunoblot analysis

Plants were grown along the surface of agar plates (pH 5.8) for 4 d and the plants were then transferred to agar plates at pH 4.3 or 5.8 and grown for an additional 3–5 d. Root tips (approximately 5 mm) were excised and homogenized in ice-cold homogenization buffer containing 50 mM MOPS-KOH (pH 7.5), 2.5 mM EDTA, 100 mM NaCl, 2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin, 10 mM NaF and 1 mM dithiothreitol (DTT) using a mortar and pestle. The homogenate was centrifuged at 14,000 r.p.m. for 1 min and the supernatant (root extract) was solubilized by adding a half volume of SDS sample buffer containing 4.5% SDS, 30% sucrose, 22.5% β-mercaptoethanol, 0.018% Coomassie Brilliant Blue, 4.5 mM EDTA and 45 mM Tris–HCl (pH 8.0). Immunoblotting of the catalytic domain and the phosphorylated penultimate threonine residue of the PM H^+-ATPase were performed as described previously (Inoue et al. 2011) with specific antibodies (Hayashi et al. 2010). The secondary antibody was goat anti-rabbit IgG–horseradish peroxidase (HRP) conjugate (Bio-Rad). The chemiluminescence signal was detected using a LightCapture system (ATTO). The signal intensities were analyzed using ImageJ software.

Measurement of PM H^+-ATPase activity in roots

Plants were grown on agar plates using media with various pH values, and the root tips were harvested from the plants. Root extracts were prepared as described above. Vanadate-sensitive ATP hydrolysis activity was measured as the PM H^+-ATPase activity as reported previously (Kinoshita and Shimazaki 1999, Kinoshita and Shimazaki 2001).

Screen for low pH-hypersensitive mutants

Five thousand seeds (M_0) were treated with EMS (Sigma-Aldrich), and EMS-mutagenized M_2 seeds were used for screening (Lightner and Caspar 1998). Approximately 40,000 M_2 seeds were screened for low pH hypersensitivity using a root-bending assay as described previously (Wu and Zhu 1996). Four-day-old seedlings grown on the vertical agar plates (pH 5.8) which had roots 1–1.5 cm in length were transferred to low pH (pH 4.3) agar plates. The seedlings were arranged in rows and the plates were orientated vertically with the roots pointing upward. Root growth was observed for three additional days, after which we isolated low pH-hypersensitive mutants based on root growth. Candidate mutants were then transferred to soil to grow to maturity.

Identification of the *loph1* locus

To identify the mutant locus, we performed genetic mapping in the *loph1* mutant. The mutant was crossed with plants of the Landsberg *erecta* (*Ler*) ecotype, and F_2 plants were obtained. F_2 plants with agravitropic roots were used for genetic mapping. Genetic mapping performed using SSLP markers indicated that the *LOPH1* locus exhibited strong linkage to the SSLP marker nga168 on chromosome 2.

Generation of transgenic Arabidopsis plants

We constructed a gene transfer vector bearing a genomic fragment containing *AUX1* to complement the *loph1* mutant with the WT *AUX1* gene. The *AUX1* genomic fragment (6,967 bp), including 5′- and 3′-non-coding sequences, was amplified by PCR from genomic WT DNA using the primers 5′-CAGAAGACGC TCTAATATACAACTG-3′ and 5′-TACTCCATATCGCATAATATGACTCAC-3′. The PCR product was cloned into pCR8/GW/TOPO (Invitrogen). The genomic AUX1 region was then introduced into the gene transfer vector pGWB1 using Gateway techniques (Invitrogen).

