Functional analysis of genes involved in the regulation of development of reproductive organs in rice (Oryza sativa)

Chen, Y.

Chapter 4

Functions of a monovalent cation-proton antiporter OsCHX14 in the flowering process of rice (*Oryza sativa*)

Yi Chen[1], Mei Wang[1, 2], Zhen Zhu[3], Pieter B.F. Ouwerkerk[1]

[1] Department of Molecular and Developmental Genetics, Institute Biology Leiden, Leiden University, Sylvius Laboratory, Sylviusweg 72, PO Box 9505, 2300 RA Leiden, The Netherlands
[2] SU BioMedicine-TNO, Utrechtseweg 48, 3704 HE Zeist, PO Box 360, 3700 AJ Zeist, The Netherlands
[3] State Key Laboratory of Plant Genomics, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, No.1 West Beichen Road, Chaoyang District, Beijing, 100101 China

Abstract

The rapid swelling and atrophy of lodicules in rice is the decisive mechanism behind the completion of the opening and closure of florets. In Chapter 3, a set of two *osjar1 Tos17* transposon mutants was described and one of the most obvious phenotypes is that the lodicules do not wither even one week after flowering started and as a result *osjar1* flowers stay open. The delay of senescence of lodicule cells after flowering in *osjar1* was confirmed by histological assays. We have verified that the gene interrupted in *osjar1* encodes for an enzyme named OsJAR1 that is capable of conjugating jasmonic acid (JA) to a selection of amino acids (AA). However through which genetic and biochenmical pathway JA-AA regulates lodicule movement is yet unknown. Further studies on *osjar1-2* in this chapter revealed that K^+ content in *osjar1-2* flowering lodicules is twice as high as wild type. Because of this finding twelve potential K^+ transporter genes were selected as candidates in searching of genes responsible for K^+ accumulation in *osjar1-2* lodicules because of their high expression level in flowers. Only one gene *OsCHX14* from the monovalent cation-proton antiporter 2 family exihibited an expression polymorphism in *osjar1-2* and wild type flowers. Moreover, only the expression of *OsCHX14* in wild type rice could be induced by exogenous JA treatment. OsCHX14 is a cytoplasm membrane-localized protein with 12 potential transmembrane domains at the N-terminus. Yeast complementation assay results showed that OsCHX14 is capable of effluxing K^+ outside of the cells. A histochemical assay of transgenic rice harbouring a promoter GUS construct revealed that *OsCHX14* is preferentially expressed in lodicules, styles and the region close to rachilla throughout the flowering process. Based on these results we hypothesize that OsCHX14 is a key factor regulating lodicule senescence in rice.

Introduction

The opening and closure of flowers involve the swelling and withering or abscission of petals and other organs. Mechanisms behind this process vary in different species and are not yet very well described. The turgidity due to osmolarity change in flower organs especially petals are believed to be the main driving force, which is often related to the metabolism of carbohydrates and ion transport (reviewed in Van Doorn and Van Meeteren, 2003). Besides the physiological factors, different plant hormones such as gibberellic acid (Raab and Koning, 1987; Takeno, 1996), ethylene (Tjosvold et al., 1995; Serek et al., 1996), indole-3-acetic acid (Takeno, 1996), trans-zeatin (Takeno, 1996), abscisic acid (Kaihara and Takimoto, 1983; Takeno, 1996), jasmonic acid (Ishiguro et al., 2001) and its methyl ester (Zeng et al., 1999) also show effects on the opening and closure of flowers in different species.

Knowledge on the molecular basis behind flower opening and closure is still very limited. However, studies on *Arabidopsis a*nd rice mutants identified several genes from the jasmonate 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 jasmonic acid (JA) biosynthesis and the defects can be rescued by the exogenous application of JA or linolenic acid. In rice, two *osjar1 Tos17* mutants that fail in floret closure were identified (Chapter 3; Riemann et al., 2008). *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 hormone. The mutants described so far are all proteins involved in jasmonate synthesis pathway, and although their effects on flower opening and closure are obvious the exact physiological and molecular mechanism is unclear.

The rice flower is composed of a pair of glumes, the palea, the lemma, two lodicules, one pistil/carpel and six stamina. Anthesis of a rice floret is a quick process which only takes 50 to 80 minutes from the opening till closure. Instead of petals as with many other plants, two lodicules at the base of the stamina on the lemma side are the crucial organs controlling the opening and closure of florets. The lodicules swell rapidly just prior to anthesis and push the lemma away from the palea resulting in floret opening. After about 30 minutes the lodicules start to wither and the lemma moves back towards the palea until the floret closes again (Matsuo and Hoshikawa, 1993). The dry weight and the 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 of the water potential 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.) demonstrated that the enlargement of lodicules is associated with rapid K^+ accumulation (Heslop-Harrison and Heslop-Harrison, 1996). On the other hand, the molecular and physiological processes behind withering of the lodicules are hardly understood. Loss of sugar and ions through the rachilla might be important since it has been shown that removal of the rachilla prolongs the period of floret closure (Wang et al., 1992, 1994). K^+ seems to be involved in loss of lodicule turgor because it decreases tremendously after floret closure while Na^+, Ca^{2+} and Mg^{2+} stay constant (Wang et al., 1991). Although flocculent and granular precipitation of calcium localized differently in lodicule cells during flowering in rice (Qin et al., 2005), there is no direct evidence in support of its role in regulation of floret opening and closure so far.

In this chapter, the *osjar1-2* mutant was further studied in order to find out the link between the JA signalling pathway and lodicule withering, as well as the molecular basis behind. To confirm the potential role of K^+ played in rice floret closure (Wang et al., 1991), we measured the K^+ content in *osjar1-2* flowering lodicules and it was found to be two times higher than in the wild type. We identified one particular gene named *OsCHX14* from a monovalent cation-proton antiporter 2 (CPA2) family which is the only one differentially expressed between *osjar1*-2 and wild type among 12 candidate genes analyzed. Our experiments demonstrated that OsCHX14 is a cytoplasm membrane localized K^+ transporter, which is preferentially expressed in lodicules and styles throughout the flowering process. Its potential function in regulation of lodicule senescence and floret closure in rice is discussed.

Results

Sencence in *osjar1-2* lodicules is delayed

In Chapter 3 we described the most obvious phenotype of the two *Tos17* mutants *osjar1-2* and *osjar1-3* which are the open staying florets. Characteristic for the two mutant alleles is that flowers open irregularly during day or even night and once opened they will not close within the normal timeframe anymore, but stay open for at least another week. The main cause for this mutant phenotype is that the withering of the lodicules which normally takes place after 30 minutes of flower opening, is strongly delayed. In order to further investigate this phenotype into more detail,

