



# Cell biology of aquaporins in rice (*Oryza sativa* L.)

Thi Thu Huyen Chu

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# THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Ecophysiologie et Adaptation des plantes

École doctorale GAIA

Unité de recherche Biochimie et Physiologie Moléculaire des Plantes

## CELL BIOLOGY OF AQUAPORINS IN RICE (*ORYZA SATIVA* L.)

Présentée par Thi Thu Huyen CHU  
Le 04 Avril 2018

Sous la direction de Doan Trung LUU

Devant le jury composé de

Bruno TOURAINE, Professeur des universités, Université de Montpellier, Montpellier

Laurent NUSSAUME, Carde scientifique des EPIC, CEA, Cadarache

Vincent VADEZ, Directeur de recherche, IRD, Montpellier

Nathalie LEBORGNE-CASTEL, Professeur des universités, Université de Bourgogne, Dijon

Nathalie LEONHARDT, Carde scientifique des EPIC, CEA, Cadarache

Doan Trung LUU, Chargé de recherche, CNRS, Montpellier

Président du jury

Membre du jury

Membre du jury

Rapporteuse

Rapporteuse

Directeur de thèse



UNIVERSITÉ  
DE MONTPELLIER

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## Abstract

As molecular players involved in the water transport through biological membranes, aquaporins (AQPs) have a role and are regulated in stress response. They were deeply investigated in plants and particularly in rice. Using functional genetic approach, we generated 5 transgenic rice lines based on Nipponbare cultivar, by overexpressing a single AQP in fusion with a fluorescent marker; among them, 3 plasma membrane AQPs (*OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*) fused with GFP and 2 tonoplast AQPs (*OsTIP1;1* and *OsTIP2;2*) fused with mCherry. Their subcellular localizations in resting condition were investigated. In rice, *OsPIP* isoforms showed typical homogeneous labelling of the plasma membrane, whereas *OsTIP* isoforms were observed localized in the tonoplast with a typical labelling of intracellular invaginations that skirted the nucleus. The behaviors of plasma membrane AQPs were tested in salt and drought stress-mimicked-conditions. Abiotic stresses triggered a re-localization of plasma membrane AQPs and salt stress enhanced endocytosis process of *OsPIP2;5* in rice root cells. Overexpressing such transgenes did not seem to affect the plant morphology and showed no beneficial effect for grain yield in both non-stress and stress conditions. We took more focus on the contribution of AQPs to rice root water transport in link with root morphology. AQPs contributed to a relatively high percentage of water transport in whole root system (44-58%) and seemed to contribute more in primary roots rather than in lateral roots.

**Key words:** Aquaporin, rice, subcellular localization, salinity, drought, root hydraulics, root morphology.

## Résumé

En tant qu'acteurs moléculaires impliqués dans le transport d'eau au travers des membranes biologiques, les aquaporines (AQP) jouent un rôle et sont régulées en réponse à des stress. Ils sont soumis à d'intenses recherches, en particulier chez le riz. En utilisant une approche de génomique fonctionnelle, nous avons généré 5 lignées transgéniques de riz dans le fond génétique Nipponbare, en sur-exprimant des AQP uniques fusionnées à un marqueur fluorescent ; parmi celles-là, figurent trois AQP de la membrane plasmique (*OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*) fusionnées à la GFP et deux AQP de la membrane tonoplastique (*OsTIP1;1* and *OsTIP2;2*) fusionnées à la mCherry. Leurs localisations subcellulaires en condition contrôle ont été observées. Chez le riz, les isoformes *OsPIP* présentaient un marquage homogène typique de la membrane plasmique, tandis que les isoformes *OsTIP* ont été trouvées avec un marquage des invaginations intracellulaires qui entourent le noyau, typique du tonoplaste. Le comportement des AQP de la membrane plasmique a été testé en conditions de stress salin et osmotique. Les stress abiotiques ont provoqué une relocalisation des AQP et le stress salin a augmenté l'endocytose de l'isoforme *OsPIP2;5* dans les cellules de la racine. Par ailleurs, la sur-expression de tels transgènes ne semblait pas affecter la morphologie des plantes et ne conférait pas un effet bénéfique sur la production de graines, aussi bien en condition contrôle que stressée. Enfin, nous nous sommes focalisés sur la contribution des AQP dans la racine de riz en relation avec la morphologie racinaire. Nous avons trouvé que les AQP contribuaient à un pourcentage relativement important dans le transport de l'eau dans la racine entière (44-58%) et que cette contribution semblait plus importante dans les racines primaires que latérales.

**Mots clés :** Aquaporine, riz, localisation subcellulaire, salinité, stress hydrique, hydraulique racinaire, morphologie racinaire.

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## Abbreviations

**AGI:** Agricultural Genetics Institute

**AQP:** Aquaporin

**At:** *Arabidopsis thaliana*

**ATSW:** Actual transpirable soil water

**BFA:** Brefeldin A

**CaMV35S:** Cauliflower mosaic virus 35S

**CFP:** Cyan fluorescent protein

**CIRAD:** Le Centre de coopération internationale en recherche agronomique pour le développement (French Agricultural Research Centre for International Development)

**CR:** Crown root

**crl1:** *crown rootless 1*

**cv.:** cultivar

**DAB:** 3,3'-diaminobenzidine

**D:** Days

**DAG:** Days after germination

**DAS:** Days after sowing

**DASA:** Days after stress application

**dpi:** Dots per inch

**DTF:** Days to flower

**DW:** Dry weight

**EC:** Electrical conductivity

**Exo:** Exodermis

**FTSW:** Fraction of transpirable soil water

**FW:** Fresh weight

**GFP:** Green fluorescent protein

**h:** Hour

**IRRI:** International Rice Research Institute

**LE:** Late endosome

**$L_0$ :** Root hydraulic conductance

**$L_{pr}$ :** Root hydraulic conductivity

**LR:** Lateral root

***lrt2:*** *lateral rootless 2*

**LSCM:** Laser scanning confocal microscopy

**LSD:** Least Significant Difference

**mCherry:** Monomeric red fluorescent protein

**Mes:** Mesodermis

**MIPs:** Major intrinsic proteins

**MVB:** Multivesicular body

**MPEM:** Multiphoton excitation microscopy

**MS:** Murashige and Skoog

**MTSW:** Maximal transpirable soil water

**NB:** Nipponbare

**NIPs:** Nodulin26-like intrinsic proteins

**NSCCs:** Non-selective cation channels

**OPR:** Outer part of the root

***Os:*** *Oryza sativa*

**BPS:** Phosphate-buffered saline

**PC:** Pot capacity

**PCC:** Pearson's correlation coefficient

**PEG:** Polyethylene glycol

**PIPs:** Plasma membrane intrinsic proteins

**PM:** Plasma membrane

**PPL:** Primary root length

**PR:** Primary root

**RDW:** Root dry weight

**ROS:** Reactive oxygen species

**rpm:** Rounds per minute

**RR:** Radicle root

**RSA:** Root surface area

**RT-PCR:** Reverse transcription-Polymerase chain reaction

**SE:** Standard error

**SIPs:** Small basic intrinsic proteins

**TC65:** Taichung 65

**TGN:** *trans*-Golgi network

**TIPs:** Tonoplast intrinsic proteins

**w/v:** weight/volume

**WC:** Water content

**WT:** Wilting point

**XIPs:** Uncategorized (X) intrinsic proteins

# Résumé de la thèse en français

## Contexte

### ***La sécurité alimentaire dans un contexte de changement climatique***

Le changement climatique et l'augmentation constante de la population mondiale mettent l'agriculture face au défi de produire suffisamment de nourriture et de bonne qualité, avec beaucoup de contraintes. Ce changement climatique va provoquer inmanquablement une disponibilité aléatoire en eau douce, affectant ainsi la production des plantes cultivées. Certaines régions subissent déjà des épisodes sévères de sécheresse, de températures extrêmes, et d'autres types de contraintes environnementales abiotiques liées à l'eau (inondations ou salinisation des sols).

### ***La culture du riz est directement affectée par le changement climatique***

Deux espèces de riz assurent à elles seules la quasi-totalité de la production mondiale: *Oryza sativa*, dans le monde entier, et *O. glaberrima* en Afrique. En 2014, 738 Mt ont été produites dans le Monde, dont 45 Mt au Vietnam, faisant de cette céréale la première dans ce pays (Sources : FAOSTAT). Cultivé principalement dans les deux deltas du Fleuve Rouge et du Mékong, le riz nécessite un apport d'eau douce et d'être protégé de la salinisation des sols. Comparés à la période 1980-2000, les scénarios climatiques prédisent une augmentation jusqu'à 4.5°C dans le pays, ainsi qu'une augmentation du niveau de la mer entre 15 – 90 cm pour 2070 (ISPONRE and UNEP 2009). La culture du riz est donc fortement impactée par le changement climatique ; ce qui peut causer une menace pour la sécurité alimentaire de ce pays, mais plus largement au niveau mondial. Sous l'impulsion d'instituts tels que l'International Rice Research Institute (IRRI) ou Africa-Rice, de nouvelles variétés élites de riz plus tolérantes à la sécheresse et à une salinité modérée des sols ont été obtenues par des programmes d'amélioration des plantes, par exemple en introgressant le locus *Salto1* pour la tolérance à la salinité. Cependant, encore plus d'efforts devront être entrepris pour une meilleure connaissance des mécanismes mis en place par les plantes pour répondre à ces stress, pour développer des variétés de plantes cultivées plus tolérantes, dans un contexte d'intensification des stress environnementaux.

## Problématiques scientifiques et hypothèse de travail

### *La sécheresse et la salinité, deux stress abiotiques majeurs pour les plantes cultivées*

La sécheresse provoque tout un ensemble de mécanismes de réponses dans la plante entière dont le raccourcissement du cycle de vie dans une stratégie d'évitement, et le renforcement de l'appareil racinaire pour une meilleure absorption de l'eau du sol. Au niveau tissulaire, les plantes limitent la perte en eau en réduisant la surface ou la conductance hydraulique foliaire (stomatique ou cuticulaire), elles prélèvent plus efficacement l'eau en modifiant la conductance hydraulique racinaire, pour maintenir le potentiel hydrique des tissus. Au niveau cellulaire, elles tentent de maintenir la turgescence cellulaire et réduisent la perte par évaporation, en accumulant des solutés compatibles (mécanisme d'ajustement osmotique). Le riz, bien qu'étant sensible à la sécheresse, possède une forte diversité génétique, source de tolérance à ce stress, et donc d'amélioration.

Le terme salinité représente tous les problèmes des sols liés à une accumulation excessive de sels, ayant pour effets les stress osmotique et ionique. Le stress osmotique est dû à un taux élevé de solutés à l'extérieur des racines, conduisant à l'inhibition de l'absorption d'eau, de l'expansion cellulaire et le développement de racines latérales. Le stress ionique est, quant à lui, lié à la toxicité de l'ion  $\text{Na}^+$  qui s'accumule en excès dans les cellules végétales, conduisant à divers effets délétères sur le métabolisme cellulaire. La production du riz, qui est l'une des céréales les plus sensibles à la salinité, est ainsi affectée par ce stress. Au niveau cellulaire et tissulaire, des mécanismes sont développés pour s'adapter au stress salin (Munns and Tester, 2008) :

1- L'ajustement osmotique fait appel aux mêmes mécanismes que pour la sécheresse.

2- L'exclusion du  $\text{Na}^+$ , des parties aériennes vers les racines permet aux premières d'échapper à sa toxicité et préserve la capacité photosynthétique.

3- La séquestration du  $\text{Na}^+$  et du  $\text{Cl}^-$ , par exemple dans la vacuole, évite que leur toxicité ne soit exprimée dans le cytoplasme où ont lieu de nombreuses activités enzymatiques essentielles pour la vie de la cellule, mais sensibles à la toxicité de ces ions.

### ***L'ajustement osmotique chez les plantes cultivées.***

L'ajustement osmotique au niveau cellulaire par la synthèse et l'accumulation de solutés compatibles a été identifié comme un mécanisme de rétention d'eau intracellulaire, permettant une adaptation à la sécheresse. Compte tenu de leur fonction dans le transport d'eau, les aquaporines (AQP) devraient avoir un rôle dans l'ajustement osmotique. Ainsi, chez le riz leurs contributions dans le transport d'eau ont été évaluées en condition normale à 75%, et en condition de sécheresse à 85%. Cependant, l'analyse génétique n'a pas établi de corrélation entre les gènes d'AQP et les traits hydrauliques (Grondin *et al.*, 2016). D'autres études relataient que la sur-expression chez le riz de façon individuelle des isoformes *OsPIP1;1* ou *OsPIP1;3* conférait une meilleure tolérance à la sécheresse (Lian *et al.*, 2004; Guo *et al.*, 2006; Liu *et al.*, 2013). Ceci reflète, sans doute, la complexité des flux d'eau dans la racine de riz et la modulation de l'absorption d'eau pour maintenir son approvisionnement dans les parties aériennes, approvisionnement qui nécessite la coordination de processus sous le contrôle de gènes régulant les AQP. L'ajustement osmotique est aussi un mécanisme central dans la tolérance à la salinité ; ce qui suggère que la fonction des AQP nécessite ici aussi d'être élucidée chez le riz.

