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Research Article

The Potassium Transporter OsHAK5 Alters Rice Architecture via ATP-Dependent Transmembrane Auxin Fluxes

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ABSTRACT

Plant HAK/KUP/KT family members function as plasma membrane (PM) H+/K+ symporters and may modulate chemiosmotically-driven polar auxin transport (PAT). Here, we show that inactivation of OsHAK5, a rice K+ transporter gene, decreased rootward and shootward PAT, tiller number, and the length of both lateral roots and root hairs, while OsHAK5 overexpression increased PAT, tiller number, and root hair length, irrespective of the K+ supply. Inhibitors of ATP-binding-cassette type-B transporters, NPA and BUM, abolished the OsHAK5-overexpression effect on PAT. The mechanistic basis of these changes included the OsHAK5-mediated decrease of transmembrane potential (depolarization), increase of extracellular pH, and increase of PM-ATPase activity. These findings highlight the dual roles of OsHAK5 in altering cellular chemiosmotic gradients (generated continuously by PM H+-ATPase) and regulating ATP-dependent auxin transport. Both functions may underlie the prominent effect of OsHAK5 on rice architecture, which may be exploited in the future to increase crop yield via genetic manipulations.

Key words: rice, potassium transporter, architecture, root, shoot, auxin

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INTRODUCTION

Potassium (K+) is the most abundant cation in plants (100–150 mM in cell sap) and an essential macronutrient cycling between the root and the shoot (Maathuis and Sanders, 2006; Cherel, 2014). Among the five families of transport proteins involved in K+ homeostasis (Nieves-Cordones et al., 2016; Li et al., 2017b; and references therein), HAK/KUP/KTs are the most prominent as they have been widely associated with K+ transmembrane transport in bacteria, fungi, and plants (Véry et al., 2014; Nieves-Cordones et al., 2016). A few plant HAK/KUP/KT K+ transporters have been extensively characterized in studies of K+ acquisition, K+ translocation, salt tolerance, and osmotic regulation (Li et al., 2017b). In Arabidopsis, both plasma membrane (PM)-located HAK5 and AKT1 participate in K+ uptake from low-K+ external solutions but only high-affinity HAK5 can facilitate K+ uptake from solutions with K+ concentrations below 10 μM (Véry et al., 2014).

The roles of some HAK/KUP/KT K+ transporters in modulating chemiosmotically-driven polar auxin transport (PAT) have long been suspected, as the Arabidopsis kup4 mutant, initially identified as tiny root hair 1 (trh1), exhibits defects in auxin-dependent root gravitropism (Rigas et al., 2001; Desbrosses et al., 2003). The trh1 mutant exhibited aberrant rates of auxin efflux and distribution of auxin reporter signals in the root apex (Vicente-Agullo et al., 2004; Rigas et al., 2013; Daras et al., 2015). Although impaired K+ transport activity could not be detected in the Arabidopsis short hypocotyl 3 (shy3)/kup2 mutant, defects in cell expansion and shoot development, possibly associated with auxin signaling, were observed in the mutant (Elumalai et al., 2002). Furthermore, the loss of function of three

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Figure 1. Effect of the OsHAK5 Expression Level on Rice Shoot Architecture.

(A) Longitudinal sections of the root–shoot conjunction photographed by a stereoscope. The red arrows indicate axillary bud primordia. pOsHAK5pro:GUS (driven by the OsHAK5 promoter) staining of a longitudinal section of a root–shoot conjunction of a WT-N plant. (B) Phenotypes of 3-week-old seedlings. White arrowheads indicate the outgrowth of tiller buds. TB, tiller bud.

(legend continued on next page)
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Arabidopsis K⁺ efflux transporter genes (KUP2, KUP6, and KUP8) resulted in enhanced auxin responses and larger root cells, along with reduced lateral root growth upon exposure to an auxin transport inhibitor (Osakabe et al., 2013). Regardless, the overexpression of Arabidopsis HAK5 could stimulate root-hair elongation under low-K⁺ conditions (Zhao et al., 2016). These reports link H⁺/K⁺ transporters with PAT regulation (Rubery and Sheldrake, 1973, 1974) as well as with programmed and plastic organ growth. Consequently, the enhanced activity of HAK/KUP/KT H⁺/K⁺ symport proteins would be expected to decrease the chemiosmotic transmembrane gradients (namely, to depolarize, i.e., lower the PM potential, and to elevate the apoplastic pH). Conversely, the suppressed activity of HAK/KUP/KT H⁺/K⁺ symport proteins would be expected to increase the chemiosmotic transmembrane gradients to affect PAT oppositely.

An obvious candidate for the study of such mechanisms is the high-affinity rice K⁺ transporter OsHAK5, as the growth phenotype in plants with inactive OsHAK5 is not fully rescued by the increased K⁺ supply (Yang et al., 2014). Our experiments, employing disruption or overexpression of OsHAK5 in rice from two different genetic backgrounds, strongly suggest that OsHAK5-mediated regulation of root morphology and shoot architecture is mediated by changes in extracellular pH, PM polarization, PM-ATPase activity, auxin (indole-3-acetic acid [IAA]) flux rates, and auxin distribution.

