Effects of nitrate and ammonium application on nitrogen accumulation in an *Arabidopsis* gene silencing mutant of vacuolar pyrophosphatase1 (AVP1)

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Summary

To investigate nitrogen accumulation in a transposon-silenced transgenic mutant of *Arabidopsis* vacuolar H^+-PPase (AVP1), ^{15}N-labeled nitrate and/or ammonium was supplied in the culture solution. When the mutant plants were supplied with $^{15}NO_3^-$, ^{15}N accumulation in the shoots and roots was almost the same as in Columbia plants (Col). However, when the mutant plants were supplied with $^{15}NH_4^+$, ^{15}N accumulated more highly in the cationic fraction in shoots compared with in Col. This increased accumulation was not detected when mutant plants were supplied with both $^{15}NO_3^-$ and $^{15}NH_4^+$. Furthermore, it was observed that transposon-containing mutant plants could survive in low pH conditions (pH 2) for 12 days, suggesting that the pH in cell might be different from that in Col. These results suggested that the metabolism and utilization of nitrogen might be related to AVP1 in addition to PPi hydrolysis.

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Key words : AVP1, silenced mutant, nitrate, ammonium, low pH

INTRODUCTION

Pyrophosphate (PPi) is universally present in higher plant cells. PPi is generated by many biosynthetic reactions, such as the synthesis of UDP-glucose, and PPi is a substrate for cellulose synthesis in plants (Dennis et al. 2000). PPi at a high concentration in cells inhibits metabolic processes; therefore, it needs to be hydrolyzed quickly by enzymes such as pyrophosphatase. Two types of H^+-translocating inorganic pyrophosphate (H^+-PPase) that use PPi as an energy source instead of ATP have been well characterized. Type I H^+-PPase is localized to the vacuolar membrane (Robinson et al. 1996), whereas type II H^+-PPase is located in the Golgi body (Segami et al. 2010). Recently, it was reported that up-regulation of H^+-PPase resulted in greater growth of *Arabidopsis* (Ferjani et al. 2011) and Romaine lettuce (Paez-Valencia et al. 2014), and it was important for tolerance to drought and salt stress in *Arabidopsis* (Gamboa et al. 2013), cotton (Pasapula et al. 2011) and barley (Rhiannon et al. 2014). In addition, type I H^+-PPase enhanced phosphorus nutrition in tomato and rice plants (Yang et al. 2007) and was related to deprivation of Pi and N in tobacco plants (Li et al. 2014). Gluconeogenesis is inhibited by elevated levels of cytosolic PPi (Ferjani et al. 2011), and *Arabidopsis* vacuolar H^+-PPase (AVP1) facilitates auxin flux, which regulates organogenesis (Li et al. 2005). Therefore, it is well-known that PPi influences many biochemical reactions in plant cells, but the physiological roles of PPi remain to be elucidated.

On the other hand, there are few reports on PPi and PPase related to nitrogen metabolism. Amidation of aspartate by glutamine or ammonium as a nitrogen source yields asparagine, a stable amino acid used to store nitrogen in plant cells and to transport it from source to sink organs (Coruzzi et al. 2000). When asparagine synthetase catalyzes the synthesis of asparagine, ATP is used and produces PPi and AMP. However, the effect of PPi on nitrogen metabolism is not well understood. In this report, we investigated the effect of the form of nitrogen supply (^{15}N-labeled nitrate or ammonium) on the accumulation of nitrogen in a transgenic mutant *Arabidopsis* line, in which a T-DNA insertion silenced the expression of AVP1.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Columbia wild type (Col) was used as the *Arabidopsis* control line, and RIKEN line psh21977 was used as a mutant plant in this study and that was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. This homozygous mutant has a transposon T-DNA-tag in the type I H^+-PPase (AVP1) gene (Ito et al. 2002; Kuromori et al. 2004).

Col and mutant seeds were sown on rock wool and cultivated in a programmed incubator (TOMY CL-301, Tokyo, Japan) in the following conditions: 18 h of illumination at 22℃, and 6 h of darkness at 22℃. After germination, the seedlings were transferred to a hydroponic system. The chemical

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composition of the culture solution except for NO_3^- and NH_4^+ was as follows (mg l^{-1}) : Na_2HPO_4•$12H_2O$ (289.10), K_2SO_4 (111.43), Fe(III)•EDTA (5.00), $MgSO_4$•$7H_2O$ (371.70), $CaCl_2$•$2H_2O$ (294.00), $MnSO_4$•$4H_2O$ (2.30), H_3BO_4 (1.85), $ZnSO_4$•$7H_2O$ (0.29), $CuSO_4$•$5H_2O$ (0.24), $(NH_4)_5Mo_7O_{24}$•$4H_2O$ (0.03), $CoCl_2$•$6H_2O$ (0.03). The plants were grown in a culture solution containing 2.5 mM sodium nitrate until 14 days after sowing and then 0.5 mM sodium nitrate, which was replaced twice per week. At 24 days after sowing, plants were treated as follows.

