Identification of rice cornichon as a possible cargo receptor for the Golgi-localized sodium transporter OsHKT1;3

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Abstract

Membrane proteins are synthesized and folded in the endoplasmic reticulum (ER), and continue their path to their site of residence along the secretory pathway. The COPII system has been identified as a key player for selecting and directing the fate of membrane and secretory cargo proteins. Selection of cargo proteins within the COPII vesicles is achieved by cargo receptors. The cornichon cargo receptor belongs to a conserved protein family found in eukaryotes that has been demonstrated to participate in the selection of integral membrane proteins as cargo for their correct targeting. Here it is demonstrated at the cellular level that rice cornichon OsCNIH1 interacts with OsHKT1;3 and, in yeast cells, enables the expression of the sodium transporter to the Golgi apparatus. Physical and functional HKT–cornichon interactions are confirmed by the mating-based split ubiquitin system, bimolecular fluorescence complementation, and Xenopus oocyte and yeast expression systems. The interaction between the two proteins occurs in the ER of plant cells and their co-expression in oocytes leads to the sequestration of the transporter in the ER. In the yeast cornichon mutant erv14, OsHKT1;3 is mistargeted, preventing the toxic effects of sodium transport in the cell observed in wild-type cells or in the erv14 mutant that co-expressed OsHKT1;3 with either OsCNIH1 or Erv14p. Identification and characterization of rice cornichon as a possible cargo receptor opens up the opportunity to improve our knowledge on membrane protein targeting in plant cells.

Key words: Cornichon, endoplasmic reticulum, Golgi, OsHKT1;3, protein–protein interaction.

Introduction

Membrane transport proteins have to be selectively targeted to specific membranes to control ion and metabolite fluxes into and within the cell. However, very little is known about the sorting mechanisms of plant transporters. During evolution, eukaryotic cells developed multiple systems to target proteins to their respective compartments. Once membrane proteins are synthesized and folded in the endoplasmic reticulum (ER), they are directed by specific protein–protein interactions through the secretory pathway to the site where they will finally dwell. Transport of membrane and secretory cargo proteins is mediated by COPII vesicles that help to select these cargoes from ER-resident proteins. In yeast, humans, and plants, there is evidence that selection of cargo proteins is mediated specifically by the Sec24 subunit of the COPII system.
COPII complex (Miller et al., 2002; Mossessova et al., 2003; Manciás and Goldberg, 2008; Faso et al., 2009; Conger et al., 2011). Conserved domains involved in cargo selection have been identified (Miller et al., 2003; Mossessova et al., 2003). Accessory proteins known as cargo participate in the selection of specific cargo that can be either soluble or membrane proteins destined for different organelle or vesicular membranes (Dancourt and Barlowe, 2010). Correct targeting of membrane proteins, in particular ion transporters, is important for controlling ion homeostasis within the cell. Participation of cargo receptors is proposed to increase the specificity of the secretory pathway, with a particular receptor recognizing a specific cargo to be delivered to a precise cellular site of residence (Dancourt and Barlowe, 2010). In yeast, the role of Erv29p (Belden and Barlowe, 2001). For example, Erv29p has been demonstrated to select soluble secretory proteins such as carboxypeptidase Y and proteinase A (Belden and Barlowe, 2001; Otte and Barlowe, 2004), while Erv26p is involved in the export of membrane proteins such as alkaline phosphatase and mannosyltransferase (Bue et al., 2006; Bue and Barlowe, 2009). In contrast, Erv14p participates in the ER export of membrane proteins through ER luminal-localized sorting signals (Powars and Barlowe, 1998, 2002). Yeast Erv14p is a well conserved protein found in eukaryotes, initially identified in Drosophila as cornichon (Roth et al., 1995), and later in mammals where it was found to be associated with glutamate receptors of the AMPA subtype (Schwenk et al., 2009). In plants, there is little information on the possible role of supplementary cargo receptors in the secretory pathway, with the exception of vacuolar cargo receptor proteins directly involved in the selection of soluble proteins for their delivery to the vacuole lumen (Sanderfoot et al., 1998; Tse et al., 2004; Wang et al., 2011). However, it has been demonstrated in plant transporters that the presence of diacidic (Mikosch et al., 2006; Dunkel et al., 2008) or dileucine motifs (Komarova et al., 2012; Wolfenstetter et al., 2012) or loops between transmembrane domains (Komarova et al., 2012) is important for the correct delivery to the target membrane.

While the acronym HKT for high-affinity K \(^+\) transporter implies that members of the family transport K \(^+\), the majority of these proteins play a more significant role in low-affinity Na \(^+\) transport and have been identified as key players in salt tolerance (Plett et al., 2010; Munns et al., 2012; Schroeder et al., 2013). Yet, knowledge of the regulation of these transporters is limited. HKT transporters form two distinct subfamilies. Members of subfamily 1 are associated with Na \(^+\) tolerance based on their expression in the parenchyma cells of the xylem where they remove Na \(^+\) from the root xylem (Uozumi et al., 2000; Ren et al., 2005; Sunarpi et al., 2005) or the leaf sheath (Cotsaftis et al., 2012; Munns et al., 2012), preventing its transport to the leaf blade. Alternatively, they may participate in Na \(^+\) recirculation from the leaves to the root along the phloem (Berthomieu et al., 2003). The specific overexpression of AthKT1;1 in the root stele of Arabidopsis thaliana (Moller et al., 2009) or rice (Oryza sativa) (Plett et al., 2010) caused a decreased Na \(^+\) transport to the shoot, with a consequent lower accumulation of Na \(^+\) in the leaves and higher fresh weight, and resulting in an increase in salinity tolerance. Members of subfamily 2 probably participate in ion absorption (Rubio et al., 1995; Horie et al., 2001, 2007; Yao et al., 2010), as indicated by their localization at the plasma membrane (Horie et al., 2007, 2011; Lan et al., 2010; Xue et al., 2011).