## Objectifs

La question principale de ma thèse est de mieux comprendre la contribution des AQP dans l'homéostasie hydrique chez le riz. Ma thèse comporte trois objectifs.

L'accumulation des transcrits et des protéines d'AQP chez le riz a été abondamment étudiée en relation avec les stress hydrique et salin. Il n'a pas été trouvé de relation cohérente entre l'accumulation de ces molécules et les propriétés hydrauliques. Cela nous conduit à considérer d'autres hypothèses, comme par exemple leur relocalisation subcellulaire en situation de stress osmotique et salin.

Comme indiqué précédemment, les manipulations génétiques de l'expression des AQP chez le riz ont été conduites sur un nombre limité d'isoformes. Le second objectif de ma thèse consistera à étudier les effets sur la tolérance à la sécheresse et à la salinité de la sur-expression des isoformes *OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;1* et *OsTIP2;2* exprimées individuellement dans des lignées transgéniques de riz.

Bien que l'absorption d'eau soit une fonction triviale de l'appareil racinaire, il est surprenant que son mécanisme soit encore mal connu, en particulier dans les contributions respectives de chaque type racinaire. Le riz présente un système racinaire fibreux possédant quatre types de racines (séminale ou « radicle » en anglais, coronaires ou adventives ou nodales ou « crown roots » en anglais, les petites et les grandes racines latérales). Ces types racinaires sont morphologiquement et anatomiquement distincts (Rebouillat *et al.*, 2008; Coudert *et al.*, 2010). La fonction respective de ces racines dans le transport d'eau n'est pas encore connue. Ici, l'objectif est de comprendre quel type de racines de jeunes plantules est le plus important pour l'absorption d'eau et sa régulation en situation de stress salin modéré (100 mM NaCl).



## Principaux résultats et discussion

### *Biologie cellulaire des AQP chez le riz*

Nous avons abordé ici l'hypothèse de la redistribution des isoformes *OsPIP* en réponse à des stress abiotiques. Un tel mécanisme a déjà été décrit chez *Arabidopsis* (Boursiac *et al.*, 2005; Luu *et al.*, 2012). Nous avons choisi les isoformes d'AQP selon des critères d'expression tissulaire et de niveau d'expression (Sakurai *et al.*, 2005, 2008; Sakurai-Ishikawa *et al.*, 2011) : *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;4*, et *OsPIP2;5*. Ces isoformes sont exprimées fusionnées à la GFP sous le contrôle du promoteur *CaMV35S*, dans des lignées de riz transgéniques. Les racines de ces dernières ont été traitées avec une solution de NaCl 100 mM ou 20% (P/V) PEG et la localisation subcellulaire des constructions a été observée par microscopie confocale à balayage laser. Nous avons associé à cette liste d'AQP de la membrane plasmique, une liste de marqueurs des compartiments intracellulaires et étiquetés avec la protéine fluorescente mCherry : deux AQP du tonoplaste *OsTIP1;1* et *OsTIP2;1*, *OsRab5a*, une petite protéine Rab fixant le GTP, impliqué dans le transport endosomal précoce (Wang *et al.* 2010), *OsGAP1*, une protéine activatrice GTPase spécifique des Rab, impliquée dans le trafic de l'appareil de Golgi vers la membrane plasmique et le Trans-Golgi Network (TGN) (Heo *et al.* 2005), *OsSCAMP1*, une protéine membranaire impliquée dans le système sécrétoire et localisée dans le compartiment endosomal précoce (Lam *et al.*, 2007). Ces marqueurs subcellulaires pourraient être utilisés pour identifier, par co-marquage, la localisation des *OsPIP* redistribués dans les compartiments intracellulaires.

Nos résultats montrent en condition contrôle une localisation conforme à l'identité des isoformes d'AQP, soit dans la membrane plasmique, soit dans le tonoplaste, aussi bien dans les couches cellulaires externes qu'internes de la racine de riz. En effet, nous avons appliqué un éclaircissement des tissus pour visualiser le marquage subcellulaire des cellules de l'endoderme et du cylindre central de la racine.

Les traitements par 100 mM NaCl ou 20% (P/V) PEG conduisent à une augmentation sensible du marquage intracellulaire. Ainsi, nous avons constaté qu'*OsPIP1;1* ne marque que 2% des cellules racinaires en condition contrôle, mais ~55% et 43% des cellules, respectivement, en situation de stress salin et osmotique. Nous avons posé la question de la spécificité de cette réponse, en utilisant une lignée transgénique exprimant la construction

CFP-LTi6a connue pour marquer la membrane plasmique. CFP-LTi6a présente une tendance plus faible à la relocalisation dans des compartiments intracellulaires; ceci suggère une spécificité de comportement des isoformes OsPIP.

Nous avons questionné la dynamique de l'endocytose, en prenant l'isoforme OsPIP2;5 comme le prototype des AQP et en traitant les racines des plantes transgéniques avec la bréfeldine A (BFA). La BFA est un antibiotique produit naturellement par le champignon *Eupenicillium brefeldianum* dont l'action d'inhibition de l'ADP-ribosylation factor-guanine-exchange factor provoque l'agrégation des vésicules endosomales incluant le TGN, dans un compartiment appelé « corps BFA ». Dans une précédente étude, la cinétique du marquage des corps BFA par un marqueur de la membrane plasmique traduisait la cinétique du cyclage constitutif et donc l'endocytose. Nous avons observé que seules 16% des cellules de l'exoderme présentaient des corps BFA, en situation contrôle, alors que sous stress salin, nous en avons observé 47%. Ce résultat suggère une dynamique de cyclage (et donc d'endocytose) des AQP accrue en situation de traitement salin.

L'ensemble de ces résultats permet d'étendre le comportement des AQP de la membrane plasmique au riz, en indiquant une redistribution et une dynamique subcellulaire accrue en réponse à un traitement salin.

### ***Effets de la sur-expression des aquaporines et des marqueurs intracellulaires sur la tolérance à la sécheresse et à la salinité***

Plusieurs approches génétiques de modification de l'expression des AQP ont été menées pour étudier leurs effets sur la tolérance aux stress abiotiques. Les résultats de ces études sont souvent contradictoires et parcellaires, car elles ne concernent que les isoformes OsPIP1;1, OsPIP1;3 ou OsPIP2;2. Nous avons choisi de mener une analyse plus complète en mettant à profit la collection de plantes de riz transgéniques exprimant sous le contrôle du promoteur *CaMV35S* les 5 AQP (cf. paragraphe précédent). Par ailleurs, à côté du rôle des AQP dans l'ajustement osmotique, la séquestration du Na<sup>+</sup> joue un rôle majeur dans la tolérance à la salinité. Des données de la littérature suggèrent que le trafic endomembranaire pourrait intervenir dans ce phénomène, puisque la perte de fonction de deux transporteurs du Na<sup>+</sup> localisés dans les compartiments endosomaux rend les plantes mutantes plus sensibles à la salinité (Bassil *et al.*, 2011). Nous avons donc aussi intégré les

plantes transgéniques de riz exprimant les protéines marqueurs des compartiments endosomaux présentés dans le précédent paragraphe. Enfin, nous avons voulu conduire les analyses jusqu'à la production de graines, paramètre que nous estimons être le plus pertinent pour mesurer la tolérance aux stress.

Notre hypothèse de travail propose que la sur-expression des AQP et des protéines marqueurs des compartiments endosomaux augmente la tolérance aux stress hydrique et salin des plantes transgéniques. Ces expériences ont été menées dans trois environnements différents :

- (Expérience A) Dans une serre du Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), localisée à Montpellier, pendant l'automne 2016.
- (Expérience B) Dans une net-house (structure de culture de plantes dans laquelle les murs sont faits de filets) de l'Agricultural Genetics Institute (AGI), à Hanoi (Vietnam), pendant l'automne 2017.
- (Expérience C) Dans une net-house de l'International Rice Research Institute (IRRI), à Los Banos (Les Philippines), pendant l'automne 2017.

L'analyse des plantes a porté sur la taille des plantes, le nombre de talles, la date de floraison, la fertilité par panicule, la masse totale des grains, le potentiel hydrique, la teneur en chlorophylle, l'accumulation des espèces actives de l'oxygène, la teneur en  $\text{Na}^+$  et  $\text{K}^+$  dans les feuilles. L'ensemble des résultats ne montre pas de différences notables entre les plantes sauvages et transgéniques. Une étude sur l'expression du transgène *OsPIP1;3* avait indiqué que les riz exprimant un faible niveau de ce transgène présentaient une meilleure tolérance à la sécheresse que les plantes le sur-exprimant (Lian *et al.*, 2004). Au vu de nos résultats, nous questionnons donc la pertinence de toutes stratégies visant à sur-exprimer des transgènes pour améliorer la tolérance aux stress abiotiques.

### ***Contribution respective des types de racines à l'hydraulique racinaire chez le riz***

L'objectif ici est de décrire une relation fonctionnelle entre les AQP exprimées et le type racinaire. Nous avons comparé les valeurs de transport d'eau entre les mutants de riz

affectés dans leurs architectures racinaires et leurs sauvages respectifs: *crown rootless 1* (*crl1*) et *lateral rootless 2* (*lrt2*), respectivement dépourvus de racines coronaires et latérales, ont pour sauvages, respectivement, Taichung 65 (TC65) et Nipponbare (NB) (Inukai *et al.*, 2005; Faiyue *et al.*, 2010b). Les mesures ont été effectuées à l'aide de chambres à pression, permettant d'obtenir les valeurs de conductivité hydraulique racinaire ( $L_{pr}$ ).

Dans un premier temps, nous avons cherché à déterminer une relation entre surface totale de l'appareil racinaire (RSA) et la masse sèche (RDW). Nous avons cultivé les plantes en conditions hydroponiques 7, 10, 13 et 21 jours après germination et prélevé les appareils racinaires. Une régression linéaire a été utilisée pour établir une telle relation pour chaque génotype et entre la RSA ( $m^2$ ) et la RDW (g) :  $y = 4.835 \times 10^{-1} x - 2 \times 10^{-4}$  pour NB ( $R^2 = 0.9038$ );  $y = 2.069 \times 10^{-1} x + 4 \times 10^{-6}$  pour *lrt2* ( $R^2 = 0.8622$ ),  $y = 4.258 \times 10^{-1} x - 3 \times 10^{-4}$  pour TC65 ( $R^2 = 0.858$ ) et  $y = 4.706 \times 10^{-1} x - 2 \times 10^{-4}$  pour *crl1* ( $R^2 = 0.9125$ ), où  $y$  et  $x$  représentent respectivement la RSA et la RDW. Nous avons obtenu des coefficients de corrélation élevés ( $R^2 > 0.85$ ). Par ailleurs, NB, TC65 et *crl1* constituent un groupe à part du mutant *lrt2*, avec des pentes distinctes. Ces relations linéaires sont particulièrement importantes pour le calcul des valeurs de  $L_{pr}$ , puisqu'après les mesures en chambres à pression, les appareils racinaires sont pesés et une correspondance avec la RSA est établie sans avoir à mesurer en détail leurs dimensions.

Les appareils racinaires des génotypes ont été caractérisés quant à la longueur de la racine séminale, le nombre de racines primaires, la longueur totale des racines primaires (PRL), le nombre de racines latérales et le nombre de racines latérales par longueur de racines primaires (LRs per PRL). Les deux génotypes sauvages présentent des longueurs de la racine séminale, des nombres de racines primaires, et des PRL similaires, ainsi que des nombre de racines latérales, des LRs per PRL, des RDW et des RSA légèrement différents. Le mutant *crl1* présente une plus grande longueur de racine séminale, mais une plus faible PRL et un nombre de racines latérales plus faible, par rapport à son génotype sauvage. Quant au mutant *lrt2*, il présente aussi une plus grande longueur de racine séminale et un nombre équivalent en racines primaires.