RESULTS

OsHAK5 Knockout Reduced Tiller Number, Overexpression Increased Tiller Number, and Both Decreased Plant Height

Previously, knockout (KO) or knockdown (KD) of OsHAK5 by T-DNA insertion or RNA-interference (hak5) or overexpression (OX) of OsHAK5 (OX) was shown to alter rice K⁺ uptake and growth (Yang et al., 2014). Here, we focus on OsHAK5-expression-related details of rice plant architecture, plant height, and tiller number, with the latter parameter having a direct impact on rice yield. Under normal K⁺ conditions, in the genetic backgrounds of Hwayoung, Dongjin, and Nipponbare, a total or partial loss of function of OsHAK5 decreased the tiller number and grain yield (Figure 1; Supplemental Table 1; Supplemental Figures 1 and 2). By contrast, OsHAK5 overexpression resulted in a greater tiller number, higher yield, and shorter plant height (Supplemental Table 2; Figure 1; Supplemental Figure 3). Morphological phenotypes observed in Dongjin and Hwayoung KO lines were highly similar and were different from Nipponbare OX lines. For this reason, a Dongjin (D) KO line and a Nipponbare (N) OX line were selected for detailed examination and comparison with their wild-type (WT) backgrounds in the remainder of this study.

OsHAK5 Knockout or Overexpression Did Not Affect Axillary Bud Formation but Suppressed Tiller Outgrowth

The pOsHAK5::GUS reporter indicated abundant OsHAK5 expression in emerging auxiliary tiller buds (Figure 1A), in addition to the expression domains described previously (Yang et al., 2014). Axillary tiller bud outgrowth is regulated by rootward auxin transport streams in combination with strigolactone signaling (Beveridge, 2006). In brief, oshak5 had suppressed, but all three OX lines had accelerated bud outgrowth (Figure 1B), which lasted for the entire period of the vegetative stage (Figure 1C and 1D; Supplemental Tables 1 and 2), consistent with OsHAK5 function in regulating auxin transport rates. By contrast, axillary bud formation was unaltered in both oshak5 and the OX lines (Figure 1A).

Changes in Root Morphology Largely Correlated with Altered OsHAK5 Expression

Seminal root growth is highly regulated by K⁺ homeostasis, whereas lateral root density is less affected by it (Gruber et al., 2014). Therefore, seminal root growth should rely on OsHAK5 (H⁺/K⁺ symport), i.e., on both OsHAK5 expression and the extracellular pH. As expected, at pH 4.5, knocking out and overexpressing OsHAK5 inhibited and enhanced seminal root elongation, respectively (Figure 2A and 2B). Unexpectedly, seminal root elongation was diminished in OsHAK5-KO plants at pH 6.5 (Figure 2B), suggesting a role for OsHAK5 in seminal root growth beyond just H⁺/K⁺ symport. In addition, at pH range from 4.5 to 6.5, OsHAK5 loss of function and overexpression decreased and increased lateral root density, respectively (Figure 2B). Interestingly, the decreased lateral root density of OsHAK5-KO at pH 5.5 was independent of external K⁺ (Figure 2C and 2D). Compared to the WT-D, OsHAK5-KO plants also had shorter lateral roots, and this phenotype was exacerbated by the absence of exogenous K⁺ (Figure 2C and 2E), suggesting that OsHAK5 regulates lateral root formation and growth.

Physiologically relevant concentrations of K⁺ have no direct effects on root-hair growth and development (Desbrosses et al., 2003) but a role of auxin in regulating root-hair elongation has been clearly demonstrated (Kubes et al., 2012; Velasquez et al., 2016). In OsHAK5-KO plants, seminal root hairs were formed, but their elongation was almost completely suppressed along the entire mutant root, irrespective of the K⁺ supply (Figure 2C and 2F). By contrast, OsHAK5-OX plants had significantly enhanced root-hair development (approximately 40% longer than those in WT-N), especially in the absence of exogenous K⁺ (roughly 9-fold longer than those in WT-N; Figure 2C and 2F).

Although the absolute concentration of the accumulated K⁺ in plants depended strongly on the K⁺ supply, there was no significant difference in the K⁺ concentration among the different parts of the root system between WT-D and KO lines or between WT-N and OX
lines under both normal K⁺ and K⁺-deficient conditions (Figure 2G), consistent with previous observations by Yang et al. (2014). In addition, limiting the K⁺ supply significantly decreased the root thickness of both WT and oshak5 mutant lines; however, there was no significant difference in root diameter between the WT and the mutant (Supplemental Figure 8). These results implicate the activity of the OsHAK5 transporter rather than the K⁺ concentration in the regulation of root morphogenesis.

Both Knockout and Overexpression of OsHAK5 Altered Auxin Distribution and Transmembrane Fluxes

The altered growth of lateral roots and root hairs appears to reflect OsHAK5 regulation of polar auxin delivery streams. For example, the observed lack of pH dependence of the lateral root phenotype of the oshak5 mutant (Figure 2A and 2B) resembles the various lateral root phenotypes of ATP-binding-cassette type B4 (ABCB4) auxin transporter mutants (Terasaka et al., 2005; Wu et al., 2007). Consistent with these findings, endogenous steady-state IAA levels in the root–shoot conjunction, including the non-elongated node, reflecting rootward transport from the shoot, was indeed lower in OsHAK5 KO plants compared with the WT and, conversely, auxin levels in the root–shoot transition zone increased with OsHAK5 overexpression (Figure 3A). Furthermore, both OsHAK5 KO and overexpression decreased auxin levels at the root tip (Figure 3A). The lower rootward transport of auxin in the KO line may explain the former and, while auxin is also synthesized in the root tip (Bhalerao and Bennett, 2003; Ljung et al., 2005), an enhanced shootward transport of auxin in the OX line may explain the latter (Figure 4).

