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Overexpression of a Plasma Membrane H⁺-ATPase Gene *OSA1* Stimulates the Uptake of Primary Macronutrients in Rice Roots

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Abstract: Plasma membrane (PM) H⁺-ATPase is a master enzyme involved in various plant physiological processes, such as stomatal movements in leaves and nutrient uptake and transport in roots. Overexpression of *Oryza sativa* PM H⁺-ATPase 1 (*OSA1*) has been known to increase NH₄⁺ uptake in rice roots. Although electrophysiological and pharmacological experiments have shown that the transport of many substances is dependent on the proton motive force provided by PM H⁺-ATPase, the exact role of PM H⁺-ATPase on the uptake of nutrients in plant roots, especially for the primary macronutrients N, P, and K, is still largely unknown. Here, we used *OSA1* overexpression lines (*OSA1*-oxs) and gene-knockout *osa1* mutants to investigate the effect of modulation of PM H⁺-ATPase on the absorption of N, P, and K nutrients through the use of a nutrient-exhaustive method and noninvasive microtest technology (NMT) in rice roots. Our results showed that under different concentrations of P and K, the uptake rates of P and K were enhanced in *OSA1*-oxs; by contrast, the uptake rates of P and K were significantly reduced in roots of *osa1* mutants when compared with wild-type. In addition, the net influx rates of NH₄⁺ and K⁺, as well as the efflux rate of H⁺, were enhanced in *OSA1*-oxs and suppressed in *osa1* mutants under low concentration conditions. In summary, this study indicated that overexpression of *OSA1* stimulated the uptake rate of N, P, and K and promoted flux rates of cations (i.e., H⁺, NH₄⁺, and K⁺) in rice roots. These results may provide a novel insight into improving the coordinated utilization of macronutrients in crop plants.

Keywords: plasma membrane H⁺-ATPase; rice; nitrogen; phosphorus; potassium; transport; noninvasive microtest technology



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1. Introduction

Plasma membrane (PM) H⁺-ATPase, belonging to the P-type ATPase superfamily, is highly conserved in plant species [1]. In rice, there are ten PM H⁺-ATPase genes (*OSA1*–*OSA10*). PM H⁺-ATPases generate membrane potential and provide proton motive force for active transport of nutrients, sugars, and other substances. PM H⁺-ATPase activities are regulated at multiple levels, including transcriptional, translational, and post-translational regulations [2]. Environmental stresses, including salinity, alkalinity, nutrient deficiency, and low pH, have been known to activate PM H⁺-ATPases [3–5]. The phosphorylation of PM H⁺-ATPase at the penultimate threonine (Thr) residue activates the pump by creating a binding site for 14-3-3 proteins [6–8]. Phosphorylation at the penultimate Thr of PM H⁺-ATPases is involved in regulating a variety of physiological processes, such as

stomatal opening, hypocotyl elongation, and root growth [8–11]. While the phosphorylation of PM H⁺-ATPase plays important roles in regulating the physiological functions of plants, the transcriptional regulation of PM H⁺-ATPase genes remains the fundamental basis for controlling PM H⁺-ATPase activity, which is essential for the transmembrane transport of mineral nutrients.

Plants require nitrogen (N), phosphorus (P), and potassium (K) to grow and maintain a variety of metabolic processes [12–14]. PM H⁺-ATPase is required for low P adaptation in plants such as soybeans [15]. As a result of P deficiency in white lupin, PM H⁺-ATPase activity is induced in cluster roots, which is associated with citrate release and rhizosphere acidification [16]. Overexpression of a tomato PM H⁺-ATPase gene, *SIHA8*, promotes N and P uptakes in tomato roots [17]. Furthermore, it has been demonstrated that K⁺ transport is chemically linked to ATP hydrolysis [18]. PM H⁺-ATPase provides a driving force for cellular K⁺ uptake mediated by K⁺ channels or H⁺/K⁺ symporters. PM H⁺-ATPase energizes multiple ion channels and various H⁺-coupled transporters, such as HAK1 (a high affinity K⁺ transporter), PHTs (H⁺-coupled phosphate transporters), and NRTs (H⁺/NO₃[−] symporters) [19], by generating H⁺ gradient across the PM and membrane potential to drive the transport of these nutrient ions [20]. Several isoforms of PM H⁺-ATPase in rice have been demonstrated to mediate N, P, and K uptakes. For example, NO₃[−] was found to induce expression of *OSA2*, *OSA5*, *OSA7*, and *OSA8* in rice roots [21]. Rice *osa8* knockout mutants showed reduced P concentration in the shoots [3]. In some cases, overexpression of *OsHAK5*, a potassium transporter gene, improved PM H⁺-ATPase activity [22]. In our previous studies, we found that overexpression of the *OSA1* gene could improve the NH₄⁺ uptake rate, increase plant growth, and enhance the total nutrient amount per plant, such as P and K [23]. However, we did not determine the P and K uptake rates in previous studies, and it is still ambiguous whether *OSA1* gene overexpression could stimulate P and K uptakes. In this study, we first estimated the N, P, and K uptake rates of *OSA1* overexpression plants and knockout mutant plants by cultivating them with hydroponic culture under different concentrations of P and K by using nutrient-exhaustion methods. We also used noninvasive microtest technology (NMT) methods to analyze the flux rates of NH₄⁺, K⁺, and H⁺ with *OSA1* overexpression plants and knockout mutant plants under the high-affinity transport conditions. We found that the overexpression of *OSA1* significantly improved P and K uptake rates in roots. This study could help to demonstrate the crucial role of PM H⁺-ATPases in coordinating N, P, and K absorptions.

2. Results

2.1. *OSA1*-Overexpression Promotes Rice Growth under Different Concentrations of P and K

Hydroponically grown *OSA1* overexpression lines (*OSA1*-oxs), *osa1* mutants, and WT rice plants were investigated for phenotypic differences under different concentrations of P and K (normal concentrations of P and K (PK treatment), 0.5-fold P and 0.5-fold K (0.5PK treatment), and 2-fold P and 2-fold K (2PK treatment)). *OSA1*-oxs exhibited improved root and shoot growth when compared with WT (Figure 1A). Under PK treatment, root biomass of *OSA1*-oxs was 30% higher than that of WT. However, when supplied with 2PK, the root biomass reached to 130% higher than that of WT (Figure 1B). In addition, *osa1* mutants showed reduced root biomass by approximately 37–44% under 0.5PK treatment when compared with WT (Figure 1B). It is interesting that the root dry weight of WT under 2PK treatment decreased by 41% compared with that of control (PK treatment), whereas *OSA1*-oxs did not exhibit any growth defects (Figure 1B). These results suggest that high concentrations of P and K inhibit root growth, but higher activities of PM H⁺-ATPases could alleviate this inhibition. The shoot biomass also significantly increased in *OSA1*-oxs under all treatments but decreased in *osa1* mutants under 2PK treatment when compared with WT (Figure 1C). Furthermore, we determined the root surface area of *OSA1*-oxs and *osa1* mutants using root scanner; the root surface area is positively correlated with root dry weight. Under PK and 2PK treatments, the root surface area of *OSA1*-oxs was significantly higher than that of WT, and the root surface area of *osa1* mutants was reduced under 0.5PK

and PK treatments (Figure 1D). These results demonstrate that overexpression of *OSA1* can improve plant growth under moderate, low, and high concentrations of P and K.

