Plant Biotechnology 31, 213–219 (2014)
DOI: 10.5511/plantbiotechnology.14.0421a

Original Paper

Functional and molecular characteristics of rice and barley NIP aquaporins transporting water, hydrogen peroxide and arsenite

Maki Katsuhara[1,*], Shizuka Sasano[1], Tomoaki Horie[2], Tadashi Matsumoto[3], Jiye Rhee[1,a], Mineo Shibasaka[1]

[1] Institute of Plant Science and Resources, Okayama University, Kurashiki, Okayama 710-0046, Japan;
[2] Division of Applied Biology, Faculty of Textile Science and Technology, Shinshu University, Ueda, Nagano 386-8567, Japan;
[3] Graduate School of Bioresource Sciences, Akita Prefectural University, Shimoshinjo, Akita 010-0195, Japan
*E-mail: kmaki@rib.okayama-u.ac.jp Tel: +83-86-434-1221 Fax: +83-86-434-1249

Received March 29, 2014; accepted April 21, 2014 (Edited by H. Shimada)

Abstract Mercury-sensitive water transport activities were detected in seven NIP (Nodulin 26-like intrinsic protein) type aquaporins among eleven NIPs examined. Amino acid substitutions in rice OsNIP3;3 revealed that mercury-sensitivity depended on a histidine (but not on a cysteine) in apoplastic loop C in plant NIP aquaporins, although the cysteine is involved in the mercury-sensitivity of animal aquaporins. Rice OsNIP3;3 was also first identified as a unique aquaporin facilitating all water, hydrogen peroxide and arsenite transports. In rice OsNIP3;2, hydrogen peroxide and arsenite transport activities were detected, but water transport was not. Barley HvNIP1;2- or rice OsNIP2;1-expressing yeast cells showed the arsenite transport activity but not the H_2O_2 transport activity. The present work revealed novel molecular mechanisms of water and other low molecular weight compounds transport/selection in barley and rice NIP aquaporins, including the histidine-related mercury-sensitivity in the water transport of aquaporins.

Key words: Arsenite, aromatic/arginine filter, hydrogen peroxide, mercury-sensitive water transport, NIP aquaporins.

The first aquaporin (CHIP28, now hAQP1) was discovered as a water channel in human erythrocyte membranes (Preston et al. 1992) and soon many aquaporins were identified widely from bacteria, animals and plants. Higher plants have diverse aquaporin proteins (Bienert and Chaumont 2011; Kaldenhoff and Fischer 2006; Katsuhara et al. 2008; Sakurai et al. 2005; Tyerman et al. 2002) and they are classified into plasma-membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), X-intrinsic proteins (XIPs) and other subgroups. In contrast to PIPs and TIPs, which normally transport water molecules with high conductivity, NIPs and XIPs (Bienert et al. 2011) are recognized to be essential for the transport of physiologically important small molecules such as glycerol (Dean 1999), ammonia (Niemietz and Tyerman 2000), silicon (Ma et al. 2006), boron (Takano et al. 2006), or lactic acid (Choi et al. 2007). Some NIPs have been intensively investigated (Bienert et al. 2008; Liu, et al. 2009; Mitani-Ueno et al. 2011), but many NIPs remain to be characterized.

In the present study, water, hydrogen peroxide and arsenite transport activities were characterized in barley and rice NIPs. Hydrogen peroxide is a multifunctional compound (Cheeseman 2007), a reactive oxygen species inducing cell death and a signal molecule controlling various cellular reactions including Ca^{2+}-channel activation (Pei et al. 2000). Arsenite is one chemical form of arsenic (As) that is toxic to living cells. Ma et al. (2008) demonstrated that arsenite is transported via NIP aquaporins. Previously some rice NIPs (OsNIP1;1, OsNIP2;1=Lsi1, OsNIP2;2=Lsi6, OsNIP3;1, OsNIP3;2) were investigated as silicon ($Si(OH)_4$) or arsenite ($As(OH)_3$) transporters (Ali et al. 2009; Bhattacharjee et al. 2008; Bienert et al. 2008; Ma et al. 2008), but their water transport activities are not yet well understood. Novel OsNIP3;3 was characterized in

Abbreviations: ar/R, aromatic/arginine filter; EGFP, enhanced green fluorescence protein; Hv, *Hordeum vulgare*; NIP, Nodulin 26-like intrinsic protein; NPA, asparagine-proline-alanine; MBS, modified Barth's solution; Os, *Oryza sativa*; P_f, water permeability coefficient; PIP, plasma-membrane intrinsic protein; TIP, tonoplast intrinsic protein; TMH, trans-membrane helix.

[a] Present address: Faculty of Sciences, University of South Bohemia in Ceske Budejovice, Czech Republic.

This article can be found at http://www.jspcmb.jp/
Published online June 5, 2014

the present study. Multiple barley NIPs were also newly identified and characterized this time. Schnurbusch et al. (2010) isolated HvNIP2;1 from a barley variety Sahara (HvNIP2;1(Sahara)), and they reported this NIP aquaporin as a boron transporter. However, the sequence data and characterized feature (present study) indicated that HvNIP2;1(Sahara) is different from the HvNIP2;1 (accession number: AB540229) previously reported in the database. Therefore we propose that HvNIP2;1(Sahara) should be renamed HvNIP2;2, and this name "HvNIP2;2" will be used below.

Materials and methods

NIP gene identification and the introduction of point mutation

Rice NIP aquaporin genes were identified according to genomic annotation by Sakurai et al. (2005) and barley NIP aquaporin genes were identified from EST database and a full length cDNA database (Matsumoto et al. 2011). Their accession numbers are listed in supplemental Table S1. Their amino acid sequences were described in supplementary Figure S1. Point mutation of the construct for the amino acid substitution was done essentially using the one-step site-directed and site-saturation mutagenesis protocol according to the previous report (Zheng et al. 2004). The primers used are listed in supplementary Table S2.

Vector construction for the expression in Xenopus laevis oocytes, the swelling (water transport) assay, and statistic analysis

The coding regions of barley and rice NIPs were subcloned into the pXβG-ev1 oocyte expression vector (Katsuhara et al. 2002). The constructs were linearized with a certain restriction enzyme whose recognition site is not included in each coding region, then subjected to cRNA synthesis with the mMESSAGE mMACHINE T3 transcription Kit (Ambion).