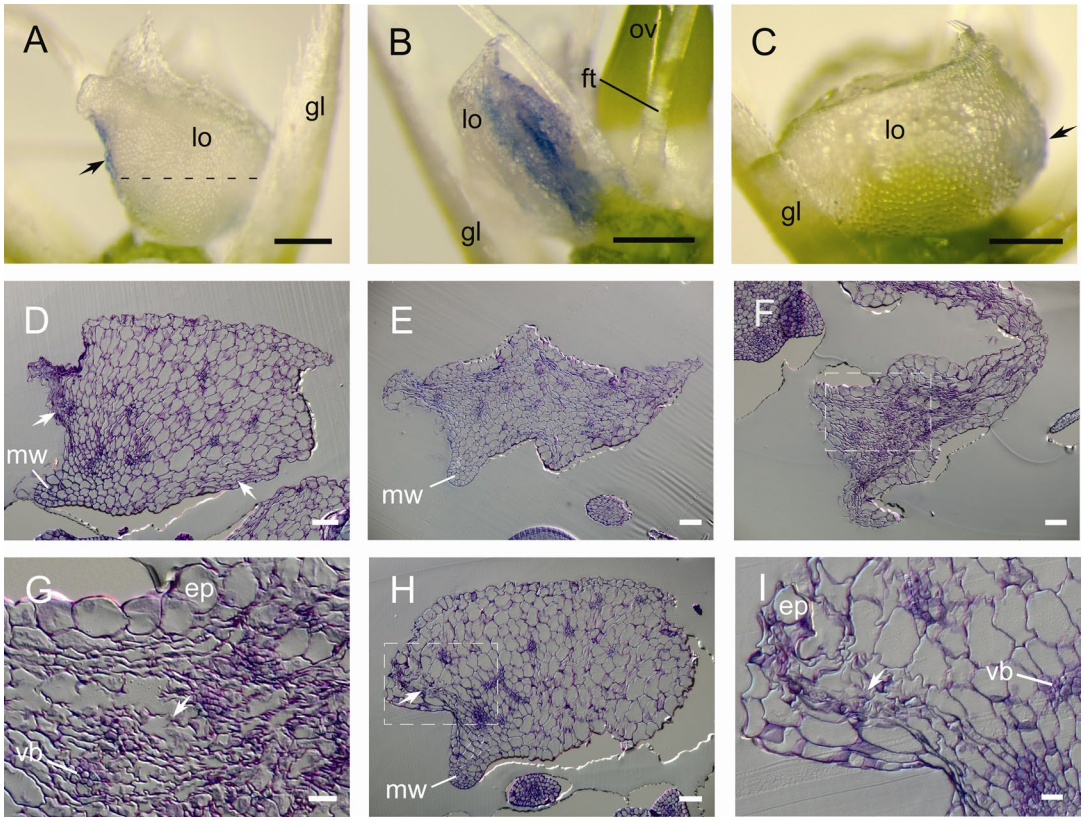


Figure 1. Histochemical characteristics of wild type and *osjar1-2* lodicules at different flowering stages. **A** to **C** lodicules were stained with 0.1% Evans Blue. Wild type of **A.** 60 minutes after flowering start and **B.** two hours after closure. **C.** *osjar1-2* of three days after flowering start. **D** to **I** Lodicules were fixed and embedded in plastic. Sections were made in 4µm at the direction indicated in **A** with dot line and stained with 1% Toluidine Blue. Wild type of **D** 60 minutes, **E** 90 minutes after flowering start and **F** two hours after closure. **G.** Close-up of the square region in **F**. **H.** *osjar1-2* of three days after flowering start. **I.** Close-up of the square region in **H**. Bar (A to C) = 500 µm; Bar (D, E, F and H) = 50 µm; Bar (G and I) = 20 µm. ep, epidermis; ft, filament; gl, glume; lo, lodicule; mw, membranous wing; ov, ovary; vb, vascular bundles.

we stained the lodicules of wild type and *osjar1-2* with Evans Blue. Staining with Evans Blue is a rapid and convenient assay to monitor viability of plant cells. Living cells retain the ability to exclude Evans Blue at the plasma membrane and are hence not stained blue, whereas damaged and dead cells are unable to exclude Evans Blue and will stain with a deep blue colour. As shown in Figure 1, 60 minutes after flowering starts, in rice flowers only a small part of the lodicule is staining blue (Figure 1A). After completion of flower opening, withering of the lodicules will start and the stained part of the lodicule is expanding until it covers most of the lodicule which is reached in about two hours after flower opening started (Figure 1B). However, lodicules of *osjar1-2* plants were only stained within a very limited region even three days after flowering started (Figure 1C). The lodicules were still enlarged like wild-type lodicules shortly after flowering started. These results clearly confirm that senscence in lodicules of *osjar1-2* flowers is severely delayed.

To study delay of senescence in the *osjar1* mutant in more detail, transverse histochemical-stained sections of wild type and *osjar1-2* lodicules at different flowering stages were compared (Figure 1D-I). At 60 minutes after flowering has started, most of the parenchymatic cells in wild type lodicules are still enlarged while two to three cell layers beneath the epidermis, cells are

showing signs of collapse and start to shrink (Figure 1D). The withering of the parenchymatic cells spreads to most of the lodicule 90 minutes after flowering has started (Figure 1E). As a result, the lodicules will lose their turgor and consequently the lemma will move back towards the palea resulting in closure of the floret. Two hours after closure, most of the parenchymatic cells are collapsed and degragation of cell walls is observed (Figure 1F and G). In contrast, parenchymatic cells of *osjar1-2* lodicules kept enlarged even three days after flowering started. Only a few cells near the membranous wing exhibit signes of collapsing (Figure 1H and I).

***osjar1-2* lodicules accumulate K^+ ions during the flowering process**

Unlike maize and rye plants (Heslop-Harrison and Heslop-Harrison, 1996) that accumulate K^+ ions during floret opening, the K^+ ion content of lodicules in rice does not change before and during flowering. However, the K^+ ion flux may play a crucial role in closure of the flowers as K^+ concentration in lodicules decreases sharply after dehiscence (Wang et al., 1991). Therefore, we speculate that one possible reason for the failure of *osjar1-2* floret closure is related to the K^+ flux in lodicules. To substantiate this hypothesis, the K^+ content of the lodicules from *osjar1-2* and wild type flowers was measured using an atomic absorption spectrometer. Thirty random lodicules from five wild type plants taken about 30 minutes after dehiscence start (when lemma and palea were fully separated) were excised from the flowers and dried out in an oven. Lodicules from *osjar1-2* plants were taken three days after flowering started. After lysis with nitric acid the K^+ content of the extracts were measured and normalized against the dry weight of lodicules. Compared to the wild type, the dry weight of *osjar1-2* lodicules was 20% higher (Figure 2A). However, the K^+ concentration of mutant lodicules was two times higher (Figure 2B).

Differential expression and JA-inducible expression of *OsCHX14* in *osjar1-2* and wild type flowers

In order to investigate if the increase in K^+ accumulation in *osjar1-2* lodicules was due to mis-expression of certain genes controlled by the JA related pathway, we searched literature and EST databases (NCBI) for candidate genes. As a result, we found that the most likely gene candidates for a function in K^+ transport in rice flowers are members of the so-called CPA2 and KT/HAK/KUP families that encode for cation/H^+ exchange proteins. Twelve genes from these two families were found with high expression levels in rice flowers (Sze et al., 2004; Gupta et al., 2008). Six of them (*OsCHX1, 3, 5, 8, 9* and *14*) are from the CPA2 family and the other six (*OsHAK1, 2, 15, 17 18* and *23*) are from the KT/HAK/KUP family respectively. RT-PCR was used to monitor expression of each gene on cDNA samples from *osjar1-2* and wild type flowers. To further determine if expression of the 12 candidate genes is controlled by the JA pathway, cDNA samples derived from 1 DAF panicles treated with 5 mM JA were made and used as templates for RT-PCR on the genes of the CPA2 family. As shown in Figure 3, only one gene, *OsCHX14* showed an expression polymorphism between *osjar1-2* and wild type panicles and has a lower expression level in *osjar1-2* flowers. The expression of *OsCHX1, 5, 8* and *9* did not differ between the mutant and wild type and *OsCHX3* is very weakly expressed in both wild type and the mutant. Interestingly, *OsCHX14* was also the only gene affected by JA treatment. Exogenous JA induced the expression of *OsCHX14* within 30 minutes and the expression level increased till one hour after treatment in wild type. Yet no induction was observed in *osjar1-2* panicles (Figure 3). We did not find any expression polymorphisms or

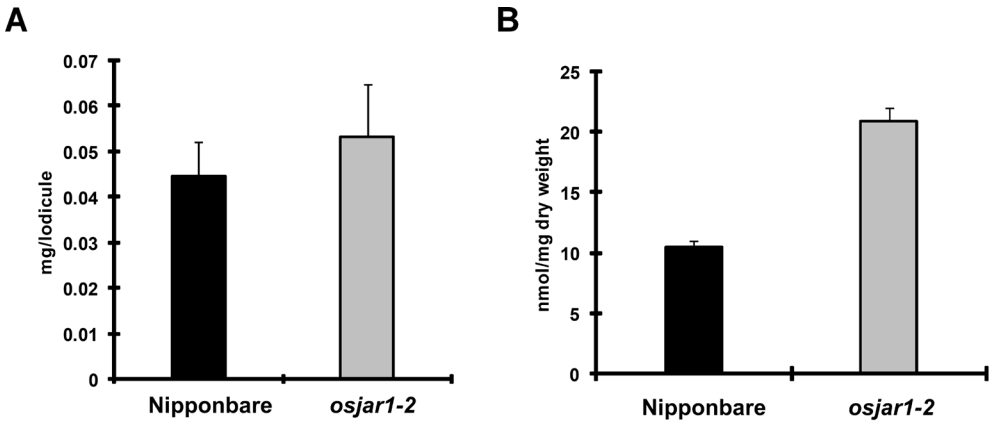


Figure 2. *osjar1-2* lodicules accumulate K^+ in flowering process. Thirty random lodicules from five flowering plants of Nipponbare and three days after flowering start of *osjar1-2* were harvested carefully, dried out and weighted. **A.** The dry weight of *osjar1-2* lodicules and Nipponbare. **B.** After lysis by 65% HNO_3, and K^+ concentration of the extracts were measured and normalized against the dry weight of lodicules.

