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OsCHX14 is Involved in the K⁺ Homeostasis in Rice (*Oryza sativa*) Flowers

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Previously we showed in the *osjar1* mutants that the lodicule senescence which controls the closing of rice flowers was delayed. This resulted in florets staying open longer when compared with the wild type. The gene *OsJAR1* is silenced in *osjar1* mutants and is a key member of the jasmonic acid (JA) signaling pathway. We found that K concentrations in lodicules and flowers of *osjar1-2* were significantly elevated compared with the wild type, indicating that K⁺ homeostasis may play a role in regulating the closure of rice flowers. The cation/H⁺ exchanger (CHX) family from rice was screened for potential K⁺ transporters involved as many members of this family in Arabidopsis were exclusively or preferentially expressed in flowers. Expression profiling confirmed that among 17 CHX genes in rice, *OsCHX14* was the only member that showed an expression polymorphism, not only in *osjar1* mutants but also in RNAi (RNA interference) lines of *OsCOI1*, another key member of the JA signaling pathway. This suggests that the expression of *OsCHX14* is regulated by the JA signaling pathway. Green fluorescent protein (GFP)-tagged *OsCHX14* protein was preferentially localized to the endoplasmic reticulum. Promoter-β-glucuronidase (GUS) analysis of transgenic rice revealed that *OsCHX14* is mainly expressed in lodicules and the region close by throughout the flowering process. Characterization in yeast and *Xenopus laevis* oocytes verified that *OsCHX14* is able to transport K⁺, Rb⁺ and Cs⁺ in vivo. Our data suggest that *OsCHX14* may play an important role in K⁺ homeostasis during flowering in rice.

Keywords: CHX transporter • Flower • Jasmonate • Lodicule • Potassium • Rice.

Abbreviations: CHX, cation/H⁺ exchanger; CPA2, cation proton antiporter family 2; cRNA, capped complementary RNA; ER, endoplasmic reticulum; EV, empty vector; GFP, green fluorescent protein; GUS, β-glucuronidase; JA, jasmonic acid; MBS, modified Barth's saline; NHX, Na⁺/H⁺ antiporter;

ORF, open reading frame; PCD, programmed cell death; RFP, red fluorescent protein; RNAi, RNA interference; TEA, tetraethylammonium; WT, wild type.

Introduction

Flowering in rice marks the onset of a period of pollination, fertilization and seed set which is crucial for determining yield. In many plant species, the opening and closure of flowers involves the swelling and withering or abscission of petals and other organs. A rice floret is composed of a pair of glumes, the palea, the lemma, two lodicules, one stamen and six pistils. Anthesis of a rice floret is a quick process which only takes 50–80 min from the opening till closure. Two lodicules at the base of the stamen on the side of the lemma, which are analogous to eudicot petals (Yoshida 2012), are crucial for controlling the opening and closure of florets. The lodicules rapidly swell just prior to anthesis and push the lemma away from the palea, resulting in opening of the floret. After about 30 min, the lodicules start to wither and the lemma moves back towards the palea until the floret closes again (Matsuo and Hoshikawa 1993). The mechanisms for opening and closing flowers varies between species and are not yet very well understood (summarized in van Doorn and van Meeteren 2003, van Doorn and Kamdee 2014). However, changes in osmolarity in petals are believed to be the main driving force for flower closure and this is thought to be related to the metabolism and flux of carbohydrates, amino acids and ions (Beauzamy et al. 2014).

In rice, the dry weight and concentration of soluble sugars in the lodicules were found to rise significantly during floret opening. Therefore, it is suggested that the movement of the lemma away from the palea is mainly caused by decreasing the water potential which in turn is regulated by the accumulation of soluble sugars in lodicules (Wang et al. 1991). However, experiments in maize (*Zea mays* L.) and rye (*Secale cereale* L.) also demonstrated that the enlargement of lodicules is associated

with rapid K^+ accumulation (Heslop-Harrison and Heslop-Harrison 1996). The metabolic processes behind withering of the lodicules are poorly investigated. Loss of sugar and ions through the rachilla might be important since it has been shown that dissection of the florets from the rachilla prolonged the period of floret closure (Wang et al. 1992, Wang et al. 1994). K^+ seems to be involved in loss of turgor in lodicules because it decreases tremendously after floret closure while Na^+ , Ca^{2+} and Mg^{2+} stay constant (Wang et al. 1991). However, flocculent and granular precipitation of calcium was found to be localized differently in lodicule cells during flowering in rice (Qin et al. 2005), which indicates that Ca^{2+} may also play a role in regulation of floret opening and closure.

In Arabidopsis, K^+ homeostasis was also shown to play a key role in the flowering process. Knocking-out of Na^+/H^+ antiporters *NHX1* and *NHX2* resulted in a significant change of vacuolar pH and intravacuolar K^+ . Filaments of the double mutant *nhx1nhx2* did not elongate and lacked the ability to dehisce and release pollen (Bassil et al. 2011).

Furthermore, several plant hormones such as gibberellic acid (Raab and Koning 1987, Takeno 1996), ethylene (Tjosvold et al. 1995, Serek et al. 1996, Celikel and van Doorn 2012), auxin (Takeno 1996, van Doorn et al. 2013), *trans*-zeatin (Takeno 1996), ABA (Kaiharu and Takimoto 1983, Takeno 1996) and jasmonate (JA; Zeng et al. 1999, Ishiguro et al. 2001, Stitz et al. 2014) also showed effects on the opening and closure of flowers in different species. Studies on Arabidopsis and rice mutants identified several genes from the JA biosynthesis and signaling pathways to be involved in the flowering process. An Arabidopsis mutant in the phospholipase A1 gene is defective in anther dehiscence and flower opening (Ishiguro et al. 2001). The gene product catalyzes the initial step of JA biosynthesis, and the defects can be rescued by the exogenous application of JA or linolenic acid. The NAC-like gene *ANTHER INDEHISCENCE FACTOR* from Arabidopsis acts as a repressor controlling anther dehiscence by regulating genes in the jasmonate biosynthesis pathway (Shih et al. 2014). A recent study using a series of mutants from JA biosynthesis and signaling pathways revealed that JA is involved in flower maturation and opening and as a means of communicating with pollinators in *Nicotiana attenuata* (Stitz et al. 2014). In rice, two *osjar1 Tos17* mutants that fail in floret closure were identified (Riemann et al. 2008, Xiao et al. 2014). *OsJAR1*, the gene transposon-mutated in *osjar1-2* and *osjar1-3*, encodes an enzyme able to conjugate JA to specific amino acids resulting in the active hormones.

The above data suggest that K^+ and JA are both important in regulation of the flowering process. Moreover, the double mutant *nhx1nhx2* which accumulated significantly less K^+ in vacuoles partially mimicked the phenotypes of *osjar1* mutants in rice (Riemann et al. 2008, Bassil et al. 2011, Xiao et al. 2014). This indicates that there might be correlations between K^+ homeostasis and JA signaling in regulation of the flowering process.

In this study, the *osjar1* mutants were used to find if there is a link between lodicule withering, K^+ homeostasis and the JA signaling pathway. To confirm the potential role of K^+ in flower

closure, we measured the K concentration in *osjar1-2* lodicules as well as flowers during flowering, and both were found to be significantly higher than in the wild type (WT). We identified one particular gene named *OsCHX14* from the cation/ H^+ exchanger (*CHX*) family which is the only one differentially expressed between *osjar1* alleles and the WT. This gene was also the only one which showed an expression polymorphism in RNA interference (RNAi) lines of *OsCOI1*, another key member of the JA signaling pathway. Our experiments also demonstrated that *OsCHX14* is an endoplasmic reticulum-(ER) localized K^+ transporter, which is preferentially expressed in lodicules and styles throughout the flowering process. The role of *OsCHX14* in flower closure is discussed.

Results

osjar1-2 flowers and lodicules accumulate K^+ during flowering

In previous studies (Riemann et al. 2008, Xiao et al. 2014), two *Tos17* mutants (*osjar1-2* and *osjar1-3*) were described. A main characteristic of the mutants is that the flowers do not close within the normal time frame, but stay open until seed set. The main cause for this phenotype is that the withering of the lodicules is strongly delayed due to loss of function of *OsJAR1* which encodes a JA-Ile synthetase (Xiao et al. 2014). However, more details on the mechanism of how the JA signaling pathway controls flower closure are still needed. The K^+ flux may play a crucial role in closure of the flowers in rice as the K concentration in lodicules decreases sharply after dehiscence (Wang et al. 1991). Thus, we speculate that the failure of *osjar1* floret closure is likely to be related to the K^+ flux and homeostasis in lodicules as well as the adjacent regions. To substantiate this hypothesis further, the K concentration in flowers of *osjar1-2* and the WT was compared. Because rice florets open and close in a relatively short period and *OsJAR1* expression is highly dynamic during the flowering process (Xiao et al. 2014), we selected flowers with the largest angle between palea and lemma to ensure that the mutant and WT were comparable in terms of stage. Compared with the WT, the K concentration in *osjar1-2* flowers was elevated by 30% ($P < 0.05$) (Fig. 1A) and reached $318.8 \mu\text{mol g}^{-1}$ DW. We also measured the concentrations of several other mineral elements in the opening flowers. In addition to K, *osjar1-2* flowers also accumulated elevated concentrations of Ca, Mg, Mn, Na and P (Fig. 1C). In contrast, the level of S, Zn and Fe was similar between the WT and the mutant. This indicates that the JA signaling pathway may also be involved in regulating the homeostasis of other mineral elements. Herein we focus on the study of K^+ which is the most abundant element in rice flowers, specifically comparing lodicules from the same developmental stage. The K concentration in lodicules of *osjar1-2* and the WT were both higher than in the flowers. The ratio of the K concentration in lodicules to that in the flowers was 1.97 in *osjar1-2* and 1.64 in the WT. Similarly to whole flowers, *osjar1-2* lodicules accumulated much more K^+ than the WT. The mean K concentration in lodicules of *osjar1-2* was 72% ($P < 0.01$) higher than that of the