The observed changes in auxin steady-state levels are consistent with altered transmembrane auxin fluxes into and out of root epidermal cells. Under conditions that maintained cell viability, IAA influx and efflux currents were measured amperometrically using the scanning ion-selective electrode technique (SIET; see Methods), similar to those previously measured in maize roots (McLamore et al., 2010). These currents were smaller in the OsHAK5-KO line and were potentiated in the OsHAK5-OX line (Figure 3B and 3C). Given that the diameters of the roots of the WT and oshak5 were similar (Supplemental Figure 8), the...
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We further examined whether exogenous free IAA could mediate the defective phenotype of KO plants. We found that adding IAA into the nutrient solution increased both root and shoot growth of the WT and oshak5 mutant (Supplemental Figure 9A). The adventitious root number, and total root and shoot dry weight of OsHAK5-KO plants treated with exogenous IAA reached values similar to those of the WT without IAA treatment. However, the IAA-treated WT and oshak5 mutant still exhibited significantly different phenotypes, although the extent of the difference was smaller (Supplemental Figure 9). Therefore, we conclude that although exogenous auxin can partially rescue the defective phenotype of the oshak5 mutant, OsHAK5 directly contributes to the difference in plant architecture, even at higher auxin levels (Supplemental Figure 9).

OsHAK5 Enhanced Both Rootward and Shootward Polar Auxin Transport in Roots

Rootward PAT was measured by applying exogenous [3H]-labeled IAA ([3H]IAA) to the cut surface of roots excised 1 cm above the root–shoot conjunction. After 6 h of incubation, 1-cm root segments were excised and collected, starting from the root apex. The [3H]IAA content of each segment was measured by scintillation counting (see Methods). During this incubation period, the radioactive [3H]IAA signal accumulated to its highest level closest to the root apex (in the 0- to 1-cm segment; Figure 4A and 4C; Supplemental Figure 4A and 4C). Based on this finding, and on the differences (Δs) between the transgenic lines and their respective WTs, the [3H]IAA signal delivered to the lowest-most segment of the root tip correlated well with OsHAK5 expression, that is, in the oshak5 line, the signal was approximately 50% of the signal in the WT (Figure 4A and Supplemental Figure 4A), and in OsHAK5-OX, it was approximately 130% of the signal in the WT (Figure 4C and Supplemental Figure 4C).

To examine the involvement of auxin efflux transporters in rootward transport, we conducted experiments in the presence and absence of the auxin efflux inhibitor N-1-naphthylphthalamic acid (NPA). NPA binds directly to ABCB auxin efflux transporters and inhibits ABCB-mediated efflux in the single cells of dicots and monocots (Noh et al., 2001; Yang and Murphy, 2009; McLamore et al., 2010; Kamimoto et al., 2012; Kubes et al., 2012). NPA also reduces auxin efflux mediated by PINFORMED (PIN) auxin efflux carriers (Teale and Palme, 2018).

In the WT-D, NPA (10 μM) reduced [3H]IAA rootward transport into the first (0–1 cm) root tip segment by approximately 25% but had no effect in Oshak5 (Figure 4B). Furthermore, NPA treatment eliminated the increased Δ signal observed in the OsHAK5-OX line (Figure 4D). These findings strongly suggest that OsHAK5 positively regulates auxin efflux transporters.

The direct impact of OsHAK5 activity on shootward auxin transport from the root apex was confirmed by tracking the movement of [3H]IAA after submerging the root apex in an agarose droplet and incubating for 5 h. The [3H]IAA content was quantified in 2-mm root sections excised from the tip (excluding the 1 mm closest to the site of application) through the elongation zone. [3H]IAA signals normalized to the mass decreased with distance and exhibited the greatest differences in the apical section (1–3 mm; Figure 4E and 4G). Evidently, the loss of OsHAK5 function resulted in the accumulation of the Δ signal at the root apex and...
decreased accumulation in more distal segments, while OsHAK5 overexpression resulted in decreased $^3$H$_2$O retention at the root apex (Figure 4E and 4G; Supplemental Figure 4E and 4G). Similar to the results of rootward assays, NPA reduced shootward transport to approximately 75% in WT and OsHAK5 loss-of-function lines. In addition, NPA reduced $^3$H$_2$O transport in the WT from the point of application in root segments collected to levels observed in the OsHAK5-OX line (Figure 4H). The overall effects of OsHAK5 inactivation appear remarkably similar to those of Arabidopsis and maize ABCB loss-of-function lines (McLamore et al., 2010; Kubes et al., 2012).

A more specific inhibitor of ABCB-mediated auxin efflux, 2-[4-(diethylamino)-2-hydroxybenzoyl]benzoic acid (BUM), has been shown to effectively inhibit ABCB-mediated, but not PIN-mediated, auxin efflux in single cells and roots (Kim et al., 2010). The effects of BUM were almost identical to those of NPA in regard to both the location of activity and the extent of transport reduction (Supplemental Figure 4A–4H), with the exception that BUM had a slightly greater impact on rootward transport into the first root segment in the OsHAK5-KO line compared with the WT (D). These results suggest that ABCB-mediated and, possibly, PIN-mediated effluxes are positively regulated by OsHAK5.