Nitrogen Utilization

^{15}N-labeled sodium nitrate (5 mM; 30.7 atom% ^{15}N) and 2.5 mM ^{15}N-labeled ammonium sulfate (30.7 atom% ^{15}N), 2.5 mM ^{15}N-labeled ammonium nitrate (^{15}N-ammonium labeled, 30.5 atom% ^{15}N and ^{15}N-both ammonium and nitrate labeled, 10.5 and 10.0 atom% ^{15}N, respectively) was applied in the hydroponic culture solution for Col and mutant plants. One day after treatment, plants were separated into shoot and root tissues. Roots were washed with deionized water, then shoots and roots were immediately frozen in liquid nitrogen and lyophilized. After drying, the samples were ground into fine powder.

Hydrophilic low molecular weight compounds were extracted from plants using 80% ethanol. Sample powder was mixed with 1 mL of 80% ethanol and treated at 60℃ for 15 min. After treatment, samples were incubated on ice for 3 min, vortexed for 10 min, and then centrifuged at 8,000*g* for 10 min at 4℃. The supernatant was collected in a tube and the precipitate was resuspended in 500 μL of 80% ethanol, vortexed for 3 min, then centrifuged at 10,000*g* for 10 min at 4℃. The second supernatant was combined with the first supernatant. The precipitate was resuspended in 500 μL of 80% ethanol, vortexed for 3 min, then centrifuged at 12,000*g* for 10 min at 4℃. The third supernatant was also combined with the first and second supernatants. The supernatants were the 80% ethanol-extracted fraction, and the precipitate was the 80% ethanol-insoluble fraction. The 80% ethanol-extracted fraction was dried using a centrifugal concentrator. After drying, the samples were dissolved with 200 μL of deionized water, then the sample was fractionated using DOWEX 50 cation exchange resin (The Dow Chemical Company, Michigan, USA), and the cation-exchanged fraction was eluted using 0.5 mL of 2 N HCl.

The nitrogen and ^{15}N concentrations of each fraction after Kjeldahl digestion were determined by the indophenol method and emission spectrometry, respectively. (Ohyama et al, 2004) ^{15}N relative abundance (percentage of N derived from an ^{15}N-labeled source) was calculated using the equation:

^{15}N relative abundance (%)=100 × (^{15}N atom%excess of sample/^{15}N atom%excess of labeled source).

Amino Acid Analysis

Sodium nitrate (5 mM) and 2.5 mM ammonium sulfate were supplied in the hydroponic culture solution for Col and mutant plants. One day after treatment, plants were separated into shoots and roots. Roots were washed with deionized water, then shoots and roots were frozen immediately in liquid nitrogen and lyophilized. After drying, the samples were ground into fine powder and hydrophilic low molecular compounds were extracted with 80% ethanol, as described previously.

Free amino acids in the sample extracted with 80% ethanol were analyzed using ACQUITY UPLC™ Amino Acid Analysis Application Solution (Waters, Massachusetts, USA). Standard and samples were derived using a Waters AccQ-Tag Ultra Derivative kit for analysis. Twenty-one amino acids (His, Asn, Ser, Gln, Arg, Gly, Asp, Glu, Thr, Ala, GABA, Pro, Cys, Lys, Tyr, Met, Val, Ile, Leu, Phe, Trp) and ammonium were included in the standard. Two μL of sample or standard was mixed with 70 μL of AccQ Fluor™ borate buffer in a tube. Four μL of internal standard (0.25 mM GABA) and 4 μL of deionized water were mixed in a tube. One mL of AccQ-Tag Ultra Reagent Diluent was added to AccQ-Tag Ultra Reagent Powder and heated on top of a heating block for 3 min at 55℃, then 20 μL of reconstituted AccQ-Tag Ultra Reagent was added to the tube, the mixture was heated on a heating block for 10 min at 55℃, and then 100 μL of sample was analyzed.

The amino acid derivatives were separated using an ACQUITY UPLC System (Waters). A Waters Empower 3FR2 was used for system control and data acquisition. The column used was an ACCQ-TAG™ ULTRA C18 (1.7 μL, 2.1 × 100 mm; Waters) set at 60℃. Derivatized amino acid standards and sample were injected into the column and separated by gradient elution consisting of 10% AccQ-Tag Ultra Eluent A (A) and AccQ-Tag Ultra Eluent B (B). The flow rate was 0.7 mL min^{-1}, and the gradient profile was: initially 0.54 min/0.1% B; 0.54–3.2 min/0.1–4.0% B; 3.2–3.8 min/4.0–6.0% B; 3.8–7.0 min/6.0–9.1% B; 7.0–8.5 min/9.1–12.1% B; 8.5–9.5 min/12.1–21.2% B; 9.5–9.8 min/21.2–59.6% B; and the column was washed with 59.6% B for 0.60 min, then the column was reconditioned with 0.1% B for 0.5 min. The injection volume of standards and sample was 1.0 μL. UV detection was set to 260 nm.

Low pH Treatment

Col and mutant seeds were sown and germinated on rock wool. At 7 days after sowing, the rock wool was dipped in culture solution adjusted to pH 2. Photos were taken by digital camera (Casio QV-2900UX, Casio, Tokyo, Japan).