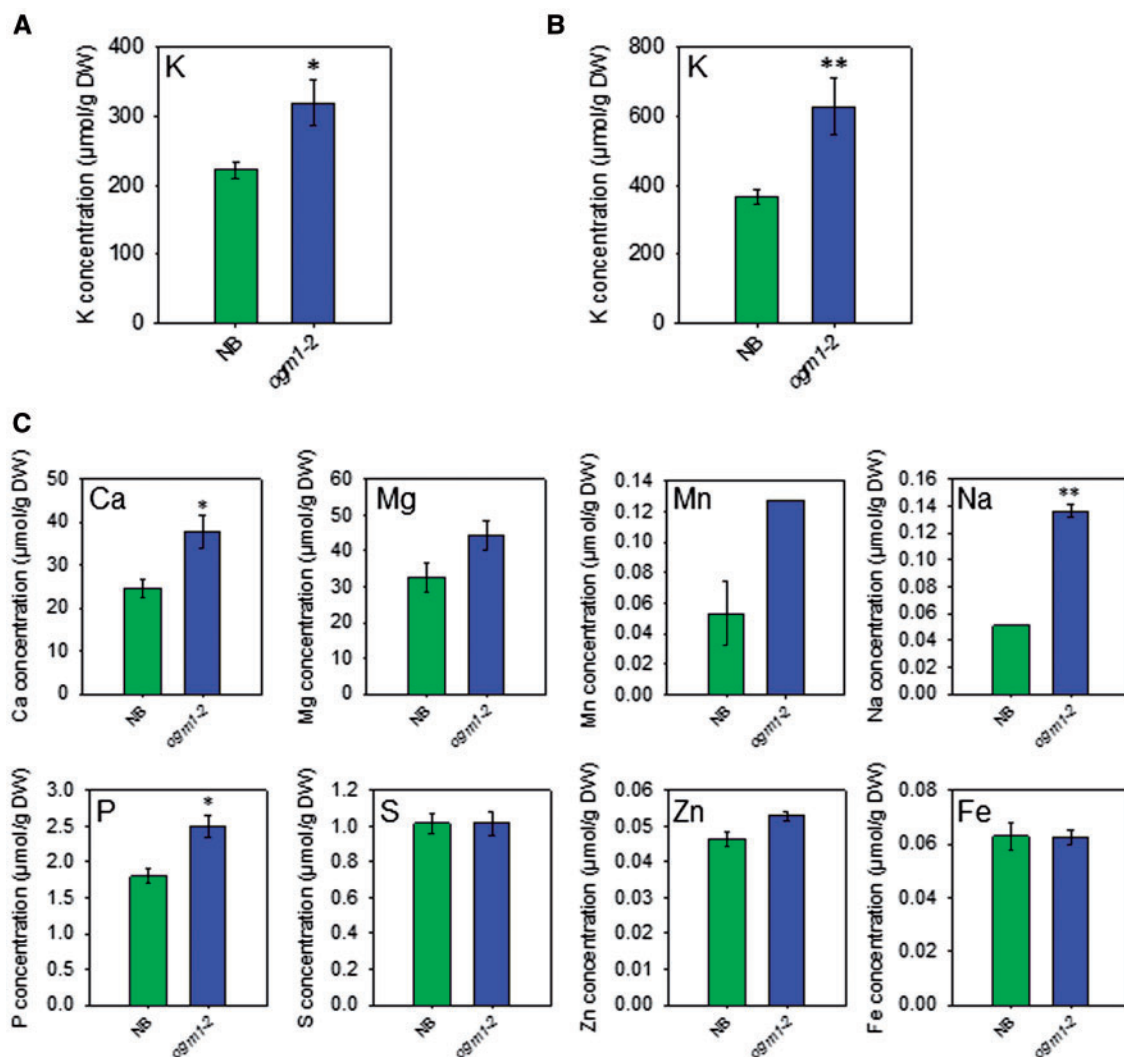


Fig. 1 *osjar1-2* flowers and lodicules accumulate K⁺ during the flowering process. Fifty flowers and 30 lodicules from five flowering plants of *osjar1-2* and the WT (Nipponbare) with the largest open angle between palea and lemma were harvested carefully, dried and weighed. After digestion, element concentrations in flowers were measured by inductively coupled plasma optical emission spectrometry and the K concentration in lodicules was measured by atomic absorption spectrometry. K concentrations are shown in (A) for flowers and in (B) for lodicules. Concentrations of other elements measured in flowers are shown in (C). NB, Nipponbare. Error bars indicate \pm SE. * P < 0.05, ** P < 0.01.

WT (Fig. 1B) and reached 628.2 $\mu\text{mol g}^{-1}$ DW, but in the WT the K concentration was only 365.4 $\mu\text{mol g}^{-1}$ DW.

Differential expression of OsCHX14 in *osjar1* and WT flowers

A large number of proteins from several families have been shown to be involved in K⁺ uptake and transport in plants (Rodriguez-Rosales *et al.* 2009, Dreyer and Uozumi 2011, Wang and Wu 2013, Adams and Shin 2014). This makes the identification of new candidate genes responsible for the abnormal K⁺ homeostasis in flowers of *osjar1-2* challenging. To start with, we focused on the CHX genes that belong to the monovalent cation proton antiporter family 2 (CPA2), and many CHX members are specifically or preferentially expressed in flower organs in *Arabidopsis* (Sze *et al.* 2004). There are 17 CHX genes identified in rice so far (Sze *et al.* 2004, Chanroj *et al.* 2012). In order to screen for CHX members regulated by the JA

signaling pathway, we studied the expression profile (NCBI GEO Accession GSE29577) in RNAi lines of the mutant *OsCOI1* (F-box protein coronatine insensitive 1) (Yang *et al.* 2012) which is a JA-Ile receptor downstream of OsJAR1 in the JA signaling pathway. Comparison of three RNAi and WT replicates showed that only *OsCHX14* exhibited significant expression polymorphisms (Supplementary Table S1). The *OsCHX14* transcripts in three RNAi replicates were four times higher than in two WT replicates but only 20% higher than in the third WT sample (Supplementary Fig. S1). This indicates that expression of *OsCHX14* might be regulated by the JA signaling pathway. We then investigated the expression of *OsCHX14* and five other rice CHX genes in the two *osjar1* mutants and WT flowers from the same stage as we used for the K concentration measurement. As shown in Fig. 2, both *osjar1-2* and *osjar1-3* had an increased *OsCHX14* transcript level which was 54% and 61% higher than in WT flowers, respectively. In the

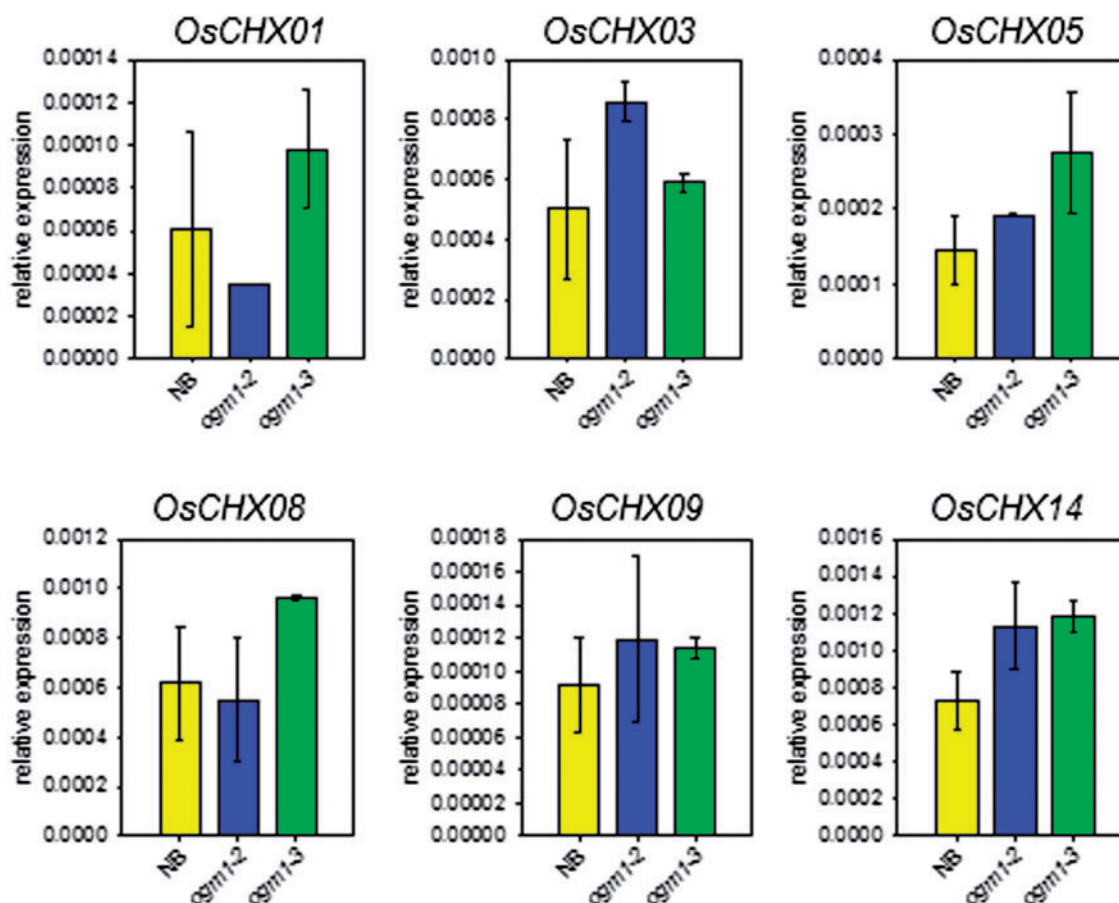


Fig. 2 *OsCHX14* expression is regulated by the JA signaling pathway. Quantitative RT-PCR was used to monitor the expression of six *CHX* genes in flowers with the largest open angle between the palea and lemma. Error bars indicate \pm SE.