To investigate whether OsHAK5 could directly contribute to auxin transport across the PM, we expressed OsHAK5 in the well-characterized yap1-1, IAA-sensitive Saccharomyces cerevisiae mutant at different extracellular pHs using OxAUX1 as an uptake control (see Methods). OsHAK5 indeed promoted IAA accumulation at pHs 4.5 and 5.5 in yeast, resulting in a stronger suppression of IAA-induced growth (Supplemental Figure 10). The yeast cells expressing the empty vector, OsAUX1, and OsHAK5 showed a similar growth rate at pH 7.5 (Supplemental Figure 10). These data indicate that OsHAK5 stimulates IAA transport activity either directly or indirectly in a manner dependent on the existence of a chemiosmotic gradient, or, at least, on external protons.

OsHAK5 Activity Diminished the PM Potential, and Elevated the Extracellular pH and PM-ATPase Activity

The immediate expected effect of the overexpression of the OsHAK5 H$^+$/K$^+$ uptake symporter would be an increase in the
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Figure 4. Effect of the OsHAK5 Expression Level on [3H]IAA Rootward and Shootward Transport in the Root.

(A–D) Assay of [3H]IAA rootward transport. The relative [3H]IAA concentration in three seminal root segments of the WT-D and KO mutant (oshak5) (A and B), and the WT-N and OsHAK5-OX line (C and D) without or with 10 μM NPA (−NPA or + NPA), respectively. The seedlings were grown for 21 days and then the [3H]IAA solution (20 μl) was applied to the root shoot junction after root excision 1 cm above the root-shoot conjunction. After 6 h of transport, three root segments (0–1 cm, 1–2 cm, and 2–3 cm from the root tip end) were obtained from 10–12 plants of each line and weighed, and the [3H]IAA concentration in each 1-cm segment was determined.

(E–H) Assay of [3H]IAA shootward transport. The relative [3H]IAA concentration in each 2-mm seminal root segment of the WT-D and KO mutant (E and F), and the WT-N and OsHAK5-OX line (G and H) without or with 10 μM NPA (−NPA or + NPA), respectively. The [3H]IAA solution (20 μl) was applied to the root tip. After 5 h of exposure, the 1-mm regions in contact with the agar droplet were discarded, and the adjacent 2-mm root segments were excised from 10–12 plants of each line. The [3H]IAA concentration in each 2-mm segment was determined.

The plant types are identical to those shown in Figure 1. Significant differences between the WT and the mutant or OsHAK5-overexpressing line in equivalent segments are indicated by different letters (P < 0.05, one-way ANOVA).

The extracellular pH and a decrease in the transmembrane PM potential, and thus, a decrease in the chemiosmotic gradient. The PM potential was monitored in protoplasts isolated from OsHAK5-OX, OsHAK5-KO, and WT roots using the ratiometric fluorescent PM potential probe di-8-ANEPPS (see Methods; Supplemental Figure 5). The protoplast PM potential was more depolarized in OsHAK5-OX and hyperpolarized in OsHAK5-KO cells compared with their respective WT cells (Figure 5A).

The extracellular pH was assayed by adding bromocresol purple to the supporting medium (Li et al., 2005). Consistent with the results observed with di-8-ANEPPS, the immediate rhizosphere of the roots of the WT and OsHAK5 loss-of-function seedlings was acidified, but this acidification was suppressed in OsHAK5-OX (Figure 5B). In addition, the pH of the extracellular fluid bathing the roots (“apoplastic sap”) of hydroponically grown OsHAK5-OX plants was also found to be significantly increased compared with the WT (Figure 5C). The ATPase activity of isolated PM vesicles from OsHAK5-KO and -OX lines decreased and increased compared with the respective WTs (Figure 5D). PM-ATPase activity is usually presumed to be largely composed of H+-ATPase activity. If PM-ATPase was an H+-ATPase, then the predicted apoplastic pH would be higher in the KO plants and lower in the OX lines, and the predicted membrane potentials would be depolarized in KO cells and hyperpolarized in the OX cells relative to their respective WTs. Accordingly, the predicted chemiosmotic gradient across the cell membrane would be lower in KO and higher in OX lines compared with their respective WTs. However, the opposite was found, that is, hyperpolarization in KO cells and depolarization in OX cells (Figure 5A), as well as predominant relative apoplastic alkalization in OX cells (Figure 5B and 5C) and slight relative acidification in KO cells (Figure 5B and 5C).
These findings imply that the chemiosmotic gradient was lower in OX and higher in KO lines compared with WT. Thus, PM-ATPase activity was not H+-ATPase activity in that it did not generate a chemiosmotic gradient. PM-ATPase activity was stimulated by OsHAK5 overexpression and inhibited by OsHAK5 inactivation, and/or it may have been inhibited by external protons.

**OsHAK5 Affected Extracellular pH-Dependent IAA Fluxes in the Root Meristematic Zone**

If the altered apoplastic H+ concentration resulting from altered OsHAK5 levels did indeed negatively regulate PM-ATPase abundance/activity, and if, in addition, PM-ATPase activity was necessary for auxin fluxes, then artificially increasing the extracellular pH in WT plants would increase auxin fluxes and lowering the extracellular pH would decrease auxin fluxes. Contrary to this expected pH effect in WT plants, auxin efflux increased at pH 4.5 relative to pH 5.5, which is similar to the detected higher net IAA effluxes in root apices of pea plants grown in acidic media (pH 4.0) (Li et al., 2018). However, in support of the notion that OsHAK5 positively regulates auxin fluxes, at extracellular pHs 4.5 and 5.5 (the norm for roots), the IAA efflux was completely suppressed in the oshak5 mutant (Figure 6 A and 6B). Similarly, the auxin influx, which (without the addition of exogenous auxin) can only be reliably monitored
act as a proton-driven, high-affinity K⁺ transporter (Scherzer-dependent manner (Figures 2A, 2B, 5, and 6). We found that Dionaea muscipula gradients (Steinacher et al., 2012). Interactions between H⁺ fluxes of auxin are strongly affected by transmembrane pH developmental processes in plants (Tsuda et al., 2011). Cellular cell manner to establish the auxin gradient that controls multiple by at least half compared with the WT (Figure 6A and 6B).