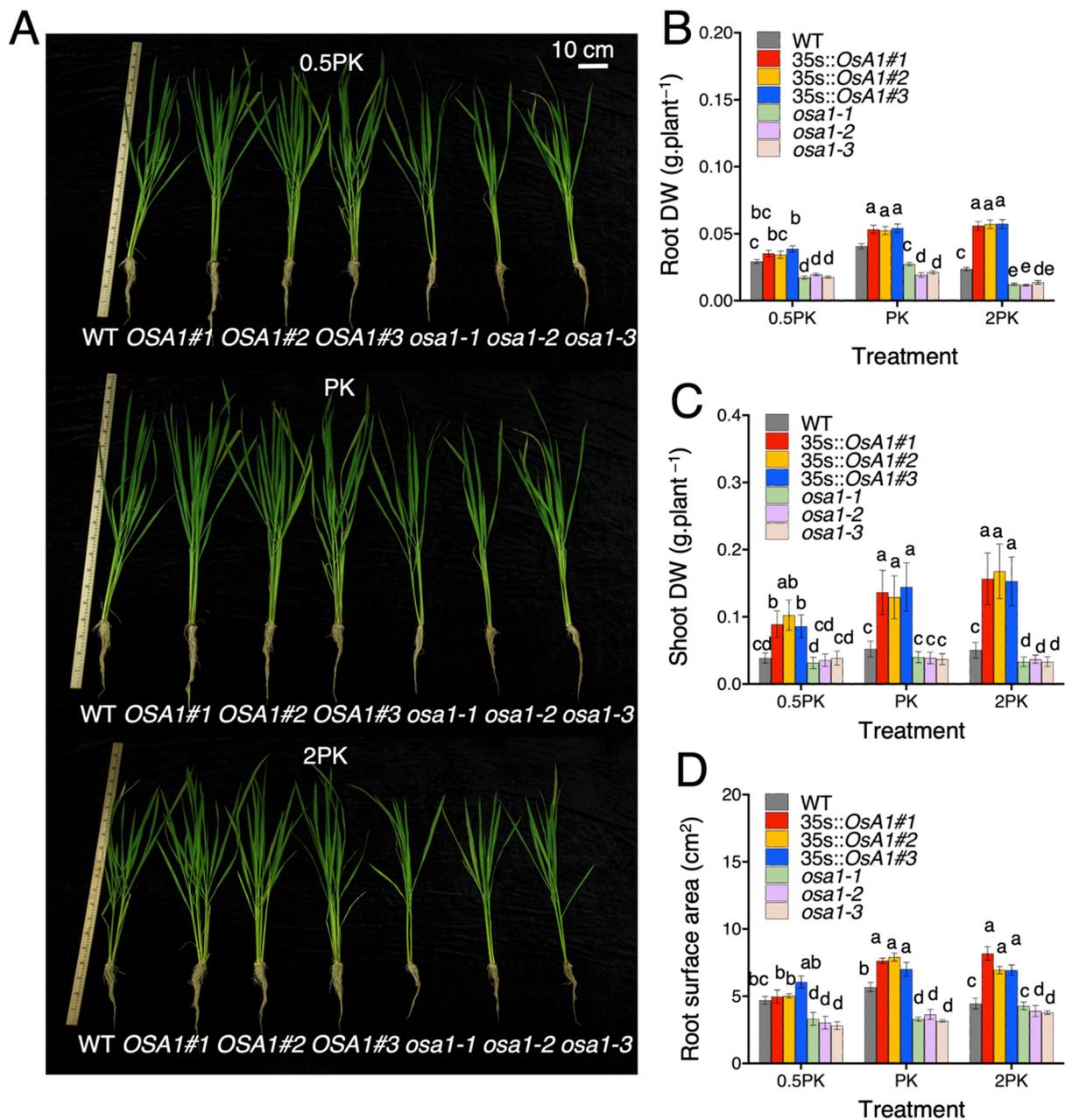


Figure 1. Phenotype of WT, *OSA1*-oxs, and *osa1* mutants. Rice plants were grown hydroponically in a greenhouse for 4 weeks under a 16 h fluorescent light ($400 \mu\text{mol m}^{-2} \text{s}^{-1}$)/8 h dark cycle at 24°C , in 55–70% relative humidity. (A) Seedlings were incubated in IRRRI nutrient solution containing 2 mM NH_4^+ as N source, and half (0.5PK), normal (PK), and twofold (2PK) concentrations of P and K. Scale bar = 10 cm. Root dry weight (B), shoot dry weight (C), and root surface area (D) were determined using 5-week-old plants grown in 1/2 IRRRI nutrient solution for 1 week, then transferred to full IRRRI nutrient solution containing different concentrations of P and K (0.5PK, PK, 2PK) for 4 weeks. Values are the mean \pm SD ($n = 9$). Different letters indicate significant differences at $p < 0.05$ according to two-way ANOVA with Tukey's multiple-comparisons test.

2.2. PM H⁺-ATPase Activity in Rice Roots of OSA1-Modified Plants under Different Concentrations of P and K

We next performed qRT-PCR assay to determine *OSA1* gene expression levels in WT, *OSA1*-oxs, and *osa1* mutants under different concentrations of P and K. Results showed that the relative expression levels of *OSA1* were significantly enhanced in the roots of *OSA1*-oxs but were decreased in *osa1* mutants as compared with WT under different concentrations of P and K (Figure 2A). Further investigation of rice root PM H⁺-ATPase activity was conducted under different concentrations of P and K. Results showed that PM H⁺-ATPase activity of WT increased by 18% under the 0.5PK treatment and was suppressed by approximately 33% under the 2PK treatment compared to that of the PK treatment (Figure 2B), which indicates that high concentrations of P and K inhibit PM H⁺-ATPase activity. In addition, *OSA1*-oxs showed significantly higher PM H⁺-ATPase hydrolysis activity under all treatments when compared with WT. By contrast, *osa1* mutants showed significantly reduced PM H⁺-ATPase activity compared to WT under the 0.5 PK and PK treatments (Figure 2B).