Oocytes were isolated from adult female *Xenopus laevis* frogs and maintained in modified Barth's solution (MBS) overnight at 18°C as described previously (Katsuhara et al. 2002). Oocytes were injected with cRNA of barley or rice *NIPs*, or with water instead of cRNA for the negative control. Injected oocytes were incubated in MBS for approximately 24 h. For the water transport (swelling) assay, an oocyte was transferred from the MBS (200 mOsm) to 5-fold diluted MBS (40 mOsm) to induce water influx, and the osmotic water permeability coefficient (P_f) was calculated as described previously (Katsuhara et al. 2002). For the mercury-sensitivity test, oocytes were incubated with MBS containing 1 mM $HgCl_2$ for 10 min, and then transferred to a 5-fold diluted MBS (40 mOsm, $HgCl_2$-free) to start the swelling assay.

Significant effects of injected aquaporins or mercury-sensitivity were analyzed by Students *t*-test, using the least significant difference at 5% level.

Yeast strains and growth

Two yeast (*Saccharomyces cerevisiae*) strains derived from BY4741 were used in this study; the ΔSKN7 strain that has an impaired oxidative stress defense response and is sensitive to H_2O_2 (Bienert et al. 2007) and the ΔACR3 strain that lacks an arsenite extrusion transporter (Maciaszczyk-Dziubinska et al. 2010) and is sensitive to arsenite (Bienert et al. 2007). Before the introduction of pYES2 (Invitrogen), cells were culture with YPAD medium (1% bacto yeast extract (Difco), 2% bacto peptone (Difco), 2% glucose and 27 μM adenine (Sigma)). After the transformation with pYES2, cells were culture with synthetic medium (SC-Ura) that was provided as 0.67% yeast nitrogen base without amino acids (Difco), pH 6.0 and supplemented with 0.077% Complete Supplement Mixture (CSM) Drop-out: -Ura +Ade (Formedium) according to the auxotrophic requirements of the strains used. Both yeast cells were grown on SC-Ura (Glu), i.e., SC-Ura supplemented with 2% glucose, or SC-Ura (Gal+Suc), i.e., SC-Ura supplemented with 2% galactose and 0.6% sucrose.

H_2O_2 transport assay

ΔSKN7 yeast cells were transformed with either an empty pYES2 (Invitrogen) as vector control or pYES2 including each NIP gene. They were precultured on SC-Ura (Glu) medium. Cells were resuspended in sterile water and 3 μl of fresh yeast cells were streaked on solid SC-Ura (Gal+Suc) medium containing 0 or 0.3 mM hydrogen peroxide. After 3 days of incubation at 30°C, differences in growth were scored. This assay was duplicated.

Arsenite transport assay

ΔACR3 yeast cells were transformed with either an empty pYES2 as vector control or pYES2 including each NIP gene. They were precultured on SC-Ura (Glu) medium. Cells were resuspended in sterile water to an A_{600} of 0.01 or 10-fold dilution series, then 3 μl of each was spotted on solid SC-Ura (Gal+Suc) medium containing As_2O_3 that is converted to $As(OH)_3$ by hydration in the solution. After 3 to 5 days of incubation at 30°C, differences in growth were scored. This assay was performed at least three times.

Results

Water transport

A significant increase of water permeability coefficient (P_f) was observed in *Xenopus* oocytes expressing barley and rice NIPs except OsNIP3;2 (Figure 1). Among the water-permeable NIPs, OsNIP2;2 and OsNIP3;1 showed weak water transport activity. Intense EGFP fluorescence was observed in the plasmamembrane of oocytes expressing EGFP-OsNIP2;2 or EGFP-OsNIP;3;2 (supplementary Figure S2), indicating that the low water transport activity of OsNIP2;2 and OsNIP3;2 was not due to the mis-localization of NIP proteins. EGFP fluorescence was weak in the plasmamembrane of

oocytes expressing EGFP-OsNIP3;1.

Incubation with $HgCl_2$, a typical water channel inhibitor, reduced the P_f of oocytes expressing seven NIPs among the ten examined. Because OsNIP3;3 showed the highest activity ($P_f = 1.6 \pm 0.2 \times 10^{-2}$ cm s^{-1} without $HgCl_2$, Figure 1) and mercury-sensitivity, we focused on OsNIP3;3 to investigate the molecular mechanism of water transport and its inhibition by mercury ion. First, Cys[124] that can be targeted by mercury ion in OsNIP3;3 was substituted by a serine residue that is the most analogous amino acid to Cys. The thiol group (–SH), a possible target by mercury ion, in Cys is replaced with the hydroxyl group (–OH) in Ser. However, both water transport activity and mercury-sensitivity remained in OsNIP3;3-C124S (Figure 2A). Next, five histidine residues in OsNIP3;3, i.e., His[94], His[120], His[125], His[139] or His[168], were substituted for phenylalanine. Some of the histidine residues were also substituted for alanine residues (Figure 2B–D). The imidazole group, another possible target by mercury ion, in His is replaced with the phenyl group or hydrogen in Phe or Ala, respectively. Water transport activity was lost in OsNIP3;3-H120F ($P_f = 0.14 \pm 0.04 \times 10^{-2}$ cm s^{-1}) or OsNIP3;3-H125F ($P_f = 0.20 \pm 0.06 \times 10^{-2}$ cm s^{-1}) and no inhibition with mercury was observed (data not shown). Both water transport activity and mercury-sensitivity remained in OsNIP3;3-H94F (Figure 2B). Water transport activity was markedly reduced with the substitution of His[139] for phenylalanine but mercury-sensitivity was still detected in OsNIP3;3-H139F (Figure 2C). Substitution of His[94] or His[139] for Ala greatly decreased the water permeability and therefore it was difficult to detect the mercury-sensitivity in these OsNIP3;3-H94A and OsNIP3;3-H139A. Water transport activity remained mostly and partially in OsNIP3;3-H168F and OsNIP3;3-H168A, respectively. However, mercury-sensitivity disappeared in OsNIP3;3-H168F and OsNIPH3;3-H168A (Figure 2D), suggesting that His[168] in the loop C (supplementary Figure S3B) is a target of

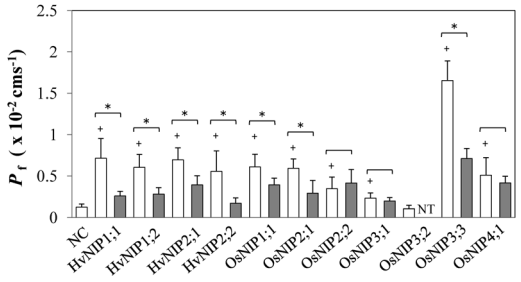

Figure 1. Water transport activities of rice and barley NIPs. Water permeability coefficient (P_f) was determined from swelling assays using *Xenopus* oocytes injected with 50 ng of each *NIP* cRNA. NC represents negative control (water-injected) oocytes. Gray bars indicate P_f of oocytes pretreated with 1 mM $HgCl_2$ for 10 min before swelling assays. NT, Not tested. Data represent mean±SD (n=6–10). +p<0.05 vs. NC, *p<0.05 between with and without 1 mM $HgCl_2$ in the same NIP, by Stutents t-test.