To express a constitutively active form of the PM H^+-ATPase in the *loph1* mutant, we constructed a gene transfer vector bearing a genomic *AHA2* gene with an *ost2* mutation (Merlot et al. 2007). An *AHA2* genomic fragment (9,151 bp), including 5′- and 3′-non-coding sequences, was amplified by PCR from genomic WT DNA using the primers 5′-CCCAAGCTTCAGATCGATGTCA AGTCAATGATG-3′ and 5′-GCGGATCCCACACATGGCCGTTTATACGTAC-3′. The amplified DNA fragment was digested with *Bam*HI and *Hin*dIII enzymes and cloned into the gene transfer vector pCAMBIA1300 (CAMBIA) using *Bam*HI and *Hin*dIII sites. Nucleotide substitution of the *ost2* mutation into the *AHA2* gene in pCAMBIA1300 was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The PCR was performed using the primers 5′-GGTTTATGTGGAATTCACTTTCATGGGTCATGGAAATGG-3′ and 5′-CCATTTCCATGACCCATGAAAGTGAATTCCACATAAACC-3′. The resulting vectors were verified by DNA sequencing.

The plant transformation vectors were introduced into *Agrobacterium tumefaciens* (GV3101) for transformation of Arabidopsis Col and *loph1* plants using the floral dip method (Clough and Bent 1998). The transgenic plants were selected based on resistance to hygromycin after germination.

Gene expression analysis by reverse transcription—PCR (RT—PCR)

Total RNAs were extracted from the root tips of Col and *loph1* mutant plants using the RNeasy Plant Mini Kit (QIAGEN). First-strand cDNAs were obtained from 1 μg of total RNA using the PrimeScript 1st strand cDNA Synthesis Kit (TAKARA). The coding regions of *AUX1* cDNAs were amplified by PCR using the primers 5′-CTTGCTTCTGATTCTTTCTCTTCTC-3′ and 5′-GACTTTTACTC CCTTCACGTATACG-3′.

Quantitative real-time PCR

The expression levels of the auxin-responsive genes, *SAUR9*, *SAUR19* and *IAA1*, and the H^+-ATPase genes, *AHA1* and *AHA2*, were determined based on quantitative real-time PCR (qRT-PCR). The reactions were performed using the PowerSYBR Green PCR Master Mix (Life Technologies) and specific primer sets: 5′-CAACGACGTGCCAAAAGGT-3′ and 5′-CACATAGCGACTTCGGTG TTGA-3′ for *SAUR9*, 5′-GGCTTAACGATCCCTTGTCCC-3′ and 5′-TTTACAAT GAATAAGTCTATTTCTAACTGAAGGA-3′ for *SAUR19*, 5′-TGAAAGGATCCG AAGCTCCTACT-3′ and 5′-TGCCTCGACCAAAAGGTGTT-3′ for *IAA1*, 5′-GAA CGTCCTGGGGCGC-3′ and 5′-GATACCCTTCACCTTTGCAAATGT-3′ for *AHA1*, 5′-TTGTTGAACGTCCTGGAGCA-3′ and 5′-AATTCCCAGTTGGCGTA AACC-3′ for *AHA2*, and 5′-GAATATCAACAGGAGGAAGAGTACTGAG-3′ and 5′-AACTACAACTGGTAGTTGAGGTGTTCC-3′ for *TUB2*. Reactions were performed in a StepOne Real Time PCR system (Applied Biosystems) using the comparative C_T method for relative quantitation of expression levels. *TUB2* was used as an internal control.

Determination of auxin accumulation in root tips using a promoter—GUS assay

Seeds of *DR5:GUS*/Col transgenic plants in which the expression of a GUS gene was driven by the auxin-responsive *DR5* promoter were kindly provided by T.J. Guilfoyle (Ulmasov et al. 1997). *DR5:GUS*/*loph1* plants were generated by crossing *DR5:GUS*/Col and *loph1* mutant plants. The homozygous transgenic plants were used for GUS staining experiments.

Plants grown on media with various pH values as described above were collected and fixed in ice-cold 90% acetone for 20 min and then incubated with GUS staining solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% Triton X-100 and 0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-glucuronide for 4 h at 37 °C. After the reaction, samples were fixed with solution containing 15% acetic acid and 85% ethanol until observation.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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