same experiment, we did not find consistent expression polymorphisms for the other *CHX* genes tested.

OsCHX14 encodes an ER-localized protein

The coding sequence of *OsCHX14* was amplified for further study from Nipponbare rice and our clone is identical to the longest open reading frame (ORF) as present in the full-length cDNA clone AK069092 from the KOME collection (Kikuchi et al. 2003). *OsCHX14* encodes a predicted protein of 790 amino acids (**Supplementary Fig. S2**). Through BLAST analysis of the sequence of this clone, *OsCHX14* was shown to be identical to LOC_Os05g19500. We also analyzed the protein structure of *OsCHX14* using the online tool ARAMEMNON (plant membrane protein database, <http://aramemnon.botanik.uni-koeln.de/index.ep>), and a total of 18 individual programs show evidence for a prediction of the transmembrane α -helix segment. Different programs give a varied number of transmembrane domains ranging from nine to 13 (**Supplementary Fig. S3**). The individual predictions are combined into a consensus prediction by Consensus TM alpha helix prediction (AramTmCon) using the Bayes' theorem. The result suggests that *OsCHX14* contains 11 putative membrane-spanning α -helical segments (**Supplementary Figs. S2, S3**). In addition, the ARAMEMNON software predicted strong secretory pathway and weak

chloroplast localizations. We further analyzed *OsCHX14* using another newly available online tool named cropPAL (<http://crop-pal.org/>) (Hooper et al. 2016). This software also predicted *OsCHX14* to be localized on the plasma membrane, vacuole and secretory pathways. To study further the subcellular localization of *OsCHX14*, we used a green fluorescent protein (GFP)-tagged construct which was analyzed transiently in *Arabidopsis* (Col-0) root protoplasts. As shown in **Fig. 3**, top row, *OsCHX14*/GFP was found co-localized with an ER marker tagged with a red fluorescent protein (RFP) (Thomas et al. 2008). The *OsCHX14*/GFP and ER/RFP signals are matching in the merged image, which strongly suggests that *OsCHX14* is an ER-localized protein. As a control, we expressed the GFP construct in protoplasts together with the same ER/RFP marker. The control GFP signal exhibited a universal localization in the cell and was not matched with the ER/RFP signal shown in the merged images (**Fig. 3**, bottom row).

OsCHX14 is preferentially expressed in rice flowers

The temporal and spatial expression pattern of *OsCHX14* was investigated in more detail using transgenic rice equipped with either of two *OsCHX14* promoter–GUS (β -glucuronidase) constructs differing in their respective length which were either 2.6 kb (construct ProLOsCHX14::GUS) or 1.1 kb (construct

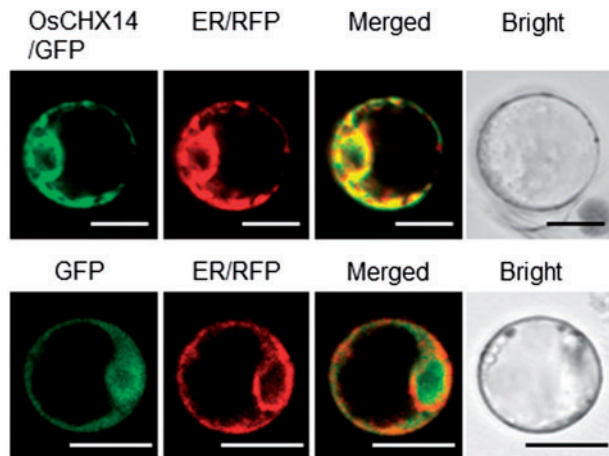


Fig. 3 OsCHX14 is localized to the endoplasmic reticulum. The OsCHX14 ORF was cloned in-frame with a GFP reporter gene and transiently expressed in Arabidopsis root protoplasts together with an ER marker tagged with an RFP reporter (top row). GFP alone and the ER/RFP were also co-transfected in protoplasts for comparison (bottom row). Expression of all constructs was driven by the CaMV (*Cauliflower mosaic virus*) 35S promoter. After culturing overnight, individual protoplasts were observed using fluorescence (left first and second panels) and bright field (fourth panel on the right) confocal microscopy. Merged pictures are shown in the third panel. Scale bar = 10 μm .

ProSOsCHX14::GUS). In total, seven ProLOsCHX14::GUS and five ProSOsCHX14::GUS independent lines were generated and analyzed. Both constructs were found to have essentially the same expression pattern with no obvious quantitative or qualitative differences between the different constructs. The OsCHX14 promoter was found to be mainly active in flower organs, predominantly in lodicules, style, stigma and the base of the ovary, implying that OsCHX14 has a crucial function in these specific tissues. GUS activity was observed before flowering till 1 d after flowering (Fig. 4A–D). The signal was especially strong at the proximal region of lodicules and the ovary close to the rachilla, 1 h after floret closure (Fig. 4C, G). Fig. 4I and J showed that GUS expression was predominantly in the vascular system of lodicules and stigmas. The expression in anther and pollen was also observed before flowering (Fig. 4K). Compared with the flowers, GUS activity in the vegetative tissues was barely detectable in the vascular cylinder of mature roots (Fig. 4L). Our data are consistent with a gene expression atlas covering the entire life cycle of rice, showing that the strongest expression of OsCHX14 was observed in flowers (Supplementary Fig. S4; Wang *et al.* 2010).

Characterization of OsCHX14 in yeast strains defective in K⁺/H⁺ antiporter

Yeast (*Saccharomyces cerevisiae*) was used for complementation assays to monitor a putative function of OsCHX14 in transporting K⁺. Yeast is a convenient tool to study ion transport due to its low complexity alkali metal cation/H⁺ antiporter system. Due to the availability of knock-outs for all three well-described antiporter genes, *Nha1p* (YLR138w), *Nhx1p*

(YDR456w) and *Kha1p* (YJL094c), and the availability of double and triple mutants for complementation, it is now relatively easy to identify heterologous proteins for putative functions in transport of alkali metal cations in yeast. To perform this experiment, we used two constructs in which the OsCHX14 ORF was either solely driven by a GAL1 promoter (construct CHX14-A) or a yeast consensus sequence (Hamilton *et al.* 1987) was added between the GAL1 promoter and the OsCHX14 coding region to improve the efficiency of translation initiation (construct CHX14-B). CHX14-A, CHX14-B as well as the empty vector (EV) were transformed into strain LMB11 (*ena1-4Δnha1Δkha1Δ*) (Maresova and Sychrova 2006), respectively. The EV was also transformed into strain BW31 (*ena1-4Δnha1ΔKHA1*) (Kinclova-Zimmermannova *et al.* 2005) carrying the yeast endogenous cation/H⁺ antiporter gene *KHA1* for comparison. Since several CHX proteins from Arabidopsis exhibited a pH-related phenotype in yeast complementation assays (Maresova and Sychrova 2006, Padmanaban *et al.* 2007, Chanroj *et al.* 2011) in the drop test, we used three media of differing pH values in combination with three different K⁺ concentrations. As shown in Fig. 5A, no significant difference was observed in strain LMB11 (*kha1Δ*) expressing OsCHX14 and strain BW31 (*KHA1*) carrying EV grown on selection medium (containing 7 mM K⁺) at pH 4.7 and pH 6.0 compared with the EV control. However, at pH 7.5 and with 7 mM K⁺, BW31 (*KHA1*) carrying the EV grew faster than LMB11 (*kha1Δ*) with the EV, which is consistent with previous data (Maresova and Sychrova 2005). CHX14-B-harboring LMB11 (*kha1Δ*) also grew slightly faster than LMB11 (*kha1Δ*) with the EV at pH 7.5 with 7 mM K⁺ but not the CHX14-A-carrying LMB11 (*kha1Δ*), which may be related to the differing OsCHX14 protein levels in CHX14-A- and CHX14-B-expressing yeast. When an extra 100 mM K⁺ was added to the media, all yeast strains grew equally under the three different pH conditions. Yet when 500 mM of extra K⁺ was added to the selection plates, BW31 (*KHA1*) with the EV and LMB11 (*kha1Δ*) harboring either of the two different CHX14 constructs all grew faster than the LMB11 (*kha1Δ*) carrying the EV control. Subtle differences were observed between the two different OsCHX14 constructs since CHX14-A-harboring yeast grew slightly faster than those with CHX14-B.