Nous avons déterminé les valeurs de conductances hydrauliques racinaires ( $L_0$ ) et avons établi qu'elles sont plus fortes pour NB comparé à TC65 (respectivement,  $1.60 \pm 0.066$

$\times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$  et  $1.30 \pm 0.063 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ ) et que celles de *crl1* and *lrt2* sont inférieures à celles de leurs génotypes sauvages (respectivement,  $0.83 \pm 0.052 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$  et  $1.02 \pm 0.071 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ ). Les réductions de la  $L_0$  chez *crl1* et *lrt2* comparées à leurs génotypes sauvages sont, respectivement, de 36.15% et 36.25%. Malgré une  $L_0$  plus faible que chez les génotypes sauvages, les mutants arrivent à développer un appareil aérien tout à fait comparable aux sauvages. Nous confirmons une hypothèse avancée par d'autres auteurs sur une meilleure efficacité d'usage de l'eau chez les génotypes sauvages (Faiyue *et al.*, 2010b). Par ailleurs, nous avons aussi déterminé la  $L_0$  des racines coronaires seules et avons estimé qu'elles contribuent à hauteur de 63.67% de la conductance hydraulique totale de la racine. Dans une étude récente sur l'orge, il avait été établi que les racines séminales contribuaient à 92% de l'absorption d'eau (Knipfer and Fricke, 2011). Toutefois, chez cette espèce, et à ce stade de développement, l'appareil racinaire comporte de 6 à 7 racines séminales et seulement 2 racines coronaires, alors que chez le riz, il n'y a qu'une racine séminale et 5 à 6 racines coronaires.

Les valeurs de  $L_{pr}$  varient de 9 à  $15 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$  et sont du même ordre de grandeur que celles déjà décrites dans la littérature, chez le riz. Elles sont aussi du même ordre de grandeur que chez le blé ( $\sim 6 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Bramley *et al.*, 2009)), *Arabidopsis thaliana* ( $\sim 5 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Sutka *et al.*, 2011)), le lupin ( $\sim 1 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Bramley *et al.*, 2009)), et plus faible que chez le maïs et le haricot ( $\sim 2\text{-}3 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Miyamoto *et al.*, 2001)). Nous constatons que le mutant *crl1* et son génotype sauvage n'ont pas de valeurs de  $L_{pr}$  significativement différentes; ceci suggère que les racines séminales et coronaires ont des capacités intrinsèques de transport d'eau identiques. Par ailleurs, la  $L_{pr}$  chez le mutant *lrt2* (qui ne dispose pas de racines latérales) reflète la capacité intrinsèque de transport d'eau des racines primaires. Du fait d'une valeur de  $L_{pr}$  plus élevée chez le mutant que chez son génotype sauvage, nous avançons l'idée que la capacité intrinsèque de transport d'eau des racines primaires est plus élevée que celle des racines latérales. Ceci a comme corolaire une plus faible contribution des AQP et/ou de la voie apoplastique dans les racines latérales. Précisément, pour tenter de mieux comprendre les contributions respectives de chacun de ces deux composants, nous avons dans un premier temps utilisé l'azide, connu pour inhiber l'activité intrinsèque des AQP chez le riz (Grondin *et al.*, 2016). Nous avons observé que la réduction de la  $L_{pr}$  était d'environ 40 à 60% selon le génotype et que l'inhibition chez le mutant *lrt2* était plus forte que chez son génotype sauvage. Ceci

suggère une plus forte contribution des AQP dans les racines primaires que dans les racines latérales et réciproquement une contribution plus faible des AQP dans les racines latérales que dans les racines primaires. Il est nécessaire d'évaluer la contribution de la composante apoplastique. Pour cela, nous avons traité les racines avec une suspension d'encre de Chine, composée de particules d'environ 85 nm de diamètre, et connue pour bloquer la voie apoplastique dans les systèmes racinaires de riz (Ranathunge *et al.*, 2004). Nous avons constaté un blocage de cette voie à hauteur de ~60% pour *cr1*, ~44% pour TC65 et NB et seulement ~16% pour *Irt2*. La faible valeur d'inhibition par l'encre de Chine chez le mutant *Irt2*, nous a suggéré une plus faible contribution de la voie apoplastique dans les racines primaires. De plus, ce résultat est en accord avec celui obtenu par l'utilisation de l'azide et soutient le modèle hydraulique que les racines primaires présentent une contribution intrinsèque des AQP plus forte (et une voie apoplastique plus faible) que dans les racines latérales.

Nous avons tenté de mieux comprendre l'hydraulique racinaire dans les étapes précoces d'un stress salin modéré (100 mM NaCl). Après 30 min de traitement, le système racinaire de NB présente une inhibition de la  $L_p$ , environ 2 fois plus forte que chez son génotype mutant *Irt2*. Nous interprétons ce résultat comme une plus faible capacité d'absorption du  $\text{Na}^+$ , provoquant une plus faible inhibition des AQP. En effet, si la question de l'entrée du  $\text{Na}^+$  dans la racine fait toujours débat, certains auteurs ont montré que l'entrée du  $\text{Na}^+$  était principalement par la voie apoplastique et liée aux sites d'émergence des racines latérales et de la région de l'apex où les bandes de Caspary et la subérine sont rompues ou immatures (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003, 2004, Krishnamurthy *et al.*, 2009, 2011). D'autres auteurs ont, quant à eux, montré que l'absorption du  $\text{Na}^+$  n'était pas aux sites d'émergence des racines latérales, mais plutôt au travers des couches corticales des racines latérales (Faiyue *et al.*, 2010*b,a*). Quelle que soit la voie principale d'entrée du  $\text{Na}^+$ , nos résultats indiquent que l'inhibition des AQP est largement dépendante de l'entrée du  $\text{Na}^+$  dans la racine.

L'ensemble de ces résultats obtenus grâce à des génotypes de riz contrastés dans leurs architectures racinaires permet une meilleure compréhension de la contribution respective des types racinaires, mais aussi de la voie apoplastique par rapport à l'activité intrinsèque des AQP.

## Conclusion générale

L'objectif central de notre thèse est de mieux comprendre le rôle des AQP chez le riz et en particulier en réponse à des contraintes de l'environnement telles que les stress hydrique et salin. Dans la partie ayant trait à l'hydraulique racinaire, grâce à une approche utilisant des mutants de l'architecture racinaire, nous avons dégagé l'idée que les racines primaires présentent une contribution intrinsèque des AQP plus forte (et une voie apoplastique plus faible) que dans les racines latérales. Cette découverte est d'importance, car cela focalise sur ce type de racines nos observations sur les AQP. Précisément, dans la partie ayant trait à l'étude de la localisation subcellulaire des AQP en réponse aux stress osmotique et salin, nous avons observé que les isoformes *OsPIP* subissaient une redistribution de la surface de la cellule vers des compartiments intracellulaires et qu'ils subissaient aussi une dynamique d'endocytose accrue en réponse au stress salin. Si l'ensemble de ces résultats décrit un comportement spécifique des AQP en réponse à des stress abiotiques, ils ne rendent pas compte d'un rôle de ces molécules dans la tolérance à ces stress. Par une approche génétique, nous n'avons pas pu décrire d'effets bénéfiques de la sur-expression des AQP (*OsPIP* et *OsTIP*) dans la tolérance à la sécheresse et à la salinité. Nous proposons l'hypothèse qu'une régulation fine de l'expression des AQP pourrait être primordial à la tolérance du riz aux stress abiotiques.

L'ensemble de ces résultats obtenus grâce à l'apport des approches de génomique fonctionnelle permet de mieux comprendre la fonction et la régulation des AQP chez le riz, en particulier en réponse à des contraintes de l'environnement. L'enjeu est d'importance pour la sécurité alimentaire de la population mondiale.

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## General introduction

Water is an indispensable element for all living cells. In the 1920s, when the lipid bilayer was discovered, the water movement across cells and subcellular compartments was hypothesized to simply diffuse across biological membrane. However, this argument is not relevant to elucidate the significant difference of water permeability among membranes and the fast flow of some physiological processes.

Koefoed-Johnsen and Ussing first proposed the transport of water across biological membranes through specialized pores rather than simple diffusion, in 1953 (Koefoed-Johnsen and Ussing, 1953), and then this hypothesis was firmly confirmed in mammalian cells by Macey and colleagues, in 1970 (Macey and Farmer, 1970). Functional expression of the Arabidopsis tonoplast intrinsic protein (AtTIP1;1) in *Xenopus laevis* oocytes, in 1993, was the first demonstration in plants of their intrinsic water transport activity (Maurel *et al.*, 1993). Then the term “Aquaporin” (AQP) was proposed by Peter Agre and colleagues (Agre *et al.*, 1993).

This part aims at introducing AQPs in rice (*Oryza sativa* L.), and more specifically their function and regulation in root system, under abiotic stresses, such as drought and salt stress, where these water channels play a major role. This short part will complete the introductions of each result chapter presented afterwards. We will introduce briefly drought and salt stress in rice, rice root system architecture, and rice AQP characteristics.

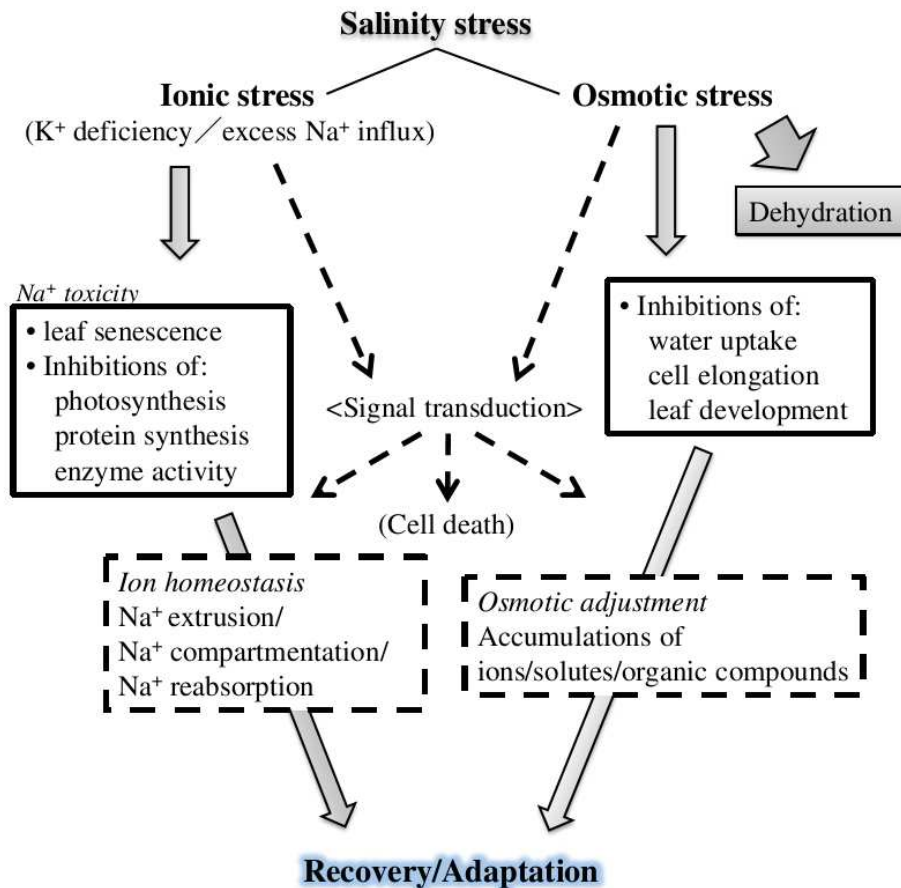
### 1. Drought and salt stress in plant with a central focus on rice

Drought is one of the most devastating abiotic stresses reducing crop yield and can provoke a range of mechanistic responses in plants. At the whole plant level, drought can induce restriction of growth and conservation of soil water uptake, earlier completion of the life cycle, and enhanced root growth to increase the supply of available soil water. At the tissue/organ level, plants can reduce water loss through reduced leaf surface or leaf (stomatal and cuticular) conductance or take up water more efficiently by altering root conductance ( $L_0$ ), in order to maintain tissue water potential. At the cell level, under severe drought stress, accumulation of osmoprotectants, antioxidants and reactive oxygen species (ROS) scavengers is involved. In drought condition, rice exhibits water uptake regulation

which depends on root architecture and root function. The latter is partially controlled by the AQPs.