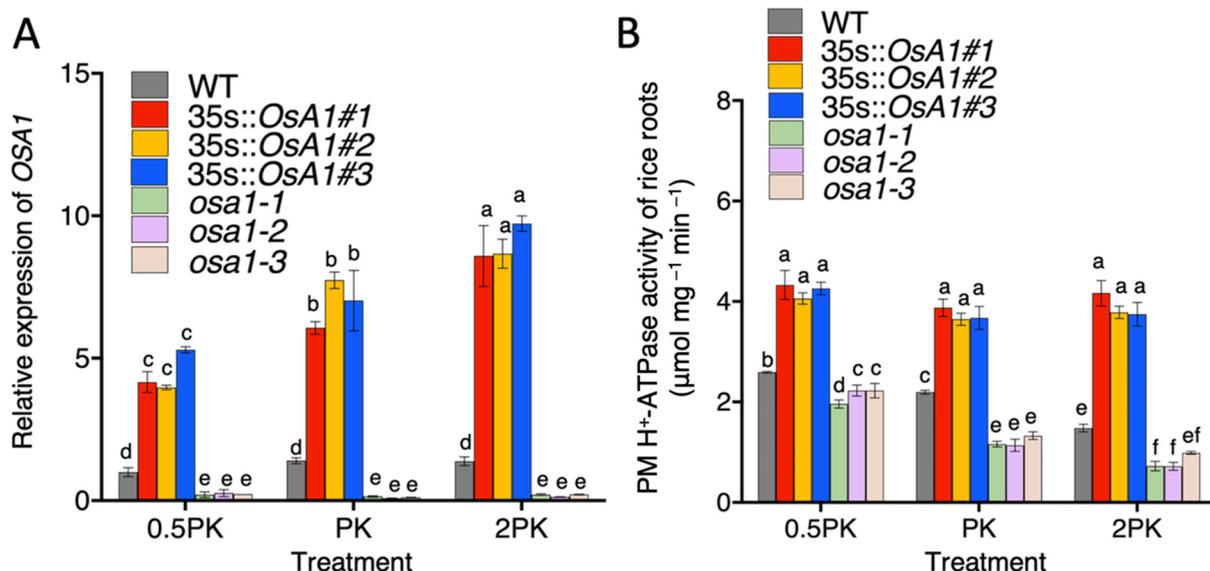


Figure 2. *OSA1* expression level and PM H⁺-ATPase activity in WT, *OSA1*-oxs, and *osa1* mutants under different concentrations of P and K (0.5PK, PK, and 2PK). (A) Relative expression of *OSA1* in WT and *OSA1* transgenic plants roots. Values indicate means ± SD ($n = 3$). (B) Hydrolytic activity of PM H⁺-ATPase in roots of *OSA1*-oxs and *osa1* mutants. Different letters indicate significant differences at $p < 0.05$ according to two-way ANOVA with Tukey's multiple-comparisons test.

2.3. Overexpression of OSA1 Stimulates P, K, and N Uptake Rates under Different Concentrations of P and K

Using the nutrient-exhaustion method, we examined the uptake rates of N, P, and K in rice roots after incubation in a nutrient solution with different concentrations of P and K for 1, 2, and 4 h. As a result of incubation for one hour, the N uptake rate averagely increased by approximately 20.7%, 22.8%, and 24% in *OSA1*-oxs roots, and averagely decreased by approximately 16.1%, 14.7%, and 13.2% in *osa1* mutant roots under the 0.5PK, PK, and 2PK treatments, respectively (Figure 3A). *OSA1*-oxs roots absorbed K with an average rate of about 14.2%, 24.5%, and 34.9% higher than that of WT under the 0.5PK, PK, and 2PK treatments, respectively, but the uptake rate showed no significant difference in *osa1* mutants (Figure 3B). The P uptake rate increased averagely by 11.5% in *OSA1*-oxs under the 0.5PK treatment and there was no significant difference between the *osa1* mutants and WT under all treatments (Figure 3C). After incubation for 2 h, the N uptake rate of *OSA1*-oxs roots averagely increased by approximately 31.3%, 38.4%, and 43.7% under the 0.5PK, PK, and 2PK treatments, respectively, and it exhibited no significant difference in *osa1* mutant

roots (Figure 3D). The average K absorption rate of *OSA1*-oxs roots was 27.4%, 20.5%, and 24.6% higher under 0.5PK, PK, and 2PK treatments, respectively, whereas there was no difference between *osa1* mutants and WT (Figure 3E). The average P uptake rate increased by 38%, 15.8, and 8.4% in *OSA1*-oxs under 0.5PK, PK, and 2PK treatments, respectively, and no significant difference was found in *osa1* mutants (Figure 3F). After incubation for 4 h, N uptake rate by *OSA1*-oxs roots averagely increased by approximately 36.7%, 60.4%, and 53.5% under 0.5PK, PK, and 2PK treatments, respectively, and no significant difference was found in *osa1* mutant roots (Figure 3G). In addition, the average K absorption rate of *OSA1*-oxs roots was 23.3%, 27%, and 17.2% higher than WT, and was reduced by 26.6%, 21.3%, and 19.5% in *osa1* mutant roots under 0.5PK, PK, and 2PK treatments, respectively (Figure 3H). As shown in Figure 3I, the average P uptake rate increased by 54%, 25.2%, and 19.9% in *OSA1*-oxs under 0.5PK, PK, and 2PK treatment, respectively. These results indicate that overexpression of *OSA1* could improve macronutrient uptake rates under moderate, low, and high P and K concentrations.

After 1, 2, and 4 h of incubation at different concentrations of P and K, we determined the total amount of N, P, and K in the whole plants. Under 0.5PK, PK, and 2PK treatments, the NH_4^+ amount averagely increased by 114%, 140%, and 165%, respectively, in *OSA1*-oxs and decreased by 42.4%, 28.5%, and 29.4%, respectively, in *osa1* mutants compared to WT (Figure S1A). P content averagely increased by 78%, 103%, and 124% in *OSA1*-oxs, and averagely decreased by 33.8%, 34.7%, and 31.7% in *osa1* mutants under 0.5PK, PK, and 2PK treatments, respectively (Figure S1B). In addition, the K content in *OSA1*-oxs averagely increased by 119%, 115%, and 112%, and averagely decreased by 24.8%, 40.3%, and 41% in *osa1* mutants when compared with WT under 0.5PK, PK, and 2PK treatments, respectively (Figure S1C).

2.4. Overexpression of *OSA1* Increases the Expression of Nutrient Transporter Genes in Roots under Different Concentrations of P and K

Next, we determined the relative expression levels of the corresponding genes related to N, P, and K uptakes under different concentrations of P and K in *OSA1* overexpression and knockout mutant plants by qRT-PCR assay. *OsAMT1;1* and *OsAMT1;2* are two critical NH_4^+ transporter genes that are highly induced by N deficiency [24]. Here, we found that the relative expression level of *OsAMT1;1* was about 2–3 times higher in *OSA1*-oxs roots and decreased significantly in *osa1* roots when compared with that of WT under all treatments of different concentrations of P and K (Figure 4A). In addition, the relative expression level of *OsAMT1;2* in *OSA1*-oxs roots was also significantly higher than that of WT under all these conditions and decreased significantly in *osa1* mutants under the PK condition (Figure 4B). *OsPHT1;1* and *OsPHT1;2*, belonging to *OsPHT* gene family, are involved in phosphate uptake in rice roots [25]. Here, we found that *OsPHT1;1* expression was significantly enhanced in *OSA1*-oxs roots but showed no significant difference in *osa1* mutants when compared with WT (Figure 4C). However, the expression of *OsPHT1;2* showed no significant difference among *OSA1*-oxs, *osa1* mutants, and WT (Figure 4D). Then, we detected expression levels of K transporter genes, *HAK1* and *HAK5* [26]. *OSA1*-oxs plants exhibited at least a two times higher expression level of *HAK1* in the roots under all these treatments, and *osa1* mutants showed a reduced expression level of *HAK1* under PK treatment (Figure 4E), but no significant difference was found for *HAK5* (Figure 5F). All these results suggest that the overexpression of *OSA1* could activate the expression of N, P, and K transporter genes under moderate, low, and high P and K concentrations.