In A. thaliana, only a single member of the HKT family, AthKT1;1, is present (Uozumi et al., 2000), while the rice genome contains nine HKT genes, one of them probably a pseudo-gene (Garcia-deblás et al., 2003). The presence of more than one HKT gene in rice, with members from the two subfamilies, indicates that the corresponding proteins must play particular roles in the physiology of the plant, either by expression in a particular tissue or organelle, or by presenting unique transport properties. Previous studies have confirmed this view, with members from the two subfamilies showing differences in ion selectivity (Horie et al., 2001, 2011; Goldack et al., 2002; Jaboune et al., 2009; Yao et al., 2010; Sassi et al., 2012) and differential gene expression profiles in plant tissues (Goldack et al., 2002; Kader et al., 2006; Jaboune et al., 2009).

OsHKT1;3 functions as a highly selective Na \(^+\) transporter and its transcript is expressed in the vascular tissue of roots and leaves. The high expression in leaf adaxial epidermal buliform cells has been used to propose its involvement in turf-gors changes for rolling and unrolling of leaves in response to environmental variations (Jaboune et al., 2009). Of particular interest is the high Na \(^+\) selectivity of OsHKT1;3 which, if not unique among HKTs, raises the question of the role of such a mechanism, in view of the toxic effects that sodium has on glycosylates, such as rice, and its widespread expression in the plant (Jaboune et al., 2009). To study the regulation of OsHKT1;3, interacting proteins that might regulate its activity/trafficking were searched for employing a large-scale protein–protein interaction screen based on the mating-based split ubiquitin system (mSUS) (Lalonde et al., 2010; Chen et al., 2012; Jones et al., 2014). In this screen, a plant homologue of the putative cargo receptor, cornichon, was identified. Here the characteristics of rice cornichon are reported at the cellular level, together with its interaction with OsHKT1;3 as a cargo protein, and the intracellular localization of OsHKT1;3 to the Golgi system where it may function as the shunt conductance for the H \(^+\) pumps that acidify this organelle.

Materials and methods

Mating-based split ubiquitin system (mSUS)

For PCR amplification of the open reading frame (ORF) for OsHKT1;3 and OsCNIH1 genes without their stop codon, the primers described in Supplementary Table S1 available at JXB online were employed. Entry clones were generated with the pENTR-TOPO vector/plasmid following the manufacturer’s instructions (Invitrogen). The integrity
of gene insertion was confirmed by sequencing and by digestion with PronBII (Roche). The LR clonase (Invitrogen) was employed to transfer the OsHKT1;3 and OsCNIH1 genes to the pMETYC_GW (Cub clones) and pXN32_GW (Nub clones) vectors, respectively. Yeast media were prepared as previously described (Lalonde et al., 2010). The THY.AP4 (MATa ura3, leu2, lexA::LacZ::trp1 lexA::HIS3 lexA::ADE2) and THY.APS (MATa URA3, leu2, trp1, his3 lexp::ade2) yeast strains were transformed with the pMETYC_GW and pXN32_GW vectors, respectively (Obrdlik et al., 2004), employing the LiAc protocol previously described (Lalonde et al., 2010). Cub clones were pre-screened to identify false-positive and false-negative fusions. Those fusions that did not interact with the soluble NubWT, that has a strong affinity for the Cub domain, corresponded to false positives. Those Cub fusions that passed the pre-screening were used with the A. thaliana membrane-linked interactome protein library Nub clones. Positive interactors were chosen by the similar growth shown to the soluble NubWT in IS-500 medium (Lalonde et al., 2010).

Transitory expression of OsHKT1;3 and OsCNIH1 tagged with fluorescent proteins in Nicotiana benthamiana leaf epidermis

For the transitory transformation of N. benthamiana leaves, plants were grown in a greenhouse from seeds in pots containing Metromix 500 soil (SunGro) at 25 °C under natural light conditions. Leaves from 4-week-old plants were infiltrated with Agrobacterium tumefaciens harbouring either the pOsHKT1;3-EYFP, pOsHKT1;3-mCherry, or pOsCNIH1-mCherry constructs under the control of the 35S promoter and/or the indicated subcellular marker gene constructions (Nelson et al., 2007). For bimolecular fluorescence complementation (BiFC) experiments, leaf infiltration was done using the constructs pYFC43-OsHKT1;3, pYFC43-OsCNIH1, pYFC43-AtP1P2A, pYFN43-OsHKT1;3, pYFN43-OsCNIH1, and pYFN43-AtP1P2A (Belda-Palazón et al., 2012). Agrobacterium tumefaciens co-transformed with each construct were grown in 30 mL of LB medium with rifampicin (50 μg ml−1) and spectinomycin (50 μg ml−1) or kanamycin (5050 μg ml−1) at 28 °C for 3 d. The vectors were transferred to A. tumefaciens strain GV3101 of Agrobacterium tumefaciens at an OD600 of 0.3. To obtain the expression clone for each gene, an LR gateway-based recombination reaction (Invitrogen) with either pX-EYFP-GW or pX-Cherry-GW for C-terminal translational fusions, or pYFC43 and pYFN43 for BiFC assays, were achieved. Transformed electroporant cells of the strain GV3101 of A. tumefaciens with each of these constructions were used for expression in tobacco. Image J software (http://imagej.nih.gov/ij/) was used to analyse the confocal images for co-localization. Pearson’s coefficient and scatter plots were obtained using the JACoP Plug-in (Bolte and Cordelières, 2006).

Functional expression of OsHKT1;3 in Xenopus oocytes

The two-electrode voltage-clamp technique was used to record the activity of OsHKT1;3 and OsCNIH1, individually or co-expressed in oocytes 2 d after cRNA injection as described (Ortiz-Ramirez et al., 2011). For all measurements, oocytes were clamped at their free running membrane potential. The reported results are the means ±SD of 5–10 oocytes. For expressing OsHKT1;3 and OsCNIH1 in Xenopus oocytes, both genes were cloned into the pPO2 oocyte expression vector (Ludewig et al., 2002) employing the primers described in Supplementary Table S1 at JXB online. An OsHKT1;3–EGFP fusion protein was made by adding the ORF of the enhanced green fluorescent protein (pEGFP-C1; Clontech) at the C-terminus of OsHKT1;3 which was then subcloned into pPO2. Clones were verified by sequencing. Capped cRNA was transcribed in vitro by SP6 RNA polymerase using the mMessage mMachine kit (Ambion), after linearization of the plasmid with Bbr P1 (Roche).