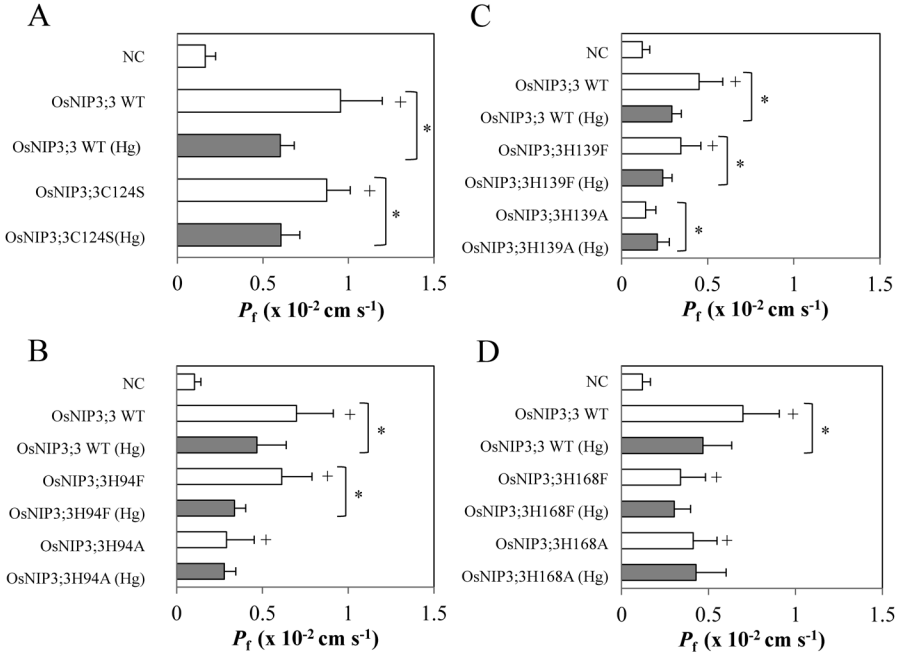

Figure 2. Water transport activities of mutated OsNIP3;3s. (A) Cys[124] was substituted for Ser, (B) His[94] was substituted for Phe or Ala, (C) His[139] was substituted for Phe or Ala, and (D) His[168] were substituted for Phe or Ala. Water permeability coefficient (P_f) was determined from swelling assays using *Xenopus* oocytes injected with 1 ng of each *NIP* cRNA. NC represents negative control (water-injected) oocytes. WT represents wild type (non-mutated) cRNA. Gray bars indicate P_f of oocytes pretreated with 1 mM $HgCl_2$ for 10 min before swelling assays. Note that each figure panel (A to D) represents an independent experiment using different batches of oocytes. Data represent mean±SD (n=6–10). +p<0.05 vs. NC, *p<0.05 between with and without 1 mM $HgCl_2$, by Stutents t-test.

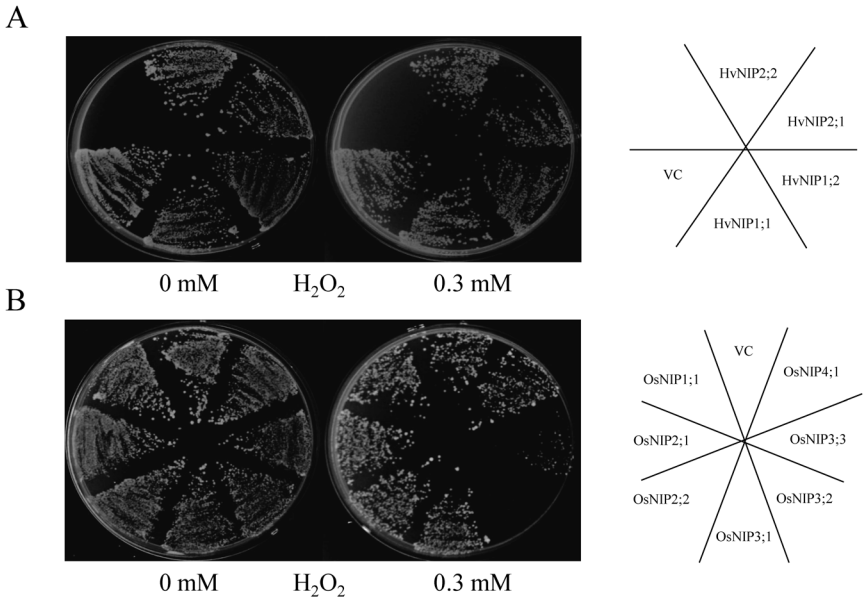

Figure 3. Growth of yeast ΔSKN7 expressing barley and rice NIPs. Yeast ΔSKN7 expressing barley (A) or rice (B) NIP aquaporins were cultured on SC-Ura (Gal+Suc) agar plates without (left) or with (right) 0.3 mM H_2O_2. Pre-cultured yeasts were freshly streaked and grown for 3 days at 30°C. VC, pYES2 empty vector.

mercury ion.