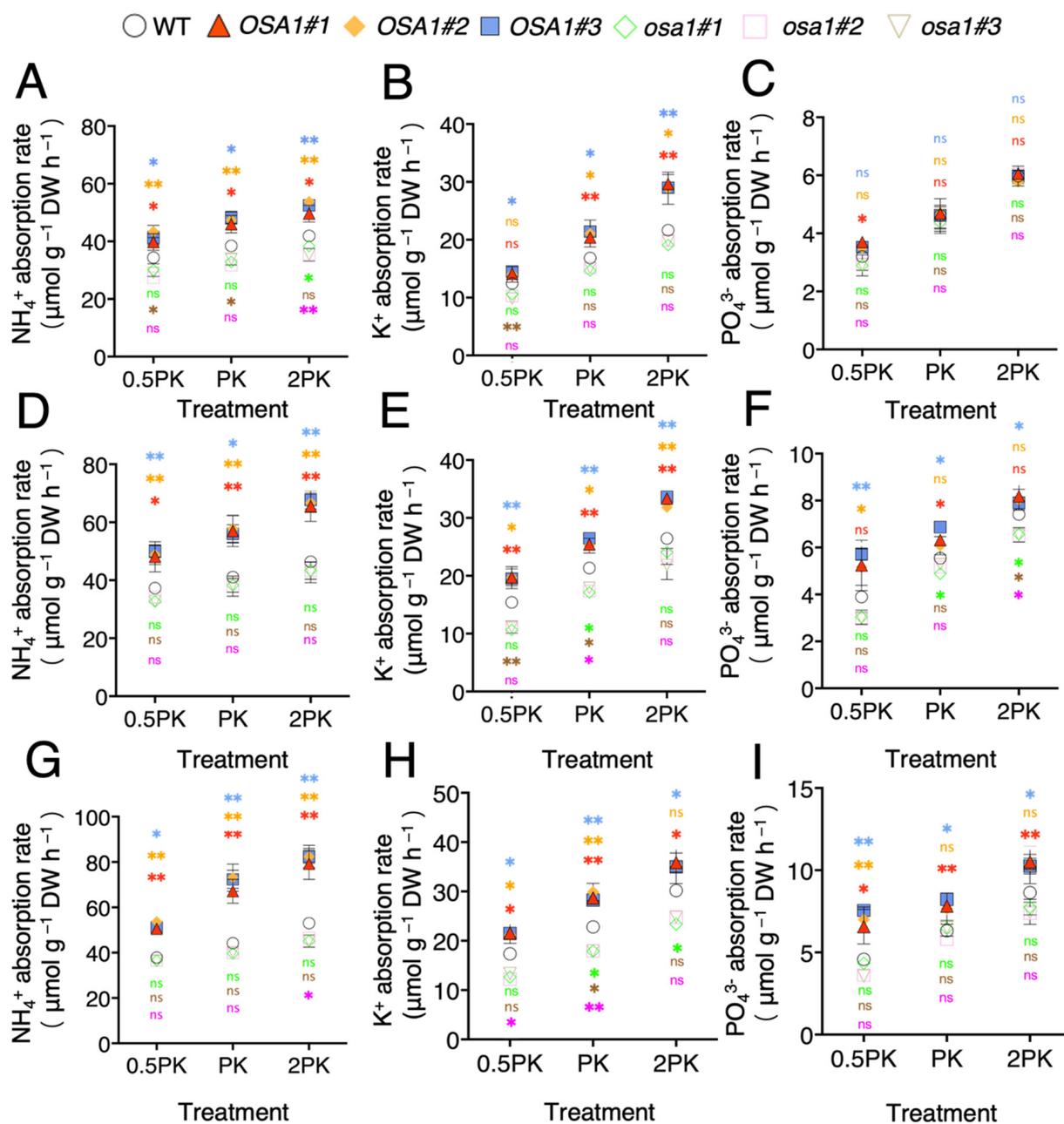


Figure 3. NH_4^+ , K^+ and PO_4^{3-} absorption rates in roots of WT, *OSA1*-oxs, and *osa1* mutants. Rice plants were grown hydroponically in greenhouse for 4 weeks. Seedlings were incubated in IRRI nutrient solutions containing 2 mM NH_4^+ as N source, and half (0.5PK), normal (PK), and twofold (2PK) concentrations of P and K for 1 h (A–C), 2 h (D–F), and 4 h (G–I). Values are the mean \pm SD ($n = 3$). Differences between *OSA1*-oxs and WT, and between *osa1* mutants and WT were assessed using the two-tailed Student’s *t*-test (ns, no significant difference, * $p < 0.05$; ** $p < 0.01$).