Heterologous expression of OsHKT1;3 in yeast

OsHKT1;3 was amplified by PCR employing the primers described in Supplementary Table S1 at JXB online. The PCR-amplified OsHKT1;3 gene was inserted into the Yep532-NHA1-URA and pGRRU1-NHA1-URA vectors (previously digested with PstI and PvuII, respectively) behind the Saccharomyces cerevisiae NHA1 promoter (Kinclova et al., 2001) using homologous recombination in S. cerevisiae BW31a cells (MATa leu2-3112 ura3-1 trp1-1 his3-1115 ade2-1 can1-100 GAL SUC2 mal10 end-4::HIS3 nla::LEU2). The resulting vectors were pYeEp352-OsHKT1;3 and pGRU1-OsHKT1;3. The correct constructions were confirmed by sequencing using oligos described in Supplementary Table S1. OsCNIH1 and ERV14 cloned in pENTR-TOPO were transferred to pDR-F1-GW-LEU by an LR reaction (Invitrogen). pDR-F1-OsCNIH1 and pDR-F1-ERV14 constructs are under control of the PMi1 promoter. Constructions were verified by PvuII digestion (Thermo Scientific). The S. cerevisiae BY4741 strain (MATa his3-1/1/leu2-2/3/kan1-103/ura3-3) was used to determine NaCl sensitivity in drop-test assays. Yeast cells were grown aerobically at 30 °C in standard YPD (to prepare competent cells for transformation) or Yeast Nitrogen Base (YNB) media without amino acids (to select and maintain transformants), with 2% glucose as carbon source and appropriate auxotrophic supplements. The growth phenotype of cells was estimated in drop tests on selective media for the auxotrophic markers. All yeast strains were transformed with the pCEN.PD5-CUB vectors, respectively. Yeast transformation was performed using the LiAc protocol previously described (Lalonde et al., 2010). Transformed yeast colonies were grown aerobically at 30 °C on selective media (to select for tagged gene expression) and on non-selective media (to ensure expression was due to the tagged gene). Growth was observed for 3–4 d.

Fluorescence microscopy

Fluorescence from EYFP was visualized by excitation with an Argon laser at 514 nm with the spectral detector set between 540 nm and 30 nm for the emission. The wavelengths employed for Citrine and mCherry were 488 nm and 543 nm (excitation), and 515/30 nm and 630/60 nm (emission), respectively. Abaxial epidermal peels of mature leaves were placed onto microscope slides in water, covered with a cover slide, and observed by fluorescence microscopy using an inverted multiphoton confocal microscope (Olympus FV1000) equipped with a ×60 oil immersion objective. Results are representative images from >10 cells from at least four different independent transformations.

Results

Analysis of protein–protein interactions between OsHKT1;3 and the Arabidopsis membrane interactome

In order to obtain additional information on the functioning of OsHKT1;3, the possible regulation of the transporter through protein–protein interactions was studied by employing the mbSUS (Obrdlik et al., 2004; Lalonde et al., 2010). Screening an A. thaliana membrane-linked interactome protein library as prey (Nub clones), with OsHKT1;3 protein used as bait (Cub clone) (Lalonde et al., 2010; Jones et al., 2014), 19 possible interactions were identified, as indicated by growth of diploid yeast cells under selective conditions (Table 1). These interactions were common to two other rice HKTs, OsHKT1;1 and OsHKT2;1 (Table 1).

OsHKT1;3 interacts with OsCNIH1 in yeast

After analysing the 19 interacting proteins, it was decided to focus on a protein homologous to Erv14p in yeast and cornichon in Drosophila and mammals, which has been shown to direct the trafficking of membrane proteins from the ER to the Golgi
apparatus (Gillingham et al., 2004), or to the plasma membrane (Schwenk et al., 2009; Herzig et al., 2012). Confirmation of the interaction between OsHKT1;3 and rice cornichon (OsCNIH1; Os06g04500) was obtained by employing OsHKT1;3 and OsCNIH1 as Cub and Nub clones, respectively, in the mbSUS. Growth of diploid yeast cells in selective medium confirmed the interaction between OsHKT1;3 and OsCNIH1 (Fig. 1; IS0), and the strength of the interaction was demonstrated by growth of the diploid cells in the presence of 0.5 mM methionine which acts as a repressor of the pMETYC promoter (Grefen et al., 2007; Fig. 1, IS500). As expected, OsHKT1;3 did not interact with soluble NubG, but it did with soluble NubWT, that were used as false-positive and false-negative controls, respectively (Fig. 1). Additional confirmation of the interaction between OsHKT1;3 and OsCNIH1 was derived from analysing LacZ activity with X-Gal as a substrate, whereby clear blue signals in the diploid cells harbouring OsHKT1;3 and OsCNIH1 clones were observed. The blue precipitate was also observed with the positive control (soluble NubWT), but not with soluble NubG, the negative control (Fig. 1, LacZ).

Sequence analysis of rice cornichon

The rice genome contains two cornichons, OsCNIH1 (Os06g04500), a small hydrophobic protein of 135 amino acids (Fig. 2A, B), and OsCNIH2; (Os12g32180), which encodes a slightly larger protein (149 amino acids; Fig. 2B). The two isoforms share 44% identity at the amino acid level. Cornichon belongs to a family of membrane proteins unique to eukaryotes that is predicted to have three membrane-spanning α-helixes (Fig. 2A, Kyte–Doolittle; Fig. 2B, TMHMM, black lines), with the N-terminus towards the cytoplasm according to the predicted positive-inside rule (von Heijne, 1992), and the C-terminus located in the ER lumen. All plant cornichon homologues possess an acidic domain in the luminal C-terminus (Fig. 2B, red line). The similarity between plants, yeast, fly, worm, zebra fish, and human cornichons is ~40%, with OsCNIH1 showing higher identity with the Arabidopsis homologues AtCNIH2, AtCNIH3, and AtCNIH4 (Fig. 2C).