Suffering from high salinity, plants have to cope with two major stresses, osmotic and ionic stress (**Figure 1**). Osmotic stress occurs immediately with an excess level of salt outside the root, leading to inhibitions of water uptake (even a water efflux can occur under severe stress), cell expansion and lateral bud development (Munns and Tester, 2008). Ionic stress comes afterwards when  $\text{Na}^+$  is over-accumulated in plant, especially in leaves, causing leaf chlorosis and necrosis, reducing essential cellular metabolism activities such as protein synthesis, enzyme activity, and photosynthesis. As a consequence, crop yield is highly affected by salinity (Yeo and Flowers, 1986). Rice is one of the most sensitive species to salt stress (Munns and Tester, 2008). Several mechanisms are developed by plants to withstand salt stress (Munns and Tester, 2008; Horie *et al.*, 2012):

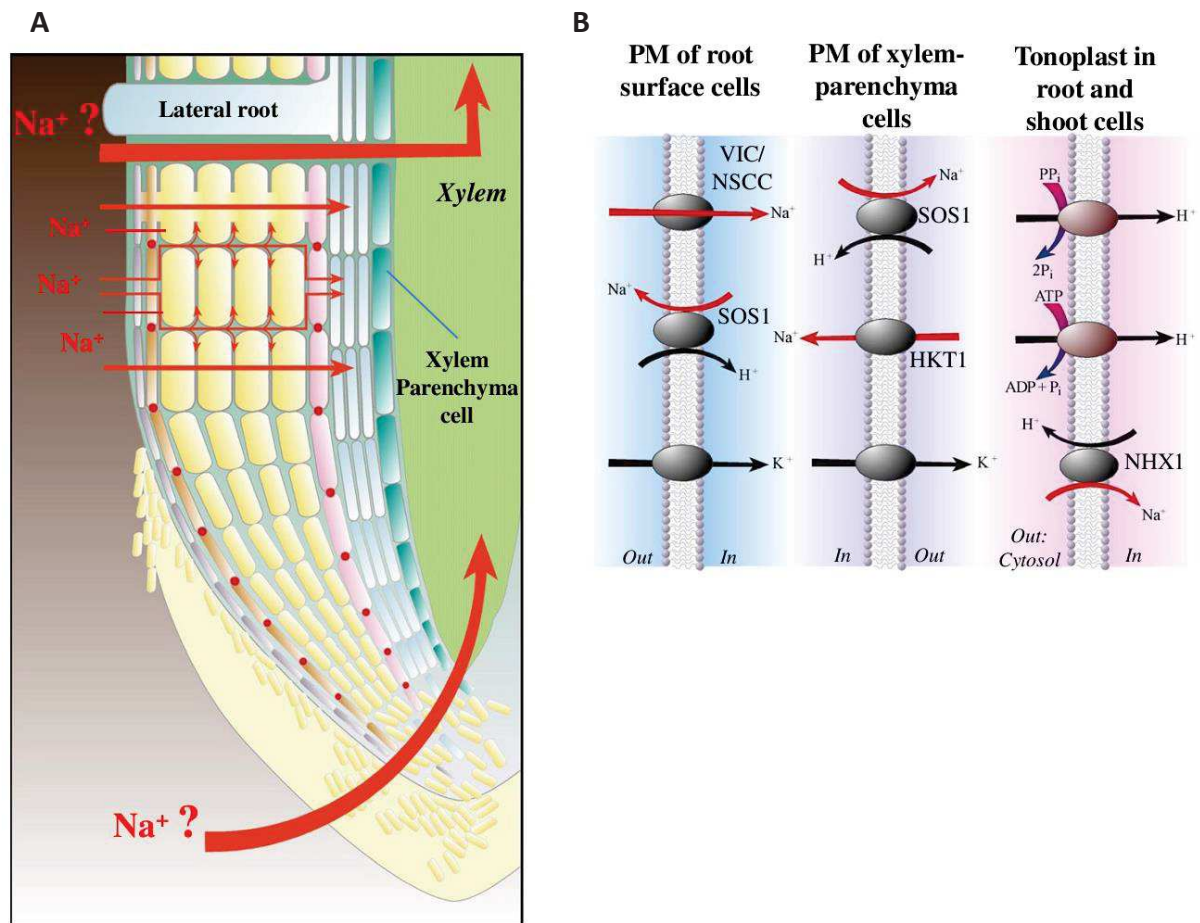
- (1) The osmotic adjustment by means of the accumulation of ions, solutes, organic compounds (also referred to as compatible solutes) maintains cell turgor and consequently water uptake by the root system. Here, water uptake is controlled by AQPs.
- (2) Restriction of  $\text{Na}^+$  accumulation in shoots by increasing root barriers (casparian strips and suberin lamellae),  $\text{Na}^+$  extrusion at the plasma membrane of soil-root interface cells,  $\text{Na}^+$  sequestration into the vacuole and  $\text{Na}^+$  reabsorption from the xylem vessels by means of  $\text{Na}^+$  transport systems.



**Figure 1.** A schematic summary of effects of high salinity and adaptation mechanisms in plants. From (Horie *et al.*, 2012).

By which way  $\text{Na}^+$  enters the root of plants in general and of rice in particular is still a controversy issue. It has been believed that abundant amount of  $\text{Na}^+$  was uptaken in rice by apoplastic pathway (so-called bypass flow) (Yeo *et al.*, 1987; Krishnamurthy *et al.*, 2009, 2011). The precise entry of  $\text{Na}^+$  bypass flow was suggested through lateral root emerging sites and root tip regions where the Casparian strips and suberin lamellae are disrupted or immature, respectively (Yeo *et al.*, 1987; Miyamoto *et al.*, 2001; Ochiai and Matoh, 2002; Ranathunge *et al.*, 2003, 2004, Krishnamurthy *et al.*, 2009, 2011) (**Figure 2**). By using different rice mutants which have different root morphological characteristics; it was concluded that bypass flow in rice is not at the sites of lateral root emergence (Faiyue *et al.*, 2010b), but rather through their cortical layers (Faiyue *et al.*, 2010a). Some studies pointed to the contribution of cell-to-cell pathway in  $\text{Na}^+$  uptake. For instance, it was reported a major participation of non-selective cation channels (NSCCs) or  $\text{K}^+$  selective channels in  $\text{Na}^+$  influx into rice root cells of salt-tolerant rice cv. Pokkali or salt-sensitive rice cv. BRRI Dhan29, respectively (Kader and Lindberg, 2005). HKT transporters such as *OsHKT2;1* and *OsHKT2;4*

may also be involved in the toxic  $\text{Na}^+$  influx, but their contribution is still disputed (Horie *et al.*, 2012).



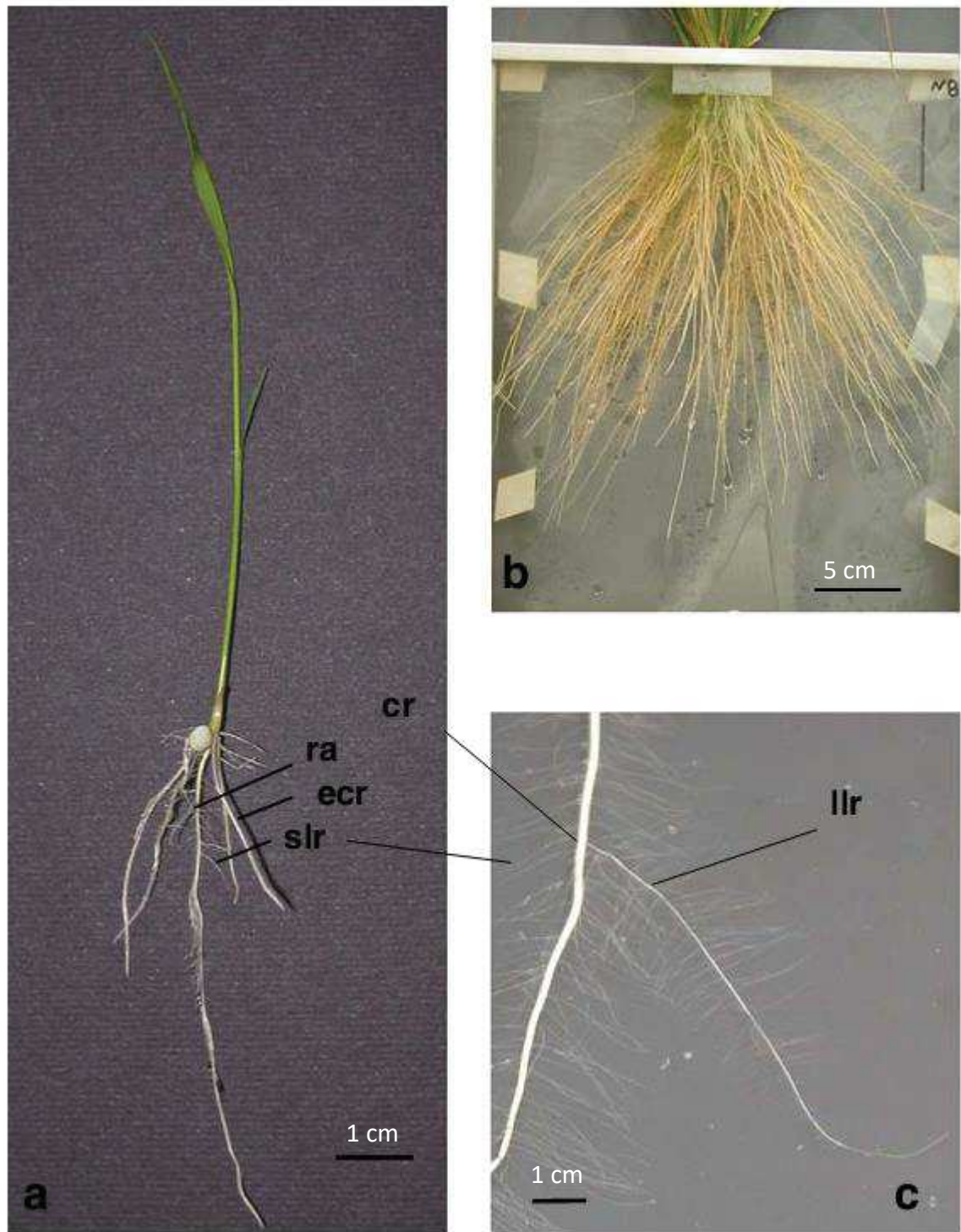
**Figure 2.  $\text{Na}^+$  influx pathways into roots and  $\text{Na}^+$  mediations by transport systems in biological membranes.** **A.** Several entries of  $\text{Na}^+$  into the roots including apoplastic and cell-to-cell pathways. **B.** Transport systems involved in  $\text{Na}^+$  extrusion in root epidermal cells,  $\text{Na}^+$  reabsorption in root xylem parenchyma cells and  $\text{Na}^+$  sequestration into the vacuole in root and shoot cells (from the left to the right, respectively). From (Horie *et al.*, 2012).

## 2. Rice root architecture and anatomy

### 2.1 Rice root architecture

Like in other cereals, rice root system consists of different root types (**Figure 3**). The radicle (so-called seminal root) is the first root which emerges from the coleorhiza. The crown roots emerge from nodes. In general, radicle and crown roots are called as primary roots to distinguish with lateral roots, which emerge from the pericycle and endodermal





**Figure 3. Root architecture.**

(a) Root system of seedling cv. Nipponbare, 1 week after germination. (b) Morphology of the rice root system 40 days after germination. (c) Detail of a crown root 40 days after germination. ra, radicle; ecr, embryonic crown root; cr, crown root; llr, large lateral root; slr, small lateral root. From (Rebouillat *et al.*, 2009)

cells of primary roots. Lateral root cap cells originate from the endodermis, whereas, others originate from the pericycle (Kawata and Shibayama, 1965). Lateral roots can be classified

into two different types. Large lateral roots are thinner than primary roots, and are able to produce small lateral roots. Small lateral roots display determinate elongation, grow laterally, never bear lateral roots and are by far the most abundant. On the other hand, primary roots and large lateral roots show indeterminate growth down-ward and produce lateral roots (Reviewed by (Rebouillat *et al.*, 2009)).