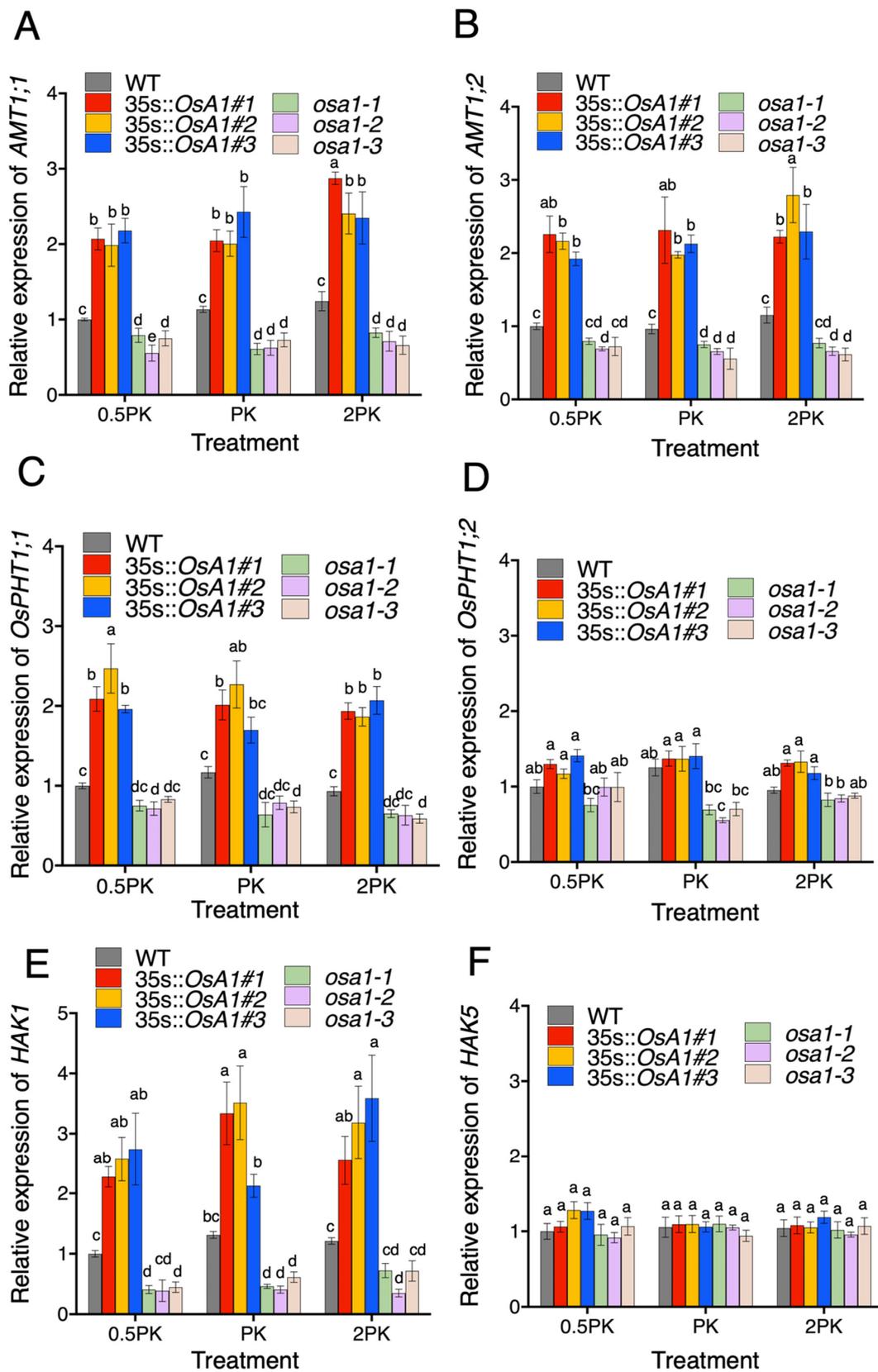


Figure 4. Relative expression level of N, P, and K transporter genes in roots of WT, *OSA1*-oxs, and *osa1* mutants. Four-week-old rice plants were cultured in IRR1 nutrient solution containing 2.0 mM NH_4^+

and half (0.5PK), normal (PK), and twofold (2PK) concentrations of P and K for 4 h. (A,B) Relative expression levels of *AMT1;1* and *AMT1;2*. (C,D) Relative expression levels of *OsPHT1;1* and *OsPHT1;2*. (E,F) Relative expression levels of *HAK1* and *HAK5*. Values are presented as the means \pm SD ($n = 3$). Different letters indicate significant differences at $p < 0.05$ according to two-way ANOVA with Tukey's multiple-comparisons test.

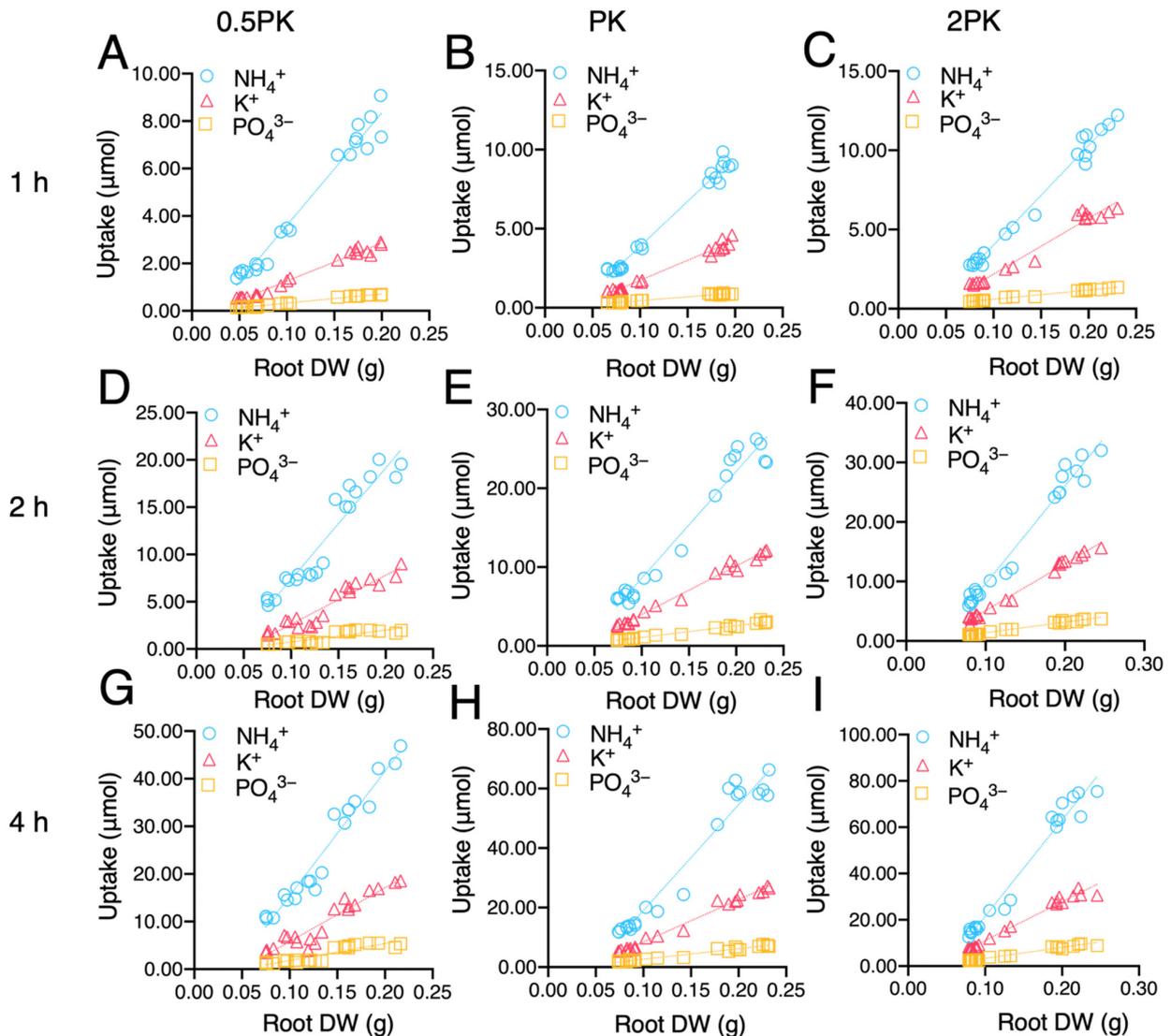


Figure 5. Correlation between N, P, and K uptake contents and root dry weight (DW). Rice seedlings including WT, *OSA1-oxs*, and *osa1* mutants were incubated under 0.5PK (A,D,G), PK (B,E,H), and 2PK (C,F,I) IRRR nutrient solutions, with 2.0 mM NH_4^+ being the N source for 1 h (A–C), 2 h (D–F), and 4 h (G–I). The difference of N, P, and K contents between blank solution and incubation solution was used to calculate for the nutrient uptake content by roots.