| OsHKT1;3 (Cub) | AGs Arabidopsis (Nub) | Description |
|----------------|-----------------------|-------------|
| AT3G17000      | Ubiquitin-conjugating enzyme 32 |
| AT3G25805      | Unknown protein       |
| AT1G17280      | Ubiquitin-conjugating enzyme 34 |
| AT1G21240      | Cell wall-associated kinase |
| AT1G34640      | Unknown protein       |
| AT1G47640      | Unknown protein       |
| AT1G63110      | Cell division cycle protein-related |
| AT2G26180      | IQ-domain 6 (IQD6)    |
| AT3G08040      | Member of the MATE family |
| AT3G10640      | VPS60 vesicle-mediated transport |
| AT3G12180      | Cornichon family protein |
| AT3G28220      | Meprin and TRAF homology domain-containing protein |
| AT4G30850      | Heptahelical transmembrane protein homologous to human adiponectin receptors and progestin receptors |
| AT5G06100      | myb family of transcription factors (MYB33) |
| AT5G06320      | Tobacco hairpin-induced gene (HIN1) |
| AT5G10450      | 14-3-3 gene family |
| AT5G37050      | Unknown protein       |
| AT5G49540      | Unknown protein       |
| AT5G52240      | Similar to progesterone-binding proteins in animals. |
Split-YFP analyses demonstrate that OsHKT1;3 and OsCNIH1 interact in the ER

To confirm the interaction between OsHKT1;3 and OsCNIH1 in planta, and to identify the site(s) of interaction, the split-YFP (yellow fluorescent protein) system was employed by fusing the N-terminal half of EYFP to the N-terminal half of OsHKT1;3 (YFN-OsHKT1;3) and the C-terminus of EYFP to the N-terminus of OsCNIH1 (YFC-OsCNIH1). Co-expressing these constructs in tobacco leaves led to fluorescence recovery of EYFP (Fig. 3A), indicating the association between OsHKT1;3 and OsCNIH1 at the ER, according to the reticulated structure highlighted by EYFP. The co-localization of the two proteins was confirmed with fusions of the N- and C-terminal halves of EYFP to the N-terminus of OsCNIH1 (YFN–OsCNIH1) or OsHKT1;3 (YFC–OsHKT1;3), respectively (Fig. 3E). As a positive control for the BiFC assay, the well-known tetramericization of the plasma membrane aquaporin AtPIP2A was used. For this, the N- or C-terminal halves of EYFP were fused to the N-terminus of the aquaporin AtPIP2A (YFN–AtPIP2A and YFC–AtPIP2A). As expected, oligomerization of the aquaporin was observed at the plasma membrane (Fig. 3I). Co-expression...
of YFN-OsCNIH1 and YFC-AtPIP2A did not lead to reconstitution of EYFP fluorescence (Fig. 3C, H). Additional analyses demonstrated fluorescence reconstitution by expressing the C- and N-termini of YFP fused independently to the N-terminus of OsCNIH1, indicating the oligomerization of the protein in intracellular structures that resembled the Golgi apparatus (Fig. 3B). In contrast, similar studies with OsHKT1;3 failed in reconstituting YFP fluorescence, suggesting that this transporter does not oligomerize (Fig. 3D). No interactions were observed between the aquaporin AtPIP2A and OsHKT1;3 or OsCNIH1 (Fig. 3C, F–H). These results demonstrated that the interaction between OsHKT1;3 and OsCNIH1 occurs at the ER, and indicated the possible oligomerization of rice cornichon. Moreover, the results suggested that BiFC did not seem to be a result of overexpression of the proteins in the same membrane, as indicated by failure in reconstituting EYFP associated with OsHKT1;3.

**Cornichon co-localizes with OsHKT1;3 and resides in the ER and Golgi**

Further support for the interaction between OsHKT1;3 and OsCNIH1 was obtained by analysing tobacco leaf epidermis co-transformed with OsCNIH1–mCherry and OsHKT1;3-EYFP. Expression of OsCNIH1–mCherry was generally observed as a reticulated structure (Fig. 4A, left), while that of OsHKT1;3–EYFP mainly appeared as bright puncta (Fig. 4A, centre). Overlapping both images showed that the puncta associated with OsHKT1;3–EYFP superimposed the reticula highlighted by OsCNIH1–mCherry (Fig. 4A, right, white dots). By plotting the pixel number for OsHKT1;3–EYFP against that from OsCNIH1–mCherry, pixel distribution was demonstrated to occur along a straight line (Fig. 4D, left), and, by applying Costes’ method (Costes et al., 2004) for image analysis using ImageJ (JACoP Plug-in), a Pearson’s coefficient (PC) and P-value of 0.68 and 100%, respectively, were calculated (Fig. 4D, left) (Bolte and Cordelières, 2006). These findings confirmed that OsCNIH1 and OsHKT1;3 partially co-localized in the ER. In view of these results, it was important to determine and confirm the site(s) of residence for OsCNIH1 and OsHKT1;3 individually, and for this co-expression studies were carried out employing several well-characterized membrane markers fused to fluorescent proteins. OsCNIH1–mCherry highlighted a reticulated structure, strongly suggesting its expression at the ER (Fig. 4B, left); this was confirmed by the comparable expression observed for the
OsCNIH1 directs OsHKT1;3 to the Golgi

ER marker AtWAK2–Citrine (Fig. 4B, centre) (Nelson et al., 2007) and by the overlapping of signals from these two markers (Fig. 4B, right, white signal). Image analysis demonstrated a high degree of co-localization between OsCNIH1 and AtWAK2 (PC=0.82; P=100%; Fig. 4D, centre). Localization of OsHKT1;3–EYFP emphasized small, highly motile punctate structures in the cytoplasm, indicating localization to potentially vesicular structures (Fig. 4C, centre). To identify this compartment, OsHKT1;3–EYFP was co-expressed with the Golgi marker GmMan1–mCherry (Nelson et al., 2007), and similar punctate cytoplasmic signals were observed for both proteins (Fig. 4C, left and centre), that when overlapped showed partial co-localization (Fig. 4C, right, white areas). Applying Costes’ method, a calculated PC of 0.62 with a P-value of 100% was obtained, confirming the co-localization of the two proteins (Fig. 4D, right) which indicated that OsHKT1;3–EYFP partially localized to the Golgi.