## **2.2 Rice root anatomy**

The radial anatomy of rice roots is typical for semiaquatic plants (**Figure 4**). Though having various size or number of cell layers in each tissue, in general, primary roots and large lateral roots include epidermis, exodermis, sclerenchyma, mesodermis/ aerenchyma, endodermis and center cylinder form the outer to the inner. Small lateral roots display much simpler internal structure with no mesodermis and aerenchyma. Each of the epidermis, exodermis, sclerenchyma or endodermis contains one single cell layer. Sclerenchyma is highly lignified but not composed of suberin, it can be a complement structure against the weakness of extensive formation of aerenchyma and limits oxygen loss from the root (Ranathunge *et al.*, 2003). Different from other tissues, mesodermis consists of several cell layers. At mature zone of roots, mesodermis differentiates into spokes and aerenchyma, which acts as an oxygen reservoir for rice to grow in flooding condition. Aerenchyma starts being established at about 1-2 cm and completed at 10 cm from the root apex (Ranathunge *et al.*, 2003, 2004). Casparian strips development and suberin lamellae deposition are two typical events occurring in the cell wall of exodermis and endodermis. Casparian strips deposit on the radial and transverse walls of the cells, whereas, suberin lamellae develop in inner tangential walls (**Figure 5**) (Clark and Harris, 1981). Casparian strips are strongly lignified (Schreiber, 1996). Suberin is a biopolymer consisting of aliphatic and aromatic domains. Casparian strips develop sooner compared to the suberin lamellae which commences at about 2 cm and is well-developed at 10 cm from the root apex in endodermis. Casparian strips develop later in exodermis, about 3 cm from the root tip. Suberin deposition in endodermal cell walls starts at about 2 cm, matures at 5-7 cm and completes at about 10 cm from the root tip with its thickness increasing along root towards the root base. Exodermal suberin also develops a bit later at about 3 cm from the root apex (Ranathunge *et al.*, 2003, 2004; Schreiber *et al.*, 2005).

**Figure 4. Radial anatomy of rice root.**

**Upper part:** The scheme represents radial anatomy of radicle root ~ 2 cm from the root tip.

**Lower part:** Radial anatomies of different rice root types. Root cell walls were visualized by their auto-fluorescence under UV illumination.

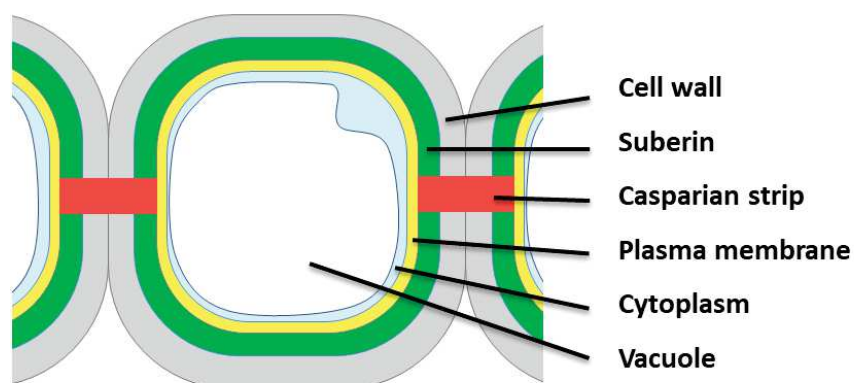
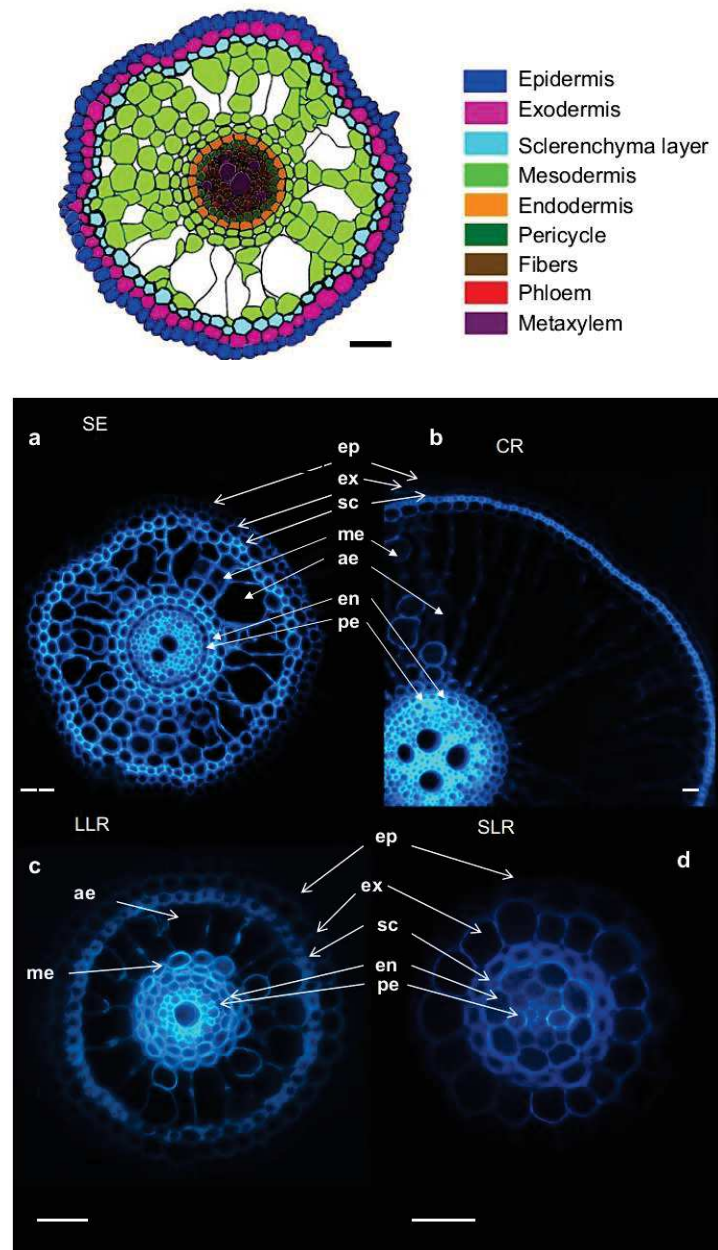
(a) Radicle cross section, ~1.5 cm from the root tip. (b) Crown root cross section, ~6 cm from the root tip.

(c) Large lateral root cross section ~2 cm from the root tip.

(d) Small lateral root transverse section. Crown and large lateral roots were collected from 40 day-old plants, while radicle and small lateral roots were collected from 1 week-old seedlings.

ep, epidermis; ex, exodermis; sc, sclerenchyma; me, mesodermis; ae, aerenchyma; en, endodermis; pe, pericycle. Scale bars: 50µm.

From (Rebouillat *et al.*, 2009)



**Figure 5. A scheme of casparian strip (in red) and suberin lamellae (in green) in plant cell.**



### 3. Diversity of AQP isoforms and their substrate specificities in rice

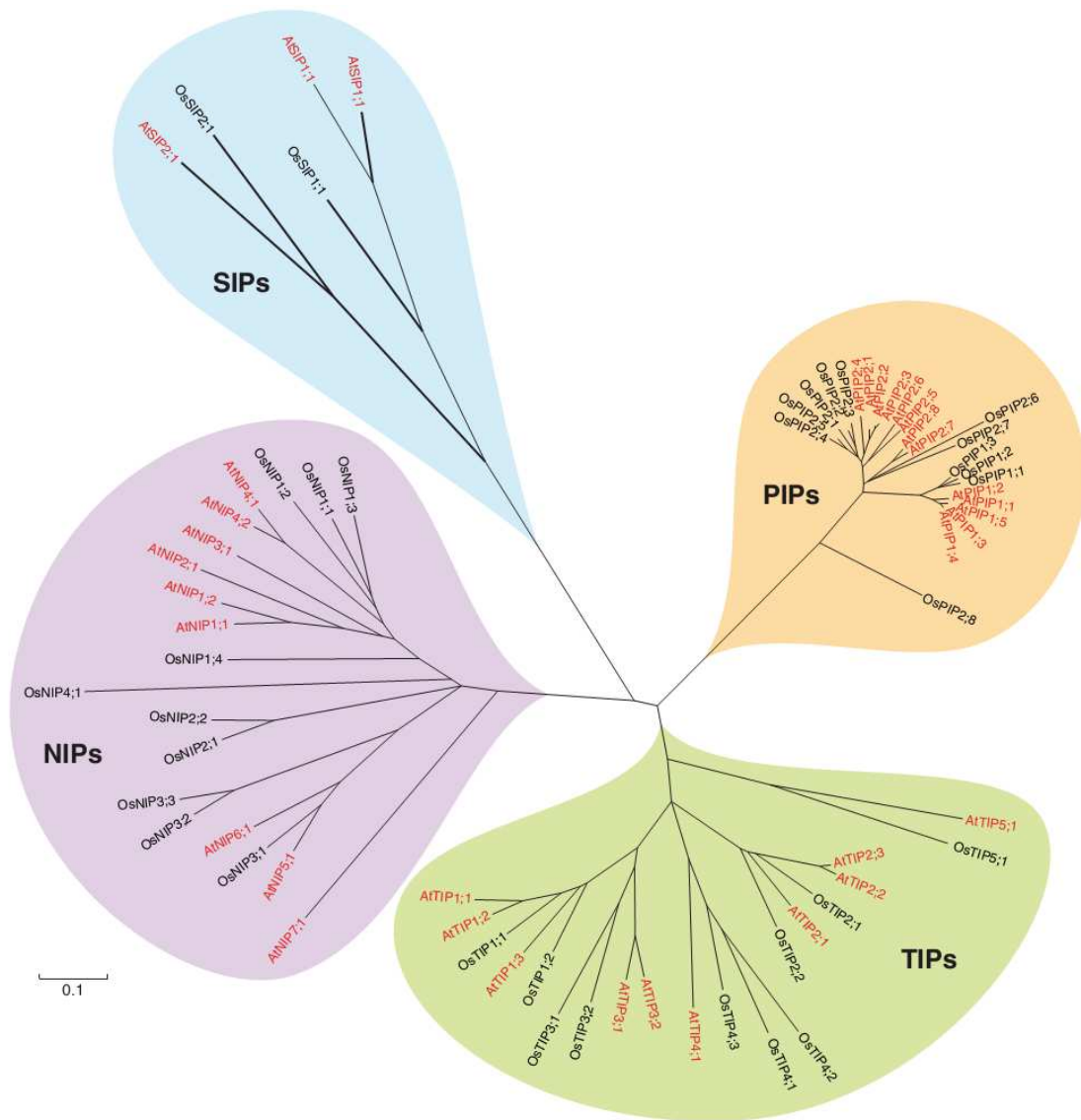
AQPs are classified into the ancient superfamily of major intrinsic proteins (MIPs) and divided into five subfamilies based on sequence homology and subcellular localization including: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and uncategorized (X) intrinsic proteins (XIPs) (Maurel *et al.*, 2015). So far, AQP genes have been already identified in several plant species (Maurel *et al.*, 2015) (**Table 1**).

**Table 1. Diversity of AQP gene family in plants.**

Species	Common Name	PIPs	TIPs	NIPs	SIPs	XIPs	Total	Reference
<i>Oryza sativa</i>	Rice	11	10	10	2		33	(Sakurai <i>et al.</i> , 2005; Nguyen <i>et al.</i> , 2013)
<i>Arabidopsis thaliana</i>	Mouse ear-cress	13	10	9	3		35	(Johanson <i>et al.</i> , 2001)
<i>Solanum lycopersicum</i>	Garden tomato	14	11	12	4	6	47	(Reuscher <i>et al.</i> , 2013)
<i>Populus trichocarpa</i>	Black cottonwood	15	17	11	6	6	55	(Gupta and Sankararamakrishnan, 2009)
<i>Glycine ma</i>	Soybean	22	23	13	6	2	66	(Zhang <i>et al.</i> , 2013)
<i>Gossypium hirsutum</i>	Upland cotton	28	23	12	7	1	71	(Park <i>et al.</i> , 2010)
<i>Zea mays</i>	Maize	13	11	3	4		31	(Chaumont <i>et al.</i> , 2001)
<i>Citrus sinensis</i> L. Osb.	Sweet orange	8	11	9	3	3	34	(de Paula Santos Martins <i>et al.</i> , 2015)
<i>Sorghum bicolor</i>	Sorghum	14	13	11	3		41	(Reddy <i>et al.</i> , 2015)

Number of isoforms identified in several plant species given as examples is indicated. From (Maurel *et al.*, 2015).

For instance, thirty-three AQP genes in the genome sequence of rice (cv. Nipponbare), including eleven *PIPs*, ten *TIPs*, ten *NIPs* and two *SIPs* were reported, based on the phylogenetic relationship with *Arabidopsis thaliana* and *Zea mays* (**Figure 6**) (Sakurai *et al.*, 2005; Nguyen *et al.*, 2013).



**Figure 6. Phylogenetic tree of rice (*Os*) and Arabidopsis (*At*) aquaporin families.**  
The scale bar of 0.10 is equal to 10% sequence divergence. From (Maurel *et al.*, 2015).