Microscope analysis about pH of root.

The pH of Roots was visualized by pH indicator (pHrodo™ Green AM Intracellular pH Indicator, Thermo Fisher, USA). The roots were obtained in hydroponically grown plants supplied with 0.5mM nitrate after 30 days after sowing. Roots were soaked into 0.1M HEPES buffer (pH 7.4) for 30 min then soaked into new 10mL of HEPES buffer with staining solution (10μL of pHrode™ diluted 100μL of PowerLoad™) for 30 min at room temperature. The roots were observed by microscope (Olympus BX-60) equipped fluorescence cube (U-FBN cube was constracted with excitation filter as 470-495 nm, absorption filter as 510 nm and dichroic mirror as 505 nm).

Statistical Processing

Each value was examined for significant differences using t-test ($p<0.05$).

RESULTS AND DISCUSSION

At 24 days after sowing (18 days after pre-hydroponically culture described as materials and methods), the plants were treated 5.0 mM sodium nitrate or 2.5mM ammonium sulfate (means 5mM NH_4^+) until 45 days after sowing. During these sampling periods, ammonium application made plants biomass restrict (Fig. 1) as same as previous report (Helali et al. 2010), both in mutant and wild plants compared with nitrate application.

To ensure nitrogen utilization in short time, ^{15}N-labeled 5 mM NO_3^- and/or NH_4^+ were supplied in the hydroponic culture solution in one day. When the plants were supplied with 5 mM ^{15}N-labeled nitrate, nitrogen concentrations of each fraction were not significantly different between Col and mutant plants both in the shoots and roots (Fig. 2a and b). In the roots, cation-exchange resin flow-through and exchanged fractions contained about 38% and 33% of the applied nitrogen $^{15}NO_3^-$ both in Col and mutant plants, respectively. On the other hand, the ^{15}N relative abundance in shoots was 20% and 40% these plant lines, respectively (Fig. 2c and d).

When the plants were supplied with 5 mM ^{15}N-labeled ammonium, nitrogen concentrations in the 80% ethanol-insoluble fraction and cation-exchange resin exchanged fraction in the roots, and cation-exchange resin flow-through fraction in the shoots were significantly increased in mutant plants (Fig. 3a). In mutant plant shoots, N from applied ^{15}N in the cation-exchange resin exchanged fraction was significant higher than in the Col (Fig. 3c). Although the ^{15}N level in roots was not significantly different (Fig. 3d), the amount of N from ^{15}N (nitrogen concentration × % of N from applied N) was significantly different ($p=0.05$, 1.5 mg g^{-1} dry weight in Col versus 3.5 mg g^{-1} dry weight in mutant plants).

When both nitrate and ammonium were supplied with mutant and Col plants, N concentration in root was relatively same as nitrate application (Fig. 4b and Fig. 5b compared with Fig 2b). When the plants were supplied with 2.5 mM ammonium nitrate (^{15}N-NH_4^+ + ^{15}N-NO_3^-) with both nitrate