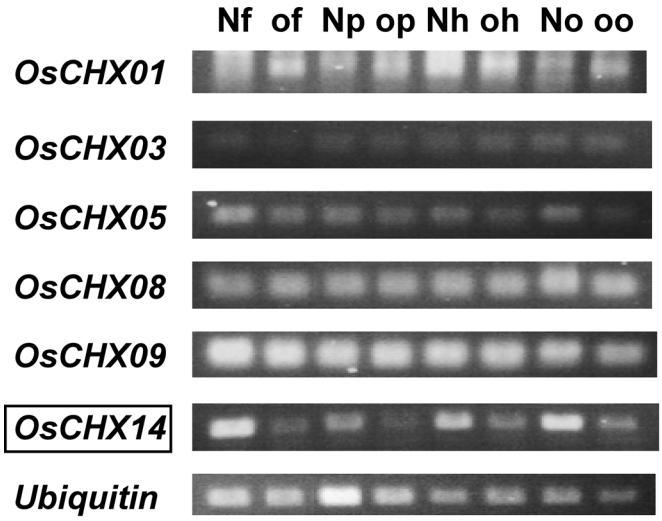
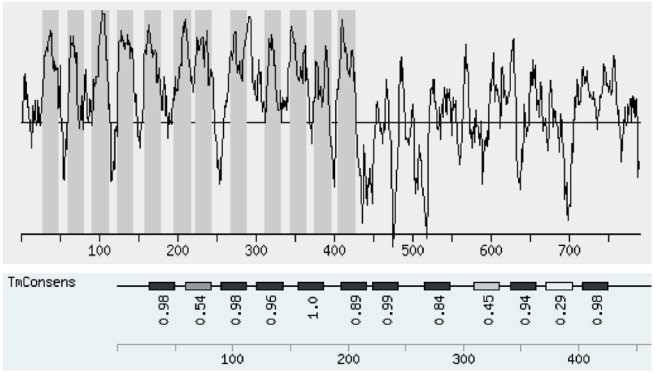


Figure 3. *OsCHX14* expression is regulated by OsJAR1. Gene specific primers were used to amplify on cDNAs isolated from *osjar1-2*, wild type flowers and wild type panicles one day after flowering treated with 5mM JA. Nf, Nipponbare flower. of, *osjar1-2* flower. Np, Nipponbare panicle one day after flowering start (DAF). op, *osjar1-2* panicle (one DAF). Nh, Nipponbare panicle (one DAF) half hour after 5 mM JA treatment. oh, *osjar1-2* panicle (one DAF) half hour after 5 mM JA treatment. No, Nipponbare panicle (one DAF) one hour after 5 mM JA treatment. oo, *osjar1-2* panicle (one DAF) one hour after 5 mM JA treatment.

A

MAPEAAAAALKPMKATSDGVFQGEDPLEAALPLAIVQICIVVVLTRVLAFFLRPLRQPRVIAEIIGGIMLGPSAIGRNSA
FINTVFPKQSLTVLDTLANIGLLFFLFLVGLELDLRAIRRTGAGALAIAVAGISLPFVLGIGTSVVLQNTVNRGVPTGPF
LVFMGVALSITAFPVLARILAELKLLTTDLGRMAMSAAAVNDVAAWILLALAIALSGSGSPFVSLWVLLSGVGFVLSSFF
FIRPLLSWMARRSPEGEPVKELYICTTLTIVLAAGFITDTIGIHALFGAFIVGIIVPKEGPFAGVLLEKVEDLISGLFLP
LYFVSSGLKTNVLTIKGGDSWGLLVLVVATACIGKIGGTVLASLIVRVPLREAVTLGVLMNTKGLVELIVLNIGKDRHVL
NDETFAILVLMALINTFITTPLVMAIYKPARRAPPYKNRAVQRPNPDDELRMMVCFHSTRNIPTMINLMESSRGTRRRGI
TVYAMHLVELSERSSAINMVHKARRNGMPFWNRRRNGDGDQLVVAFETYQQLSHVSIRAMTAISDLHTIHEDVVTSAHQK
RAALIVLPFHKLHQMDGHMESLGDEYQHINQRVLHHAPCSVGILVDRGLGGAAQVAASDVSYNIVVLFFGGRDDREALSY
ATRMVEHPGIALHVIRFVPESGGGGANDKAAADDAFLAEFRGKVAGGNDSIRYEERTSRGKADVVEAIKAMGPTNLFLVG
QGSPCAPLMEPSAECPELGPVGSYLAMPDFSTVASVLVMKQYDPTAEHYELVEEVADTAVDIDTPGPRRG

B



C

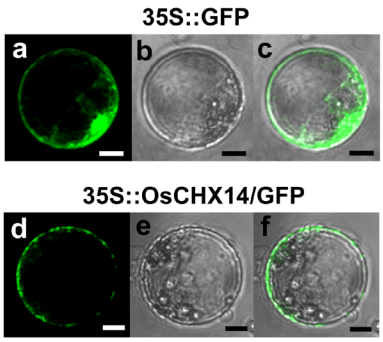


Figure 4. OsCHX14 has a transmembrane structure and is localized on the cytoplasm membrane. The protein structure was analyzed on the ARAMEMNON website. Subcellular localization was analyzed in rice seedling protoplast by tagging *OsCHX14* ORF in frame to a *GFP* reporter gene. **A.** Protein structure of OsCHX14. The potential membrane-spanning regions were marked with lines underneath. **B.** TMconsens analysis result. Twelve transmembrane domains were discovered at the N-terminus. **C.** *OsCHX14* coding region was fused in framed with a *GFP* reporter gene and transformed into rice seedling protoplast. OsCHX14/GFP signal is predominantly visualized on cytoplasm membrane. After culturing overnight, cells were observed using fluorescence (a, d) and bright field (b, e) microscopy. Merged picture of a and b, d and e are shown in c and f respectively. Bar = 10 μm

induction by JA treatment in the candidates selected from the KT/HAK/KUP family (data not shown) and therefore we did not further analyze members from this gene family.

***OsCHX14* encodes a cytoplasm membrane localized protein**

A full length cDNA sequence of 2,731 bp for *OsCHX14* is represented by Genbank Accession AK069092 from the KOME collection (Kikuchi et al., 2003) and encodes a predicted protein of 790 amino acids (Figure 4A). By BLASTing the protein sequence of Genbank Accession AK069092, *OsCHX14* is found identical with locus LOC_Os05g19500. The *OsCHX14* gene has two exons and only one intron. We amplified the ORF of *OsCHX14* from cDNA derived from Nipponbare rice. Then we analyzed protein structure of OsCHX14 in ARAMEMNON (plant membrane protein database, http://aramemnon.botanik.uni-koeln.de/). A total of eighteen individual programs show evidence for a prediction of the transmembrane alpha helix segment. Different programs give a

varied number of *trans*-membrane domains from 9 to 13 (data not shown). The individual predictions are combined to a consensus prediction by Consensus TM alpha helix prediction (TMconsens) using the Bayes' theorem. The result shows that the OsCHX14 protein contains 12 putative membrane-spanning α-helical segments at the N-terminus (Figure 4A and B). The probability scores are relatively high, except for the eleventh putative *trans*-membrane domain (0.29, Figure 4B). To confirm localization of OsCHX14 on the membrane we used a GFP-tagged OsCHX14 construct which was analyzed in a transient protoplast transformation system based on rice cells. As shown in Figure 4C, the GFP signal was predominantly localized on the cytoplasm membrane which confirms the presence of *trans*-membrane domains and suggests a function in the membrane. As a control we used a Pro35S-GFP construct which localized aspecifically in both the membrane and cytosol.