DISCUSSION

The membrane-localized polar auxin transporters, including ABCB, and PIN efflux and AUX1 influx carriers (summarized in our model shown in Figure 7A) mediate auxin fluxes in a cell-to-cell manner to establish the auxin gradient that controls multiple developmental processes in plants (Tsuda et al., 2011). Cellular fluxes of auxin are strongly affected by transmembrane pH gradients (Steinacher et al., 2012). Interactions between H⁺ pumps, H⁺-coupled K⁺ carriers, and K⁺ channels at the PM help to maintain the cellular pH and membrane potential gradient (∆ψ) (Sze and Chanroj, 2018), which can alter the localized auxin concentration and distribution at developing organs. Plant HAKs have long been assumed to be H⁺/K⁺ cotransporters (Maathuis and Sanders, 2006). For example, DmHAK5, identified in Dionaea muscipula, has been shown to act as a proton-driven, high-affinity K⁺ transporter (Scherzer et al., 2015). Our findings that OsHAK5-OX and OsHAK5-KO affected the chemiosmotic gradient, PM polarization, and extracellular (apoplastic) pH as predicted (Figure 5A–5C) strongly support the notion that OsHAK5 is an electrogenic K⁺/H⁺ symporter resembling DmHAK5.

HAK/KUP/KT transporters have long been suspected to function in the maintenance of plant architecture (Rigas et al., 2001; Elumalai et al., 2002; Desbrosses et al., 2003). Here, we provide direct evidence that OsHAK5 in rice controls shoot architecture (Figure 1 and Supplemental Figures 1–3) and root morphology (Figure 2) by modulating PAT and dynamic auxin distribution (Figures 3 and 4; Supplemental Figure 4) in an extracellular pH-dependent manner (Figures 2A, 2B, 5, and 6). We found that OsHAK5 decreased the chemiosmotic gradient (Figure 5A and Supplemental Figure 5) and increased ATPase activity in the PM (Figure 5D). In addition, OsHAK5 overexpression increased both rootward and shootward transport of [³H]IAA, which was inhibited by the ABCB transporter inhibitor, NPA, and the specific inhibitor, BUM (Figure 4 and Supplemental Figure 4). Taken collectively, our results indicate that OsHAK5 alters rice architecture mainly via ABCB-driven auxin fluxes, a notion also supported by a feedback mechanism (from altered OsHAK5 expression levels), which significantly affected the transcript levels of OsABCB family genes in the roots of OX and KO lines relative to their respective WTs (Supplemental Figure 11).

Notably, the decreased tiller number and grain yield caused by the partial or total loss of function of OsHAK5 resembled the phenotype of a loss-of-function mutant of OsABCB14, an auxin transporter (Xu et al., 2014). Moreover, the oshak5 mutant displayed pH independence of the lateral root phenotype (Figure 2A and 2B), which resembles the various lateral root phenotypes of the abcb4 mutant (Terasaka et al., 2005; Wu et al., 2007). A rootward auxin transport stream is required for lateral root outgrowth (Wu et al., 2007). The impairment of rootward [³H]IAA transport (Figure 4 and Supplemental Figure 4) by OsHAK5 inactivation can explain why net endogenous steady-state IAA levels at both the root apex and root–shoot conjunction in the oshak5 line were decreased (Figure 3A). Moreover, similar to the artifactual increases in cellular gradients and rootward auxin transport resulting from the global overexpression of AVP1, a vacuolar pyrophosphatase (Li et al., 2005), OsHAK5 overexpression increased auxin transport at the root-shoot conjunction (Figure 3A). The consistency in the alterations of both rootward and shootward auxin transport (Figure 4) and root growth (Figure 2) reflects the positive regulation of polar auxin delivery streams by OsHAK5.

OsHAK5 appears to negatively regulate chemiosmotic gradients (it depolarized the shoot cell membrane [Figure 5A] and alkalined the root apoplast [Figure 5B and 5C, top]) but to positively regulate root PM-ATPase activity (Figure 5D).
Figure 7. Model Linking the Activity of the OsHAK5 Transporter to Auxin Fluxes across the Cell Plasma Membrane and to Root Morphology and Shoot Architecture in Rice.

(A) Association of OsHAK5 activity with trans-PM auxin fluxes. OsHAK5 affects PM polarization via K⁺/H⁺ symport, chemiosmotic gradients, and ATPase activity, which determines the activity of ABCB, in addition to the activities of AUX and PIN.
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Protonated IAA can diffuse freely across the cell PM and remain trapped in the cytosol due to its high pH, while the electrogenic efflux of negatively charged IAA\(^{-}\) (Geisler et al., 2017) is affected by the membrane potential (Figure 7A, auxin and H\(^{+}\) transport schematic). A computational auxin/pH feedback model has predicted that an altered extracellular or cytosolic pH can affect (increase or decrease) simultaneously both the auxin influx and efflux (Steinacher et al., 2012). In line with this, we found that the extracellular pH could alter the auxin flux rate and direction in the rice root (Figure 6A and 6B). Compared with the WT, both the influx and efflux of auxin were further reduced in the OsHAK5-KO line (Figures 3B, 3C, 6A, and 6B).