Hydrogen peroxide transport

The activity for hydrogen peroxide transport was tested using the ΔSKN7 strain of *Saccharomyces cerevisiae* in the present study. The SKN7 protein is a transcriptional factor for thioredoxin and thioredoxin reductase genes in yeast, which are redox stress responsive (Lee et al. 1999). The SKN7 deficient mutant (ΔSKN7) is therefore more sensitive to ROS than wild type. Introducing an empty vector pYES2 showed no inhibitory effect on the growth of yeast ΔSKN7 strain on the SC-Ura (Gal+Suc) plate with 0.3 mM H_2O_2, although 0.4 mM or more H_2O_2 inhibited the growth of yeast ΔSKN7 strain with pYES2 (data not shown). ΔSKN7 yeast heterologously expressing OsNIP3;2 or OsNIP3;3 did not grow on the SC-Ura (Gal+Suc) plate with 0.3 mM H_2O_2 (Figure 3) indicating that these 2 NIPs enhanced the influx of H_2O_2 in to ΔSKN7. No HvNIPs were detected, indicating a negative effect on the growth of yeast ΔSKN7 strains on the SC-Ura (Gal+Suc) plate with 0.3 mM H_2O_2.

Arsenite transport

The ACR3 protein in yeast is an As(III) extrusion transporter (Bienert et al. 2007) and therefore its defective mutant the ΔACR3 strain cannot grow if arsenite influx is enhanced. As reported previously (Bienert et al. 2008), expression of OsNIP2;1 or OsNIP3;2 reduced the growth of yeast ΔACR3 in the presence of 5 μM $As(OH)_3$ (=As(III)). In addition to these two OsNIPs, increased sensitivity to 5 μM $As(OH)_3$ was obviously detected in yeast ΔACR3 expressing

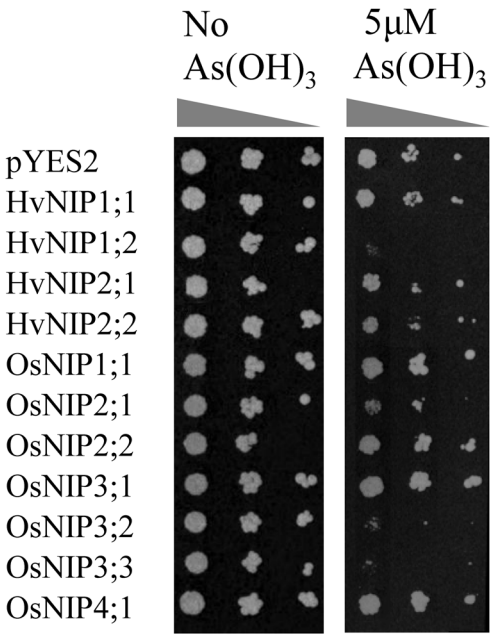

Figure 4. Growth of yeast ΔACR3 expressing barley and rice NIPs. Yeast ΔACR3 expressing various NIP aquaporins were cultured on SC-Ura (Gal+Suc) agar plates with no or 5 μM $As(OH)_3$. Pre-cultured yeast cells (A_{600}=0.01) were inoculated with 3 μl in 10-fold dilution and grown for 3 days at 30°C.

HvNIP1;2 or OsNIP3;3 (Figure 4). A slight reduction of growth was observed in yeast ΔACR3 expressing HvNIP2;1 or 2;2, in contrast to HvNIP1;1 showing no sensitivity to $As(OH)_3$.

Discussion

Mercury-sensitive water transport activities were detected in seven NIPs among eleven NIPs examined. Because OsNIP3;3 most enhanced P_f in *Xenopus* oocytes among the NIPs examined in the present study, the molecular mechanisms of water transport activity and mercury-sensitivity were investigated in detail using mutated OsNIP3;3 aquaporins. In mammalian aquaporins, a cysteine located exactly three-residues before the 2nd NPA motif in loop E was revealed to be the mercury-sensitive residue (Jung et al. 1994). In plant PIPs, on one hand, a cysteine in loop A was involved in the disulfide bond formation between monomers and, in particular, the mercury-sensitivity in the case of maize PIP (Bienert et al. 2012). On the other hand, mercury did not inhibit but increased water permeability via a non-cysteine mechanism in spinach SoPIP2;1 (Frick et al. 2013). In fact, there is no cysteine in loops A and E of barley and rice NIPs (supplementary Figure S1). However, two cysteine residues are present in OsNIP3;3 in the cytoplasmic N-terminal and loop B, respectively (supplementary Figure S1). The corresponding Cys to Cys^{35} in the N-terminal of OsNIP3;3 does not exist in other NIPs and therefore this cysteine in the N-terminal seemed unlikely to be involved in the mercury-sensitivity in NIPs. Also the corresponding cysteine to Cys^{124} in loop B of OsNIP3;3 (supplementary Figure S3A) does not exist in other NIPs except OsNIP3;2. Water transport activity and mercury-sensitivity remained in OsNIP3;3-C124S (Figure 2A), indicating that a cysteine is not responsible for the mercury-sensitivity in the water transport activity in barley and rice NIPs.

Next we focused on histidine. It contains imidazole that can be attacked by metal ions. The presence of 5 histidine residues was deduced in the amino acid sequence of the OsNIP3;3 (supplementary Figure S3A), and each histidine was substituted. Mutated OsNIP3;3s were subjected to the swelling assay. Our results indicated a high possibility that His^{168} is involved in the mercury-sensitivity. His^{168} of OsNIP3;3 is located in apoplastic loop C (supplementary Figure S3) where an external mercury ion may easily attack histidine(s) to modify the path for water transport. Histidine residues were found in loop C in all NIPs except for OsNIP2;2 (supplementary Figure S1). OsNIP2;2 has no histidine in loop C and its water transport activity was very low although OsNIP2;2 is located in the plasmamembrane of oocytes (supplementary Figure S2), supporting the hypothesis that histidine(s) in loop C is/are involved in the water transport activity and the mercury-sensitivity in NIPs. Previously a histidine in cytoplasmic loop D was revealed as a pH-sensitive residue in PIPs (Tournaire-Roux et al. 2003). The present study revealed for the first time that a histidine in the apoplastic loop C has an important role in water transport and mercury-sensitivity in NIP aquaporins. For other plant aquaporins, we examined the mercury-sensitivity in HvPIPs (barley plasma membrane type aquaporins) in which no corresponding histidine in loop C nor cysteine in loop E is present. No significant mercury-sensitivity was detected in the water transport activities of HvPIPs when they were injected and expressed in oocytes (data not shown).