Work in yeast and mammals have demonstrated that cornichon homologues did not locate exclusively to one specific compartment, but rather circulated actively between the Golgi and the ER, in association with COPII vesicles (Powers and Barlowe, 1998; Harmel et al., 2012). To identify more clearly the plant cornichon location, GmMan1–Citrine was used as a Golgi marker and AtSec24–YFP as a marker for ER exit sites (ERES)/COPII (Hanton et al., 2009; Langhans et al., 2012). Co-expression of OsCNIH1–mCherry (Fig. 5A, left) with GmMan1–Citrine (Fig. 5A, centre) showed that both proteins highlighted punctate structures with properties similar to that of the Golgi, with OsCNIH1–mCherry also highlighting the network associated with the ER, particularly surrounding the nucleus (Fig. 5A, left). Quantitative analyses of the overlapped images (Fig. 5A, right) showed a relatively low correlation (PC=0.40, P=100%; Supplementary Fig. S2A at JXB online), indicating that OsCNIH1 was also present in the Golgi apparatus. When OsCNIH1 was co-expressed with AtSec24–YFP, OsCNIH1 labelled the ER (Fig. 5B, left), while AtSec24 appeared as bright puncta dispersed throughout the cytoplasm (Fig. 5B, centre). AtSec24–YFP

Fig. 4. Intracellular co-localization of OsCNIH1 with OsHKT1;3 in plants. (A) Expression of OsCNIH1–mCherry (left) and OsHKT1;3–EYFP (centre), and co-localization of the two proteins (right) in tobacco leaves. (B) Expression of OsCNIH1–mCherry (left) and the ER marker AtWAK2–Citrine (centre), and co-localization of the two proteins (right). (C) Expression of OsHKT1;3–EYFP (centre) and the Golgi marker GmMan1–mCherry (left), and co-localization of the two proteins (right). (D) Scatter plots of pixel distribution of the magenta (y-axis) and green (y-axis) channels employing the Costes algorithm for images shown in (A, left), (B, centre), and (C, right). Scale bar=25 μm.
co-localized with the reticulate OsCNIH1–mCherry fluorescence (Fig. 5B, right) as indicated by the calculated PC=0.66 and P=100% (Supplementary Fig. S2B), corresponding to the proposed structure for the ERES/COP II compartment. Together, these imaging data indicated that rice cornichon located to the Golgi and ER.

Subcellular localization of OsHKT1;3

The intracellular localization of OsHKT1;3 (Fig. 4A, C) differed from that of plasma membrane-localized HKT isoforms (Horie et al., 2007, 2011; Lan et al., 2010; Xue et al., 2011). To explore the localization of OsHKT1;3 in more detail, co-localization analysis was performed with the same membrane markers used for OsCNIH1 (Figs 4, 5). Co-expression of OsHKT1;3–mCherry with AtSec24–YFP demonstrated that both proteins were visualized as punctate structures that did not locate to the same compartment (Fig. 6A). Confirmation that co-localization of OsHKT1;3–mCherry and AtSec24–YFP was low (Fig. 6A, right) was supported by the low PC of 0.32 (P=100%; Supplementary Fig. S2C at JXB online). Co-expression of OsHKT1;3 and AtPIP2A–mCherry in tobacco leaf epidermal cells verified that the puncta corresponding to OsHKT1;3–EYFP localized to the cytoplasm (Fig. 6B, left), while expression of AtPIP2A–mCherry was limited to the plasma membrane (Fig. 6B, centre). Merging of the two channels yielded few co-localization points (Fig. 6B, right); quantitative analysis revealed no co-localization between OsHKT1;3 and AtPIP2A, as indicated by the low PC of 0.14 (P=100%; Supplementary Fig. S2D). Additional evidence supporting the Golgi as the site of residence for OsHKT1;3 was obtained with use of the fungal toxin brefeldin A (BFA). BFA blocks the activation of the GTPase Arf1 by causing a non-productive complex with its Sec7 GTP exchange factor (Chardin and McCormick, 1999), resulting in the formation of Golgi aggregates described as BFA bodies (Ritzenthaler et al., 2002). Incubation of tobacco leaf epidermis transformed with OsHKT1;3–EYFP for 15 min with 25 μM BFA caused the appearance of relatively large fluorescent aggregates (Fig. 6C, right) that resembled BFA bodies, in comparison with the smaller puncta from OsHKT1;3–EYFP in the untreated epidermis (Fig. 6C, left). Measurement of the rapid movement of the fluorescent puncta associated with OsHKT1;3–EYFP gave a mean velocity of 0.14±0.02 μm s⁻¹ (mean ±SD, n=9; Supplementary Fig. S3, Supplementary Video S1), similar to the rates that have been reported for the highly motile Golgi apparatus (Boevink et al., 1998). Although some puncta did not move, corresponding to stationary Golgi stacks (Lerich et al., 2012), others moved at a lower speed that varied between 0.081 μm s⁻¹ and 0.061 μm s⁻¹. All these observations provided further evidence that OsHKT1;3–EYFP locates to the Golgi apparatus of plant cells.

Co-expression of OsCNIH1 and OsHKT1;3 in Xenopus oocytes modified the cellular localization of the transporter and inhibited its activity