Functional characterization of OsCHX14 in yeast strains defective in K^+ and Na^+ efflux systems

Yeast (*S. cerevisiae*) was used for complementation assays to monitor a putative function of OsCHX14 in transporting K^+ and Na^+ ions. Yeast is a convenient tool to study ion transport due to its low complexity alkali-metal-cation/H^+ antiporter system. By knocking-out either the three well described antiporter genes, *Nha1p* (YLR138w), *Nhx1p* (YDR456w) and *Kha1p* (YJL094c) and the availability of double and triple mutants, it is now relatively easy to study other transgenes for putative functions in transport of alkali metal cations. To perform this experiment, the ORF of *OsCHX14* was cloned into vector pYES2 under control of the galactose inducible *GAL1* promoter resulting into construct pYES2/OsCHX14. This construct was then transformed into three yeast stains W303-1A (Wallis et al., 1989), BW31 (*ena1-4Δ nha1Δ KHA1*) (Kinclova-Zimmermannova et al., 2005) and LMB11 (*ena1-4Δ nha1Δ kha1Δ*) (Maresova and Sychrova, 2006) respectively. Strain BW31 and LMB11 were originally derived from W303-1A and are double and triple mutants respectively. Strains with or without construct pYES2/OsCHX14 were grown equally on 0 or 100 mM KCl containing medium. As shown in Figure 5A, pYES2/OsCHX14 transformed BW31 and LMB11 strains were more tolerant than control strains BW31 and LMB11 on 500 mM KCl medium supporting our hypothesis that OsCHX14 has a function in transport of metal ions. Nonetheless, pYES2/OsCHX14 harbouring strains BW31 and LMB11 are still less tolerant on 500 mM of KCl compared to the pYES2 transformed W303-1A, which may be because OsCHX14 is not as efficient as the endogenous K^+ efflux system (Figure 5A).

In order to verify if OsCHX14 has a function in Na^+ transport too, pYES2/OsCHX14 and pYSE2 transformed strains were also spotted on medium containing 100 or 500 mM of NaCl. No differences were found for any strains when grown on 100 mM NaCl medium. However, BW31 cells, transformed with pYES2/OsCHX14 and control vectors were totally blocked by 500mM NaCl while pYES2 transformed W303-1A was growing still well on the same medium, which indicates that overexpression of OsCXH14 could not complement the function of yeast endogenous Na^+ efflux genes (Figure 5B). On the other hand, pYES2/OsCHX14 harbouring strain LMB11 grew slightly better than BW31 transformed cells. In previous publications *KHA1* was found preferentially localized intracellularly and not to have a function in mediating efflux of alkali metal cations from yeast cells (Ramirez et al., 1998; Maresova and Sychrova, 2005). It is possible that KHA1 in BW31 over-accumulated Na^+ inside certain intracellular organelles which could not been excluded from

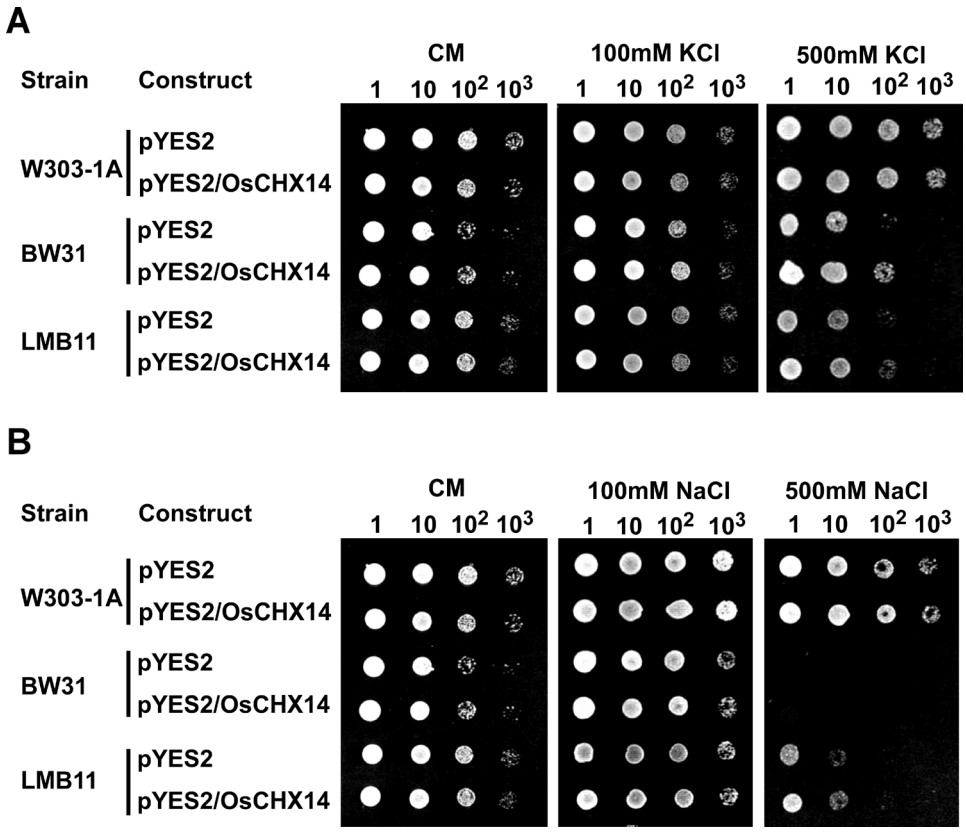


Figure 5. OsCHX14 is able to efflux K^+ outside of yeast (*S. cerevisiae*) cells. *OsCHX14* ORF was amplified and cloned into vector pYES2 under control of a *GAL1* galactose inducible promoter to form a pYES2/OsCHX14 construct. The construct was then transformed into three yeast stains W303-1A, BW31 (*ena1-4Δ nha1Δ KHA1*) and LMB11 (*ena1-4Δ nha1Δ kha1Δ*). The overnight cultures were harvested and adjust to OD600 ~1.0 with water. Three microliters of 10-fold series dilution were spotted on the selection medium supplied with 2% galactose and different concentration of KCl and NaCl. **A.** Yeast strains grown on 0, 100 and 500 mM of KCl. **B.** Yeast strains grown on 100 and 500 mM of NaCl.

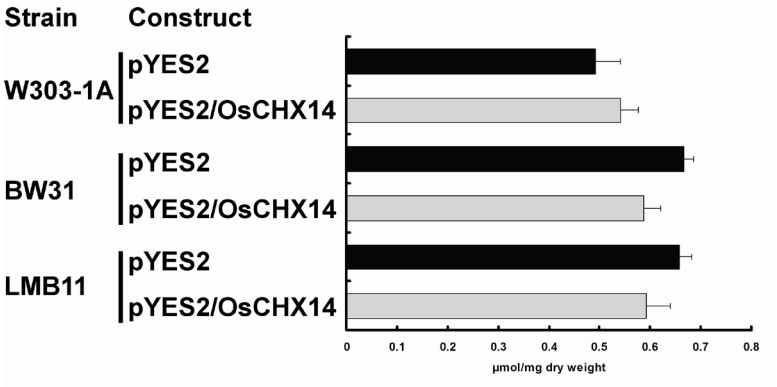


Figure 6. K^+ concentration in the pYES2/OsCHX14 and pYES2 transformed yeast strains. All yeast strains were grown in liquid selection medium containing 2% galactose and 300 mM of KCl till OD600 ~0.4. The cells were harvested, washed and dried out. The pellets were weighted and lysed with HNO_3. The extracts were diluted properly against the dry weight of the pellets and K^+ concentration was measured by atomic absorption spectrometer.

such organelles by other transporters in W303-1A. The presence of OsCHX14 in LMB11 slightly increased tolerance on medium with 500 mM of NaCl. However, it has no influence on BW31. In conclusion, from these results we did not find evidence in support of OsCHX14 being capable of transporting of Na^+.