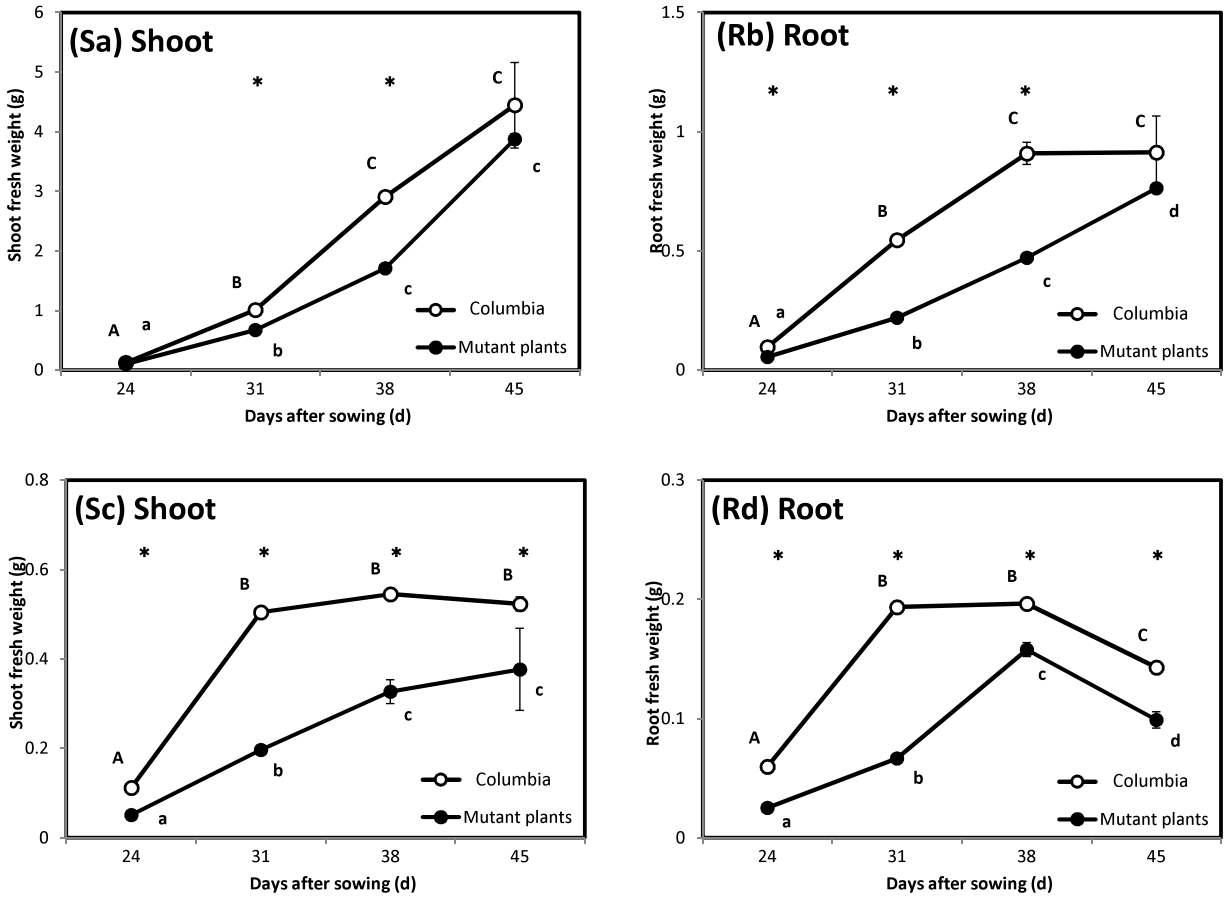

Fig 1. Shoot and root fresh weight of Col and transgenic mutant plants. The plants were grown with 5mM sodium nitrate (Sa and Rb) and 2.5mM ammonium sulfate (Sc and Rd) at 24 days after sowing till 45 days. Bars indicate standard deviation (n=3). The letters represent significant differences between Col (A, B, C and D) and mutant plants (a, b, c and d). Asterisks indicated significant differences, as determined using t test ($p \leq 0.05$).

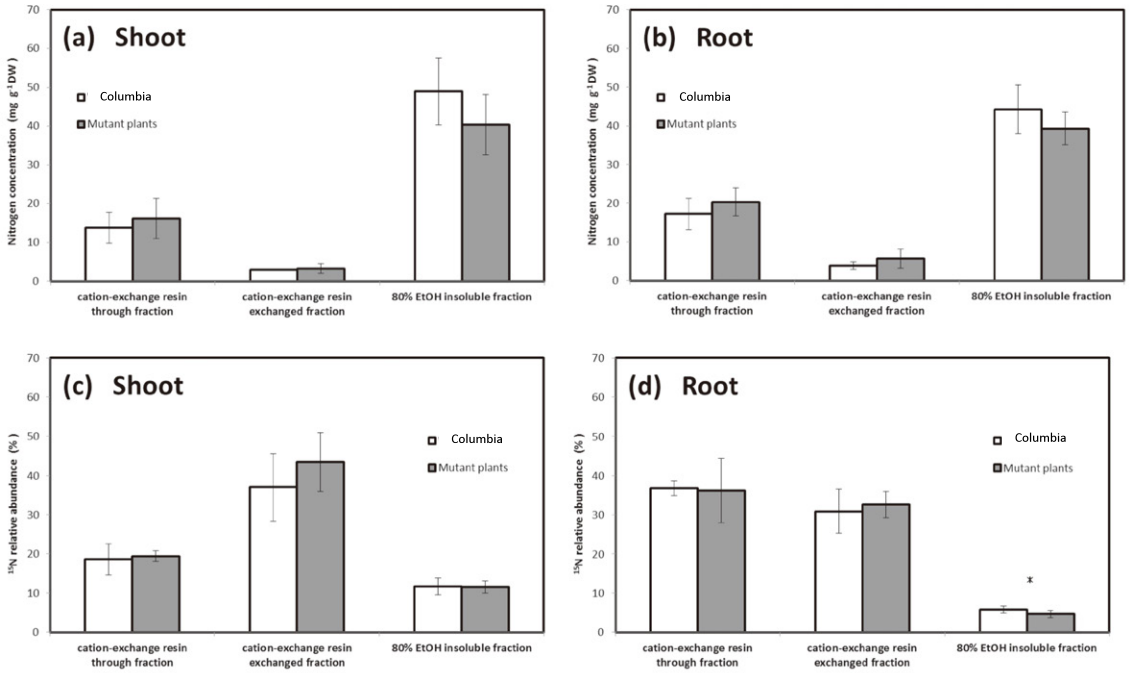

Fig 2. Nitrogen concentrations (a and b) and ^{15}N relative abundance (c and d) in Col (a and c) and mutant plants (b and d). The plants were grown with 5 mM nitrate and treated with 5 mM ^{15}N-labeled sodium nitrate at 24 days after sowing. After 1 day, nitrogen concentrations and ^{15}N relative abundance in root and shoot fractions were determined, as described in the Materials and Method. Bars indicate standard deviation (n=3). Asterisks indicated significant differences, as determined using *t* test ($p \leq 0.05$).