To find the reason for the improved growth of LMB11 (*kha1Δ*) expressing OsCHX14 at high K⁺ concentration, we compared the K concentration of LMB11 (*kha1Δ*) carrying CHX14-A and EV, as well as BW31 (*KHA1*) harboring the EV grown in selection medium supplied with 300 mM of extra K⁺ at pH 4.7. However, 500 mM K⁺ supply was not used because at this concentration the growth of the yeast strains was severely affected in liquid medium. As shown in Supplementary Fig. S5, the net K change among strains was small with the K concentration in OsCHX14-A-harboring LMB11 (*kha1Δ*) reduced by 9% compared with the other two stains, but these differences were not statistically different.

To verify further the observation that OsCHX14 can improve the tolerance of yeast to high concentrations of K⁺, we used Cs⁺, an analog of K⁺ which can be toxic in the drop test. We only tested pH 4.7 as OsCHX14-expressing LMB11 (*kha1Δ*)

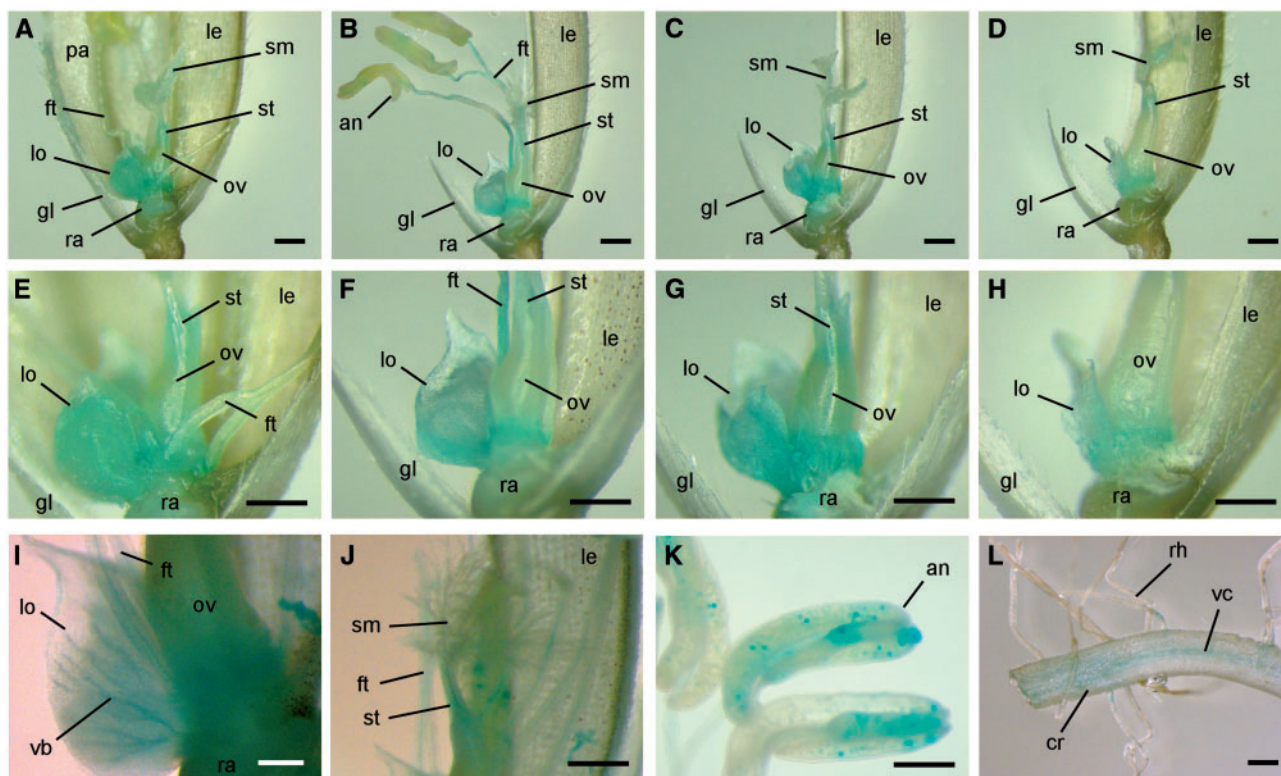


Fig. 4 *OsCHX14* is predominantly expressed in flower organs. The temporal and spatial expression pattern of *OsCHX14* was analyzed by using ProLO*OsCHX14*::GUS and ProSO*OsCHX14*::GUS transgenic rice. Tissues were stained with X-Gluc and incubated at 37 °C overnight. (A) Flowers at 2 h before dehiscence. (B) Flowering. (C) 1 h after floret closure and (D) 1 d after flowering. (E–H) Close up of flowers in (A–D). (I) Close-up of flowering lodicules. (J) Close-up of style and stigma at flowering. (K) Anthers about 2 h before dehiscence. (L) Mature root. an, anther; cr, crown root; ft, filament; gl, glume; le, lemma; lo, lodicule; ov, ovary; pa, palea; ra, rachilla; rh, root hair; sm, stigma; st, style; vb, vascular bundles; vc, vascular cylinder. Scale bar = 500 μm except for Bar (i) = 200 μm .

exhibited a similar phenotype under the three different pH conditions with 500 mM K^+ supplied. As shown in **Fig. 5B**, both CHX14-A- and CHX14-B-expressing LMB11 (*kha1Δ*) strains grew faster on selection medium (7 mM K^+) supplied with 30 mM Cs^+ compared with LMB11 (*kha1Δ*) carrying the EV control. Although loss of function of *KHA1* led to a decreased Cs^+ accumulation in yeast (Heuck et al. 2010), in our experimental conditions with 30 mM Cs^+ no obvious differences in growth were observed on BW31 (*KHA1*) with EV compared with LMB11 (*kha1Δ*) carrying the EV.

OsCHX14 transports K^+/Rb^+ in *Xenopus laevis* oocytes

We further characterized the function of *OsCHX14* in *Xenopus laevis* oocytes using Rb^+ , which is another analog of K^+ and widely used in previous studies. We first verified the expression of *OsCHX14* in oocytes by tagging a GFP reporter gene with the *OsCHX14* ORF. Capped complementary RNA (cRNA) of GFP/*OsCHX14* synthesized in vitro was injected into oocytes and GFP cRNA was used as a control. As shown in **Supplementary Fig. S6**, a GFP/*OsCHX14* fluorescence signal was observed and no signal was identified in the oocytes without any injection. However, the signal intensity was much weaker than the GFP cRNA-injected oocytes, which might be due to most of the

OsCHX14/GFP protein becoming localized to an endomembrane, as also suggested by the results obtained with *Arabidopsis* root protoplasts (**Fig. 3**).

OsCHX14 cRNA (without the GFP tag) was used for all the physiological assays described below, and water was injected as a control. We employed a time course assay by exposing the oocytes to low (10 mM) and high (0.2 mM) affinity ranges of Rb^+ , respectively, and Rb^+ concentrations inside oocytes were determined. As shown in **Fig. 6A**, water-injected oocytes accumulated Rb^+ , which was most probably due to the function of the endogenous K^+ uptake systems in the oocytes (Sobczak et al. 2010). However the *OsCHX14*-expressing oocytes deposited significantly more Rb^+ than the controls, strongly suggesting that *OsCHX14* is able to mediate Rb^+ uptake in oocytes. At 0.5 h, the Rb^+ concentration in the *OsCHX14*-expressing oocytes was 1.7 times higher than in the control exposed to 10 mM Rb^+ . This concentration then increased to 2.1-fold at 6 h, but decreased back to 1.6-fold at 8 h. In the 0.2 mM Rb^+ exposure, *OsCHX14*-expressing oocytes accumulated 1.6 times more Rb^+ than the control at 0.5 h, and this difference increased to 1.8 times at 8 h.