OsNIP3;3 was found to be a unique aquaporin facilitating all water, hydrogen peroxide and arsenite transports. Previously some AtTIPs and AtPIPs were shown to exhibit H_2O_2 permeability using the ΔSKN7 strain of yeast (Bienert et al. 2006, Bienert et al. 2007, Hooijmaijers et al. 2012). The activity for H_2O_2 transport has hardly been examined in NIPs except for AtNIP1;2 reported to be a H_2O_2 permeable NIP (Dynowski et al. 2008). In the present study, OsNIP3;2 and OsNIP3;3 were characterized as H_2O_2-permeable NIPs. As for arsenite transport, OsNIP3;3 and HvNIP1;2 were identified to show arsenite transport activity in addition to previously reported NIPs from rice, *Arabidopsis*, or *Lotus japonicus* (Ali et al. 2009; Bhattacharjee et al. 2008; Bienert et al. 2008; Isayenkov and Maathuis 2008; Kamiya et al. 2009; Ma et al. 2008). Because OsNIP3;3 was found as an arsenite transporter, the effects of $As(OH)_3$ on the expression of OsNIP3;3 in rice plants were investigated (supplementary Figure S4). In both roots and shoots, however, the presence of 5 μM $As(OH)_3$ in the growth solution resulted in no difference in expression compared with the control plants up to 4

Table 1. Summary of H_2O, H_2O_2 and As transport activities of HvNIPs and OsNIPs.

	HvNIPs				OsNIPs						
	1;1	1;2	2;1	2;2	1;1	2;1	2;2	3;1	3;2	3;3	4;1
H_2O[a)]	◎	◎	◎	◎	◎	◎	○	○	ND	◎	○
H_2O_2[b)]	ND	ND	ND	ND	ND	ND	ND	ND	○	○	ND
As [c)]	ND	○	INT	INT	ND	○	ND	ND	○	○	ND
ar/R[d)]	WVAR	WVAR	GSGR	GSGR	WVAR	GSGR	GSGR	AIGR	AAAR	AIAR	AGGR

a) H_2O transport activities with (double circles) or without (single circles) mercury-sensitivity were determined in the *Xenopus* oocyte swelling assay system. ND represents that transport activity was not detected in the present assay. b) H_2O_2 transport activity was assessed with growth yeast ΔSKN7 strain in the presence of 0.3 mM H_2O_2. ○ and ND represent that transport activity was detected or not, respectively. c) As transport activity was assessed with the growth of yeast ΔACR3 strain in the presence of 5 μM $As(OH)_3$. ○ and ND represent that transport activity was detected, or not, respectively. INT represents the intermediate activity. d) Amino acids in ar/R (aromatic/arginine) filters.

days in 3-week old rice plants grown in the hydroponic culture.

Growth of yeast ΔACR3 expressing HvNIP2;1 (=HvLsi6) and HvNIP2;2 were intermediate in the presence of 5 μM $As(OH)_3$. Arsenite uptake activities were previously measured in OsNIP2;2 (=Lsi6), OsNIP1;1 and OsNIP3;1 (Ma et al. 2008) in oocytes, but we could not detect their arsenite uptake activity in the present yeast ΔACR3 system, probably because of the low sensitivity of present assay system than the oocyte system. Arsenite uptake activities of OsNIP2;2, OsNIP1;1 and OsNIP3;1 were lower than OsNIP2;1 (Ma et al. 2008), and therefore, they might show no obvious effect in the present yeast system.

Although an aromatic/arginine (ar/R) sequence is suggested to be a putative selective filter (Mitani-Ueno 2011; Wallace and Roberts 2005), there was no correlation between first 3 amino acids in ar/R and transport specificity among substrates (H_2O, H_2O_2 and $As(OH)_3$) selectivity in NIPs examined in the preset study. A second Ser in ar/R was proposed to be required for As, B and Si transport in OsLsi1(=OsNIP2;1) (Mitani-Ueno 2011). However, all the amino acids in ar/R of OsNIP3;3 differed from those of OsNIP2;1 except for the 4th constitutional arginine. Also Bienert et al. (2007) found that the H_2O_2-permeable hAQP8, AtTIP1;1, and AtTIP1;2 share the same first 3 amino acids (HIG) in ar/R but AtTIP2;1 possessing the same amino acids has no H_2O_2-permeability. This HIG in the first 3 amino acids in ar/R was not found in H_2O_2-permeable NIPs in the present study, either (Table 1). These results suggest that not only ar/R but also other structural features must be involved in the substrate selectivity in NIPs.

In conclusion, the molecular characteristics of NIPs transporting water and other neutral low molecular weight compounds are revealed in the present study. Novel high water and arsenite transport activities were identified in OsNIP3;3 although its expression was not induced in rice plants with arsenite treatment. Further works are required to reveal the physiological roles of NIPs in various environmental conditions and stress tolerances. The present data also indicate that amino acid substitution can modify water permeability in NIPs. A better molecular understanding of NIP functions might allow us to improve the transport properties of water and other low molecular weight compounds in plants via NIP aquaporin engineering.

Acknowledgements

We thank Dr. Sakurai (NARO Tohoku Agricultural Research Center, Japan) for the information on real time PCR to quantify the amount of OsNIP3;3 transcript. Part of the arsenite transport in the present work was supported by JSPS KAKENHI Grant Number 20580063, and another part of this work by the Program for Promotion of Basic Research Activities for Innovative Biosciences to M.K.

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Functional and molecular characteristics of rice and barley NIP aquaporins transporting water, hydrogen peroxide and arsenite

Maki Katsuhara[1, *], Shizuka Sasano[1], Tomoaki Horie[2], Tadashi Matsumoto[3], Jiye Rhee[1,a] and Mineo Shibasaka[1]

MATERIALS AND METHODS

Multiple sequences alignment analysis

The program ClustalW (http://www.genome.jp/tools/clustalw/, version 2.1) was used to perform multiple amino acid sequence alignments of full-length NIPs. Regions of trans-membrane helixes (TMHs) and inter-TMH loops in barley and rice NIPs were estimated by SWISS-MODEL (http://swissmodel.expasy.org/, Arnold et al. 2006) using the amino acid sequence of hAQP5 (accession number BC032946, h means *Homo sapiens*) as a model.

Subcellular localization of EGFP-NIPs in *Xenopus* oocytes

Rice NIPs were fused with EGFP at the N-terminal. They were subcloned into the pXβGev1 and their cRNAs were synthesized as described in the main body. One day after the injection, GFP fluorescence in the sliced oocyte was analyzed using a BioZero microscope (BZ-8000, KEYENCE Corporation, Japan) as described previously (Mahdieh 2008).