Many studies have proved that AQPs are multifunctional channels, which can transport not only water but also various small neutral molecules and/or physiological substrates such as carbon dioxide, ammonia, urea, glycerol, metalloids like boron and silicon, and ROS (Maurel *et al.*, 2015; Afzal *et al.*, 2016) (**Table 2**), but it is widely accepted that PIPs and TIPs are the two most abundant AQPs in plant cells and mainly function as water channels in plant roots. Osmotic water permeability of individual rice AQPs was tested in several expression systems such as proteoliposome, yeast, *Xenopus* oocyte or protoplast (Lian *et al.*, 2004; Sakurai *et al.*, 2005, 2008; Liu *et al.*, 2013). For instance, expressed in yeast heterologous expression system, *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5* and

*OsTIP2;2* stimulated significantly the osmotic water permeability, but not *OsPIP1;1*, *OsPIP1;2*, *OsTIP1;1*, or *OsTIP1;2* (Sakurai *et al.*, 2005, 2008). *OsPIP1;3* (RWC3) was reported

**Table 2. Functional expression and substrate specificity of representative plant AQPs**

Subclass	Isoform	Substrate	Expression System	Transport Assay
PIP			Proteoliposome	
	<i>OsPIP1;1</i>	Water	<i>Xenopus</i> oocyte	Shrinkage
	<i>OsPIP1;3</i>	Water	<i>Xenopus</i> oocyte	Shrinkage
	<i>OsPIP2;1</i>	Water	Proteoliposome	Shrinkage
	<i>OsPIP2;2</i>	Water	Proteoliposome	Shrinkage
	<i>OsPIP2;3</i>	Water	Proteoliposome	Shrinkage
	<i>OsPIP2;4</i>	Water	Proteoliposome	Shrinkage
	<i>OsPIP2;5</i>	Water	Proteoliposome	Shrinkage
	<i>AtPIP2;1</i>	Water	Proteoliposome	Shrinkage
	<i>AtPIP2;1</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Toxicity growth assay
	<i>AtPIP2;2</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>HvPIP2;1</i>	CO <sub>2</sub>	Transgenic rice	Gas exchange
	<i>NtAQP1</i>	Glycerol	<i>Xenopus</i> oocyte	Radiolabeling
	<i>NtAQP1</i>	CO <sub>2</sub>	<i>Xenopus</i> oocyte	Intracellular pH
	<i>NtAQP1</i>	CO <sub>2</sub>	Yeast	Intracellular pH
	<i>NtAQP1</i>	CO <sub>2</sub>	Planar lipid bilayer	Local pH
TIP	<i>OsTIP2;2</i>	Water	Proteoliposome	Shrinkage
	<i>AtTIP1;1</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>NtTIPa</i>	Urea	<i>Xenopus</i> oocyte	Radiolabeling
	<i>NtTIPa</i>	Glycerol	<i>Xenopus</i> oocyte	Radiolabeling
	<i>AtTIP1;2</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Intracellular fluorescence
	<i>TaTIP2</i>	NH <sub>3</sub>	Yeast	Extracellular pH
	<i>ZmTIP1;1</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Toxicity growth assay
	<i>AtTIP2.3</i>	NH <sub>3</sub>	<i>Xenopus</i> oocyte	Radiolabeling
NIP	<i>OsNIP1;1</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>OsNIP2;1</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>OsNIP2;1</i>	As(OH) <sub>3</sub>	Yeast	Toxicity growth assay
	<i>OsNIP2;1</i>	Si(OH) <sub>4</sub>	<i>Xenopus</i> oocyte	Ge-radiolabeling
	<i>OsNIP2;1</i>	As(OH) <sub>3</sub>	Rice mutant	Dosage
			Yeast	Toxicity growth assay
	<i>OsNIP2;1</i>	Na <sub>2</sub> SeO <sub>4</sub>	Rice mutant	Dosage
			<i>Xenopus</i> oocyte	Intracellular dosage
			Yeast	Intracellular dosage
	<i>OsNIP2;2</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>OsNIP2;2</i>	Si(OH) <sub>4</sub>	<i>Xenopus</i> oocyte	Ge-radiolabeling
	<i>OsNIP3;1</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>OsNIP3;2</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Toxicity growth assay
	<i>OsNIP3;2</i>	As(OH) <sub>3</sub>	Yeast	Toxicity growth assay
	<i>OsNIP3;3</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Toxicity growth assay
	<i>OsNIP3;3</i>	As(OH) <sub>3</sub>	Yeast	Toxicity growth assay
	<i>OsNIP4;1</i>	Water	<i>Xenopus</i> oocyte	Swelling
	<i>AtNIP5;1</i>	B(OH) <sub>3</sub>	<i>Xenopus</i> oocyte	Intracellular dosage
	<i>AtNIP5;1</i>	As(OH) <sub>3</sub>	<i>Xenopus</i> oocyte	Intracellular dosage
	<i>ZmNIP2;1</i>	GeO <sub>2</sub>	Yeast	Toxicity growth assay
	<i>BjNOD26</i>	Water	Proteoliposome	Shrinkage
	<i>BjNOD26</i>	NH <sub>3</sub>	Proteoliposome	Internal pH
SIP	<i>VvSIP1</i>	Water	Yeast	Shrinkage
	<i>VvSIP1</i>	Water	Proteoliposome	Shrinkage
XIP	<i>NtXIP1;1</i>	H <sub>2</sub> O <sub>2</sub>	Yeast	Toxicity growth assay
	<i>PtXIP2;1</i>	Water	<i>Xenopus</i> oocyte	Swelling

*OsNIP2;1* and *OsNIP2;2* are known as Lsi1 and Lsi6, respectively. Adapted from (Maurel *et al.*, 2015).

to facilitate water movement across oocyte membranes but 4 times less than mammalian AQP2 (Lian *et al.*, 2004). The low and high water channel activity of *OsPIP1;1* and *OsPIP2;1*, respectively, were also reported when expressed in *Xenopus* oocytes (Liu *et al.*, 2013). Interestingly, co-expression with *OsPIP2;1* significantly enhanced the water transport activity of *OsPIP1;1*, probably thanks to the interaction of these two AQPs, leading to a re-localization of *OsPIP1;1* to the plasma membrane. This hypothesis was initially presented in another monocot. Indeed, assembly of *ZmPIP1s* and *ZmPIP2s* in heterotetramers leading to the targeting of *ZmPIP1* isoforms to the plasma membrane was reported in maize (Zelazny *et al.*, 2007). Consistently, the high water permeability of liposome membranes reconstituted with *OsPIP1;1* was reported (Liu *et al.*, 2013; Scalera *et al.*, 2014).

## 4. Rice AQP expression in normal conditions

Reverse transcription-PCR (RT-PCR) was a technique among others applied to estimate rice AQP transcript abundance (Sakurai *et al.*, 2005; Guo *et al.*, 2006; Sakurai-Ishikawa *et al.*, 2011; Grondin *et al.*, 2016). Additionally, isoform-specific antibodies were developed for immuno-chemistry applications (Sakurai *et al.*, 2008; Sakurai-Ishikawa *et al.*, 2011).

### 4.1 Tissue-specific expression

In rice, as in other plant species, AQPs were reported to be organ-specifically expressed at different levels, depending on growth stage, and variety (**Table 3**). For instance, in cv. Akitakomachi, several AQPs were expressed predominantly in root system such as *OsPIP1;3*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP2;1* and *OsNIP2;1*, while others were more expressed in leaf blades including *OsPIP2;7*, *OsPIP2;8*, *OsTIP1;2*, *OsTIP3;1*, *OsTIP3;2*, *OsTIP4;2*, *OsTIP4;3*, *OsTIP5;1*, *OsNIP1;1*, *OsNIP1;2*, *OsNIP1;4*, *OsNIP3;2*, *OsNIP3;3* and *OsNIP4;1* (Sakurai *et al.*, 2005, 2008). Interestingly, eight genes (*OsPIP1;1*, *OsPIP1;2*, *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;6*, *OsTIP2;2*, *OsTIP4;1* and *OsSIP1;1*) were detected almost equally in both roots and leaf blades (Sakurai *et al.*, 2005, 2008; Sakurai-Ishikawa *et al.*, 2011). In contrast, *OsPIP2;1* and *OsPIP2;4* were not detected in cv. Moroberekan roots, neither *OsPIP2;4* in cv. Azucena roots (Grondin *et al.*, 2016), while this latter had the highest transcript level in cv. Zhonghua 11 roots (Guo *et al.*, 2006). *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;6*, *OsPIP2;7* and *OsTIP4;1* could not be detected in roots of Giza 178, Sakha 101, IR64 and PLS2

cv. (Nada and Abogadallah, 2014). Moroberekan, a moderate drought tolerance rice cv., showed higher relative transcript abundance of *OsPIP1;1*, *OsPIP1;2*, *OsPIP2;2*, *OsPIP2;6* and *OsPIP2;8* in roots compared to the drought susceptible IR64 (Grondin *et al.*, 2016).

**Table 3. Expression in leaves or roots of *OsPIPs* and *OsTIPs* in rice**

Subclass	Isoform	Organ level		Level	Methodology	References
		Leaves	Roots			
PIPs	<i>OsPIP1;1</i>	++	++	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP1;2</i>	++	++	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP1;3</i>	+/-	++	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP2;1</i>	++	++/-	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP2;2</i>	++/-	++	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP2;3</i>	+	++	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011)
	<i>OsPIP2;4</i>	+	++	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP2;5</i>	+	++	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011)
	<i>OsPIP2;6</i>	+	+/-	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014; Grondin <i>et al.</i> , 2016)
	<i>OsPIP2;7</i>	++/-	+/-	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Guo <i>et al.</i> , 2006; Nada and Abogadallah, 2014)
	<i>OsPIP2;8</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Grondin <i>et al.</i> , 2016)
TIPs	<i>OsTIP1;1</i>	++/+	+/++	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Li <i>et al.</i> , 2008; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014)
	<i>OsTIP1;2</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Li <i>et al.</i> , 2008; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014)
	<i>OsTIP2;1</i>	+	++	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Sakurai-Ishikawa <i>et al.</i> , 2011)
	<i>OsTIP2;2</i>	++	++/-	mRNA protein	RT-PCR immunoblotting	(Sakurai <i>et al.</i> , 2005, 2008; Li <i>et al.</i> , 2008; Sakurai-Ishikawa <i>et al.</i> , 2011; Nada and Abogadallah, 2014)
	<i>OsTIP3;1</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005)
	<i>OsTIP3;2</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005)
	<i>OsTIP4;1</i>	++	++/-	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Li <i>et al.</i> , 2008; Nada and Abogadallah, 2014)
	<i>OsTIP4;2</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Li <i>et al.</i> , 2008)
	<i>OsTIP4;3</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005; Li <i>et al.</i> , 2008)
	<i>OsTIP5;1</i>	++	+	mRNA	RT-PCR	(Sakurai <i>et al.</i> , 2005)

(-) indicates non-detected; (+) or (++) indicates the individual AQP was expressed relatively less or more in the specific organ, respectively.