2.5. Correlation between N/P/K Uptakes and Root Morphology

A correlation analysis was conducted between N, P, and K uptakes and root morphology. These nutrient uptakes were all positively correlated with root dry weight and root surface area (Figure 5 and Figure S2). Interestingly, the increment trend of nutrient uptake with root dry weight or root surface area was in the order of $\text{N} > \text{K} > \text{P}$ under all treatments (Figure 5 and Figure S2). The R^2 values and regression equations for their correlations were shown in Tables S1 and S2. The correlation coefficients were all significant at p value < 0.001 . According to these results, improved NH_4^+ , PO_4^{3-} , and K^+ uptake could

not only attribute by the enhanced PM H^+ -ATPase activity, but also by the increased root growth conferred by *OSA1* overexpression.

2.6. *OSA1* Overexpression Promotes Cation Fluxes in Rice Roots under High-Affinity Transport Concentrations

To measure the net flux rates of NH_4^+ , K^+ , and H^+ in roots of WT and *OSA1* transgenic plants under high-affinity transport concentrations of NH_4^+ and K^+ (0.1 mM NH_4^+ and 0.1 mM K^+), we used a noninvasive microtest system (NMT) to perform the microelectrode ion flux measurement (MIFE) with intact roots of 5-day-old seedlings. The nutrient flux rate was measured along the root tip, around 150 μ m from the root cap (Figure 6A). As a result, the NH_4^+ influx rate, K^+ influx rate, and H^+ efflux rate increased in three *OSA1*-oxs by 64.2%, 28.3%, and 96.1%, respectively (Figure 6B–D), and decreased significantly in the roots of three *osa1* mutants by approximately 35.7%, 27.3%, and 21%, respectively, when compared with that of WT (Figure 7A–C). These results suggest that *OSA1* plays a positive role in controlling NH_4^+ and K^+ uptake rates, as well as the H^+ excretion rate in rice roots under a high-affinity transport system.

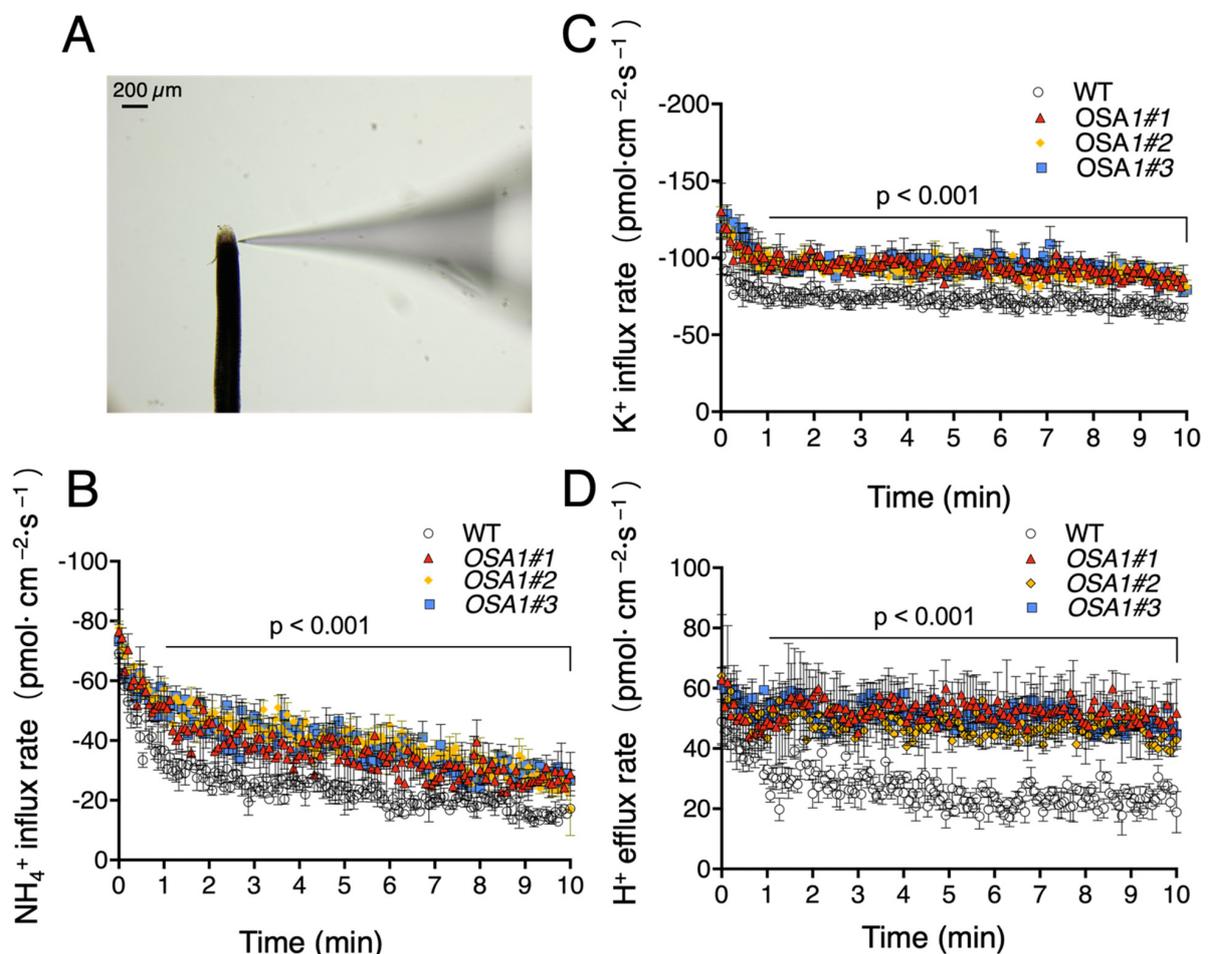


Figure 6. Quantification of NH_4^+ influx, K^+ influx, and H^+ efflux rates of WT and *OSA1*-oxs roots. The 5-day-old seedlings of WT and *OSA1*-oxs were treated with hydroponic solution lacking NH_4^+ or K^+ for 24 h. Intact roots were equilibrated in the measuring solution (0.1 mM NH_4^+ and 0.1 mM K^+ are in the test solution) for 10 min. (A) The nutrient flux rate was measured along the root tip, around 150 μ m from the root cap. Black-colored object is a rice root and the right one is a microelectrode probe used to determine the change of ion flux in the root surface. The NH_4^+ influx rate (B), K^+ influx rate (C), and H^+ influx rate (D) were calculated. Values are mean \pm SD ($n = 3$). Significant difference was evaluated using the two-tailed Student's *t* test.