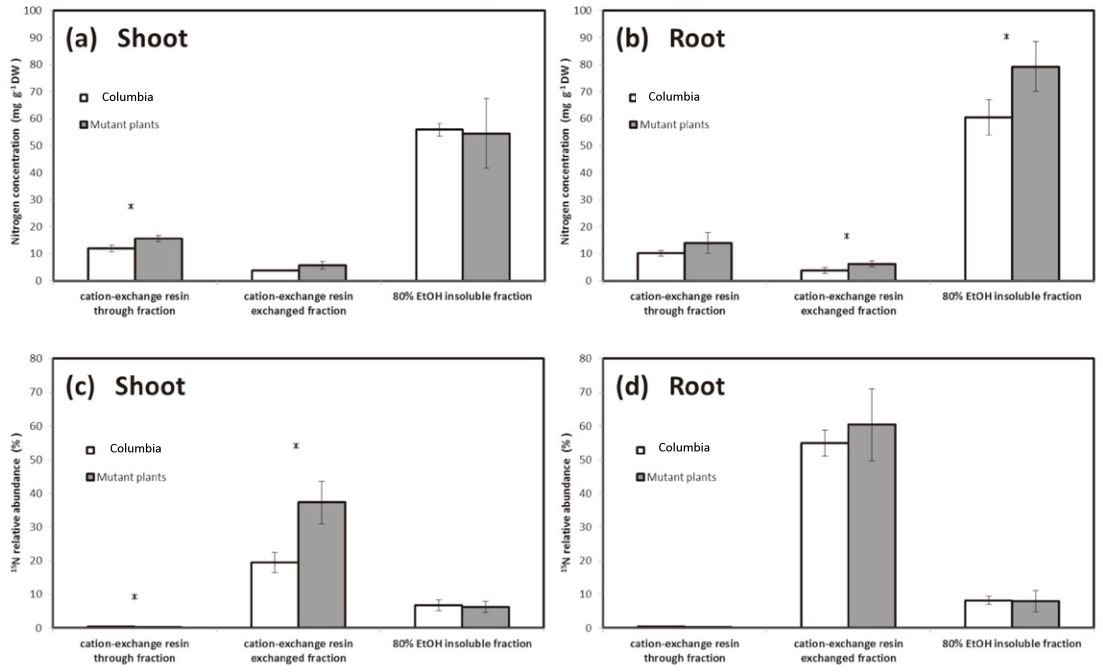

Fig 3. Nitrogen concentrations (a and b) and ^{15}N relative abundance (c and d) in Col (a and c) and mutant plants (b and d). The plants were grown with 5 mM sodium nitrate and treated with 2.5 mM ^{15}N-labeled ammonium sulfate at 24 days after sowing. After 1 day, nitrogen concentrations and ^{15}N relative abundance in root and shoot fractions were determined, as described in the Materials and Method. Bars indicate standard deviation (n=3). Asterisks indicate significant differences, as determined using *t* test ($p \leq 0.05$).