In view of the results that indicated the interaction and co-localization of OsCNIH1 and OsHKT1;3 in plant cells, the oocyte expression system was employed to study the potential effects of OsCNIH1 on the transport activity of OsHKT1;3. Initially, the properties of the transporter reported as an Na⁺-selective transport mechanism were confirmed (Jabnoune et al., 2009). Oocytes injected with OsHKT1;3 cRNA showed the activation of inward currents when exposed to Na⁺ solutions (Fig. 7A, right), but not when the cation was absent (Fig. 7A, centre). A control water-injected oocyte exposed to sodium failed to activate any measurable currents (Fig. 7A, left). Confirmation that the inward currents corresponded to Na⁺ movement was derived from the increasing current magnitude recorded with increasing concentrations of Na⁺, together with clear shifts in the reversal potential (Eᵣ) (Fig. 7B, arrows) activated by voltage.
OsCNIH1 directs OsHKT1;3 to the Golgi ramps. Similar experiments with a water-injected oocyte did not show changes in current magnitude or in $E_r$ with variable Na$^+$ concentrations (Fig. 7C). Plotting the $E_r$ values against external Na$^+$ concentrations gave a linear relationship with a slope of 54.2 mV per decade Na$^+$ (Fig. 7D, circles). A similar relationship was observed for $E_r$ values obtained in the presence of 1 mM K$^+$ (Fig. 7D, squares). These results clearly indicated that OsHKT1;3 functions as an Na$^+$-selective transporter/channel and that OsHKT1;3 does not function as an Na$^+$/K$^+$ symporter, as has been reported for some members of the subfamily 2 of HKT transporters (Rubio et al., 1995; Gassmann et al., 1996; Horie et al., 2001). Kinetic properties of the transporter were obtained by plotting current magnitude from voltage ramps against external Na$^+$ concentrations at different holding potentials, observing saturation at concentrations >50 mM (Fig. 7E). Data analysis with the Michaelis–Menten equation showed that the apparent transport affinity constant ($K_m$) for Na$^+$ increased at less negative holding potentials, with values of 6.4, 9.6, 17.1, and 42.6 mM obtained at –180, –160, –140, and –120 mV, respectively (Fig. 7E). Assuming a single binding site for sodium, Equation 1 was employed to evaluate the voltage dependence of $K_m$, where $\delta$ is the fractional electrical distance, $e$ is the elementary charge, $V$ is the membrane potential, $k$ is Boltzmann’s constant, and $T$ is the absolute temperature (Woodhull, 1973).

$$K_m(\delta) = K_m^{(0mV)} \ast \exp(\delta \ast e \ast \frac{V}{k \ast T})$$ (1)

From this analysis, the putative Na$^+$ binding site was calculated to be located at (b) 65% within the membrane electrical field (Fig. 7F, fitted line). Having confirmed the basic properties of OsHKT1;3 (Jabnoune et al., 2009), the effects of co-expressing OsHKT1;3 together with OsCNIH1 in the oocyte system were then analysed. Voltage-clamp recordings showed that the activity of the transporter was inhibited
by the presence of OsCNIH1, as indicated by the absence of inward currents at all extracellular Na⁺ concentrations tested (Fig. 8A, right). In comparison, an oocyte injected with only the cRNA for OsHKT1;3, activated large inward currents in response to voltage ramps, similar to those previously observed (Fig. 8A, left; see also Fig. 7D). The inhibition of OsHKT1;3 by co-injection of OsCNIH1 in the oocytes could be the result either of direct inhibition of the transporter activity or of the interaction between the two proteins preventing the default targeting of OsHKT1;3 to the oocyte membrane. To distinguish between these two possibilities, EGFP was fused to the C-terminus of OsHKT1;3 and then the construct was expressed in albino Xenopus oocytes, individually or together with OsCNIH1, and the expression was observed under epifluorescence microscopy. When expressed alone, OsHKT1;3–EGFP was observed as punctate structures at the membrane of the oocyte (Fig. 8B, left). In contrast, when co-injected with OsCNIH1, OsHKT1;3–EGFP fluorescence was observed in the interior of the oocyte as a reticulated structure, resembling the ER (Fig. 8B, centre). Autofluorescence from a control water-injected oocyte was minimal (Fig. 8B, right).

**Deletion of ERV14 in yeast modifies the intracellular location of OsHKT1;3 which is restored upon expression of OsCNIH1 or ERV14**

To gain further insight into the interaction between OsCNIH1 and OsHKT1;3, the effect of mutating ERV14, the yeast cornichon homologue, on the heterologous expression of OsHKT1;3 tagged with GFP (pGRU1-OsHKT1;3) in BY4741 yeast cells was tested. Transformation of BY4741 yeast cells with the pGRU1-OsHKT1;3 vector showed that localization of OsHKT1;3 was intracellular, highlighting several round bodies that indicated the presence of the transporter at the Golgi apparatus (Fig. 9A; BY4741).
A different localization for OsHKT1;3–GFP was observed in the BY4741Δerv14 mutant cells, as indicated by the diffused fluorescence observed around the nucleus and throughout most of the cytoplasm, a distribution that has been associated with the ER (Fig. 9A; BY4741Δerv14 (Manford et al., 2012)). Confirmation that localization of OsHKT1;3 at the Golgi apparatus depended on the presence of OsCNIH1 was obtained by co-transforming BY4741Δerv14 cells with OsCNIH1 and OsHKT1;3–GFP and observing the localization of the latter as round structures corresponding to the Golgi apparatus (Fig. 9A; BY4741Δerv14+OsCNIH1). Similar results were observed when rice cornichon was replaced with ERV14 in the co-transformation of the BY4741Δerv14 cells (Fig. 9A; BY4741Δerv14+ERV14), indicating that both rice cornichon and yeast Erv14p can direct the transporter to the Golgi apparatus. Additional evidence was gathered by investigating the effects of expressing OsHKT1;3 in BY4741 and BY4741Δerv14 yeast cells upon salt stress. Figure 9B (top) shows that transformation of BY4741 cells with OsHKT1;3 and the empty pDR-F1 vector (lower row) reduced cell growth between 0.8 M and 1.5 M NaCl, when compared with the parental cells transformed with the two empty vectors pGRU1 and pDR-F1 (Fig. 9B, upper row). Co-transformation of BY4741Δerv14 cells with OsHKT1;3 and the empty vector pDR-F1, in contrast, did not affect cell growth in the presence of NaCl (Fig. 9B, bottom, third row). Co-transformation of the BY4741Δerv14 mutant cells with OsCNIH1 and OsHKT1;3 partially restored the sensitivity of the cells towards NaCl (Fig. 9B, bottom, second row), a similar response to that caused by the co-transformation of the BY4741Δerv14 cells with OsHKT1;3 and ERV14 (Fig. 9B, bottom, fourth row).