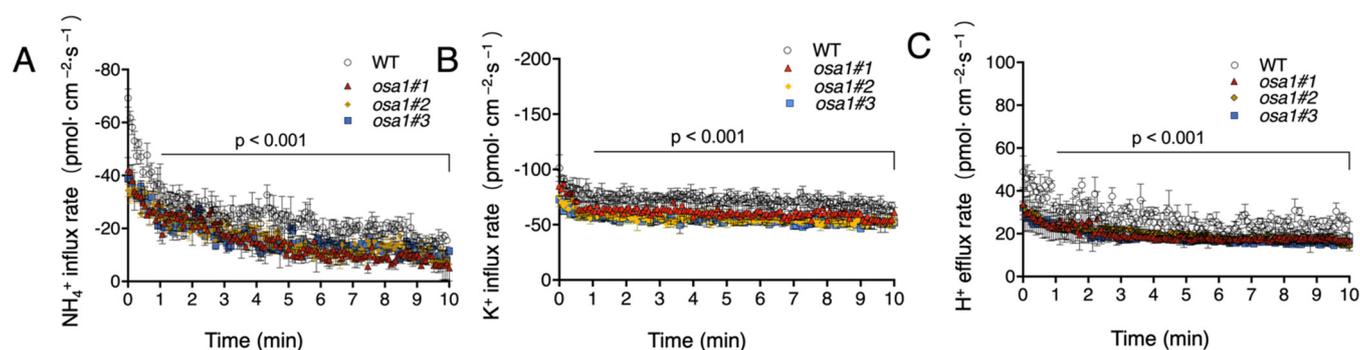


Figure 7. Quantification of NH_4^+ influx, K^+ influx, and H^+ efflux rates of WT and *osa1* mutant roots. The 5-day-old seedlings of WT and *osa1* mutants were treated with a hydroponic solution lacking NH_4^+ for 24 h. Intact roots were equilibrated in the measuring solution for 10 min. The NH_4^+ influx rate (A), K^+ influx rate (B), and H^+ efflux rate (C) were tested. Values are means \pm SD ($n = 3$). Significant difference was evaluated using the two-tailed Student's *t*-test.

3. Discussion

Numerous studies have been conducted on the effects of N, P, and K on crop growth and development [27,28]. Our previous study demonstrated that the overexpression of *OSA1* significantly enhanced NH_4^+ uptake and improved grain yield in rice [21]. The present result showed that the overexpression of *OSA1* in rice significantly increased the PM H^+ -ATPase activity (Figure 2B), and activated the expression of several nutrient transporter genes, such as *OsAMT1;1*, *OsAMT1;2*, *OsPHT1;1*, and *OsHAK1* (Figure 4), and improved the uptake rates of macronutrients such as N, P, and K by roots under different concentrations of P and K (Figure 3).

Notably, the 0.5PK treatment activated PM H^+ -ATPase in WT roots compared with that of normal conditions (PK treatment) (Figure 2B), which is consistent with previous findings that low K^+ or low P concentrations in apoplast could significantly improve PM H^+ -ATPase activity [3,29]. In addition, we found higher concentrations of P and K improved NH_4^+ uptake rates in WT, and this effect was even enhanced in *OSA1*-oxs plants (Figure 3A,D,G). Previous studies showed that a high K concentration reduced the NH_4^+ uptake rate in rice due to the competitive absorption between K^+ and NH_4^+ [30]. It is possible that the increased uptake of phosphate could help to maintain the charge balance in the cytoplasm and further facilitate the uptake of K^+ . Theoretically, absorption of N, P, and K will increase H^+ concentrations in root cytoplasm, which need to be pumped out of cells by PM H^+ -ATPase to maintain the membrane potential and cytosolic pH. Interestingly, overexpression of *OSA1* activated the expression of several transporter genes related to N, P, and K uptakes in rice under low-affinity P and K conditions (Figure 4). However, the underlying molecular mechanism of the linkage between PM H^+ -ATPase activity and the expression of nutrient transporter genes deserves to be investigated in the future.

PM H^+ -ATPase has been known to be involved in auxin-induced root growth in the model plant *Arabidopsis* [9,10,31,32]. In rice, it has been demonstrated that overexpression of *OSA1* enhanced root biomass under different concentrations of NH_4^+ [21]. In this study, we found that biomass also increased significantly under different concentrations of P and K in *OSA1*-oxs and decreased in *osa1* mutants (Figure 1). Interestingly, we found that 2PK treatment significantly inhibited root growth in WT and *osa1* mutants, but the growth inhibition was alleviated in *OSA1*-oxs (Figure 1). Increases in the uptakes of P and K may lead to cytosolic H^+ accumulation and even H^+ toxicity in the cytoplasm of rice roots, which could negatively affect root growth [33,34]. However, improved PM H^+ -ATPase activity by overexpression of *OSA1* could alleviate H^+ accumulation in root cells and then contribute to the tolerance to high concentrations of P and K. In addition, N, P, and K contents in *OSA1*-oxs showed linear relationships with root biomass and root surface area (Figures 5 and S2), which is consistent with previous studies which found that

nutrient uptake amounts are correlated with root biomass [35,36]. However, the underlying mechanism of how PM H⁺-ATPase activity is linked to the root growth of rice under different nutrient concentrations needs to be investigated in the future, which may possibly be associated with auxin signaling.

In the high-affinity transport system, it has been well documented that PM H⁺-ATPase is involved in nutrient uptakes by supplying proton motive force to facilitate ion transports across the PM [37]. However, the detailed information is unclear about the effect of PM H⁺-ATPase overexpression on nutrient transportation in rice roots. Here, we performed MIFE experiment with NMT system to measure the net ion fluxes around the root tips of *OSA1*-modified plants and WT. In the root tip region, comprising the meristem and elongation zone (0–600 μm from the tip), a strong ion flux can be detected [38]. In this study, we detected one at the position of 150 μm far from the root tip, which belongs to the elongation region close to the meristem region (Figure 6A). The results showed that the net NH₄⁺ and K⁺ influx rates were significantly increased in *OSA1*-oxs and were significantly reduced in *osa1* mutants under the high-affinity transport system (0.1 mM NH₄⁺ and 0.1 mM K⁺) when compared with that of WT (Figures 6 and 7). NMT uses specialized flux sensors that are derived from microelectrodes to measure the dynamic ion/molecule activity (i.e., diffusion flux). Diffusion flux is the amount of substance per unit area per unit time around an intact sample [39]. Therefore, the overexpression of *OSA1* improved nutrient uptake rate directly, possibly by generating more polarized membrane potential, which is independent of the root biomass. However, to our knowledge, NMT methods are not permitted for determining phosphate influx rates in rice roots, which is expected to be explored in the future.

In conclusion, we demonstrated that the overexpression of a rice PM H⁺-ATPase gene, *OSA1*, promoted root growth and stimulated N, P, and K uptake rates by roots under different concentrations of P and K in rice. Actually, this report firstly provided evidence that the engineering of a PM H⁺-ATPase gene in rice could improve plant growth under moderate, low, and high P and K concentrations. This study could also provide a novel insight into the cultivating crop plants growing in agricultural soil, either with inadequate abundance of nutrients or with excessive abundance of nutrients. Further studies are required to explore the underlying mechanism by which *OSA1* overexpression enhances root growth and nutrient uptake under moderate, low, and high concentrations of P and K.