Homology modeling

Homology modeling was performed by the Workspace at the Swiss-Model website, URL: http://swissmodel.expasy.org/ (Arnold et al. 2006).

Plant growth and quantitative PCR analysis

Sterilized seeds of rice (var. Nipponbare) were germinated in 1 mM $CaSO_4$ and 10 mM KCl for a week and grown hydroponically in a nutrient solution (4 mM KNO_3, 1 mM $NH_4H_2PO_4$, 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4 \cdot 7H_2O$, 295 μM Fe-citrate, 46 μM H_3BO_3, 9.1 μM $MnCl_2 \cdot 4H_2O$, 0.32 μM $CuSO_4 \cdot 5H_2O$, 0.77 μM $ZnSO_4 \cdot 7H_2O$, and 0.10 μM Na_2MoO_4, pH 5.5 with NaOH). The seedlings were grown under controlled conditions (1000 μmol m^{-1} s^{-1}, 12L/12D, 28 °C during the light period and 25 °C during the dark period). Three-week-old seedlings were transferred to a nutrient solution containing no or 5 μM As_2O_3 that is converted to $As(OH)_3$ by hydration in water. After 0, 1, 2, 4 days, root or shoot samples were collected, washed and immediately ground in liquid nitrogen. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN).

OsNIP3;3 cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and its expression was analyzed by a 7300 real-time PCR machine (Applied Biosystems) using gene specific primers,

forward; 5'-CTCTGTCTTTTTACAGGTAACTTG-3'

reverse; 5'-CATTCTGGCGAAATGGTTATATC-3'

The quantification was performed using a control root sample grown without arsenite as a standard. One experimental series included 3 to 4 samples at each time point and 3 independent experiments were performed.

Discussion

Water transport activity and NPA motives

Amino acid sequences were compared among 4 NIPs from barley (HvNIPs) and 7 NIPs from rice (OsNIPs) (supplementary Figure S1). A large divergence of the amino acids sequences was observed in both N- and C-terminals of NIPs in the present study, but a common feature of 6 trans-membrane helixes (TMHs) and 5 inter-TMH loops (Forrest and Bhave 2007) was detected among the NIPs. Two NPA-motifs are the most characteristic features of the MIP gene family, although some exceptions are recognized such as AtNIP5;1 (Takano et al. 2006) and OsNIP3;1 (present study) in which two NPA are replaced with NPS and NPV. Slightly enhanced P_f was observed in *Xenopus* oocytes expressing OsNIP3;1, but its water transport activity was very low (Figure 1) probably because of the non-conserved NPA-motifs. This result suggests that typical NPA-motifs are very likely required for the high water transport activity in NIPs as well as PIPs and TIPs.

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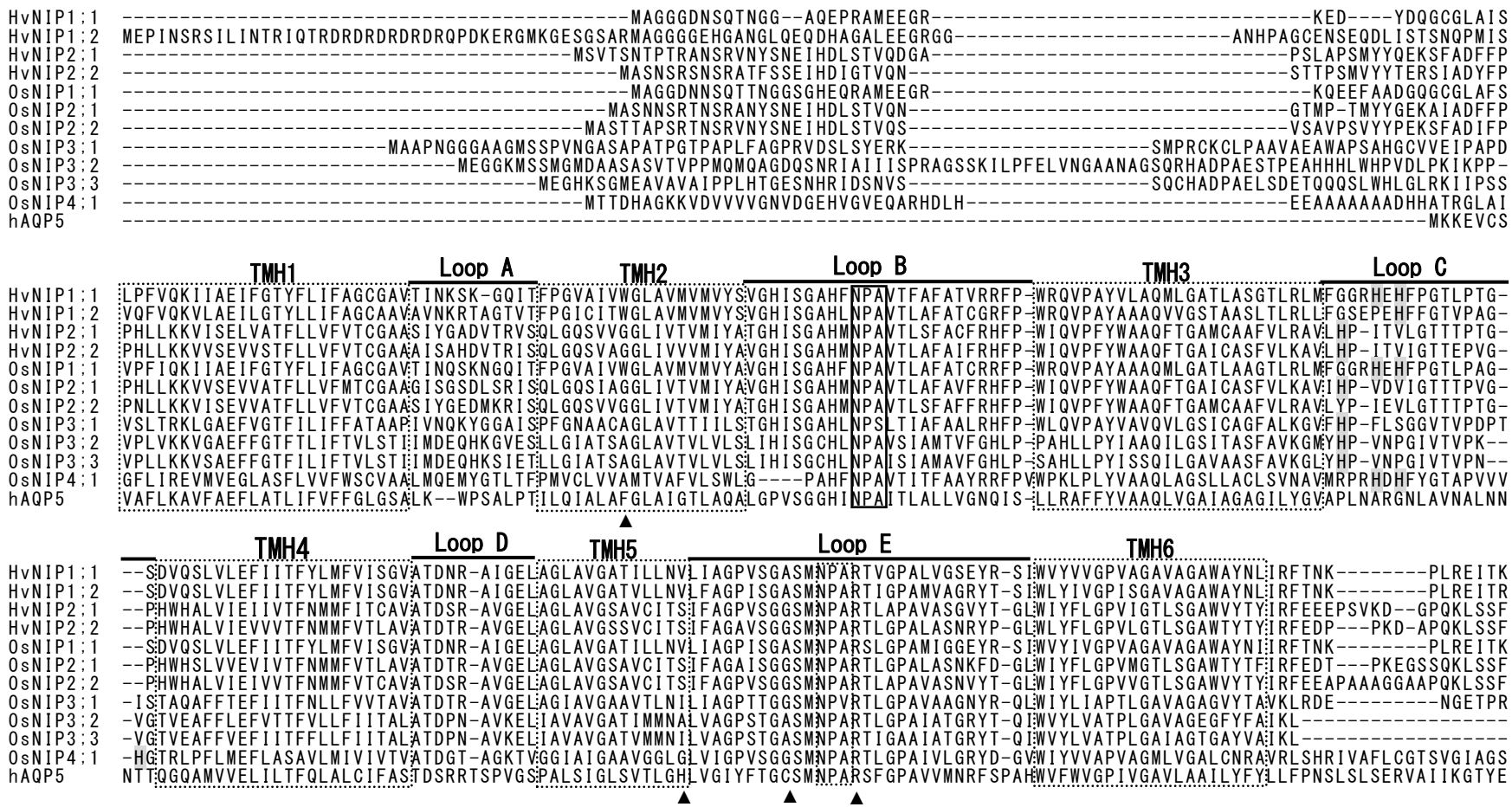


Figure S1 Alignment of amino acid sequences of HvNIPs and OsNIPs investigated in the present study

hAQP5 is a human aquaporin used as the model to estimate trans-membrane helixes (TMH1 to 6) and inter-TMH loops (Loop A to E). NPA-motives are boxed and histidine residues in Loop C were shadowed. Amino acids as the aromatic/arginine filter are indicated by black triangles.