### Discussion

The putative cargo receptor OsCNIH1 interacts with the Na⁺ transporter OsHKT1;3

Protein–protein interactions play an essential role in cell structure and function, and have been shown to be important for protein function, regulation, and targeting (Lee et al., 2009; Geiger et al., 2010). The mbSUS with the Arabidopsis interactome (Lalonde et al., 2010; Chen et al., 2012; Jones et al., 2014) was employed to identify proteins that might modulate the activity of OsHKT1;3 by direct protein–protein interactions. A total of 19 potential membrane protein interactions from Arabidopsis were identified (Table 1), and, from these, a protein which shows homology to Drosophila and human cornichon (CNIH) (Bökel et al., 2006; Schwenk et al., 2009) and yeast Erv14p (Powers and Barlowe, 1998, 2002) was selected for further analysis. In all these biological systems, the cornichon homologues have been described as cargo receptors for membrane proteins that may (Bökel et al.,...
Rosas-Santiago et al. 2006; Schwenk et al., 2009) or may not (Powers and Barlowe, 1998, 2002; Herzig et al., 2012) remain attached to the cargo. However, as far as could be assessed from the literature, there is no knowledge of the cornichon homologues in plants. The interaction between the rice homologue OsCNIH1 and the Golgi membrane Na\(^{+}\) transport protein OsHKT1;3 was identified. Evidence for the interaction between OsHKT1;3 and OsCNIH1 was derived from growth of diploid yeast cells on selective media (IS0 and IS500) and activation of the reporter LacZ (Fig. 1). This interaction was confirmed in planta by bimolecular complementation of EYFP in tobacco cells where intracellular fluorescence signals were observed from reticulated structures reminiscent of the ER (Fig. 3A, E). This result was observed with either the C- or N-terminus of EYFP fused to the N-terminus of either OsCNIH1 or OsHKT1;3 (Fig. 3A, E). These observations were corroborated by co-localization studies between OsCNIH1 and OsHKT1;3 which also demonstrated the intracellular occurrence of both proteins in co-transformed tobacco leaves, where OsHKT1;3 was observed as small puncta superimposed on the ER highlighted by OsCNIH1 (Fig. 4A). Together, these results may be used to propose that direct interaction between OsCNIH1 and OsHKT1;3 occurs at the ER (Fig. 3A, E) and is required to direct the Na\(^{+}\) transporter to the Golgi (Fig. 4C). The absence of fluorescence complementation when tobacco leaves expressed the YFN–OsCNIH1 and YFN–ArPIP2A protein chimeras indicated that the aquaporin does not interact with rice cornichon (Fig. 3C), and suggests that the interaction observed between OsCNIH1 and OsHKT1;3 was specific and not a general occurrence between cornichon and membrane proteins. Moreover, lack of EYFP fluorescence complementation by co-expression of YFN–OsHKT1;3 and YFC–OsHKT1;3 (Fig. 4D) indicates that overexpression of the protein at the same membrane does not, by itself, lead to reconstitution of the fluorescent protein, discarding the possibility that the results obtained with the interaction between OsCNIH1 and OsHKT1;3 are an artefact. This result was also supported by the absence of interaction observed with the mbSUS that indicated that OsHKT1;3 does not oligomerize (Supplementary Fig. S4 at JXB online). Corroboration of the interaction of OsCNIH1 with OsHKT1;3 was obtained independently by employing Xenopus oocytes co-expressing the two proteins, resulting in the retention of the transporter in internal structures (Fig. 8B, centre), and the inability to measure OsHKT1;3-dependent Na\(^{+}\) currents (Fig. 8A, right). In the absence of OsCNIH1, the transporter followed the

![Figure 9](image-url)
default pathway for exogenous proteins to the oocyte plasma membrane, similarly to what was observed for other heterologous expressed organelle-localized proteins, including aquaporins from plants. This response resembles the unregulated transport of the glutamate receptor (AMPAR) to the plasma membrane observed in the Caenorhabditis elegans cni1 mutant (Brockie et al., 2013), and may be a result of an imbalance in the assembly of the COPII system caused by the lack of cornichon which leads to the uncontrolled targeting of OsHKT1;3 to the oocyte plasma membrane. Only when the cargo receptor is present is the transporter retained in the endomembrane system (Fig. 5B, centre). Alterations in COPII-mediated ER membrane protein export have also been observed upon overexpression of Sec12p and were proposed to be a result of Sar1p titration (D’Enfert et al., 1989; DaSilva et al., 2004). Yet another result that further supported the interaction between OsCNIH1 and OsHKT1;3 as being responsible for the proper targeting of the transporter to the Golgi membrane was the restoration of the location of OsHKT1;3 in the yeast mutant BY4741Δerv14 upon co-expression with either of the cornichon homologues, OsCNIH1 or Erv14p (Fig. 9A). Parallel to these results, restoration of salt sensitivity to the BY4741Δerv14 yeast mutant by co-expression of the rice sodium transporter together with either of the cornichon homologues, OsCNIH1 or Erv14p (Fig. 9A), showed that OsHKT1;3 was not delivered to the Golgi membrane and, thus, did not disturb the functioning of the cell, allowing yeast growth in the presence of Na⁺.

The presence of OsCNIH1 in the ER and the Golgi apparatus (Figs 4, 5) is comparable with that reported for the homologues in yeast (Powers and Barlowe, 1998) and mammals (Harmel et al., 2012). CNIH’s homologues are associated with the transport of membrane proteins, including Axl2p in yeast (Powers and Barlowe, 1998, 2002; Gillingham et al., 2004), Gurken in Drosophila (Bökel et al., 2006), and GluRα, in mammals (Schwenk et al., 2009; Kato et al., 2010; Harmel et al., 2012; Herring et al., 2013), and, it is argued, OsHKT1;3 in rice. OsCNIH1 conserves three out of five amino acids (I96, F97, and L100; Fig. 2B, arrows) in the cytoplasmic loop between TMD2 and TMD3 that effect the binding of Erv14p to COPII vesicles (Powers and Barlowe, 2002), suggesting that in plants, transport of membrane proteins in the early secretory pathway may also involve the association of OsCNIH1 with COPII vesicles. This is supported by the co-localization of OsCNIH1 with the bona fide COPII marker AtSec24 in tobacco leaves (Fig. 5B), a site where OsCNIH1 would be functioning as a cargo receptor for OsHKT1;3 (Fig. 4A). Targeting of OsHKT1;3 to the Golgi apparatus by OsCNIH1 can be compared with the Erv14p-dependent localization of Rud3p to the Golgi in yeast that also involves the participation of the GTP-binding protein Arf1p (Gillingham et al., 2004). Recent results in yeast have revealed the central role played by Erv14p as a cargo receptor for a number of membrane proteins including the flipases Dnf1p and Dnf2p, the hexose transporters Hxt3p, Hxt4p, and Hxt5p, as well as the Na⁺/H⁺ exchanger Nha1p, among others (Herzig et al., 2012). As indicated by the BiFC results (Fig. 3B), OsCNIH1 seems to form oligomers. This feature seems to be shared with cargo receptors such as ERGIC-53 and p24 protein families (Dancourt and Barlowe, 2010) and may be important for its functioning. In particular, Emp47p, a Type I membrane protein with homology to ERGIC-53, has been demonstrated to oligomerize as a requisite for its exit from the ER (Sato and Nakano, 2003). Further studies will help to confirm this result with OsCNIH1.