To further confirm a function of OsCHX14 in K^+ efflux, the intracellular K^+ content of all yeast strains grown in liquid medium containing 2% galactose and 300mM KCl was determined. After dried out in an oven and lysis with nitric acid, the K^+ content of the yeast pellets was measured and standardized for dry weight. As shown in Figure 6, in the pYES2 transformed strains we observed that loss-of-function of *ENA1* and *NHA1* increased accumulation of K^+. Strain BW31 contains 35% higher K^+ concentration compared to untransformed W303-1A. Similar to an early report, KHA1 (Maresova and Sychrova, 2005), has no role in K^+ efflux which is demonstrated by pYES2-transformed LMB11 that has the same K^+ level as control strain BW31. However, overexpression of OsCHX14 in BW31 and LMB11 reduced K^+ accumulation 10% and 13% relative to the controls. These results further confirmed the results of drop tests and support a role of OsCHX14 in K^+ transport. On the other hand, strain W303-1A transformed with empty vector pYES2 contains 10% less K^+ than in presence of plasmid pYES2/OsCHX14. Obviously in this particular case there was no additive effect observed when OsCHX14 was added. On the contrary it reduced the K^+ efflux efficiency, but this effect was nevertheless not strong enough to cause any differences in tolerance in the drop assays with the transformed W303-1A strains.

***OsCHX14* is preferentially expressed in rice flowers**

The temporal and spatial expression pattern of *OsCHX14* was investigated in more detail using transgenic plants equipped with either of two *OsCHX14* promoter GUS constructs differing in their respective length which were either 2.6 kb (construct ProLOsCHX14::GUS) or 1.1 kb (construct ProSOsCHX14::GUS) upstream of the ATG. The constructs were introduced into japonica cultivar Zhonghua 11 by *Agrobacterium*-mediated transformation. In total, seven of ProLOsCHX14::GUS and five of ProSOsCHX14::GUS independent transgenic lines were generated and analyzed respectively. Both constructs were found to have essentially the same expression pattern and there was no obvious quantitative or qualitative difference in expression pattern observed between the different constructs (results not shown). 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 one day after flowering (Figure 7A to D). The signal was especially strong at the proximal region of lodicules and ovary close to the rachilla one hour after floret closure (Figure 7C and G). Figure 7I and J showed that the GUS expression was predominantly in the vascular system of lodicules and stigmas. The expression in anthers and pollen was also observed before flowering (Figure 7K). Compared to the flowers, GUS activity in the vegetative tissues was merely detectable in the vascular cylinder of mature roots at a very weak level (Figure 7L).

Discussion

Previous studies revealed that JA biosynthesis and conjugation pathways are involved in the regulation of the flowering process in rice (Ishiguro et al., 2001; Riemann et al., 2008). Yet very

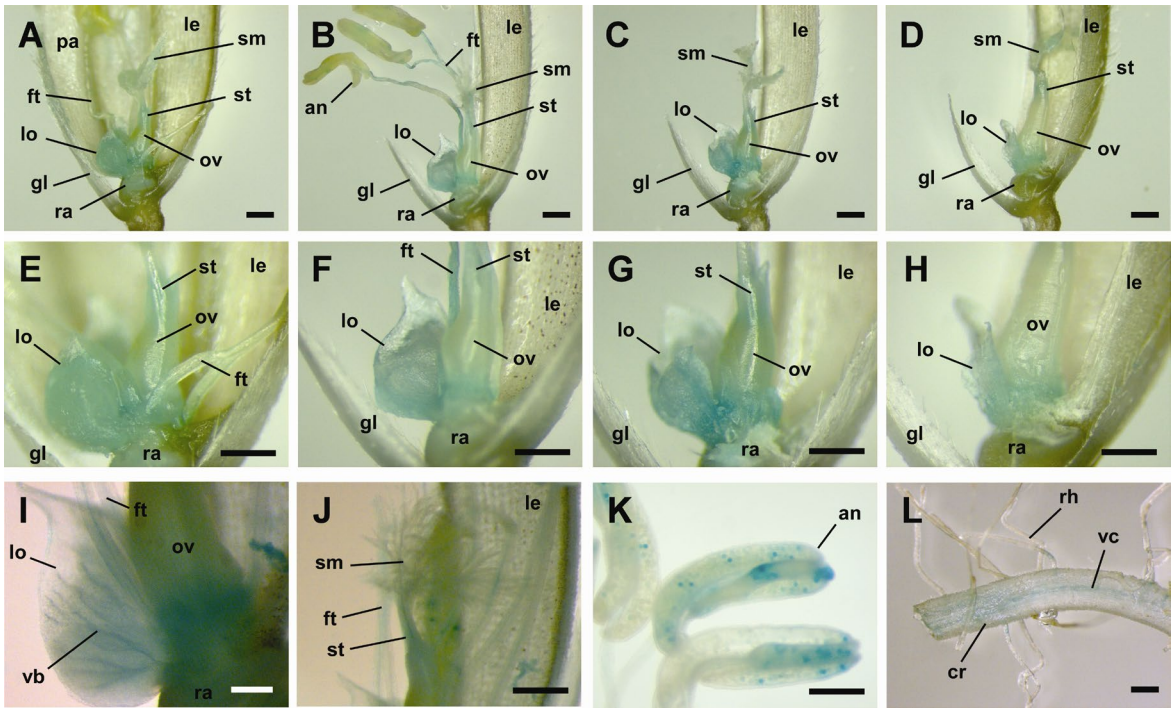


Figure 7. OsCHX14 is predominantly expressed in rice flower organs. Temporal and spatial expression pattern of *OsCHX14* was analyzed by ProLOsCHX14::GUS and ProSOsCHX14::GUS transgenic rice. The materials were stained with X-Gluc solution and incubated at 37°C overnight. Flowers at **A** two hours before dehiscence, **B** flowering, **C** one hour after floret closure and **D** one day after flowering. **E** to **H**, close up of flowers in A to D. **I.** close up of flowering lodicules. **J.** close up of style and stigma at flowering. **K.** anthers about two hours 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. Bar = 500 µM except for Bar (I) = 200 µM.

limited knowledge is available. The opening and closure of rice florets is controlled by the rapid swelling and withering of a pair of lodicules at the base of the stamina. In this chapter we describe a potential function for a CPA2 family member named OsCHX14 in regulation of flower closure in rice. Using transgenic rice plants harbouring a promoter GUS construct we observed that *OsCHX14* was specifically expressed in lodicules during flowering. Moreover, the expression of *OsCHX14* in rice flowers was controlled by the JA pathway. Importantly, lodicules of *osjar1-2* contain a higher level of K^+ than wild type lodicules during flowering and OsCHX14 was found to be able to efflux K^+ outside of yeast cells in functional assays. These data suggest a function of OsCHX14 in the transport of K^+ from lodicules during flowering and thus implicate a function in closure of rice florets.

Potassium is the most abundant alkali cation in plants and has many important regulatory roles in development and signal transduction. The opening and closure of grass flowers may relate to the accumulation and outward bound transport of K^+ in lodicules (Wang et al., 1991; Heslop-Harrison and Heslop-Harrison, 1996). Florets of *osjar1-2* failed to close because the lodicules do not wither for several additional days, whereas they normally close again within 60 minutes after opening. We monitored the K^+ level in *osjar1-2* lodicules during flowering and found o*sjar1-2* lodicules contain twice as much K^+. However the molecular basis behind and how JA is involved in this phenomenon

is unclear.

We analyzed the expression of *OsCHX14* and five other family members because previous studies (Sze et al., 2004; Bock et al., 2006) showed that many genes of CPA2 family are specifically expressed in flowers and may be involved in the regulation of osmolarity change and K^+ homeostasis in certain flower organs. In Chapter 3, OsJAR1 was confirmed to function as a JA-Ile synthetase. JA-Ile is a signaling molecule in the COI1-mediated JA-response, and acts by promoting the physical interaction between COI1 and JAZ proteins and initiates SCF^{COI1} dependent proteasome degradation of JAZ repressor proteins. In turn, this results in release of transcription factors AtMYC2 and subsequent activation of JA response target genes (Chini et al., 2007; Santner and Estelle, 2007; Thines et al., 2007; Staswick, 2008). Our results strongly suggest *OsCHX14* is down-stream regulated by OsJAR1 and the JA pathway. We showed that *osjar1-2* contains a significantly reduced level of JA-Ile and that *OsCHX14* is lower expressed in panicles of *osjar1-2*. Furthermore, the induction of JA-Ile production, *OsCHX14* expression and floret opening by JA treatment were impaired in *osjar1-2* panicles. In wild type plants, JA treatment results in a sharp increase of JA-Ile but in contrast, treatment of *osjar1-2* panicles results in a 25-fold lower level of JA-Ile than in wild type panicles. Besides, the induction of flower dehiscence and *OsCXH14* expression by JA treatment in wild type flowers occurred within 30 minutes. However, both phenomena were lost in *osjar1-2*. Taken together, these results indicate the importance of JA-Ile in regulation of *OsCHX14* expression.