In order to verify that *OsCHX14* is also able to mediate K^+ transport, we employed a K^+/Rb^+ competition assay (see **Fig. 6B**). Addition of K^+ reduced the Rb^+ concentration in water-injected oocytes, suggesting that there was competition

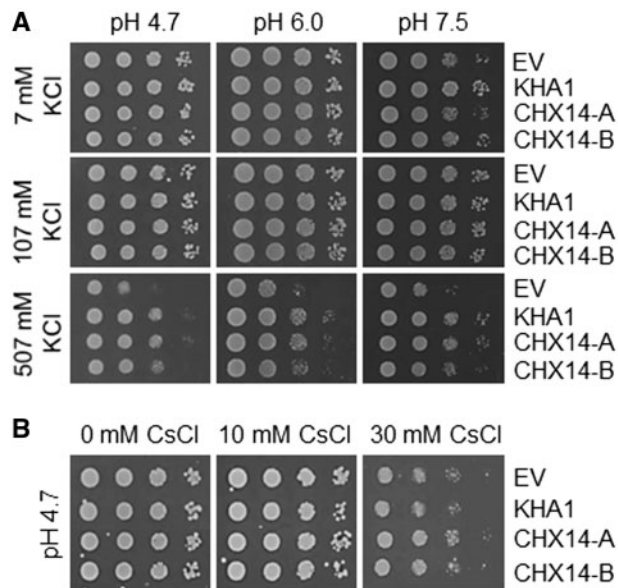


Fig. 5 OsCHX14 improves tolerance of yeast (*S. cerevisiae*) to high concentrations of K⁺. Two constructs were used in the yeast complementation assays, namely CHX14-A in which the *OsCHX14* ORF was solely driven by a *GAL1* promoter and CHX14-B in which a yeast consensus sequence was added between the *GAL1* promoter and the *OsCHX14* ORF to improve translation initiation. Empty vector (EV) and yeast endogenous cation/H⁺ antiporter *KHA1* were used as controls. The overnight cultures were harvested and adjusted to OD₆₀₀ 1.0 with water. A 4 µl aliquot of a 10-fold series dilution was spotted on the selection medium (containing 7 mM KCl) supplied with 2% galactose and different concentrations of KCl (A) and CsCl (B), respectively. EV, CHX14-A, CHX14-B represent strain LMB11 (*kha1Δ*) transformed with empty vector, CHX14-A, CHX14-B, respectively. KHA represents strain BW31 (*KHA1*) transformed with the EV.

between the two elements for uptake by the endogenous K⁺ uptake system in oocytes. Without K⁺, *OsCHX14*-expressing oocytes accumulated significantly more Rb⁺; 42.4% higher than in the control. However, the Rb concentration in *OsCHX14*-expressing oocytes was only 31.2% higher than in the control with K⁺ added. With added K⁺, the difference between *OsCHX14*-expressing oocytes and the control was 26.4% lower than without added K⁺. This result suggests that K⁺ is competing with Rb⁺ for transport by *OsCHX14* in addition to the competition for uptake by the endogenous K⁺ uptake systems in oocytes.

Because *OsCHX14* belongs to the cation/H⁺ antiporter family which carries out proton-coupled transport of substrates, we then further studied the Rb⁺ uptake under different pH conditions. At pH 5.5 and pH 7.5 there was no difference in water-injected controls, but the Rb concentration was significantly increased at pH 9.5, which suggests that oocytes favor more alkaline conditions for Rb⁺ uptake. The *OsCHX14*-expressing oocytes accumulated significantly more Rb⁺ compared with the controls under all three pH conditions. However, the uptake mediated by *OsCHX14* was optimized at pH 7.5, which was 4.8 times higher than the controls, whereas at pH 5.5 and pH 9.5 the differences were only 3.1 and 2.3 times,

respectively, between the *OsCHX14*-expressing and water-injected oocytes (Fig. 6C).

We also tested if *OsCHX14* was able to mediate the Rb⁺ efflux in oocytes by injecting Rb⁺ into oocytes and then measured the Rb concentration inside oocytes after a further incubation in the Barth's saline solution. As shown in Fig. 6D, 8 h after the Rb⁺ injection there was no difference between *OsCHX14*-expressing oocytes and the controls. However, *OsCHX14*-expressing oocytes contained 31.2% less Rb⁺ when compared with the control after 24 h, indicating that the protein can also mediate the efflux of Rb⁺ from oocytes.

We also performed a pharmacological study of *OsCHX14* transport expressed in oocytes. To do so, three widely used K⁺ channel inhibitors, TEA (tetraethylammonium), Ba²⁺ and quinine, were first tested to check if these treatments could decrease the high background Rb⁺ influx by the endogenous K⁺ uptake systems in oocytes. When compared with the controls, the inhibitors exhibited no significant influence on the Rb concentration in *OsCHX14*-expressing oocytes. However, none of the three inhibitors suppressed Rb⁺ accumulation in the controls. On the contrary, Ba²⁺ and quinine treatment significantly increased the Rb concentration by 28.8% and 169.3%, respectively (Supplementary Fig. S7), perhaps indicating that these inhibitors can suppress Rb⁺ efflux by endogenous K⁺ transport systems in oocytes. Therefore, we were not able to perform a complete kinetic characterization of *OsCHX14*.

Discussion

Turgor loss in petals is normally accompanied by senescence (reviewed in van Doorn and van Meeteren 2003). However, the molecular and physiological mechanisms are not fully understood. JA as well as other hormones have been shown to be involved in the programmed cell death (PCD) in plants (reviewed in Kuriyama and Fukuda 2002, Pennell and Lamb 1997). In Arabidopsis, JA was demonstrated to be related to leaf senescence in a COI1-dependent manner (He *et al.* 2002). Furthermore, several studies have also revealed the involvement of F-box proteins and proteasomes, which are important members in the JA signaling pathway, in senescence and resistance-related PCD (Woo *et al.* 2001, Kim *et al.* 2003, Hatsugai *et al.* 2009). Our previous data showed that cell death in parenchymal cells of *osjar1* lodicules was severely delayed, which confirmed that the JA-Ile signaling pathway is essential for PCD in rice lodicules (Xiao *et al.* 2014).

Through an ionic analysis, we found that the homeostasis of several mineral elements including K, Ca, Mg, Mn, Na and P in *osjar1-2* flowers was misregulated, indicating that the defect in the JA signaling pathway affects multiple downstream activities. This might be one of the physiological factors linking the JA signaling pathway to PCD in lodicules and consequently to flower closure. The roles of K⁺, Ca²⁺ and Na⁺ in PCD have been well studied in animals (reviewed in Bortner and Cidlowski 2007, Lang and Hoffmann 2012, Kondratskyi *et al.* 2015, Orrenius *et al.* 2015). Furthermore, K⁺ (Petrucci *et al.* 2001, Balague *et al.* 2003, Peters and Chin 2007, Demidchik *et al.*

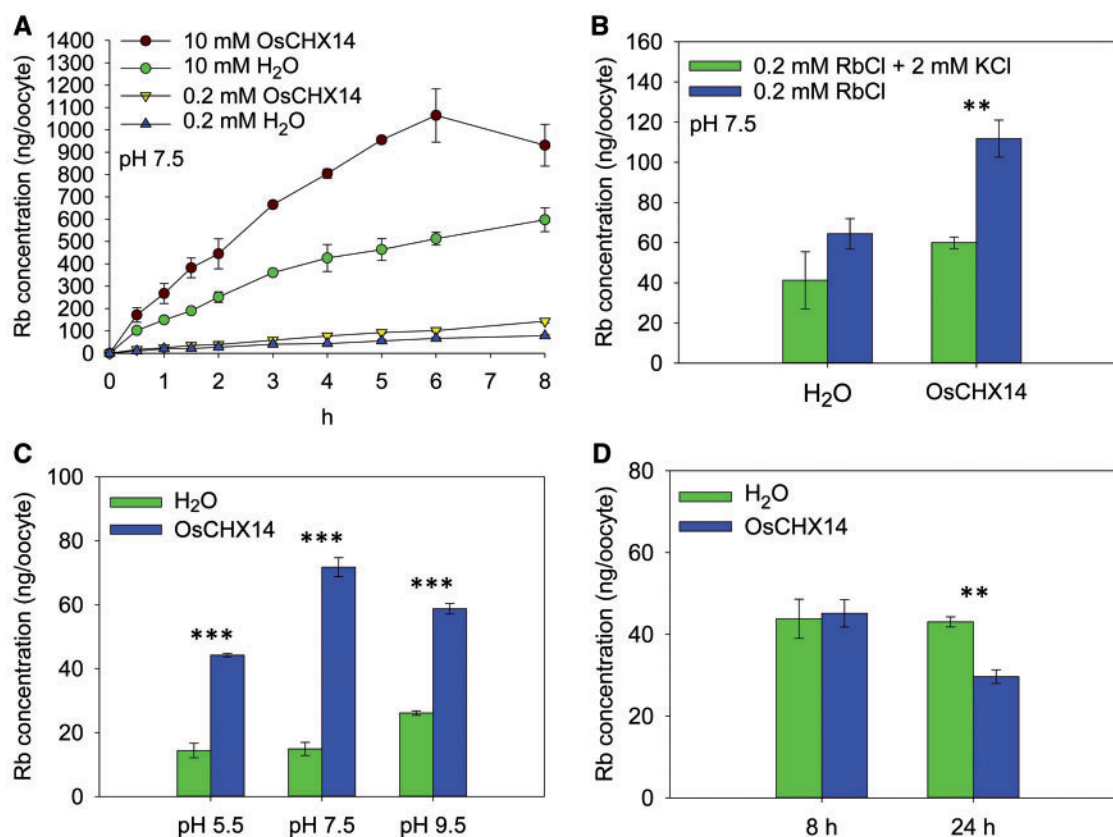


Fig. 6 Characterization of OsCHX14 expressed in *Xenopus laevis* oocytes. A 50 nl aliquot of OsCHX14 cRNA synthesized in vitro was injected into oocytes, and water-injected oocytes were used as controls. The injected oocytes were incubated in modified Barth's saline solution (MBS, pH 7.5) for 2 d before assays. (A) Oocytes were exposed to 10 and 0.2 mM RbCl in MBS (containing 1 mM KCl, pH 7.5), respectively, for different times as indicated in the figure. (B) Oocytes were pre-treated with K^+ -free MBS (pH 7.5) overnight and exposed to 0.2 mM RbCl and 2 mM KCl or 0.2 mM RbCl in K^+ -free MBS (pH 7.5) for 4 h. (C) Oocytes were exposed to 0.2 mM RbCl in MBS (containing 1 mM KCl) for 4 h at pH 5.5, 7.5 and 9.5, respectively. (D) Oocytes were injected with 50 nl of 10 mM RbCl and further incubated in MBS for 8 and 24 h. Oocytes were digested by HNO_3 at $90^\circ C$ after washing thoroughly with MBS. Rb concentration was measured by inductively coupled plasma mass spectrometry. Error bars indicate \pm SE. ** $P < 0.01$, *** $P < 0.001$.