In view of the evidence that cornichons may act as cargo receptors in plants, the Arabidopsis Membrane-based Interactome Network Database (MINDB; Jones et al., 2014) was analysed. It was found that the Arabidopsis cornichon AtCNIH1 (At3g12180) interacts with 535 proteins (Supplementary Table S3 at JXB online), with 30% of these corresponding to membrane transport proteins (Supplementary Fig. S5), strengthening the proposed role of CNIH as a membrane protein cargo receptor in plants.

OsHKT1;3 localizes to the Golgi system

Co-localization of OsHKT1;3-EYFP with the Golgi membrane marker GmMan1 (Fig. 4C), its apparent aggregation in BFA bodies caused by BFA (Fig. 6C; Ritzenthaler et al., 2002), and the high motility of the punctate structures in which it is observed (Supplementary Fig. S3, Supplementary Video S1 at JXB online) are evidence consistent with localization of this transporter at the Golgi membrane. OsHKT1;3 does not localize to the plasma membrane (Fig. 6B) as no co-localization with the aquaporin AtPIP2A was observed, distinguishing this HKT from other members of the gene family (Horie et al., 2007, 2011; Munns et al., 2012). Similar protein fusions of GFP to AtAMT1;1 (a plasma membrane resident transporter) and to another rice HKT, OsHKT1;4, demonstrated that these two proteins localized to the plasma membrane (unpublished results), discarding the possibility that tagging of the protein might have modified its membrane of residence (Figs 4C, 6A, C, D). In plants, transporters belonging to large gene families, as is the case for HKT, have membrane localized to multiple cellular locations, which can include the Golgi. In particular, NHX5 and NHX6, two Na⁺/H⁺ exchangers (Bassil et al., 2011), as well as a phosphate transporter, PHT4;6 (Cubero et al., 2009), have been localized to the Golgi, whereas other family members are either plasma membrane (i.e. NHX7, NHX8, and PHT1) or tonoplast localized (NHX1–NHX4). Moreover, members of the aquaporin family of proteins have diverse subcellular localizations, being present in several intracellular compartments (Wudick et al., 2009). Therefore, it is likely that HKT transporters may be located in different cellular compartments.

OsHKT1;3 is an Na⁺ transporter

Functional characterization in two different heterologous expression systems; Xenopus oocytes and yeast, demonstrated that OsHKT1;3 is an Na⁺ transporter (Figs 7–9). Electrophysiological data from the oocyte system showed...
that the reversal potential changed according to Nernst with extracellular Na$^+$ (54.2 mV per decade) (Fig. 7D), indicative of the high selectivity of OsHKT1;3 for sodium, confirming results reported previously (Jabnoune et al., 2009). Expression of OsHKT1;3 in the yeast BY4741 rendered the colonies more susceptible to Na$^+$ (Fig. 9B), probably by generating an ionic imbalance in the cell and further confirming the selectivity of the transporter for this cation.

Although no direct evidence is available, it is proposed that OsHKT1;3 in the Golgi membrane could catalyse the downhill transport of Na$^+$ towards the cytoplasm and, thus, may function as an alternative shunt conductance for the H$^+$ pumps located in this organelle (Mitsuda et al., 2001; Shimaoaka et al., 2004; Dettmer et al., 2005; Strompen et al., 2005). The Golgi-located NHX5 or NHX6 Na$^+$ exchangers (Yokoi et al., 2002; Bassil et al., 2011) would lead to Na$^+$ accumulation within the Golgi, establishing the Na$^+$ gradient required for the functioning of OsHKT1;3. Recently, an Na$^+$-selective mechanism has been demonstrated to play a similar role in the lysosomal membrane of mammalian cells (Wang et al., 2012).

Together, the results demonstrate that the highly selective Na$^+$ transporter/channel OsHKT1;3 unexpectedly is located at the Golgi membrane, targeted to this endomembrane by its interaction with a newly described cargo receptor in plants, OsCNIH1. Finding OsHKT1;3 at the Golgi raises the possibility of new functions for the HKT family in addition to those previously described. Future research would demonstrate the biological significance of OsCNIH1 and OsHKT1;3 in the rice plant.

### Supplementary data

Supplementary data are available at JXB online.

**Figure S1.** Deletion of ERV14 in *S. cerevisiae*.

**Figure S2.** Quantification of co-localization between OsCNIH1 and OsHKT1;3 and other membrane markers.

**Figure S3.** Dynamics of OsHKT1;3-EYFP in tobacco epidermal cells.

**Figure S4.** Rice HKT transporters do not interact with each other, indicating the absence of oligomerization.

**Figure S5.** Classification of AtCNIH-interacting proteins.

**Table S1.** Primers used for gene cloning into the Gateway (TOPO), pYeP352, and pO02 plasmids.

**Table S2.** Primers used for the deletion of ERV14 in *S. cerevisiae*.

**Table S3.** *Arabidopsis thaliana* proteins interacting with AtCNIH1.

**Video S1.** Dynamics of OsHKT1;3–GFP-labelled bodies that resemble the movement associated with the Golgi apparatus.

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