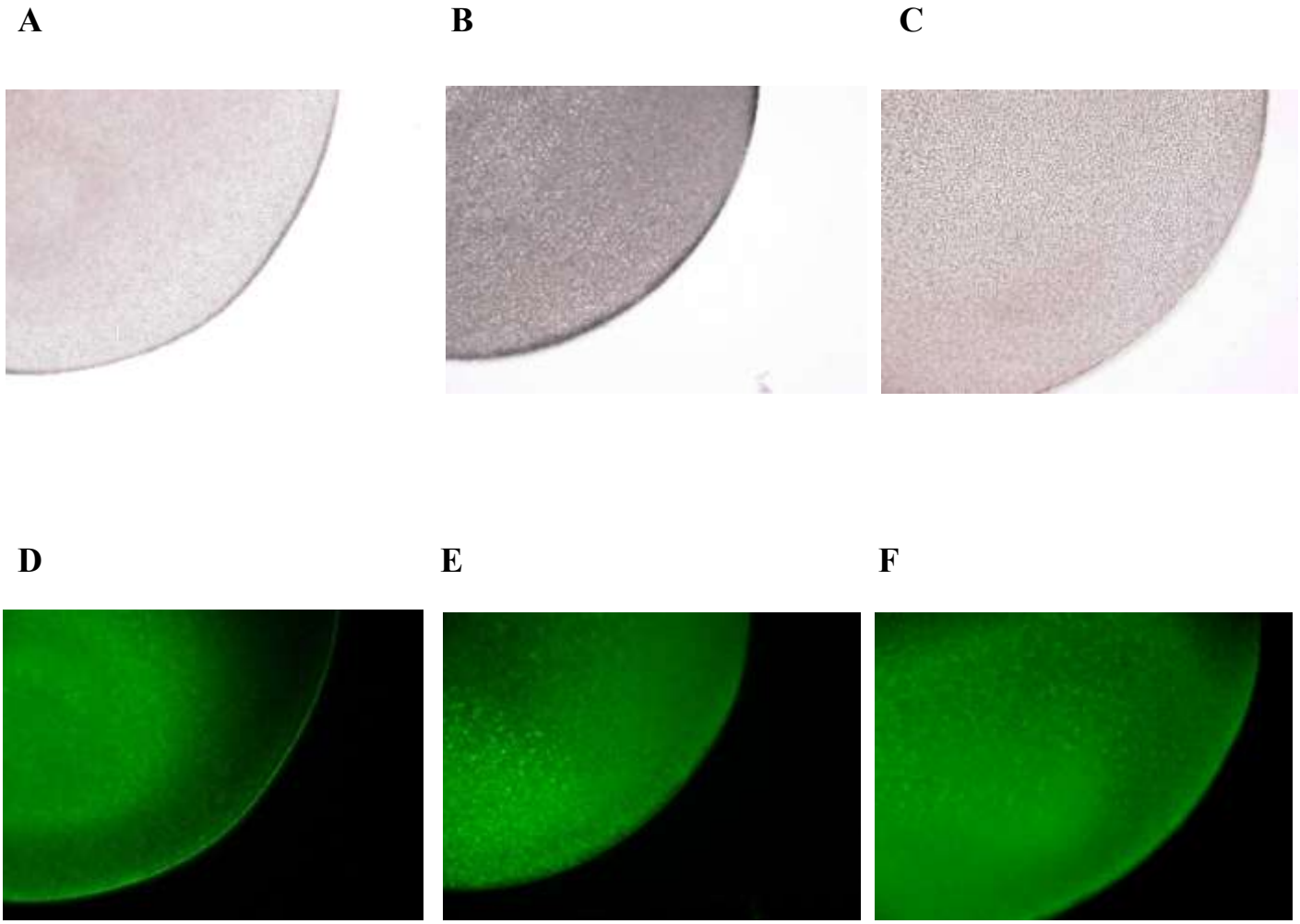
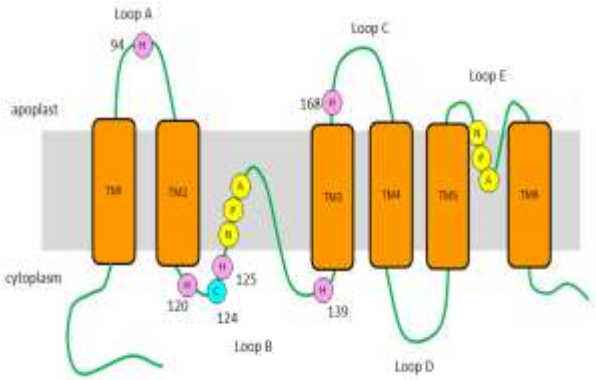


Figure S2 Microscopic images of *Xenopus* oocytes

Oocytes were injected with either 50 ng of EGFP-OsNIP2;2 (A, D), EGFP-OsNIP3;1 (B, E) or EGFP-OsNIP3;2 (C, F) cRNA. Bright-field (A, B, C) and fluorescent (D, E, F) images were recorded 24 h after the injection.