2010, Shabala et al. 2014) and Ca^{2+} (Levine et al. 1996, Rubinstein 2000, Zuppin et al. 2004) have also been suggested to function in PCD in plants. Conversely, the role of Na^+ in PCD has not been described in plants to our knowledge. Furthermore, a double mutant of *nhx1nhx2* in Arabidopsis with affected K^+ homeostasis (Bassil et al. 2011) partially mimicked the phenotypes of *osjar1* mutants in rice (Riemann et al. 2008, Xiao et al. 2014). This indicates that JA signaling and K^+ homeostasis may be functioning in the same pathway in regulating PCD. In addition, the JA signaling pathway was shown to be related to the K^+ status and homeostasis of other tissues (Armengaud et al. 2004, Armengaud et al. 2010, Ma et al. 2012, Takehisa et al. 2013). Taken together, our hypothesis is that metal homeostasis regulated by the JA signaling pathway plays a key role in PCD of lodicules, which in turn controls flower closure in rice.

OsJAR1 disrupted in the *osjar1* mutants encodes a JA-Ile synthetase, and JA-Ile is a signaling molecule in the COI1-mediated JA response that acts by promoting the physical interaction between COI1 and JAZ proteins initiating the SCF^{COI1}-dependent proteasome degradation of JAZ repressor

proteins. These steps will then release transcription factors such as MYC2, which change the expression of downstream genes (reviewed in Memelink 2009, Gfeller et al. 2010, Pauwels and Goossens 2011, Wasternack and Hause 2013). It is very possible that transporters of the misregulated elements in *osjar1-2* flowers are regulated by this pathway, and loss of function of *OsJAR1* will affect the expression of these downstream genes. Indeed, the study of publicly available microarray data for RNAi lines of *OsCOI1* and the WT, as well as our qPCR results both verified that *OsCHX14* is expressed differentially in loss-of-function plants for two key members of the *OsJAR1*/JA-Ile pathway. Together these data provide strong evidence that the expression of *OsCHX14* is regulated by the JA signaling pathway. It is very likely that the elevated K^+ accumulation in *osjar1-2* lodicules is at least partly related to the function of *OsCHX14*. However, there are many different types of K^+ channels and transporters that could possibly mediate the changes in K^+ accumulation. The identification and characterization of *OsCHX14* is important to better understand the role of K^+ in the regulation of flower closure in rice via the lodicules.

Little is known about the biological functions of most of the genes from the rice *CHX* family. However, several *CHX* genes from Arabidopsis, *AtCHX13* (Zhao *et al.* 2008, Zhao *et al.* 2015), *AtCHX14* (Zhao *et al.* 2015), *AtCHX17* (Cellier *et al.* 2004, Maresova and Sychrova 2006, Chanroj *et al.* 2011), *AtCHX20* (Padmanaban *et al.* 2007, Chanroj *et al.* 2011), *AtCHX21* and *AtCHX23* (Lu *et al.* 2011), have been characterized using heterologous expression systems as well as studies in transgenic plants. They are involved in K⁺ acquisition and homeostasis in a range of different tissues including roots, pollen and stomata guard cells. Arabidopsis microarray data revealed that 18 out of 28 *AtCHX* genes are specifically or preferentially expressed in the male gametophyte (Sze *et al.* 2004, Bock *et al.* 2006), suggesting functions in pollen development. Recently, two *Physcomitrella patens* genes *PpCHX1* and *PpCHX2* have been characterized which are able to transport K⁺ (Mottaleb *et al.* 2013). A soybean protein GmsALT3 which shares 59% similarity with *AtCHX20* has been shown to be an Na⁺ transporter and is involved in salt tolerance (Guan *et al.* 2014). Unfortunately no description of the functions of the 17 *CHX* family members in rice is yet available.

Here we report that OsCHX14 is a K⁺ transporter preferentially expressed in flowers. Phylogenetically, OsCHX14 is grouped with *AtCHX16*, *AtCHX17*, *AtCHX18* and *AtCHX19*, but not with *AtCHX20* (Sze *et al.* 2004). Unlike *AtCHX17*, *AtCHX18* and *AtCHX19* which are all localized to the pre-vacuolar compartment in protoplasts (Chanroj *et al.* 2011), GFP-tagged OsCHX14 is found associated with the ER when transiently expressed in protoplasts, which resembles the subcellular localization of *AtCHX16* and *AtCHX20* (Chanroj *et al.* 2011). However, recent studies revealed that *AtCHX16*, *AtCHX17*, *AtCHX18* and *AtCHX19* are also localized on the plasma membrane (Chanroj *et al.* 2013), and *PpCHX2* in *P. patens* is localized to both the tonoplast and plasma membrane (Mottaleb *et al.* 2013). This suggests that *CHX* proteins may move between the plasma membrane and intracellular membrane systems. Although the GFP-tagged OsCHX14 is found localized to the ER in Arabidopsis protoplasts, this could be due to the overexpression of the gene in the cells and we cannot eliminate the possibility of OsCHX14 localization to the plasma membrane in rice like the other *CHX* proteins mentioned above or even localized to other endomembranes such as the vacuole or endosome.

OsCHX14 transcript is mainly found in flower tissues, especially lodicules, with weak expression in the root. *AtCHX17* was also expressed in floral tissues, dominantly in anthers, but no expression was detected in petals. The activity of *AtCHX17* in root (except the root tip) is much stronger than in flower tissues (Cellier *et al.* 2004). Both OsCHX14 and *AtCHX17* are not expressed (or very weakly expressed) in leaf. However, *AtCHX20* expression is strong in stomata guard cells in leaves, as well as in the root cap and anthers. This expression pattern indicates that these genes play variable biological roles in planta.

OsCHX14 shares similarity, but also exhibited some unique characters when compared with closely related Arabidopsis genes when heterologously expressed in the K⁺/H⁺ antiporter mutant of yeast. In standard growth medium, *AtCHX16*,

AtCHX17, *AtCHX18*, *AtCHX19* and *AtCHX20* have all been shown to promote growth of yeast mutants on alkaline medium, which can complement the *kha1* deletion phenotype (Maresova and Sychrova 2006, Padmanaban *et al.* 2007, Chanroj *et al.* 2011). Similarly, OsCHX14 also improves the growth of the *kha1* mutant at pH 7.5 with 7 mM K⁺. Interestingly, OsCHX14 and *AtCHX20* are both associated with the ER in protoplasts. However, with a supply of 500 mM K⁺, OsCHX14 expression in the *kha1* background results in higher tolerance (increased growth) than with the control under all three pH conditions. In contrast, the *AtCHX20*-expressing yeast in the *kha1* background is more sensitive to 500 mM K⁺ compared with the controls under four pH conditions ranging from pH 4.5 to 7.5 (Padmanaban *et al.* 2007). There are no reports of *AtCHX16*, *AtCHX17*, *AtCHX18* and *AtCHX19* being used in yeast complementation experiments in the *kha1* mutant background on 500 mM K⁺. Furthermore, the K concentrations of yeast expressing OsCHX14 in the *kha1* mutant growing in liquid medium supplied with 300 mM K⁺ are not significantly different from either positive (strain carrying WT *KHA1*) or negative (*kha1* mutant harboring an EV) controls. These data suggest that OsCHX14 plays an important role in regulating the homeostasis of K⁺ inside the cells, which may result in an optimized pH in the cell for yeast growth at high K⁺ concentrations.

Previously yeast strain B31 (*ena1-4Δnha1ΔKHA1*), which is isogenic to the BW31 strain used in our study, carrying a WT *KHA1* gene was shown to be unable to improve the growth of LMB01 (*ena1-4Δnha1Δkha1Δ*), which is a mutant for *kha1*, in high K⁺ conditions (Maresova and Sychrova 2005). The strain LMB11 we used was prepared from LMB01 by removing the *kanMX* cassette (Maresova and Sychrova 2006) so the two strains share the same genotype with respect to cation/proton antiporter genes. However, the authors used undiluted cell suspensions in the drop test (Maresova and Sychrova 2005). In our experiments, the EV control strain BW31 (*KHA1*) did not show any differences when compared with strain LMB11 (*kha1Δ*) harboring the EV control when the undiluted cells were spotted on plates with 500 mM extra K⁺ at all three different pH values. However, clear improvements in growth were observed compared with the controls when 10, 100 and 1,000 times diluted cell suspensions were spotted on the same plates (see Fig. 5). This result was highly reproducible in several independent transformants. In publications for other closely related *CHX* genes, we noticed that either *KHA1* was not used as a control in the yeast complementation drop test for high K⁺ conditions (Padmanaban *et al.* 2007, Mottaleb *et al.* 2013) or 500 mM K⁺ was not employed in the drop test (Maresova and Sychrova 2006, Chanroj *et al.* 2011). Therefore, we cannot compare our results with these published data.