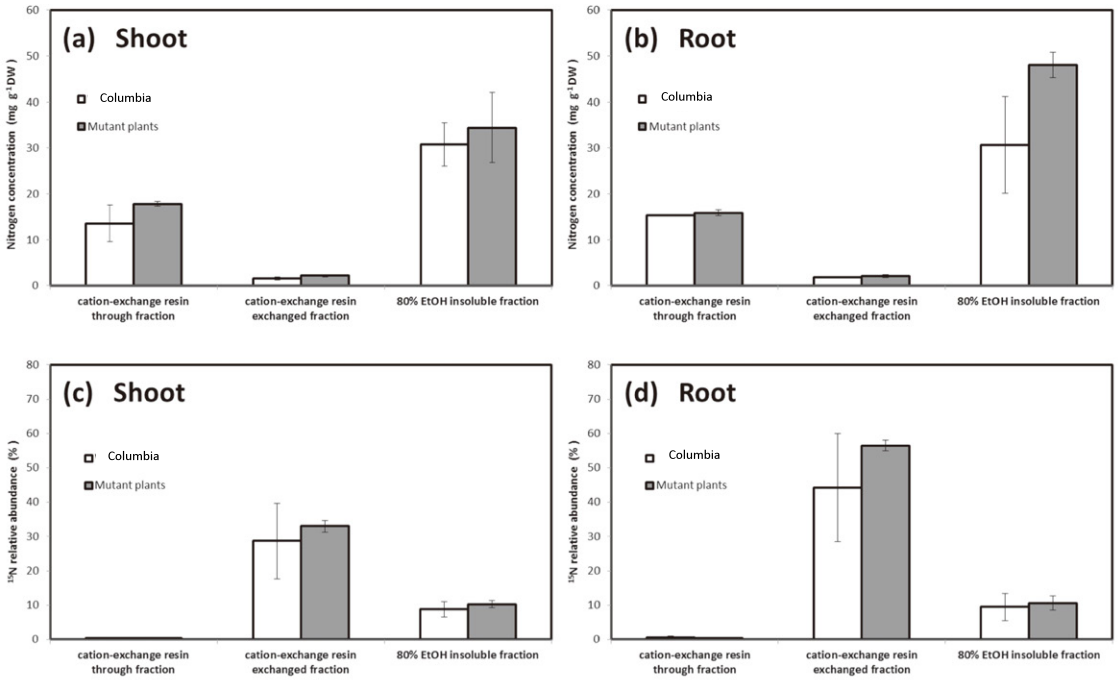

Fig 4. Nitrogen concentrations (a and b) and ^{15}N relative abundance (c and d) in Col (a and c) and mutant plants (b and d). The plants were grown with 5 mM sodium nitrate and treated with 2.5 mM ^{15}N-labeled ammonium nitrate (ammonium labeled) at 24 days after sowing. After 1 day, nitrogen concentrations and ^{15}N relative abundance in root and shoot fractions were determined, as described in the Materials and Method. Bars indicate standard deviation (n=3).

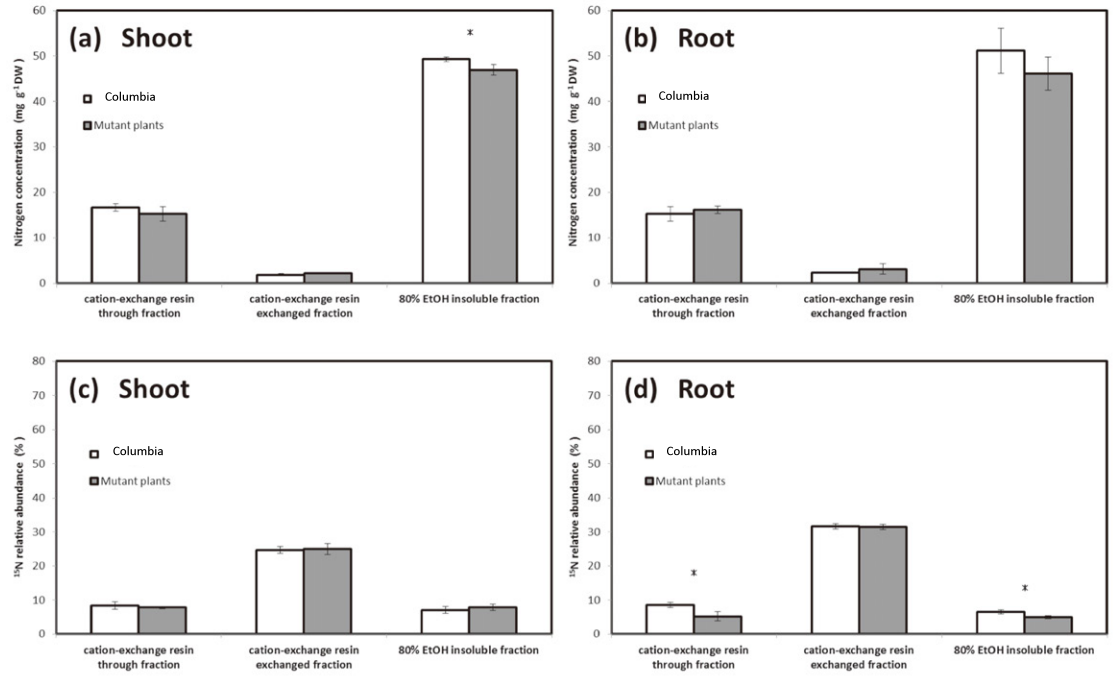

Fig 5. Nitrogen concentrations (a and b) and ^{15}N relative abundance (c and d) in Col (a and c) and mutant plants (b and d). The plants were grown with 5 mM sodium nitrate and treated with 2.5 mM ^{15}N-labeled ammonium nitrate (ammonium and nitrate double labeled) at 24 days after sowing. After 1 day, nitrogen concentrations and ^{15}N relative abundance of root and shoot fractions were determined, as described in the Materials and Method. Bars indicate standard deviation (n=3). Asterisks indicate significant differences, as determined using *t* test ($p \leq 0.05$).