4. Materials and Methods

4.1. Plant Cultivation

All the rice seeds used in this study were kept in our own lab [21]. Seeds of WT (*Oryza sativa* L. ssp. japonica cv. Nipponbare); overexpression lines of *OSA1*#1, *OSA1*#2, and *OSA1*#3; and mutant lines *osa1-1* (TOS17 line ND3017), *osa1-2* (TOS17 line ND3025), and *osa1-3* (TOS17 line ND3033) were surface-sterilized in 10% H₂O₂ for 30 min and then soaked in water before germination. Rice seedlings were grown in a greenhouse under a light intensity of 400 μmol m⁻² s⁻¹, a relative humidity of approximately 60–80%, and a 14 h light (30 °C)/10 h dark (22 °C) photoperiod. Pots with PO₄³⁻ and K⁺ treatments were filled with a minor modified IRRI nutrient solution as follows: 0.5PK treatment contains 0.25 mM K₂SO₄, and 0.15 mM NaH₂PO₄; 2PK treatment contains 1 mM K₂SO₄, and 0.6 mM NaH₂PO₄, and PK treatment (control) contains 0.5 mM K₂SO₄ and 0.3 mM NaH₂PO₄. The full chemical composition of the IRRI nutrient solution (pH 5.5) is listed below: 1 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 0.3 mM NaH₂PO₄, 1 mM CaCl₂, 1 mM MgSO₄, 9 μM MnCl₂, 0.39 μM Na₂MoO₄, 20 μM H₃BO₄, 0.77 μM ZnSO₄, 0.32 μM CuSO₄, and 20 μM EDTA-Fe [21]. Solutions were changed every 3 days. Rice seedlings were grown in 1/2 IRRI nutrient solution for 1 week, then transferred to full IRRI nutrient solution containing different concentrations of P and K (0.5PK, PK, 2PK) for 4 weeks.

4.2. Nutrient Uptake Rate Determination

Three plants exhibiting similar growth in each treatment were used for the exhaustion test according to [40,41] with some modifications. The rice seedlings were placed in a N-, P-, and K-free nutrient solution for 24 h, then plants were transferred into 250 mL IRRI nutrient solutions with different concentrations of P and K to analyze nutrient uptake rates. All experiments were performed in a growth chamber at 28 °C with a light intensity of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and relative humidity of 60%. The culture medium was sampled at 1 h, 2 h, 4 h. After that, one mL of exhaustion fluid was used for the determination of the N, P, and K concentration and was replenished with 1 mL of deionized water at the same time to ensure that the volume of depleted fluid was constant. Control solution was used as negative control, which contained the same nutrient solution with the treatment but without root incubation. At the end of the exhaustion test, the fresh root weight was calculated after the moisture was removed with filter paper. K concentration in the exhaustion fluid and control solution was measured by flame emission photometry. P concentration was determined using the molybdate yellow method. The concentration of NH_4^+ was determined by the continuous-flow analytical system AA3 (SEAL, Germany) (The detection limit is 0.0165 $\text{mg}\cdot\text{L}^{-1}$).

4.3. Measurements of Net Proton, K^+ , and NH_4^+ Fluxes Rate in Roots

A BIO-IM Series NMT Physiolyzer[®] system (YoungerUSA, MA, USA) was applied to determine the net H^+ , K^+ , and NH_4^+ fluxes in the root tip based on a previous study [42]. Five-day-old rice roots were subsequently placed in 50 mL of growth solution with 2 mM NH_4^+ for 12 h. After washing with deionized water, the roots were placed in Petri dishes containing 20 mL detection buffer containing 0.2 mM CaCl_2 , 0.1 mM KCl, 0.1 mM MgCl_2 , 0.5 mM NaCl, 0.3 mM MES (2-morpholinoethanesulfonic acid sodium salt), and 0.2 mM Na_2SO_4 (pH 6.0) for the determination of the H^+ and K^+ fluxes. For the NH_4^+ flux assay, the detection buffer containing 0.1 mM NH_4NO_3 , 0.1 mM KCl, 0.1 mM CaCl_2 , and 0.3 mM MES (pH 6.0) was used. The fluxes of rice plants were measured along the root tip around 150 μm from the root cap. The microelectrodes in the NMT system were positioned $0 \pm 2 \mu\text{m}$ away from the samples. At least three individual plants were analyzed for each sample.

4.4. Quantitative Reverse-Transcription PCR

qRT-PCR was performed using the Step One Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), as described previously [43]. Briefly, total RNA was extracted from the roots of 4-week-old WT and transgenic plants using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNAs were synthesized from total RNA using the PrimeScript II First Strand cDNA Synthesis Kit using oligo (dT) primers (TakaRa). Relative quantification was performed using an SYBR Premix Ex Taq II (Perfect Real Time) Kit (TaKaRa Biotechnology, Dalian, China) on a Step One Plus Real-Time PCR System (Applied Biosystems, Bio-Rad, CA, USA). For amplification of *OSA1* (Os03g0689300), *OsAMT1;1* (Os04g0509600), *OsAMT1;2* (Os02g0620600), *OsPHT1;1* (Os03g0150600), *OsPHT1;2* (Os03g0150800), *OsHAK1* (Os04g0401700), and *OsHAK5* (Os01g0930400), the gene-specific primers were used (Table S3). Relative gene expression levels were normalized to that of the internal control gene, *OsActin* (Os03g0718100), using the comparative cycle threshold ($\Delta\Delta\text{Ct}$) method [44]. All analyses were repeated at least three times.

4.5. Measurement of PM H^+ -ATPase Activity

The detection of ATP hydrolytic activity of PM H^+ -ATPase was completed mainly according to [16], with a few modifications. Root tissues of 4-week-old WT and transgenic plants treated with different concentrations of P and K (0.5 PK, PK, and 2PK) were ground in ice-cold homogenization buffer to isolate the PM in a two-phase partitioning method. The protein concentration of the membrane vesicle was determined by using Bio-Rad protein assay dye reagent concentrate (BIO-RAD, Hercules, CA, USA) according to the manufacturer's instruction. Na_3VO_4 (0.1 mM) was used as an inhibitor of H^+ -ATPase in

0.5 mL reaction solution, which contained 30 mM BTP/MES, 5 mM MgSO₄, 50 mM KCl, 50 mM KNO₃, 1 mM Na₂MoO₄, 1 mM NaN₃, 0.02% (*w/v*) Brij 58, and 5 mM disodium-ATP (substrate for PM H⁺-ATPase). Firstly, 30 µL of a membrane vesicle suspension containing 1–2 µg total protein was added to the reaction solution for 30 min at 30 °C; the inorganic phosphate was liberated after the hydrolysis of ATP. The reaction was stopped by adding 1 mL reagent (2% (*v/v*) concentrated H₂SO₄, 5% (*w/v*) sodium dodecyl sulfate, and 0.7% (*w/v*) (NH₄)₂MoO₄), followed by 50 µL 10% (*w/v*) ascorbic acid. After 10 min, 1.45 mL arsenide-citrate reagent (2% (*w/v*) sodium citrate, 2% (*w/v*) sodium arsenide, and 2% (*w/v*) glacial acetic acid) was added. The color will be stable after 30 min and measured spectrophotometrically at 720 nm. In each test, H⁺-ATPase activity was calculated as the amount of phosphate liberated within 30 min mg⁻¹ membrane protein in excess of the boiled-membrane protein control.

4.6. Statistical Analysis

Data were analyzed for statistics and significance by two-tailed student's *t* test and two-way ANOVA test with a significant difference at *p* < 0.05 using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms232213904/s1>.

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