More evidence supporting OsCHX14 as a K⁺ transporter comes from our functional expression data in *X. laevis* oocytes showing that OsCHX14-expressing oocytes accumulate more Rb⁺ than the controls when exposed to high and low concentrations of Rb⁺, and K⁺ competes with Rb⁺ for uptake by OsCHX14-expressing oocytes. These data are comparable with the results acquired by expressing *AtCHX17*, *AtCHX20* and

AtCHX23 in *Escherichia coli* defective in K^+ uptake in liquid medium (Chanroj et al. 2011, Lu et al. 2011). OsCHX14 is associated with the ER in protoplasts and it is likely that most of the OsCHX14 protein is also localized on the endomembrane in the oocytes, which is confirmed by the GFP/OsCHX14 localization in oocytes. Therefore, we speculate that the uptake of Rb^+/K^+ is mainly mediated by endogenous K^+ uptake systems in oocytes and then internally compartmentalized from the cytosol. This endomembrane vesicle activity of OsCHX14 in oocytes creates a Rb^+/K^+ concentration gradient between the cytosol and the Barth's saline solution when compared with control oocytes without OsCHX14. However, it is also possible that OsCHX14 protein travels between the plasma membrane and endomembrane, as suggested by the results with several Arabidopsis CHX genes (Chanroj et al. 2013) in which case the plasma membrane-localized OsCHX14 will contribute directly to uptake. The uptake of Rb^+ by OsCHX14-expressing oocytes is pH dependent, which may confirm that the function of OsCHX14 is related to the proton co-transport. However, the differing transport ability mediated by OsCHX14 in oocytes under different pH conditions could be a combined effect of the proton balance required in the various Barth's saline solutions. At these differing external pH conditions, the cytosol and lumen of the intracellular compartment may be changing and this could influence transport by endomembrane-localized OsCHX14 in oocytes. Intriguingly, the Rb^+ efflux is also observed by injection of Rb^+ inside oocytes and after a long duration of further incubation. AtCHX17 and AtCHX20 have a function in protein sorting (Chanroj et al. 2011, Chanroj et al. 2013). It could well be that the efflux is mediated through Rb^+ transported into the ER by OsCHX14 and then excreted together with certain proteins through a secretory pathway in oocytes. The K concentration in the yeast *kha1* mutant expressing OsCHX14 and grown in liquid medium with 300 mM K^+ is 9% lower than that in the controls. This might also be related to the efflux mediated by OsCHX14 through a secretory pathway.

Our data strongly suggests that OsCHX14 is an H^+ -driven K^+ transporter regulated by the JA signaling pathway which is predominantly localized in rice lodicules, but the mechanism of OsCHX14 in regulation of flower closure is still not fully clear. We propose two possible explanations of how PCD in lodicules is regulated by OsCHX14. First, ER stress is one of the crucial factors for PCD in both animals and plants, and it is tightly related to Ca^{2+} homeostasis in the ER (reviewed in Williams et al. 2014, Korner et al. 2015, Krebs et al. 2015). In mammalian systems, K^+ fluxes across the ER membranes are essential for maintaining Ca^{2+} homeostasis (reviewed in Kuum et al. 2015). The pH of the ER is near neutral and is unaffected during release of Ca^{2+} (Kim et al. 1998), and K^+/H^+ antiporter activity is important for Ca^{2+} uptake by the ER (Kuum et al. 2012). Furthermore, *osjar1-2* accumulates elevated levels of Ca^{2+} and K^+ in the lodicules, and our yeast data indicate that OsCHX14 is able to adjust the pH in the cells. Thus, OsCHX14 may play a role in Ca^{2+} homeostasis and storage in the ER through maintaining the pH in the cytosol and ER by K^+/H^+ exchange. Another hypothesis for OsCHX14 in regulation of PCD comes

from the previous reports that the cytosolic K^+ pool is related to the activity of caspase-like proteases and endonucleases, which regulates PCD in plants (reviewed in Shabala et al. 2014). OsCHX14 is localized to the ER, and our data in oocytes indicate that it mediates K^+ (Rb^+) efflux out of the cell, possibly through transport of cytosolic K^+ into the ER following a secretory pathway. In this way, the cytosolic K^+ pool is reduced during the flowering process which will trigger PCD through acidification of the cytoplasm (Walker et al. 1998). However, both hypotheses need to be further studied and tested in a systematic way. Furthermore, whether the evidence acquired in heterologous systems such as yeast and *X. laevis* oocytes represents the actual function of the gene in plants needs to be verified further. To do so, it is key to study transgenic plants with loss-of-function mutations of OsCHX14 and compare them with the *osjar1* mutants as well as with the WT in detail. However, there are currently no T-DNA mutated rice lines available, thus such lines have to be developed alternatively by RNA silencing or the CRISPR–Cas system. High resolution imaging methods such as synchrotron X-ray fluorescence and NanoSIMS can be used to compare the cellular and sub-cellular distribution of the elements in lodicules of these plants. Data obtained using these methods might be precisely matched to the expression pattern of OsCHX14. In addition, fluorescence-based biosensors can be developed to monitor the dynamics of the elements in the lodicules during the flowering process. Furthermore, the study of the expression profile of ER stress-related genes as well as the activity of proteases and endonucleases in lodicules during flowering will provide more clues to uncover the mechanism behind PCD in lodicule closing during flowering and the functions of OsCHX14 in this process.

Materials and Methods

Plant materials

Japonica rice cultivars used in this study were Nipponbare, Zhonghua 11 as well as the mutants *osjar1-2* and *osjar1-3* in the background of Nipponbare. Rice plants were grown in 10×10 cm plastic pots in the greenhouse at 28 °C and 80% humidity with a photoperiod of 12 h day and 12 h night. Plant materials for DNA and total RNA extraction were frozen in liquid nitrogen immediately after harvesting and stored at –80 °C until use.

Determination of mineral element concentrations in rice flowers and lodicules

From both Nipponbare and *osjar1-2*, 50 flowers and 30 lodicules from during flowering (when the palea and lemma were fully separated) were collected and analyzed. Flowers were ground into fine powder in liquid nitrogen and the lodicules were picked carefully with a sharp forceps. The material was dried out at 65 °C for 2 d. A 100 mg aliquot of the dried flower powders was digested with 5 ml of high purity $HNO_3/HClO_4$ (87/13, v/v). The concentrations of mineral elements were determined by inductively coupled plasma optical emission spectrometry (Optima 7300 DV, PerkinElmer). For analysis of the lodicules, all the materials harvested were used. After weighing, the lodicules were lysed with HNO_3 for 48 h. Debris was removed by centrifugation at 14,000 r.p.m. for 15 min. Measurements were done with an atomic absorption spectrometer (AAAnalyst 100, PerkinElmer). The K content of the lodicule extracts was normalized against the dry weight of lodicules. The assays were performed using three biological replicates.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from flowering flowers (three biological replicates) of Nipponbare, *osjar1-2* and *osjar1-3* with the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized with SuperScript[®] III Reverse Transcriptase (Invitrogen) and qPCR was performed on a ABI Prism 7500 (Applied Biosystems) with SYBR[®] Green JumpStart[™] Taq ReadyMix[™] (Sigma). Rice *Actin* and *Histone H3* were used as reference genes. The primer sets required for this experiment (Supplementary Table S2) were based on the corresponding cDNA sequences present in the KOMÉ collection (Kikuchi et al. 2003).

Subcellular localization of OsCHX14

The *OsCHX14* ORF was amplified on cDNA prepared from flowers of cultivar Nipponbare using the forward and reverse primers, 5'-CCGCTCGAGATGGCTCCTGAGGCGGGCGG-3' and 5'-CATGCCATGGCCCTCTACGAGGCCCCGGTG-3'. The PCR product was first cloned into *EcoRV*-digested pBluescript SK II (+) (Stratagene) to form construct SK/*OsCHX14* and the sequence was determined (ServiceXS, Leiden, The Netherlands). Next, the *OsCHX14* ORF was subcloned in-frame with the N-terminus of a GFP reporter gene in vector pTH2 (Chiu et al. 1996, Niwa et al. 1999) between *XhoI* and *NcoI*. Protoplast isolation and transfection was modified from the method described in Yoo et al. (2007). Arabidopsis (Col-0) seedlings were grown on 1× Murashige and Skoog (MS) medium in square Petri dishes for 14–21 d after germination. Root tissues were harvested, cut into fine pieces and digested in enzyme solution [20 mM MES pH 5.7, 20 mM KCl, 0.4 M mannitol, 3.0% cellulase R10 (Yakult), 0.7% macerozyme (Yakult)], placed at 55 °C for 10 min and 10 mM CaCl₂ and 0.1% bovine serum albumin (BSA) were added. The digestion process was carried out for 6 h at 25 °C with shaking at 50 r.p.m. The mixture was harvested and filtered with a 40 µm nylon sieve. Protoplasts were collected by spinning down the liquid which passed through the sieve at 350 rcf for 2 min. Supernatant was removed and the pellet was washed in 500 µl of ice-cold W5 solution [2 mM MES (pH 5.7), 154 mM NaCl, 125 mM CaCl₂, 5 mM KCl] twice. Protoplasts were then suspended in 500 µl of ice-cold W5 solution and placed on ice for 30 min, then collected by spinning down at 350 rcf for 2 min. The supernatant was removed and the pellet was resuspended into MMg solution [4 mM MES (pH 5.7), 0.4 M mannitol, 15 mM MgCl₂]. The protoplast density was counted by hemocytometry and adjusted to 2–5 × 10⁵ by MMg solution. A 5 µg aliquot of plasmid in a total volume of 20 µl was added to 2 ml Eppendorf tubes. Then 100 µl of protoplasts and 120 µl of polyethylene glycol (PEG) 4,000/Ca solution (40% PEG 4,000, 0.2 M mannitol, 100 mM CaCl₂, H₂O to 10 ml) were added and mixed well by gentle inversion. The samples were incubated for 10 min with occasional gentle inversion. Samples were then diluted with 500 µl of W5 solution; protoplasts were collected by spinning down at 350 rcf for 2 min and washed twice with W5 solution. Protoplasts were then suspended in 150 µl of W5 solution and transferred to a 48-well cell culture plate (pre-coated with 1% BSA) which was placed in the dark and incubated overnight. The images were taken with a Leica TCS SP5 (II) confocal microscope. The experiment was performed independently twice, and 20 protoplasts were observed each time.