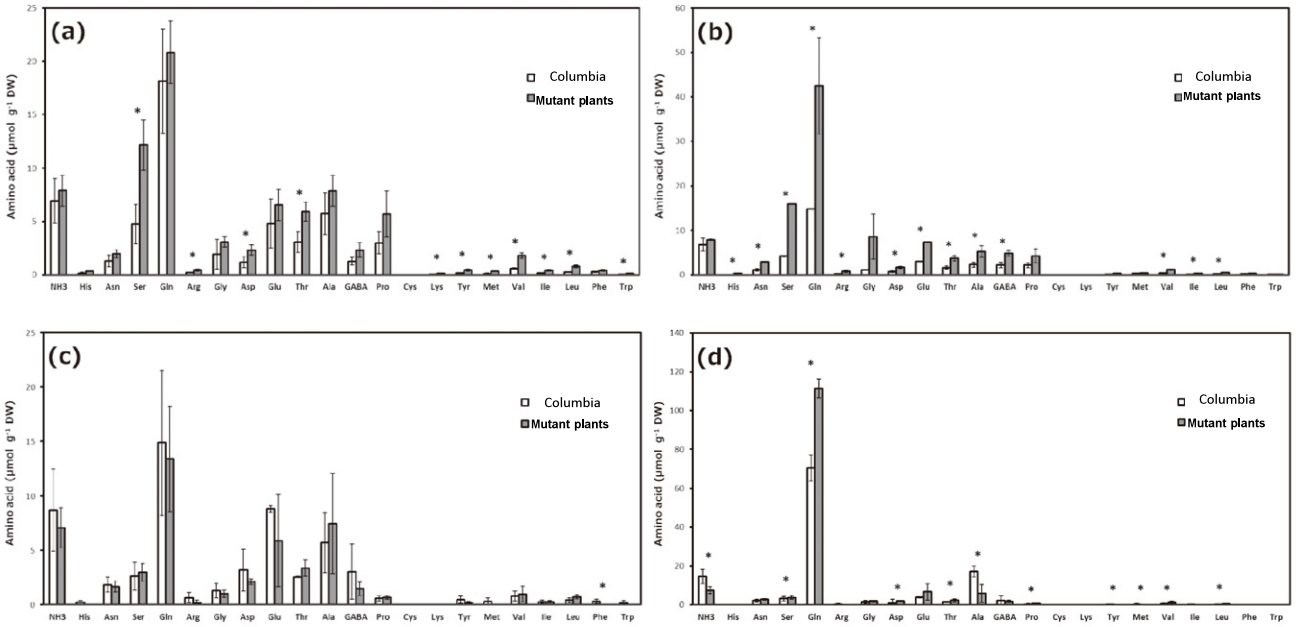

Fig 6. Amino acid concentration of Col and mutant plant shoots (a and b) and roots (c and d). The plants were grown with 5 mM sodium nitrate (a and c) or 2.5 mM ammonium sulfate (b and d). The plants at 24 days after sowing were treated with 5 mM sodium nitrate or 2.5 mM ammonium sulfate. After 1 day, amino acid concentrations were determined, as described in the Materials and Methods. Bars indicate standard deviation (n = 3). Asterisks indicate significant differences, as determined using *t* test ($p \leq 0.05$).

and ammonium labeled (Fig. 5c and d), unlike in the application of only ammonium (Fig 4c and d), the nitrogen concentration of the 80% ethanol-insoluble fraction was not significantly different between Col and mutant plants. The N % from ^{15}N was also not significantly different between Col and mutant plants for each organ.

The concentrations of free amino acids in the shoots and roots are shown in Figure 6. For nitrate application to the mutant plants, the concentrations of amino acids such as Ser, Thr and Asp showed little difference compared with Col plants. When ammonium was supplied to the mutant plants, Gln and Ala concentrations were markedly increased in both roots and shoots compared with Col plants. Increases in the amino acid C4 (Asp and Asn) to C5 (Glu and Gln) ratio more than with nitrate nutrition were also reported by Ueda et al. (2008).

V-PPase functions as a H^+ transporter from the cytosol into the vacuole, and deletion of this function effects responses to some environmental conditions. When the mutant plants were cultivated at low pH, Col plants showed dark-purple leaf color caused by pH stress, but low pH little influence the mutant plants (Fig. 7). This different response of medium pH condition might be reflected its cell pH condition indicated previously reports that the vacuole pH (Ferjani et al. 2011) or apoplast pH (Li et al. 2005) was higher in H^+-PPase mutant plants.

Furthermore, to investigate the different respons to supplied N type coused by the pH statue of root. The pHrode^{TM} Green was weekly fluorescence at neutral condition

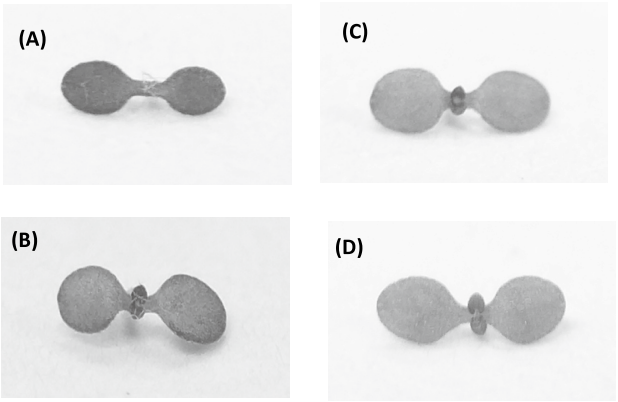

Fig 7. Effect of pH on Col (A and B) and mutant (C and D) seedlings grown on rock-wool. Plants were treated with culture solution adjust to pH 2.0 (A and C) and pH 2.0 (B and D) from 7 till 12 days after sowing.

and the intensity increased with H^+ concentration. The observation of the whole root in microscopy analysis could not discuss about cytoplasmic or vacuoles pH. When the application of nitrate showed almost same fluorescence both Col and mutant plants, the application of ammonium showed slightly faint in mutant plants than Col (Fig. 8). This different of pH in root might reflect to ammonium ability (Fig. 3) or the amino acid composition (Fig. 6) in the mutant plants.