PM-localized H\(^{+}\)-ATPase activity buffers the pH of both the cytosol and apoplastic spaces (Haruta et al., 2010). The increased PM-ATPase activity, combined with the apparent decrease in the chemiosmotic gradient observed in the OsHAK5-OX line (Figure 5), is likely to result in the increased activity of H\(^{+}\)-ATPase, stimulated indirectly by the increased import of H\(^{+}\) into the cytosol (Rienmüller et al., 2012), but otherwise masked by the activity of the overexpressed H\(^{+}\)/K\(^{+}\) symporter and, probably, the H\(^{+}\)/IAA\(^{-}\) symporter (AUX1/LAX). However, notwithstanding such a possibility, even if it is part of the underlying auxin-flux-driving mechanism in WT rice plants, the decreased chemiosmotic gradient in OX plants is unlikely to contribute to the increased auxin fluxes. Indeed, the NPA- and BUM-mediated inhibition of the increased ABCB transport of auxin by OsHAK5 overexpression (Figure 4 and Supplemental Figure 4) supports the role of OsHAK5 in regulating ABCB activity in rice. The model we propose links OsHAK5 function to trans-PM auxin fluxes (Figure 7A) and to rice root and shoot architecture (Figure 7B).

The activity of the OsHAK5 transporter positively affected the expression of OsAUX1 and several OsPIN genes (Supplemental Figure 6). We speculate that the underlying mechanism is the increase in the apoplastic pH by OsHAK5 overexpression (Figure 5C), which reduced the protonated free IAA diffusion into the cytosol (Figure 7A) and enhanced the expression of OsAUX1, thereby restoring/elevating the IAA influx rate (Figure 3C). The resemblance of the oshak5 root phenotype (Figure 2A and 2C) with that of oaux1 (Zhao et al., 2015; Giri et al., 2018) further supports this notion. To exclude the possibility that the strong mutant phenotype was caused indirectly and non-specifically by the K\(^{+}\)-status-induced deregulation of IAA transporters, we measured the transcript levels of OsPIN2 and OsAUX1 in the presence or absence of K\(^{+}\). OsHAK5-induced changes in both OsPIN2 and OsAUX1 expression levels were similar under both K\(^{+}\) conditions (Supplemental Figure 12), further confirming that the activity of the OsHAK5 transporter, not the K\(^{+}\) status, affected the PAT-controlled rice architecture.

ABCBB and PIN transport proteins have been shown to coordinate auxin efflux by stabilizing transporter activity (Titiapiwatanakun et al., 2009). Thus, the OsHAK5-induced increase in OsPIN expression helped to compensate for the effects of the decreased chemiosmotic gradients on PAT. Unsurprisingly, an increased tiller number and a reduced height were observed in OsHAK5-OX lines (Figure 1B and 1C, Supplemental Figure 3), similar to those observed after OsPIN2 overexpression in rice (Chen et al., 2012), indicating that PIN transporters are also involved in the regulation of PAT-controlled rice architecture by OsHAK5.

OsHAK5 is not unique among K\(^{+}\) transporters in performing both functions, namely K\(^{+}\) transport and auxin transport control. Notably, root and shoot growth defects in Arabidopsis kup4/trh1, shy3-1/kup2 (Rigas et al., 2001; Elumalai et al., 2002; Desbrosses et al., 2003), and rice oshak5 (Yang et al., 2014; Figure 2 in this study) could not be rescued by culturing in high K\(^{+}\) media, and none of them displayed K\(^{+}\) starvation symptoms in low K\(^{+}\) media. Knockout of OsHAK1, the OsHAK5 homolog, has revealed a significantly lower K\(^{+}\) concentration over a broad range of K\(^{+}\) supply (Chen et al., 2015); however, unlike oshak5, the oshak1 phenotype (lateral root length and density) was not significantly affected under a normal K\(^{+}\) supply (Supplemental Figure 7). The expression of these two transporters does not overlap in the primordia of lateral roots and axillary buds (Yang et al., 2014; Chen et al., 2015), indicating that they contribute differently to K\(^{+}\) distribution in plants, and also to rice plant architecture. It has also been shown that the vacuole K\(^{+}\)/H\(^{+}\) antiporter, ZIFL1.1, was specifically required at the emergence of lateral roots, at which time it indirectly modulated the cellular auxin efflux during shootward auxin transport at the root tip (Remy et al., 2013). Furthermore, NRT1.5/NPF7.3 has been shown to function as an H\(^{+}\)/K\(^{+}\) antiporter (Li et al., 2017a) and to participate in lateral root development and auxin homeostasis under K\(^{+}\) deprivation (Zheng et al., 2016). Therefore, we conclude that not only the bulk K\(^{+}\) supply, but also the precise spatial control of K\(^{+}\) transport along the route of PAT both toward the root and toward the shoot, determine which cells or tissues are expanded.

In conclusion, OsHAK5 plays a crucial role in maintaining rice architecture by altering cellular chemiosmotic gradients and regulating ATP-dependent PAT. The activity of the OsHAK5 transporter acts as a convergence point between the developmental pathways of tillering, lateral root growth, root-hair growth, and the auxin transport pathway. This strategic positioning may in
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the future render OsHAK5 a target for manipulation to increase
the crop yield in the field.