Binary vector construct and plant transformation

To check for the expression pattern using promoter–GUS constructs, a 2,591 bp and a 1,100 bp *OsCHX14* 5' regulatory fragment preceding the ATG start codon were amplified from genomic DNA from Nipponbare using the two forward primers, 5'-GGAATTCTCAACGCTAGAGATTTCTTG-3' and 5'-GGAATTCAGTTCTAATGGGTGAACATG-3', and a reverse primer 5'-ACGACGACGATGCA AATCTG-3', respectively. The resulting PCR products were digested with *EcoRI* and *NcoI* and cloned into binary vector pCambia-1391Z (GenBank accession No. AF234312; Hajdukiewicz et al. 1994) for translational fusion to the *GUS* gene and sequenced (BaseClear, Leiden, The Netherlands). Transformation of Japonica rice cultivar Zhonghua 11 was performed as previously described (Scarpella et al. 2000) except that *Agrobacterium tumefaciens* strain LBA 4404 was used instead of LBA 1119. Histochemical detection of GUS activity, cytological techniques and microscopy were performed as previously described (Scarpella et al. 2000). Samples were observed under a Leica MZ12 stereo microscope and pictures were taken with a Sony 3CCD Digital Photo Camera DKC-5000.

Yeast complementation assays

For yeast complementation assays, the *OsCHX14* ORF was amplified on vector SK/*OsCHX14* using the primer set, forward 5'-GGAATTCATGGCTCTGAGGCGGGCGG-3' and reverse 5'-GCTCTAGACCTCTACGAGGCCCGGTG-3'. A PCR product was cloned into the pCR-Blunt II TOPO vector (Invitrogen) to generate pTOPO/*OsCHX14*, and the sequence was verified. Next, the *OsCHX14* ORF was subcloned into the URA3-selectable vector pYES2 (Invitrogen) between the *EcoRI* and *XbaI* sites to produce construct CHX14-A. For CHX14-B, the same *EcoRI* and *XbaI* fragment containing the *OsCHX14* ORF was ligated into *BamHI*- and *XbaI*-digested pYES2 together with an adaptor containing a yeast consensus sequence (Hamilton et al. 1987) by annealing of two oligonucleotides: top 5'-GATCCAACACAATGTCTG-3' and bottom 5'-AATTCAGACATTGTGTGG-3'. Yeast strains used were BW31 (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2*) (Kinclova-Zimmermann et al. 2005) and LMB11 (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2 kha1Δ::loxP*) (Maresova and Sychrova 2006). All yeast handling was done as described earlier (Ouwerkerk and Meijer 2001, Ouwerkerk and Meijer 2011). Drop tests were done as described (Maresova and Sychrova 2006) with minor adjustments. Transformed strains were first selected on CM medium [0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate, 0.5% (w/v) ammonium sulfate, 2% glucose, 2% Microagar (Duchefa)] supplied with 20 mg l⁻¹ adenine (Ade), histidine (His), leucine (Leu) and tryptophan (Trp), respectively. Overnight cultures of positive strains were collected and resuspended in water and adjusted to OD₆₀₀ approximately 1.0. Drop tests were done on CM (+Ade +His +Leu +Trp) plates (pH 4.7) supplied with 2% (w/v) galactose instead of glucose. Solutions of pH 6.0 and pH 7.5 were produced by adding 20 mM MES in CM medium and adjusted with arginine. A 4 µl aliquot of a 10-fold dilution series of yeast cell suspension was spotted on the selection medium supplied with different substrates as indicated in the text and figures. The plates were incubated for 3 d at 28 °C before being scanned by an Epson Perfection V550 Photo scanner. Six independent yeast strains were assayed.

The measurement of the intracellular K⁺ content in yeast growing in liquid medium was performed as previously described (Xu et al. 2008) with modifications. CM medium (+Ade +His +Leu +Trp, pH 4.7) supplemented with 2% (w/v) galactose was supplied with 300 mM KCl. Liquid cultures of 100 ml were grown at 30 °C with shaking (200 r.p.m.) until an OD₆₀₀ of approximately 0.4. Cells were harvested by centrifugation at 3,000 × g for 5 min and washed three times with 50 ml of ice-cold 10 mM MgCl₂, 10 mM CaCl₂ and 1 mM HEPES. The pellets were dried out at 65 °C for 72 h, weighed and lysed with HNO₃ overnight. The extracts were diluted properly with MQ water (volume standardized by the dry weight of pellets) and centrifuged at 14,000 r.p.m. for 15 min to remove the debris. After filtering through a Millex-GV 0.22 µm filter unit (Millipore Cooperation), the K concentration was measured by an atomic absorption spectrometer (AAAnalyst 100, PerkinElmer). Three replicates were analyzed for each yeast strain.

Characterization in *Xenopus laevis* oocytes

For the GFP-tagged construct, the *OsCHX14* ORF was subcloned from the pTOPO/*OsCHX14* vector into an *EcoRI/XbaI*-digested pBluescript SK II+ (Stratagene). Then the *OsCHX14* ORF was excised with *EcoRV/XbaI* (*EcoRV* was before and in-frame with *EcoRI*) and ligated in-frame to the N-terminus of a GFP reporter gene in the vector pT7TS/GFP between the *EcoRV* and *SpeI* site. pT7TS/GFP is derived from the oocyte expression vector pT7TS (Cleaver et al. 1996) by adding a GFP reporter gene without the stop codon between *BglII* and *EcoRV*. For the construct without GFP, the *OsCHX14* ORF was amplified on vector SK/*OsCHX14* using the primer set, forward 5'-GAAGATCTATGGCTCTGAGGCGGGCGG-3' and reverse 5'-GGACTAGTTCACCTCTACGAGGCCCGGTG-3'. The PCR products were digested with *BglII/SpeI* and ligated into vector pT7TS. The sequence was confirmed (Eurofin). A 6 µg aliquot of oocyte expression constructs carrying the *OsCHX14* coding region was digested thoroughly with *BamHI* followed by purification with phenol/chloroform and precipitation by sodium acetate and ethanol. A 1 µg aliquot of linearized vector was used in the synthesis of cRNA. Full-length cRNAs were synthesized using a T7 mMESSAGE mMACHINE in vitro transcription kit (Ambion) following the manufacturer's instructions. Oocytes were removed and treated as described previously (Zhou et al. 1998). Stage V or VI oocytes were chosen for injection with 50 nl of cRNA (1 µg µl⁻¹) or an equal volume of

RNase-free water as negative control. The injected oocytes were incubated in modified Barth's saline [MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 0.82 mM MgSO₄, 15 mM HEPES] with 10 µg ml⁻¹ sodium penicillin and 10 µg ml⁻¹ streptomycin sulfate. The solution is adjusted to pH 7.5 using 1 M NaOH for 2 d before assay. For the Rb⁺ uptake time course assay, oocytes were exposed to MBS (pH 7.5) containing 0.2 and 10 mM RbCl, respectively, for the different durations indicated in the figures. The K⁺/Rb⁺ competition assay was done by pre-treating the injected oocytes with K⁺-free MBS (pH 7.5) for 3 h and then exposing them to 0.2 mM RbCl and 2 mM KCl or 0.2 mM RbCl alone for 4 h. For the control, we used just 0.2 mM RbCl as the MBS already contained 88 mM NaCl so Na⁺ was not added to replace K⁺. For determination of Rb⁺ uptake under different pH conditions, the oocytes were exposed to 0.2 mM RbCl for 4 h in three media with different pH values as indicated in the figures. For the Rb⁺ efflux assay, oocytes were injected with 50 nl of 10 mM RbCl and then incubated for 8 and 24 h in MBS (pH 7.5) respectively. The K⁺ channel inhibitor assay was done by pre-treatment of oocytes with 500 µM TEA, 500 µM BaCl₂ and 500 µM quinine, respectively, in MBS (pH 7.5) for 2 h and then exposure to 1 mM RbCl with the same concentrations of K⁺ channel inhibitors in MBS (pH 7.5) for 1 h. The oocytes were then washed six times in MBS (pH 7.5) and digested with HNO₃ at 90 °C for 1 h. The Rb concentration was measured by inductively coupled plasma mass spectrometry (NexION 300, PerkinElmer).

Statistical analysis

The significance of the difference was determined by one-way ANOVA using GenStat 18th edition (VSN International).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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