When the plants were grown with NO_3^- as the sole nitrogen source, absorbance and assimilation of nitrate to

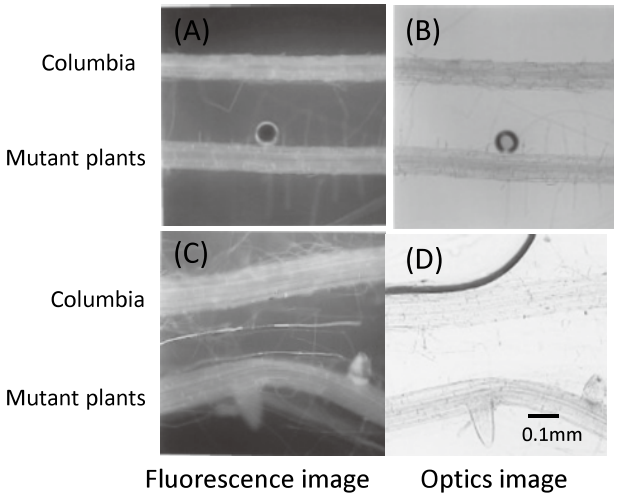

Fig 8. Microscopy image of Col and mutant plant roots treated with pH indicator. Fluorescence image was obtained using U-FBN cube equipped excitation filter as 470-495 nm, absorption filter as 510 nm and dichroic mirror as 505nm. The plants were grown with 5 mM sodium nitrate (A and B) or 2.5 mM ammonium sulfate (C and D). The plants at 24 days after sowing were treated with 5 mM sodium nitrate or 2.5 mM ammonium sulfate.

ammonium is the limiting point of nitrogen metabolism (Campbell 2001). This might be reflected in the mutant plants accumulating ^{15}N to the same degree as the Col plants.

On the other hand, when ammonium was supplied to the mutant plants as their nitrogen source, carbon supply for nitrogen assimilation might be higher than in Col plants, because of enhanced PPi-PFK activity. Although NH_4^+ assimilation was enhanced, the metabolism of nitrogen might be repressed by increasing pH in the cytosol. Thus, this resulted in increased ^{15}N accumulation in the cation exchange resin-exchanged fraction and in the free amino acid concentration of amino acids such as Gln or Ala.

When the plants were supplied with both nitrate and ammonium, the accumulation of N from ammonium in the cation exchange resin exchanged fraction was not enhanced. The addition of nitrate alleviates ammonium toxicity, as reported previously (Hachiya et al. 2012). In this article, the high level accumulation of amino acids due to NH_4^+ supply in the mutant plants was prevented by simultaneously supplying nitrate. This might be explained by nitrate assimilation (assimilation of nitrate into nitrite: NO_3^- + NAD(P)H + H^+ → NO_2^- + NAD(P)$^+$ + H_2O) or H^+ discharge by H^+-ATPase from the cytosol of the plant roots.

In this report, we used Columbia as control because this type plants were used to cultivate and its phenotype. The background ecotype of transgenic plants was Nossen, while genotypes of two Ds donor lines, namely Ds2-389-2 and Ds6-393-19, were inconsistent with that of Nossen (Ito et al. 2002, Kuromori et al. 2004). While the differences between Col and Nossen were reported such as robust growth, enhanced thermo sensory flowering, and enhanced temperature-induced hypocotyl elongation (Gangappa et al. 2017), further experiment would be needed to compare Col with Nossen in the point of view of nitrogen utilization and V-PPase activity.

In this report, we showed that plants with suppressed V-PPase activity had enhanced free amino acid concentrations when ammonium was used as the nitrogen source and mutant plants could be survived in a low pH environment during 12 days. These results suggest that there might be some relationship with cytosolic pH related with PPi, and amino acid metabolism.

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液胞型プロトン輸送性ピロフォスファターゼ（AVP1）遺伝子抑制変異体アラビドプシスの窒素集積に及ぼす硝酸およびアンモニア供給の効果

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要　約

アラビドプシスのトランスポゾン挿入による液胞型 H^+-PPase（AVP1）遺伝子抑制変異株を用い、水耕培地に与えた^{15}N で標識した硝酸態あるいはアンモニア態窒素の供給が窒素集積に及ぼす影響を調査した。遺伝子抑制変異株に^{15}N 標識硝酸を与えると、地上部および根における^{15}N の集積はコロンビアと同様であった。しかし、^{15}N 標識アンモニウムを遺伝子抑制変異株に与えると、^{15}N はコロンビアよりも地上部の陽イオン画分に多く集積した。この^{15}N 集積の増加は、遺伝子抑制変異株に^{15}N 標識硝酸と^{15}N 標識アンモニウム両方を供給した場合には認められなかった。さらに遺伝子抑制変異株は、低い pH 状態での12日間の培養でも生き残ることから、細胞内の pH がコロンビアと異なっていることが考えられた。これらの結果から、AVP1はピロリン酸の加水分解のみならず、窒素の利用や代謝にも関連することが示唆された。

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