Biological functions of most of the genes from the CAP2 family have not been clarified. Three CHX proteins from Arabidopsis, AtCHX13 (Zhao et al., 2008), AtCHX17 (Cellier et al., 2004; Maresova and Sychrova, 2006) and AtCHX20 (Padmanaban et al., 2007), have been analyzed using complementation assays in yeast (*S. cerevisiae*). They may be involved in K^+ acquisition and homeostasis of different tissues. Microarray data revealed that 18 among 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. CHX proteins may allow osmotic adjustment and K^+ homeostasis during pollen maturation and then rehydrate at pollen tube growth after fertilization. Unfortunately no description concerning the functions of 17 CHX family members in rice is yet available.

Here we report on OsCHX14 as a K^+/H^+ antiporter preferentially expressed in flowers. Observations with GFP-tagged OsCHX14 showed localization on the plasma membrane which is consistent with the occurrence of membrane-spanning domains and its proposed function as transporter. Furthermore, complementation experiments in yeast with OsCHX14 in specific mutants for Na^+ (K^+) / H^+ antiporter genes (Δnhx1 Δnha1 Δkha1) and a plasma membrane Na^+ pump (Δena1-4) showed increased tolerance towards high concentrations of K^+. These data strongly suggest that OsCXH14 is able to efflux K^+ outside of the cell. The highest expression of *OsCHX14* in rice is in lodicules and style, especially in the region close to the rachilla, which has been demonstrated to be essential for transportation of sugar and ions from lodicules (Wang et al., 1991). Our findings highlight the potential roles of OsCXH14 in the transport of K^+ from lodicules and style after flowering.

Though the evidences suggest that K^+ has essential roles in the regulation of floret opening and closure in rice and that OsCHX14 is one of the candidate key transporters involved, the relation between K^+ levels and lodicule withering is still indirect. We hereby propose two models to explain our observations. The first model is that K^+ is involved in osmoregulation of lodicule cells during

floret closure. In many other plants the flow of K^+ into and out of cells, with water following by osmosis, plays a crucial role in regulating the movement of certain organs, such as the thigmonastic response of leaf folding, seismonastic responses to vibration (in the active traps of Venus flytrap plants), and nyctinastic behavior (night, or sleep movements). The significance of osmosis to plant function is best understood by describing its role in the regulation of guard cells. Certain environmental signals such as changes in light intensity, humidity, and carbon dioxide concentration, stimulate the guard cells to take up K^+ which causes their osmotic potential to decrease and water moves in. As a consequence, the guard cells swell and the stomata open. Reverse flow leads to cell shrinkage and stomata close. The failure of floret closure and K^+ accumulation in *osjar1-2* lodicules indicates that the swelling and withering of cells in rice lodicules may also follow the same mechanism as the movement of guard cells. However, turgor change in petals due to osmotic regulation is mainly associated with organ movement by circadian rhythms without the collapse of cells, but according to our results with the Evans Blue stainings, lodicules (petals) undergo senescence shortly after flowering. Previous studies showed that turgor loss in petals is normally accompanied by senescence (reviewed in Van Doorn and Van Meeteren, 2003). Therefore, we believe a second and more appropriate hypothesis is that a decrease in K^+ levels induces programmed cell death (PCD) and that the consequent senescence of lodicules is due to the loss of cellular content. The potential roles of K^+ in PCD have been reported in both animals (reviewed in (Bortner and Cidlowski, 2007)) and plants (Petrussa et al., 2001; Balague et al., 2003; Peters and Chin, 2007). JA and other plant hormones are also involved in the PCD processes in plants (reviewed in (Pennell and Lamb, 1997; Kuriyama and Fukuda, 2002). Our results showed that the collapse in parenchyma cells of *osjar1-2* lodicules is delayed, a mutant with abnormal levels of K^+ and JA-AA compounds. Since the JA-Ile content in leaves of *osjar1* plants is much lower (Chapter 3), it is likely that the pathway of JA-Ile-mediated SCF^{COI1}/proteasome degradation of JAZ repressor is impaired. This result in the misexpression of certain downstream transporter genes like *OsCHX14*, thus changed the K^+ level in the lodicules of *osjar1-2*. Previous studies revealed the involvement of F-box protein and proteasome in the senescence and resistance related PCD of plant cells (Woo et al., 2001; Kim et al., 2003; Hatsugai et al., 2009). In Arabidopsis, JA was also demonstrated to be related to leaf senescence in a COI1 dependent manner (He et al., 2002). Based on these findings we propose that loss of K^+ after flowering induced the death of lodicule cells and closure of the flowers and that OsCHX14 is a key factor responsible for the change of K^+ level which is the controlled by the JA-Ile pathway.

Materials and methods

Plant materials

Japonica rice cultivars Nipponbare, Zhonghua 11 and mutant *osjar1-2* (in the background of Nipponbare) were used in this study. Rice plants were grown in 10 × 10cm plastic pots in the green house at 28°C and 80% humidity. The light regime was 12hr day and 12hr night. Plant material for DNA and total RNA extraction were frozen in liquid nitrogen immediately after harvesting and stored at -80°C until use.

Evans Blue staining and microscopic observations

Histochemical staining was done by vacuum infiltration of materials in 0.1% Evans Blue for five minutes and washing in water for one hour. Pictures were taken under a LEICA MZ 12 stereo microscope with a LEICA DC 500 camera. For sectioning, materials were fixed in 4% formaldehyde and embedded in Technovit 7100 (Heraeus Kulzer GmbH) after dehydrated with a series of ethanol. Next the materials were cut into 4 µm slices with a Leica RM 2165 microtome and stained with 1% Toluidine Blue. Samples were viewed under a ZEISS Axioplan2 imaging microscope and pictures were taken with a ZEISS AxioCam MRc 5 digital camera.

Determination of K^+ content in rice lodicules

Thirty randomly harvested lodicules at flowering stage of Nipponbare rice (half hour after flowering started) and *osjar1-2* (3 days after flowering starts) were picked carefully with a sharp forceps and dried out at 65°C for three days. After weighing, the lodicules were lysed with 65% HNO_3 for 48 hours. The extracts were then diluted with MilliQ (MQ) water based on the dry weight of the original lodicules. Debris was removed by centrifugation at 14,000 rpm for 15 min. Measurements were done with an atomic absorption spectrometer (AAnalyst 100, PerkinElmer). The assays were performed with three biological replicates.

RNA isolation and RT-PCR

Total RNA from flowers and panicles were isolated with TRIzol Reagent (GIBCOBRL) according to the manufacturer's instructions. JA treatment was done by immersing the whole panicles one day after dehiscence into 5mM of JA solution for 30 s. cDNA was synthesized by SuperScript™ III Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. RT-PCR was done with program 98°C 5 min, 98°C 30 sec, 58°C 30 sec, 72°C 30 sec, 35 cycles, 72°C 10 min, 4°C stop. The primer sets required for this experiment (Supplemental Table 1) were based on the corresponding cDNA sequences present in the KOME collection (Kikuchi et al., 2003).

Subcellular localization of OsCHX14

OsCHX14 ORF was amplified on cDNA prepared from flowers of cultivar Nipponbare using the forward and reverse primers, 5'-CCGCTCGAGATGGCTCCTGAGGCGGCGGC-3' and 5'-CATGCCATGGCCCCTCTACGAGGCCCCGGTG-3'. The PCR product was first cloned into *Eco*RV-digested pBluescript SK II+ to form construct SK/OsCHX14 and the sequence was determined (ServiceXS, Leiden, Netherlands). Then *OsCHX14* ORF was subcloned in frame before the N-terminus of a green fluorescent protein *(GFP*) reporter gene into vector pTH2 (Chiu et al., 1996; Niwa et al., 1999) between *Xho*I and *Nco*I. Construct pTH2/OsCHX14 vector was transformed into Nipponbare seedling protoplasts as previously described (Chen et al., 2006). Pictures were taken with a LSM 5 *Exciter* on AXIO Observer microscope (ZEISS).