METHODS

Plant Materials and Growth Conditions
Three cultivars of rice (Oryza sativa L. ssp. japonica) were used in this
work, namely, Nipponbare (NB), Dongjin (DJ), and Hywaung (HY) (Yang
et al., 2014). Similar 10-day-old healthy rice seedlings of each WT and
transgenic line were selected and transferred to IRRI nutrient solution
(pH 5.5) in a greenhouse for normal K+ and K+-deficient treatments as
described previously (Yang et al., 2014) and in the figure legends. For
pot experiments, rice plants were grown in flooded soil at 30°C–32°C
with a 16-h-light/8-h-dark photoperiod in a greenhouse at the Pailou
Experimental Research Base of Nanjing Agricultural University. The pro-
erties of the soil and conditions of the normal K+ treatment were identical
to those described previously (Chen et al., 2015). For quantification of the
K+ concentration in plants, the ground powder was digested with concen-
trated sulfuric acid and diluted with deionized water before determining
K+ concentration by flame emission photometry (Cing
 et al., 2009). For hormone treatment assays, the rice seeds were
sterilized with 15% H2O2 for 20 min, washed with deionized water, and
cultured in an incubator at 30°C for 2 days under dark conditions. After
germination, similar 6-day-old healthy seedlings were fed with IRRI
nutrient solution (Yang et al., 2014) in the presence or absence of 1 μM
IAA for 5 weeks. The solution was replaced every 2 days to avoid IAA
degradation. All plants were grown in a greenhouse with a 16-h-light
(30°C)/8-h-dark (22°C) photoperiod, and the relative humidity was
controlled at 60%–70%.

Gene Expression Analysis by Quantitative Real-Time PCR and
Immunohistochemical Localization of the GUS Reporter
The real-time qPCR conditions for target genes were similar to those
described previously (Tang et al., 2012). All primers used for real-time
qPCR are listed in Supplemental Table 3. All PCR products were
confirmed by sequencing. For the identification of GUS-positive cells,
GUS was immunohistochemically stained as described by Yang et al.
(2014).

Determination of the Number and Length of Total Roots, Lateral
Roots, and Root Hairs
The number and length of root hairs of different rice plants were measured
after 7 days of growth in normal K+ or K+-deficient hydroponic solutions.
The number of lateral roots, as well as that of all emerging lateral roots,
was determined according to Sun et al. (2012). The roots were
photographed using a color CCD camera attached to an Olympus
MVX10 stereomicroscope (http://www.olympus-global.com). The exper-
iment was replicated three independent times, and values are means ± SE of ten roots.

Quantification of IAA Content and Flux in Rice Plants
Endogenous free IAA in rice plant root tips and root–shoot conjunc-
tion segments were quantified by gas chromatography–mass spectrometry
(GC–MS) as described in Henrichs et al. (2012). The measurements
were carried out using a GC–MS system at the Experimental
Research Center in the Institute of Botany, Chinese Academy of
Sciences.

IAA fluxes were monitored non-invasively in the roots of plants grown for
7 days in hydroponic solutions using SIET (model BIO-003A; Younger
USA Science and Technology, Falmouth, MA, USA and Xu-Yue Science and
Technology, Beijing, China; http://www.xuyue.net) containing an IAA-
sensitive amperometric sensor based on a carbon nanotube-coated
external oxidizing platinum microelectrode as described previously
(Mancuso et al., 2005; Henrichs et al., 2012). The net influx current was
defined as the difference between currents recorded in the absence
and presence of exogenous 10 μM IAA. Fluxes were measured in
the roots of at least 6–10 individual plants in two independent
experiments. Cell viability was assessed using trypan blue and carbox-
dichlorodihydrofluorescein diacetate (DCFDA) as described in
McLamore et al. (2010).

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OsHAK5 Functionality Assays for Auxin Acquisition in the IAA-
Sensitive Yeast Strain yap1-1
The whole open reading frames of OsHAK5 and AIAUX1 were amplified by
PCR from cDNA of rice and Arabidopsis using forward primers OsHAK5F
(5′-GGG AAT TCA TGA CCG ACG CTC TGC ACA CAA GGA GCA-3′) and
AIAUX1F (5′-GGG AAT TCA TGA CCG ACG CTC TGC ACA CAA GGA
TAG-3′), with an EcoRI site incorporated at the 5′ end, and reverse primers
OsHAK5R (5′-CCG CTC GAG CTA GAT CTC GTA CGT CAG TAG CAG
GGA-3′), with an XhoI site incorporated at the 3′ end. After EcoRI and
XhoI double enzyme digestion, the fragment was inserted between the
EcoRI and XhoI sites of yeast expression vectors pYES2 and
pDR196 (Yang et al., 2014). Subsequently, plasmids of pYES2, pDR196,
pYES2-AIAUX1, pYES2-OsHAK5, pDR196-AIAXU1, and pDR196-OsHAK5
were transformed into the IAA-sensitive mutant strain (S. cerevisiae)
yap1-1 (Prusty et al., 1998) as described previously (Yang et al., 2014). Positive transformants were selected on glucose-
containing solid SD-U medium without uracil, and single colonies were
grown in liquid SD-U medium supplemented with 2% galactose or
glucose. For functionality assays, transformants grown in liquid SD-U me-
dium to an OD600 of approximately 0.6 were washed and diluted to OD600
in deionized water. Cells were diluted 10-fold three times, and 3 μl of each
dilution was spotted onto an SD-U medium plate supplemented with the
indicated concentrations of IAA or 50 μM 5-fluorooridole. The plates
were incubated at 30°C for 3–5 days. The assays were performed on
three independent transformants.