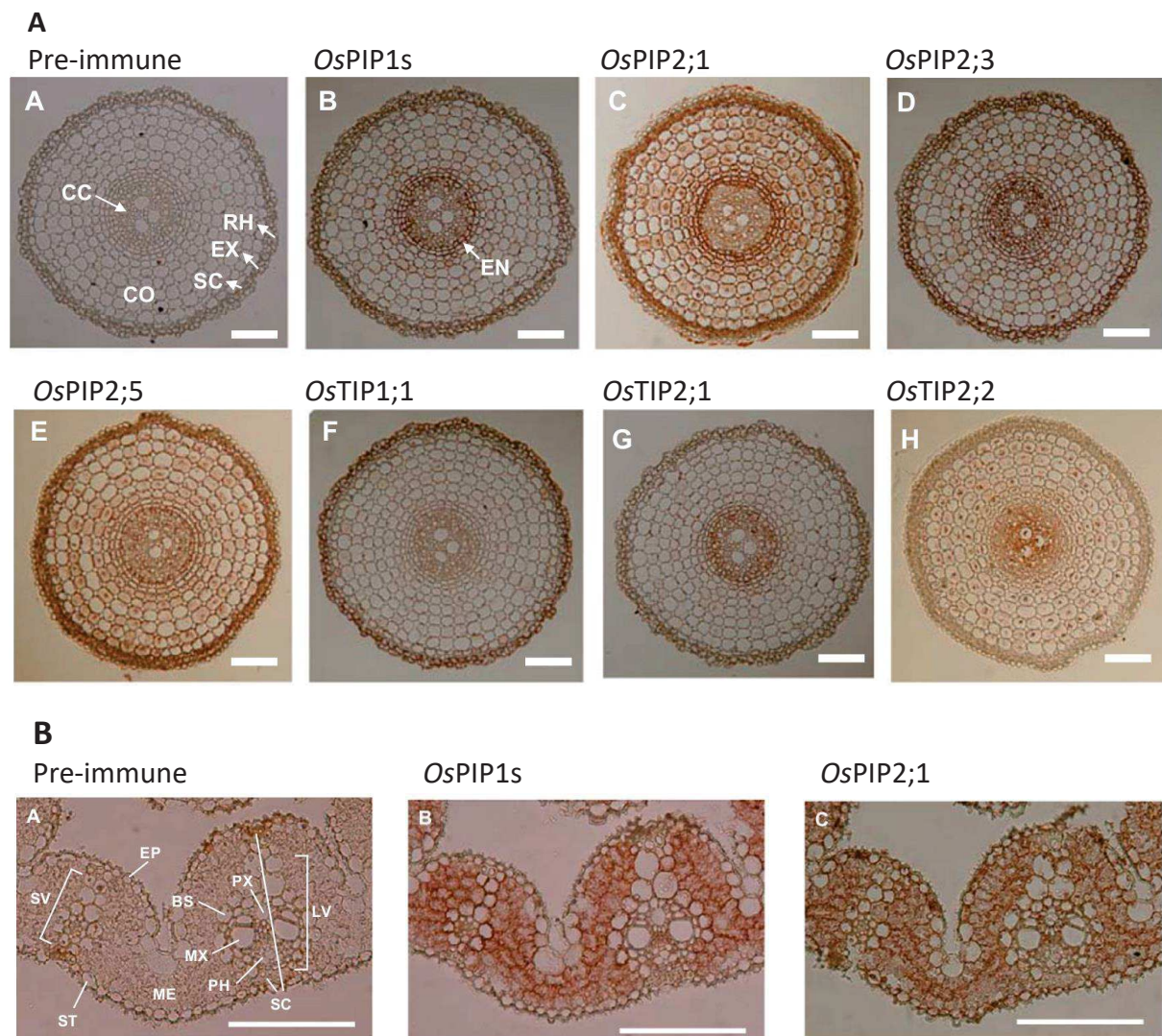
In rice, younger root zones exhibit the highest AQP expression. In other words, AQP expression gradually decreased from the tip adjacent zone to the basal zone following the transformation of cortex cells to aerenchyma (Sakurai *et al.*, 2008; Sakurai-Ishikawa *et al.*, 2011). In primary root, rice plasma membrane AQPs accumulated at high level in endodermis, *OsPIP2* isoforms expressed in all root cells while *OsPIP1* isoforms showed less accumulation in outer parts of the roots (**Table 4, Figure 7**)



**Table 4. Localization of *OsPIPs* and *OsTIPs* in rice root cell layers observed by means of immuno-cytochemistry.**

Subclass	Isoform	Epidermis	Exodermis	Sclerenchyma	Endodermis	Central cylinder	References
<b>PIPs</b>	<i>OsPIP1s</i>	+	+	+	+++		(Sakurai <i>et al.</i> , 2008)
	<i>OsPIP2;1</i>	++	++	++	+++		(Sakurai <i>et al.</i> , 2008; Sakurai-Ishikawa <i>et al.</i> , 2011)
	<i>OsPIP2;3</i>	++	++	++	+++		(Sakurai <i>et al.</i> , 2008)
	<i>OsPIP2;5</i>	++	++	++	+++	++	(Sakurai <i>et al.</i> , 2008; Sakurai-Ishikawa <i>et al.</i> , 2011)
<b>TIPs</b>	<i>OsTIP1;1</i>	+++	+++				(Sakurai <i>et al.</i> , 2008)
	<i>OsTIP2;1</i>				++	++	(Sakurai <i>et al.</i> , 2008; Matsunami <i>et al.</i> , 2016)
	<i>OsTIP2;2</i>					++	(Sakurai <i>et al.</i> , 2008)

(+), (++) or (+++) indicates the individual AQP was expressed relatively less or more in the specific organ, respectively.



**Figure 7. Tissue localization of rice AQPs in roots (A) and leaf blades (B).**

Cross-sections at about 4mm from the root tip of the primary root of 38-day-old rice plants (A) or at the middle part of the uppermost leaves of 18-day-old rice plants (B) were visualized after reactions with AQP specific antibodies. **A:** CC, central cylinder; CO, cortex; EN, endodermis; EX, exodermis; RH, rhizodermis; SC, sclerenchyma. **B:** BS, bundle sheath; EP, epidermis; LV, large vascular bundle; ME, mesophyll; MX, metaxylem; PH, phloem; PX, protoxylem; SC, sclerenchymatous cell; ST, stomata; SV, small vascular bundle. Scale bar: 100μm. Adapted from (Sakurai *et al.*, 2008).

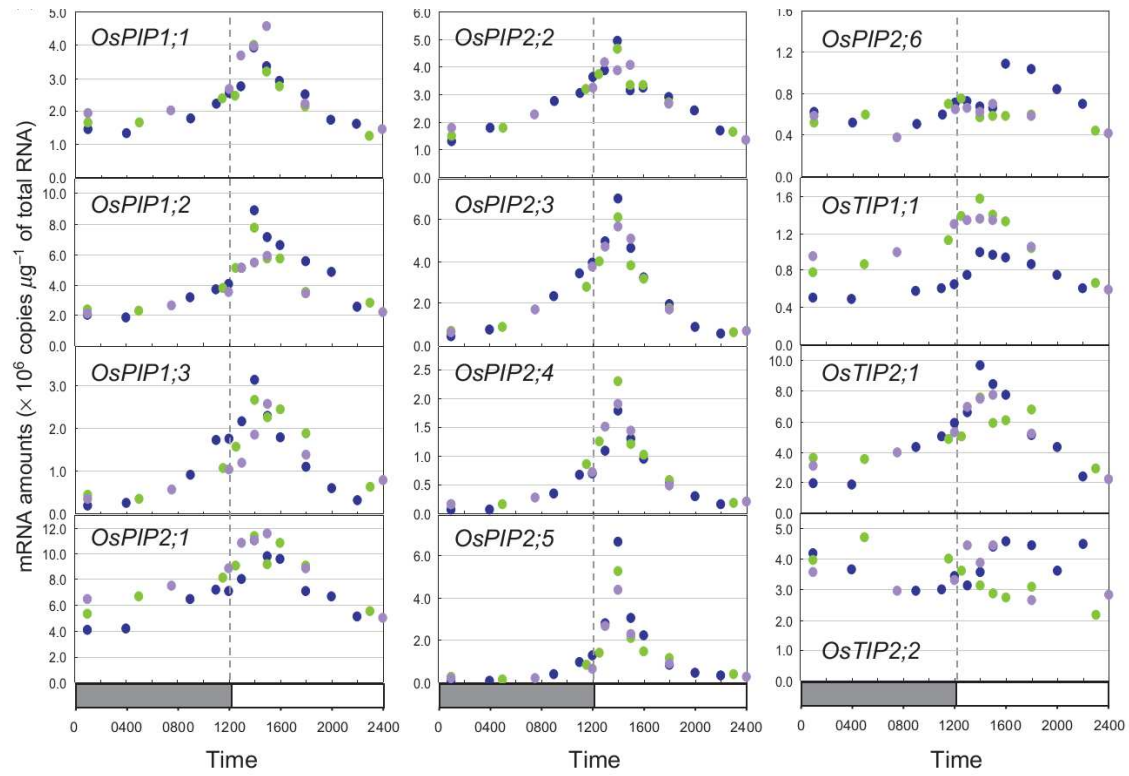
(Sakurai *et al.*, 2008; Sakurai-Ishikawa *et al.*, 2011; Matsunami *et al.*, 2016). *OsTIPs* showed different localizations, for instance, the isoform *OsTIP1;1* distributed predominantly in the epidermis and exodermis, whereas *OsTIP2;1* and *OsTIP2;2* isoforms are present in the endodermis and central cylinder (Sakurai *et al.*, 2008; Matsunami *et al.*, 2016).

In leaves, *OsPIP1s* and *OsPIP2;1* accumulated abundantly in mesophyll cells compared to epidermis and bundle sheath cells (Figure 7) (Sakurai *et al.*, 2008). At variance with this observation, high abundance of *AtPIP2;1* was reported in vascular bundles in *Arabidopsis* (Prado *et al.*, 2013).

Putative contribution of *OsTIP3;1* to rice seed width has been found by genome-wide association studies (Huang *et al.*, 2012). At last, genetic evidence stressed the importance of AQPs in seeds, by knockout and/or overexpression of *OsPIP1;1* and *OsPIP1;3* which alter both the rate and speed of germination (Liu *et al.*, 2007, 2013). Nitric oxide was reported to hormonally control their expression.

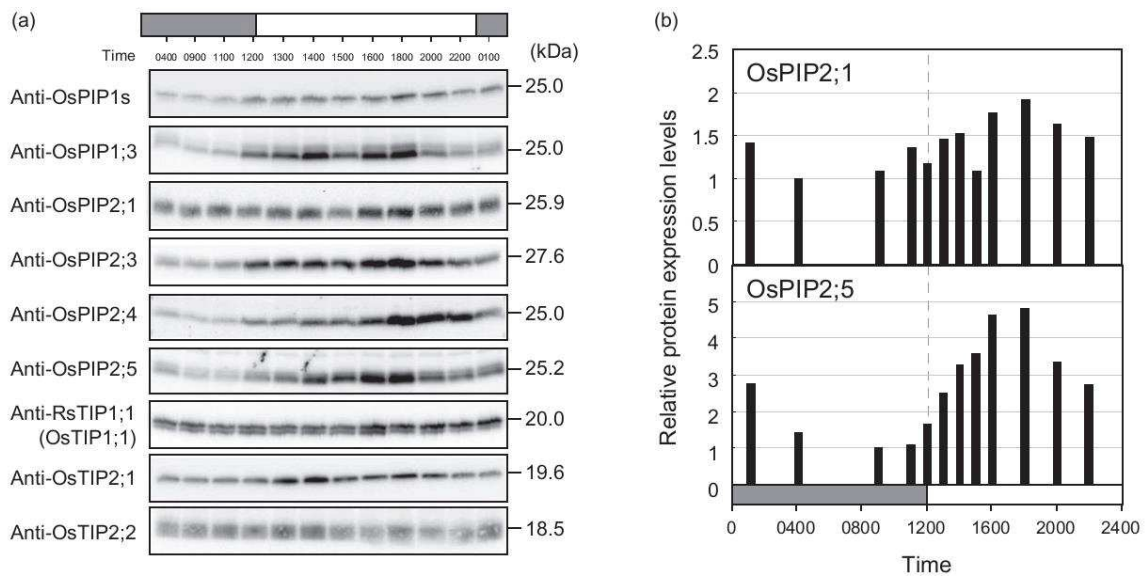
#### **4.2 Diurnal-specific expression**

The expression levels of rice AQPs also vary during the day (**Figures 8 and 9**). The mRNA levels of *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;2* and *OsTIP2;1* changed diurnally with a large amplitude which reached a peak in 1-3 h after light-on and the minimum level at night time, while *OsPIP2;6*, *OsTIP1;1*, *OsTIP2;2* showed little change in roots (Sakurai *et al.*, 2005; Sakurai-Ishikawa *et al.*, 2011; Nada and Abogadallah, 2014). Interestingly, protein levels of *OsPIP2;4* and *OsPIP2;5* in roots peaked 6 h after light initiation, suggesting a time lag of about 4 h between the peak of mRNA accumulation and appearance of proteins (Sakurai-Ishikawa *et al.*, 2011). Analysis of AQPs in field conditions reported that *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;2*, *OsPIP2;4*, *OsTIP1;1* and *OsTIP1;2* had the highest transcription level at dawn (9:00), then down-regulated during the day in roots of two japonica cvs. Giza 178 and Sakha 101 and one indica cv. PSL2, except in the indica cv. IR64 in which AQP expression peaked at noon (13:00). This expression pattern was more remarkable in leaves with exception of *OsPIP1;3*, *OsTIP1;1* and *OsTIP1;2* which expressed the strongest at 13:00. *OsPIP2;1*, *OsPIP2;7* and *OsTIP4;1* transcription increased in leaves from 9:00 to 13:00 (Nada and Abogadallah, 2014).



**Figure 8. Diurnal changes in AQP gene expression levels in rice roots of 16 day-old plants.**

Blue, green and purple dot mean results from three independent experiments. From (Sakurai-Ishikawa *et al.*, 2011).