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 *OsCHX1*4 5' regulatory fragments preceding the ATG starting code were amplified from genomic DNA from Nipponbare using two forward primer 5'-GGAATTCTCAACGCCTAGAGATTTTCTTG-3' and 5'-

GGAATTCAGTTCTAATGGGTGAACATG-3' and one reverse primer 5'-ACGACGACGATGCAAATCTG-3', respectively. The resulting PCR products were digested with *Eco*RI and *Nco*I and cloned into binary vector pCAMBIA-1391Z (Genbank Accession AF234312, (Hajdukiewicz et al., 1994)) for translational fusion to the ß-glucuronidase (*GUS*) gene and sequenced (ServiceXS, Leiden, The Netherlands). Japonica rice cultivar Zhonghua 11 was used for making the transgenic plants. The transformation, histochemical detection of GUS activity, cytological techniques and microscopy were performed as previously described (Scarpella et al., 2000) except that *A. tumefaciens* strain LBA 4404 was used instead of LBA 1119. Samples were observed under a Leica MZ12 stereo microscope and pictures were taken with a Sony 3CCD Digital Photo Camera DKC-5000.

Yeast plasmid construct and complementation drop tests.

For yeast complementation assays, the *OsCHX14* ORF was amplified on vector SK/OsCHX14 using primer set, forward 5'-GGAATTCATGGCTCCTGAGGCGGCGGC-3' and reverse 5'-GCTCTAGACCCTCTACGAGGCCCCGGTG-3'. A PCR product was cloned into pCR-Blunt II TOPO vector (Invitrogen) and sequenced commercially (ServiceXS, Leiden, The Netherlands). Next the *OsCHX14* ORF was subcloned into vector pYES2 (Invitrogen) between the *Eco*RI and *Xba*I sites. Yeast strains used were W303-1A (*MAT*a *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade 2-1 can1-100 GAL SUC2 mal10)* (Wallis et al., 1989), BW31 (*ena1Δ::HIS3::ena4Δ nha1Δ::LEU2* (Kinclova-Zimmermannova et al., 2005)) and LMB11 (*ena1Δ::HIS3::ena4Δ* nha1Δ::LEU2 kha1Δ::loxP (Maresova and Sychrova, 2006)). BW31 and LMB11 were both derived from W303-1A. All handlings with yeast and transformations were done as described earlier (Ouwerkerk and Meijer, 2001, 2011). Drop tests were done as previous described (Maresova and Sychrova, 2006) with minor adjustments. Transformed strains were first select on CM medium (0.17% Yeast Nitrogen Base without amino acids and ammonium sulphate, 0.5% (w/v) ammonium sulphate, 2% glucose; 2% Microagar (Duchefa)) supplied with 20 μg/ml of adenine (Ade), histidine (His), leucine (Leu) and tryptophan (Trp). Overnight culture of positive strains were collected and resuspended in MQ water and adjust to OD600~1.0. Drop test was done on CM (+Ade+His+Leu+Trp) plates supplied with 2% (w/v) galactose instead of glucose. Three microliters of ten fold series of dilutions were spotted on the selection medium supplied with 0, 100 or 500 mM of KCl or NaCl.

Determination of yeast intracellular K^+ content

The measurement of the intracellular K^+ content in yeast was performed as previously described (Xu et al., 2008) with modifications. CM medium (+Ade, +His, +Leu, +Trp) supplemented with galacatose was supplied with or without 300mM KCl. Liquid cultures of 100 ml were grown at 30°C with shaking (200 rpm) till OD600 of ~0.4. Cells were harvested by centrifugation at 3,000g for 5 min and washed three times with 50ml of ice-cold 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mM HEPES. The pellets were dried out at 65°C for 72 hours and weighted; then lysis with 65% HNO_3 overnight. The extracts were diluted properly with MQ water (volume standardized by dry weight of pellets) and centrifuged at 14,000 rpm 15 min to get rid of debris. After filter through a Millev-GV 0.22 μm filter unit (Millipore Cooperation), the K^+ content were measured by an atomic absorption spectrometer (AAnalyst 100, PerkinElmer). Three replicates were analyzed for each yeast strain.

Acknowledgement

We are very much grateful to Dr. Lydie Maresova (Institute of Physiology, Academy of Sciences of the Czech Republic) for generously providing yeast strain BW31 and LMB11; Dr. Paul van der Heusden (Institute Biology Leiden, Leiden University, The Netherlands) for providing yeast strain W303-1A; Dr. Henk Schat and Rudo Verweij, (Faculty of Earth and Life Sciences, Vrije Universiteit, The Netherlands) for helping of ion content determination. We thank Gerda Lamers for technical support in microscopy and Ward de Winter for tissue culture media. We thank Elly Schrijnemakers and Jan Vink for plant caretaking. This research was supported by the Programme for Strategic Scientific Alliances (04-PSA-BD-04) between China and the Netherlands of the Royal Netherlands Academy of Arts and Sciences (KNAW) for MW, PBFO, ZZ and the KNAW China Exchange Programme (04CDP022, 06CDO033, 07CDP005, 08CDP042) for YC, MW, ZZ and PBFO.

Reference

Balague C, Lin B, Alcon C, Flottes G, Malmstrom S, Kohler C, Neuhaus G, Pelletier G, Gaymard F, Roby D (2003) HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide-gated channel ion channel family. Plant Cell **15:** 365-379

Bock KW, Honys D, Ward JM, Padmanaban S, Nawrocki EP, Hirschi KD, Twell D, Sze H (2006) Integrating Membrane Transport with Male Gametophyte Development and Function through Transcriptomics. Plant Physiol **140:** 1151-1168

Bortner CD, Cidlowski JA (2007) Cell shrinkage and monovalent cation fluxes: role in apoptosis. Arch Biochem Biophys **462:** 176-188

Cellier F, Conejero G, Ricaud L, Luu DT, Lepetit M, Gosti F, Casse F (2004) Characterization of AtCHX17, a member of the cation/H^+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K^+ homeostasis. Plant J **39:** 834-846

Chen S, Tao L, Zeng L, Vega-Sanchez M, Umemura K,, Wang GL (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein-protein interactions in rice. Mol Plant Pathol **7**: 417-427

Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, Garcia-Casado G, Lopez-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666-671

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol **6:** 325-330

Gupta M, Qiu X, Wang L, Xie W, Zhang C, Xiong L, Lian X, Zhang Q (2008) KT/HAK/KUP potassium transporters gene family and their whole-life cycle expression profile in rice (*Oryza sativa*). Mol Genet Genomics **280:** 437-452

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation. Plant Mol Biol **25:** 989-994

Hatsugai N, Iwasaki S, Tamura K, Kondo M, Fuji K, Ogasawara K, Nishimura M, Hara-Nishimura I (2009) A novel membrane fusion-mediated plant immunity against bacterial pathogens. Genes & Development **23:** 2496-2506

He Y, Fukushige H, Hildebrand DF, Gan S (2002) Evidence Supporting a Role of Jasmonic Acid in Arabidopsis Leaf Senescence. Plant Physiol **128:** 876-884

Heslop-Harrison Y, Heslop-Harrison JS (1996) Lodicule function and filament extension in the grasses: Potassium ion movement and tissue specialization. Annals of Botany **77:** 573-582

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The deffective in anther dehiscence gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in Arabidopsis. Plant Cell **13:** 2191-2209

Kaihara S, Takimoto A (1983) Effect of Plant Growth Regulators on Flower-Opening of Pharbitis nil. Plant Cell Physiol. **24:** 309-316

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H,

Ooka H, Hotta I, Kojima K, Namiki T, Ohneda E, Yahagi W, Suzuki K, Li CJ, Ohtsuki K, Shishiki T, Otomo Y, Murakami K, Iida Y, Sugano S, Fujimura T, Suzuki Y, Tsunoda Y, Kurosaki T, Kodama T, Masuda H, Kobayashi M, Xie Q, Lu M, Narikawa R, Sugiyama A, Mizuno K, Yokomizo S, Niikura J, Ikeda R, Ishibiki J, Kawamata M, Yoshimura A, Miura J, Kusumegi T, Oka M, Ryu R, Ueda M, Matsubara K, Kawai J, Carninci P, Adachi J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Hayatsu N, Imotani K, Ishii Y, Itoh M, Kagawa I, Kondo S, Konno H, Miyazaki A, Osato N, Ota Y, Saito R, Sasaki D, Sato K, Shibata K, Shinagawa A, Shiraki T, Yoshino M, Hayashizaki Y, Yasunishi A (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science **301:** 376-379