[3H]IAA Transport Assay
Acropetal and basipetal auxin transport was assayed in excised seminal
roots as described in Lewis and Muday (2009) with minor modifications.
For assessment of acropetal auxin transport, solutions containing 0.7%
gar, 100 mM [3H]IAA, 10 μM prechilled IAA, 2% dimethyl sulfoxide
(DMSO), and 25 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH
5.5) with or without 10 μM NPA were prepared in 4-ml scintillation vials.
Subsequently, a 20-μl droplet of the [3H]IAA solution was applied to the
cut surface, following a shoot excision 1 cm above the root–shoot
conjunction. After 6 h of incubation at 25°C in 60%–70% humidity and
darkness, root segments were excised at 0–1, 1–2, and 2–3 cm distances
from the root apex and weighed (respective segments from 10–12 seed-
lings were pooled). The root segments were immediately placed in 3 ml
of scintillation solution for 1 day. The amount of 3H radioactivity in
each sample was determined using a Beckman LS6500 scintillation counter
for 2 min. The calculation of 3H radioactivity in rootward transport was
divided by the fresh weight of each 1-cm root sample.

Basipetal auxin transport was measured in the roots of 21-day-old seed-
lings. Solutions containing 0.7% agar, 100 mM [3H]IAA, 2% DMSO, and 25
mM MES (pH 5.5) with or without 10 μM NPA were prepared in 4-ml scintil-
lration vials. The lowest-most end of the root tip was covered with a 20-μl
droplet of the [3H]IAA solution, and the excised root was maintained vertically
in an incubator at 25°C in high humidity and darkness to avoid IAA degra-
dation. After 5 h of incubation, the 1-mm regions in contact with the agar
droplet were discarded, and the adjacent 2-mm root segments were
excised (respective segments from 10–12 roots were pooled) before
placing in 3 ml of scintillation solution for 1 day. The amount of
3H radioactivity in each sample was determined using a Beckman
LS6500 scintillation counter for 2 min. The calculation of 3H radioactivity in
shootward transport in 2-mm root segments (total content) was
calculated.
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Rhizosphere Acidification Assays of Rice Plants

Rice rhizosphere acidification assays were performed as described previously (Pacheco-Villalobos et al., 2016). For assessment of rhizosphere acidification by different plant lines, we transferred healthy 7-day-old rice seedlings to plates containing half-strength Murashige and Skoog agar supplemented with 0.15 mM bromoresol purple (Sigma-Aldrich; sensitivity range, pH 5.2–6.8). The plates were incubated in an artificial climate chamber with a 16-h-light (30°C)/8-h-dark (22°C) photoperiod and 60%–70% relative humidity for 24 h and then scanned.

Measurement of the Lateral Root Density, Seminal Root Length, and Root Diameter

Shelled rice seeds of different lines were germinated on agar plates. The plates were placed in an artificial climate chamber with a 16-h-light (30°C)/8-h-dark (22°C) photoperiod and 60%–70% relative humidity for 7 days. An emergent lateral root primordium longer than 0.5 mm (visible to the naked eye) was considered a lateral root (Sun et al., 2012). Subsequently, the seminal root length was measured using a ruler, and the lateral root density was calculated as the number of lateral roots divided by the seminal root length. The average root diameter was analyzed using WinRHIZO (Version 2009b; Regent Instruments, Montreal, QC, Canada) in Lagarde’s mode (Bouma et al., 2003).

Plasma Membrane Isolation and Hydrolytic Activity of Plasma Membrane H+-ATPase

Healthy 10-day-old rice seedlings of four lines, namely, WT(DJ), WT(NB), oshak5(DJ), and OsHAK5-OX(NB), were cultured in nutrient solution for 2 weeks. Roots and shoots were excised from the plants and washed with deionized water. The PM of roots was isolated as described in Zhu et al. (2009). Hydrolytic H+-ATPase activity was measured in roots and shoots of rice plants as described in Zhu et al. (2009) with minor modifications. The 0.5-mL reaction solution contained 5 mM MgSO4, 30 mM Bis-Tris propane/MES, 50 mM KCl, 50 mM KNO3, 50 mM K2SO4, 1 mM Na2MoO4, 1 mM NaN3, 0.5% ascorbic acid, and 2% glacial acetic acid was added to prevent further ATP hydrolysis during 30 min of color development, the A600 was measured using a spectrophotometer. H+-ATPase activity was calculated based on the amount of phosphate liberated in excess of the inorganic phosphate liberated from a boiled-membrane control. The root apoplastic pH was measured as described in Felle (1998). Healthy 10-day-old rice seedlings were cultured in nutrient solution for 4 weeks and then the roots were excised just below the root-shoot conjunction. Subsequently, the roots were washed several times with deionized water and then blotted with absorbent paper to remove the surface water. The roots were immediately placed in a 5-ml centrifuge tube with a small hole at the bottom, which was then placed in a 10-ml centrifuge tube. After 2–4 min of centrifugation at 500 rpm, the presumably apoplastic solution was collected in the 10-ml centrifuge tube. A second centrifugation at 900 rpm for 5 min using a new 10-ml centrifuge tube collected more apoplastic solution. The total volume of the apoplastic solution (sap) was approximately 10–20 μl. The root apoplastic sap pH was immediately determined using a blunt pH-sensitive microelectrode.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at Plant Communications Online.

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AUTHOR CONTRIBUTIONS

L.Y., G.X., T.Y., and N.M. conceived and designed the research; T.Y. performed the experiments together with H.F., S.Z., H.X., Q.H., W.X., and T.Y. G.X., L.Y., T.Y., and N.M. analyzed the data and wrote the article.

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