A



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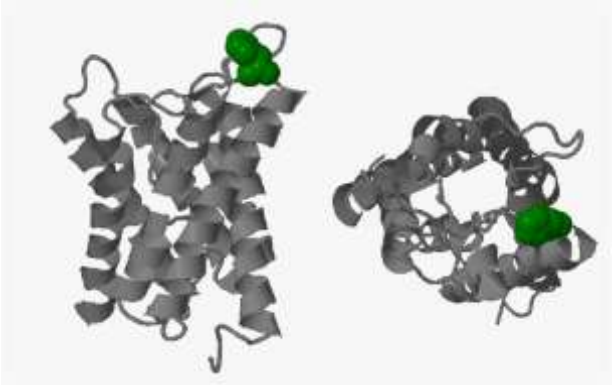

Figure S3 Molecular structure of OsNIP3;3

(A) Schematic representation of the OsNIP3;3 monomer structure. Histidine and serine substituted in the present study are displayed as pink and blue colors, respectively. NPA motives are marked in yellow. (B) Three dimensional homology modeling of OsNIP3;3 molecule. The model was constructed based on an X-ray diffraction structural model of spinach SoPIP2;1. Green ball shape indicates His^{168} in the loop C.

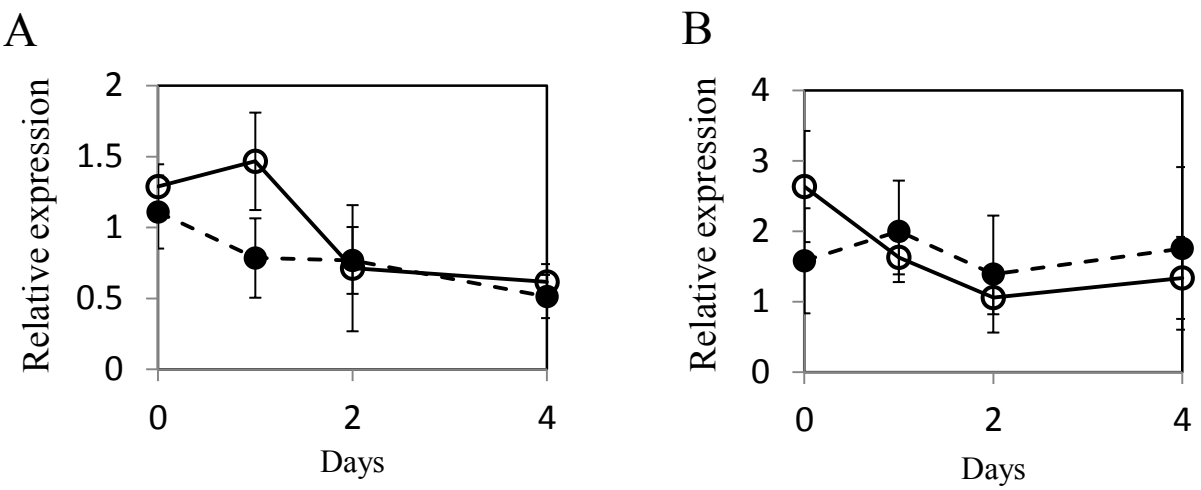

Figure S4 Effects of arsenite on the expression of *OsNIP3;3* in rice

OsNIP3;3 mRNAs were quantified using real-time PCR in 3-week old rice roots (A) or shoots (B). No (open circles with a solid line) or 5 μM of $As(OH)_3$ (filled circles with a dotted line) was applied to the hydroponic solution at day 0. Data represent means of 3 independent experiments ± SD.

Table S1

Accession numbers of rice and barley NIP aquaporins examined in the present study

Name	Accession	Other names/numbers
HvNIP1;1	AB540230	
HvNIP1;2	AB540231	
HvNIP2;1	AB540229	AB292848, HvLsi6(AB447484)
HvNIP2;2	AB710142	HvNIP2;1(GQ496520), HvLsi1(AB447482)
OsNIP1;1	AB856419	
OsNIP2;1	AK069842	OsLsi1(Ma et al., 2006)
OsNIP2;2	AK112022	OsLsi6(AB253627)
OsNIP3;1	AB856420	
OsNIP3;2	AB710140	
OsNIP3;3	AB710141	
OsNIP4;1	AB856421	

Table S2

Primers for the construction of mutants used in the present study

Mutant name	Primer name	Sequence
OsNIP3;3-C124S	ON33C124S-Sen	CACATATCAGGATCTCATCTGAACCCTG
	ON33C124S-Ant	CAGGGTTCAGATGAGATCCTGATATGTG
OsNIP3;3-H94F	ON33H94F-Sen	CATGGATGAACAATTTAAAAGTATCGAGAC
	ON33H94F-Ant	GTCTCGATACTTTTAAATTGTTCATCCATG
OsNIP3;3-H94A	ON33H94A-Sen	CATGGATGAACAAGCTAAAAGTATCGAGAC
	ON33H94A-Ant	GTCTCGATACTTTTAGCTTGTTCATCCATG
OsNIP3;3-H120F	ON33H120F-Sen	CTGTCCCTCATCTTTATATCAGGATGCC
	ON33H120F-Ant	GGCATCCTGATATAAAGATGAGGGACAG
OsNIP3;3-H125F	ON33H125F-Sen	CATATCAGGATGCTTTCTGAACCCTGCAATC
	ON33H125F-Ant	GATTGCAGGGTTCAGAAAGCATCCTGATATG
OsNIP3;3-H139F	ON33H139F-Sen	CCGTCTTTGGTTTTCTCCCTTCTGCTCATC
	ON33H139F-Ant	GATGAGCAGAAGGGAGAAAACCAAAGACGG
OsNIP3;3-H139A	ON33H139A-Sen	CCGTCTTTGGTGCTCTCCCTTCTGCTCATC
	ON33H139A-Ant	GATGAGCAGAAGGGAGAGCACCAAAGACGG
OsNIP3;3-H168F	ON33H168F-Sen	CAAAGGTCTGTATTTTCCGGTGAACCCCG
	ON33H168F-Ant	CGGGGTTCACCGGAAAATACAGACCTTTG
OsNIP3;3-H168A	ON33H168A-Sen	CAAAGGTCTGTATGCTCCGGTGAACCCCG
	ON33H168A-Ant	CGGGGTTCACCGGAGCATACAGACCTTTG
HvNIP2;2-G216M	HN22G216M-Sen	GCGGTGTCAGGTATGTCGATGAACCCG
	HN22G216M-Ant	CGGGTTCATCGACATACCTGACACCGC
HvNIP2;2-G216Y	HN22G216Y-Sen	GCGGTGTCAGGTTACTCGATGAACCCG
	HN22G216Y-Ant	CGGGTTCATCGAGTAACCTGACACCGC