Kim M, Ahn JW, Jin UH, Choi D, Paek KH, Pai HS (2003) Activation of the programmed cell death pathway by inhibition of proteasome function in plants. J Biol Chem **278:** 19406-19415

Kinclova-Zimmermannova O, Zavrel M, Sychrova H (2005) Identification of conserved prolyl residue important for transport activity and the substrate specificity range of yeast plasma membrane Na^+/H^+ antiporters. J Biol Chem **280:** 30638-30647

Kuriyama H, Fukuda H (2002) Developmental programmed cell death in plants. Curr Opin Plant Biol **5:** 568-573

Maresova L, Sychrova H (2005) Physiological characterization of Saccharomyces cerevisiae kha1 deletion mutants. Mol Microbiol **55:** 588-600

Maresova L, Sychrova H (2006) Arabidopsis thaliana CHX17 gene complements the kha1 deletion phenotypes in Saccharomyces cerevisiae. Yeast **23:** 1167-1171

Matsuo T, Hoshikawa K (1993) Science of the Rice Plant. Volume One: Morphology, Vol 1, Tokyo

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. Plant J **18:** 455-463

Ouwerkerk PBF, Meijer AH (2011) Yeast one-hybrid screens for detection of transcription factor DNA interactions. Methods in Molecular Biology **678:** 211-227

Ouwerkerk PBF, Meijer AH (2001) Yeast one-hybrid screening for DNA-Protein interactions. Current Protocols in Molecular Biology 12.12.1-12.12.22

Padmanaban S, Chanroj S, Kwak JM, Li X, Ward JM, Sze H (2007) Participation of endomembrane cation/H^+ exchanger AtCHX20 in osmoregulation of guard cells. Plant Physiol **144:** 82-93

Pennell RI, Lamb C (1997) Programmed cell death in plants. Plant Cell **9:** 1157-1168

Peters J, Chin CK (2007) Potassium loss is involved in tobacco cell death induced by palmitoleic acid and ceramide. Arch Biochem Biophys **465:** 180-186

Petrussa E, Casolo V, Braidot E, Chiandussi E, Macri F, Vianello A (2001) Cyclosporin A induces the opening of a potassium-selective channel in higher plant mitochondria. J Bioenerg Biomembr **33:** 107-117

Qin Y, Yang J, Zhao J (2005) Calcium changes and the response to methyl jasmonate in rice lodicules during anthesis. Protoplasma **225:** 103-112

Raab M, Koning R (1987) Changes in responsiveness to ethylene and gibberellin during corolla expansion ofIpomoea nil. Journal of Plant Growth Regulation **6:** 121-131

Riemann M, Riemann M, Takano M (2008) Rice JASMONATE RESISTANT 1 is involved in phytochrome and jasmonate signalling. Plant Cell Environ **31:** 783-792

Santner A, Estelle M (2007) The JAZ proteins link jasmonate perception with transcriptional changes. Plant Cell **19:** 3839-3842

Scarpella E, Rueb S, Boot KJ, Hoge JHC, Meijer AH (2000) A role for the rice homeobox gene Oshox1 in provascular cell fate commitment. Development **127:** 3655-3669

Serek M, Sisler EC, Reid MS (1996) Ethylene and the Postharvest Performance of Miniature Roses. Acta Horticulturae **424:** 145-150

Staswick PE (2008) JAZing up jasmonate signaling. Trends Plant Sci **13:** 66-71

Sze H, Padmanaban S, Cellier F, Honys D, Cheng NH, Bock KW, Conejero G, Li X, Twell D, Ward JM, Hirschi KD (2004) Expression patterns of a novel AtCHX gene family highlight potential roles in osmotic adjustment and K^+ homeostasis in pollen development. Plant Physiol **136:** 2532-2547

Takeno K (1996) Influences of plant hormones on photoperiodic flowering in Pharbitis nil: re-evaluation by the perfusion technique. Plant Growth Regulation **20:** 189-194

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661-665

Tjosvold S, Wu M, Reid M, Stroome E (1995) Reduction of post-production quality loss in potted miniature roses. Acta Horticulturae **405:** 408-414

Van Doorn WG, Van Meeteren U (2003) Flower opening and closure: a review. J Exp Bot **54:** 1801-1812

Wallis JW, Chrebet G, Brodsky G, Rolfe M, Rothstein R (1989) A hyper-recombination mutation in S. cerevisiae identifies a novel eukaryotic topoisomerase. Cell **58:** 409-419

Wang Z, Gu Y, Gao Y (1991) Studies on the Mechanism of the Anthesis of Rice III. Structure of the Lodicule and Changes of Its Contents during Flowering. Acta Agronomic Sinica **17:** 96-101

Wang Z, Gu Y, Gao Y (1994) Studies on the Mechanism of the Anthesis of Rice V. Comparison of Lodicule and Filament Structure between Sterile Line and Fertile Line. Acta Agronomic Sinica **20:** 14-20

Wang Z, Jin Y, Xu X, Gu Y, Pu L, Gao Y (1992) On the Delayed Lemma-Closing of the Wild Abortion Male Sterile Line Floret of Rice. Seientia Agricuitura Sinica **25:** 22-26

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell **13:** 1779-1790

Xu H, Jiang X, Zhan K, Cheng X, Chen X, Pardo JM, Cui D (2008) Functional characterization of a wheat plasma membrane Na^+/H^+ antiporter in yeast. Arch Biochem Biophys **473:** 8-15

Zeng X, Zhou X, Zhang W, Murofushi N, Kitahara T, Kamuro Y (1999) Opening of Rice Floret in Rapid Response to Methyl Jasmonate. J Plant Growth Regul **18:** 153-158

Zhao J, Cheng NH, Motes CM, Blancaflor EB, Moore M, Gonzales N, Padmanaban S, Sze H, Ward JM, Hirschi KD (2008) AtCHX13 is a plasma membrane K^+ transporter. Plant Physiol **148:** 796-807

Supplemental Table 1 Primers used in RT-PCR

Gene	Locus code Tigr	Primer sequence	
OsCHX01	LOC_Os02g58660	forward	5’-GCTGCTCAACAGGCTCAAG-3’
		reverse	5’-AGCGAGAGGGCGATGATG-3’
OsCHX03	LOC_Os09g37300	forward	5’-TCGTCGGCAGCATCATAAG-3’
		reverse	5’-TACGCCTCCTTCTCCTTCC-3’
OsCHX05	LOC_Os05g39600	forward	5’-GGCTACGACCTTGTGCTC-3’
		reverse	5’-CGCTCCAATCCGACATCC-3’
OsCHX08	LOC_Os08g02450	forward	5’-CCGCTCTCCGTCTATCTC-3’
		reverse	5’-CTTGCCGTGCTTGAAGTG-3’
OsCHX09	LOC_Os12g02840	forward	5’-CGATGGCGGAGAGGACAG-3’
		reverse	5’-CCTACATCTATCTCAACGGTCAC-3’
OsCHX14	LOC_Os05g19500	forward	5’-GACACCGCCGTGGACATC-3’
		reverse	5’-AAACACATTGGAGGATCAAGGTC-3’
OsHAK01	LOC_Os04g32920	forward	5’-TGATGATGCTGATGTTGGAAGAG-3’
		reverse	5’-CCTTCTACTCAAGTTGGCACAC-3’
OsHAK02	LOC_Os01g70940	forward	5’-GCACATTCCACATATTAGTCTG-3’
		reverse	5’-CCTCTTCATCTGTACCAACTG-3’
OsHAK15	LOC_Os04g52120	forward	5’-CGGCAGAAGTTATTCCTGAG-3’
		reverse	5’-AGATAGACGGCTCCAGATTG-3’
OsHAK17	LOC_Os09g27580	forward	5’-TGTTGTTCCTCGCTCCTC-3’
		reverse	5’-ACATTGCTATCATCGGTCTC-3’
OsHAK18	LOC_Os09g38960	forward	5’-TTCCTAACATCTCACCATCTCTTC-3’
		reverse	5’-AACAGCAATCCTCCGAAACC-3’
OsHAK23	LOC_Os09g21000	forward	5’-TCACCGAAGGCAATACAC-3’
		reverse	5’-CGTAGAAGTAATTGATGATGAAC-3’