**Figure 9. Diurnal changes in aquaporin protein levels in roots.**

Crude membrane fractions were prepared from the root samples and subjected to immunoblotting using specific antibodies. From (Sakurai-Ishikawa *et al.*, 2011).

## 5. Role of AQPs in the response of rice to drought and salt stress



As in other plant species, many studies reported that in rice, AQP expression varied according to stimuli of the environment. For instance, exposure of roots to low temperature for a long period (2–5 days) induced a compensatory increase in root hydraulic properties of rice root system, correlated with enhanced expression of *OsPIP2;5* in root. Since shoot has to be maintained in its integrity at control temperature, it was suggested the involvement of a shoot-to-root signal (Ahamed *et al.*, 2012). Importantly, in agreement with the role of AQPs in osmoregulation, effects of water and salt stresses were mostly reported (Maurel *et al.*, 2015).

### **5.1 Inhibition of root hydraulic properties upon drought and salt stress**

Because osmotic stress reduces water uptake, plant should balance the situation by enhancing root hydraulic conductivity ( $Lp_r$ ). However, reported data indicated different strategies. Hence, the contribution of AQPs, represented by the percentage of  $Lp_r$  inhibition by azide ( $\text{NaN}_3$ ), in three moderate drought tolerance rice cultivars, Azucena, Moroberekan and Dular, was either significantly decreased, not changed or significantly increased by drought stress, respectively (Grondin *et al.*, 2016). Under salt treatment,  $Lp_r$  of Azucena and Bala rice cultivars were reported to significantly be reduced (Meng and Fricke, 2017). Reduction of  $Lp_r$  under salt stress was also reported in other plant species, such as *Arabidopsis* (Boursiac *et al.*, 2005), and barley (Horie *et al.*, 2011); the  $Lp_r$  reduction exhibiting a shutdown of water transport to minimize water loss (Horie *et al.*, 2011). Since AQPs mediate water transport and are major components of  $Lp_r$ , their regulation may reflect a strategy of plants in response to drought and salt stress.

### **5.2 Regulation of AQP expression in rice upon drought and salt stress**

A likely number of articles studied the regulation of rice AQPs under drought (osmotic) and salt stress at mRNA level. There was a large variation of expression patterns of particular AQP genes, it may be attributed to differences in cultivars, growth stages, level and duration of treatments. Nevertheless, the results suggested a functional coordination between *OsPIPs* and *OsTIPs* in water deficit and salt stress.

Many *OsPIPs* and *OsTIPs*, analyzed at transcript abundance level, were reported to be upregulated in both leaves and roots of rice grown in low air humidity (Ku wagata *et al.*, 2012). In response to drought stress, mimicked by polyethylene glycol (PEG) treatment, in

leaves, most of *OsPIP* gene expression was not altered or down-regulated; only the expression of *OsPIP1;2*, *OsPIP1;3* and *OsPIP2;3* was up-regulated by 6 h of treatment in some cultivars. Meanwhile, the expression of *OsTIPs* (*OsTIP1;1*, *OsTIP1;2*, *OsTIP2;2* and *OsTIP4;1*, *OsTIP4;2*) was up-regulated and peaked at 4-8 h, then down regulated at 10 h; *OsTIP4;3* expression was slightly up-regulated. However, in roots, the expression of many *PIP* (such as *OsPIP1;3*, *OsPIP2;1*, *OsPIP2;5* and *OsPIP2;7*) and *TIP* (such as *OsTIP1;1*, *OsTIP1;2*, *OsTIP4;1* and *OsTIP4;2*) genes was up-regulated upon dehydration stress (Liu *et al.*, 1994; Lian *et al.*, 2004, 2006; Guo *et al.*, 2006; Li *et al.*, 2008; Henry *et al.*, 2012). Dry-down soil experiments showed the upregulation of mRNA levels of almost all *PIPs* and *TIPs* in leaves, with a higher effect in indica compared to japonica rice, whereas down-regulations of expression were observed in roots (Nada and Abogadallah, 2014; Grondin *et al.*, 2016).

In response to salt stress, *OsPIPs* transcripts were decreased in leaves in the first 2 h then increased and reached a peak at 6 h of treatment. Differently in roots, *OsPIP2* transcripts were not changed or even slightly decreased during one day of salt stress; while *OsPIP1* transcripts showed an increase, especially at the first 2 or 6 h (Guo *et al.*, 2006). However, almost all *OsTIP* transcripts were upregulated, except *OsTIP2;2* and *OSTIP4;3* in both leaves and roots (Liu *et al.*, 1994; Li *et al.*, 2008).

These data obtained on the transcript accumulation have to be balanced with their absence or weak relationship with rice root hydraulic properties. Therefore, it is difficult to identify a steady pattern for the role of AQPs in response to abiotic stress based on transcriptomic data (Grondin *et al.*, 2016; Meng and Fricke, 2017). Analysis at protein level may reflect more reliably the functional activity of AQPs in response to stress. Thus, study in parallel with two rice cultivars Puluik Arang and Akitakomachi, in which the former is the most drought tolerant, showed that Puluik Arang had better lateral root development and higher level of accumulation of the isoform *OsTIP2;1* under prolonged osmotic stress. This accumulation was detected in almost all root cells at lateral root initiation zone and abundantly in the endodermis of radicle and lateral roots at mature zone (Matsunami *et al.*, 2016). Moreover, osmotic stress markedly enhanced the *OsPIP* protein abundance in the roots of both lowland (Xiushui 63) and upland (Zhonghan 3) rice cultivars and in leaves of upland cultivar (Lian *et al.*, 2006). However, a remarkable reduction of osmotic  $L_p$  of two rice cultivars upon both drought and salt stress was reported (Meng and Fricke, 2017).

Because osmotic  $Lp_r$  likely represents the intrinsic activity of AQPs, the inconsistency between AQP protein level and  $Lp_r$  may be explained properly by post-translational modifications such as gating or subcellular redistribution (Maurel *et al.*, 2015). For instance, salt treatment caused a fast (halftime = 45 min) and strong (-70%) inhibition of  $Lp_r$  in Arabidopsis, but no marked degradation of AtPIP2;1 at early stage (Boursiac *et al.*, 2005). This is concomitant with the phosphorylation of AtPIP2;1, its enhanced lateral mobility in the plasma membrane, enhanced cycling and labelling of intracellular structures (Prak *et al.*, 2008; Li *et al.*, 2011; Luu *et al.*, 2012).

### **5.3 Genetic manipulation of AQPs in rice**

Strategies with transgenic plants overexpressing AQPs were developed in rice to manipulate their expression and analyze their contribution in plant response to abiotic stress. Though no AQP genes were annotated in the three chromosome regions highly correlated with hydraulic traits of the OryzaSNP panel, except OsNIP2;2 in region 2 (Grondin *et al.*, 2016), some studies highlighted the enhanced-drought tolerance of rice overexpressing AQPs.

Overexpressing OsPIP1;3 made transgenic plants with higher osmotic  $Lp_r$ , leaf water potential and relative cumulative transpiration under drought stress compared to wild-type plants; this suggests a role of this AQP in drought avoidance (Lian *et al.*, 2004). Moreover, overexpressing either OsPIP1;1 or OsPIP2;2, in Arabidopsis gave transgenic plants a better elongation of roots compared to the control plants in both drought and salt stress (Guo *et al.*, 2006). The role of the level of expression of OsPIP1;1 in salt tolerance was investigated by analyzing transgenic rice with high or lower expression of a transgenic OsPIP1;1 (Liu *et al.*, 2013). High expression level ( $\geq 4 \times 10^5$  copies/mg total RNA) promoted vegetative growth of rice in control, but not in salt stress (150 mM NaCl) condition, and even made the plant more sensitive when exposed to a more intense stress (200 mM NaCl). Transgenic seedlings with middle to low levels of transgene expression ( $\leq 4 \times 10^5$  copies/mg total RNA) exhibited tolerance to salt (100 mM NaCl), and showed longer roots and shoots compared to wild-type plants.

## 6. Context and thesis objectives

Because of global changes and steady increase in world population, agriculture is facing challenges for producing sufficient amounts of good quality food. Global changes are predicted to dramatically increase the variability of fresh water supply, both in space and time, and will thus affect crop yield. Several regions are already under severe risk of drought, extreme temperatures, and of other types of abiotic stresses linked to water (e.g. as a result of periodic flooding or salinization). Understanding plant response to these stresses can contribute to the development of more stress-tolerant crop varieties.

As shown in the previous parts of this general introduction, AQPs have roles in multiple levels of rice physiology, particularly in drought and salt stress. The aim of my PhD was to understand their contribution to the water homeostasis in rice. To address their contribution, three objectives were considered.

AQP transcript and protein abundance has been extensively studied in rice upon drought and salt stress (§5.2). The inconsistency between this molecule accumulation and hydraulic properties led us to consider the hypothesis of the involvement of the regulation of their subcellular localization. Hence, we analyzed their subcellular localization upon osmotic and salt stress in root cells by means of cell biology approaches.

Genetic manipulation of AQPs in rice was performed on a limited number of isoforms (§5.3). The second objective addressed the effects of the overexpression of AQPs on drought and salt tolerance. Here, we will consider the isoforms *OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;1*, *OsTIP2;2*, individually overexpressed in transgenic rice plants.

Although water uptake is dependent on root function, little is known on the respective contribution between root characteristics (architecture and anatomy, §2) and root function (water uptake per length of root and  $Lp_r$ ). The third objective was to bring more data on the contribution of root system architecture in root hydraulics.

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## **Chapter I:**

**Sub-cellular markers highlight intracellular dynamics of membrane proteins in response to abiotic treatments in rice**

# **Sub-cellular markers highlight intracellular dynamics of membrane proteins in response to abiotic treatments in rice**

**Thi Thu Huyen Chu<sup>1,5</sup>, Thi Giang Hoang<sup>2,5,6</sup>, Duy Chi Trinh<sup>1,5</sup>, Charlotte Bureau<sup>3,4</sup>, Donaldo Meynard<sup>3,4</sup>, Aurore Vernet<sup>3,4</sup>, Mathieu Ingouff<sup>7</sup>, Nang Vinh Do<sup>2,6</sup>, Christophe Périn<sup>3,4</sup>, Emmanuel Guiderdoni<sup>3,4</sup>, Pascal Gantet<sup>5,6,7</sup>, Christophe Maurel<sup>1</sup> and Doan-Trung Luu<sup>1</sup>**

<sup>1</sup> BPMP, CNRS, INRA, Montpellier SupAgro, Univ Montpellier, Montpellier, France

<sup>2</sup> National key laboratory for Plant Cell Biotechnology, Agricultural Genetics Institute, Pham Van Dong, Co Nhue, Tuliem, Hanoi, Vietnam

<sup>3</sup> CIRAD, UMR AGAP, 34398 Montpellier, France

<sup>4</sup> Université de Montpellier, CIRAD-INRA-Montpellier SupAgro, 34000 Montpellier France

<sup>5</sup> University of Science and Technology of Hanoi, LMI RICE, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam

<sup>6</sup> IRD, LMI RICE, Agricultural Genetics Institute, Pham Van Dong road, Co Nhue, Tuliem, Hanoi, Vietnam

<sup>7</sup> Université de Montpellier, UMR DIADE, 911 Avenue Agropolis, 34394 Montpellier Cedex 5, France

## **Corresponding author:**

### **Dr. Doan-Trung Luu**

Biochimie et Physiologie Moléculaire des Plantes

Institut de Biologie Intégrative des Plantes

UMR 5004 CNRS/UMR 0386 INRA/Montpellier SupAgro/Université de Montpellier

2, Place Viala

F-34060 Montpellier Cedex 2

France

E-mail : [luu@supagro.fr](mailto:luu@supagro.fr)

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## Abstract

Cell biology approach using membrane protein markers tagged with fluorescent proteins highlights the dynamic behaviour of plant cell membranes, not only in the standard but also in changing environmental conditions. In the past, this strategy has been extensively developed in plant models such as Arabidopsis. Here, we generated a set of transgenic lines expressing membrane protein markers to extend this approach to rice, one of the most cultivated crop in the world and an emerging plant model. Lines expressing individually eight membrane protein markers including five aquaporins (*OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;1*, *OsTIP2;2*) and three endosomal trafficking proteins (*OsRAB5b*, *OsGAP1*, *OsSCAMP1*) were obtained. Importantly, we challenged in roots the aquaporin-expressing transgenic lines upon salt and osmotic stress and uncovered a highly dynamic behaviour of cell membrane.

**Key words:** *Oryza sativa*, subcellular markers, intracellular dynamics, abiotic stress.

## Introduction

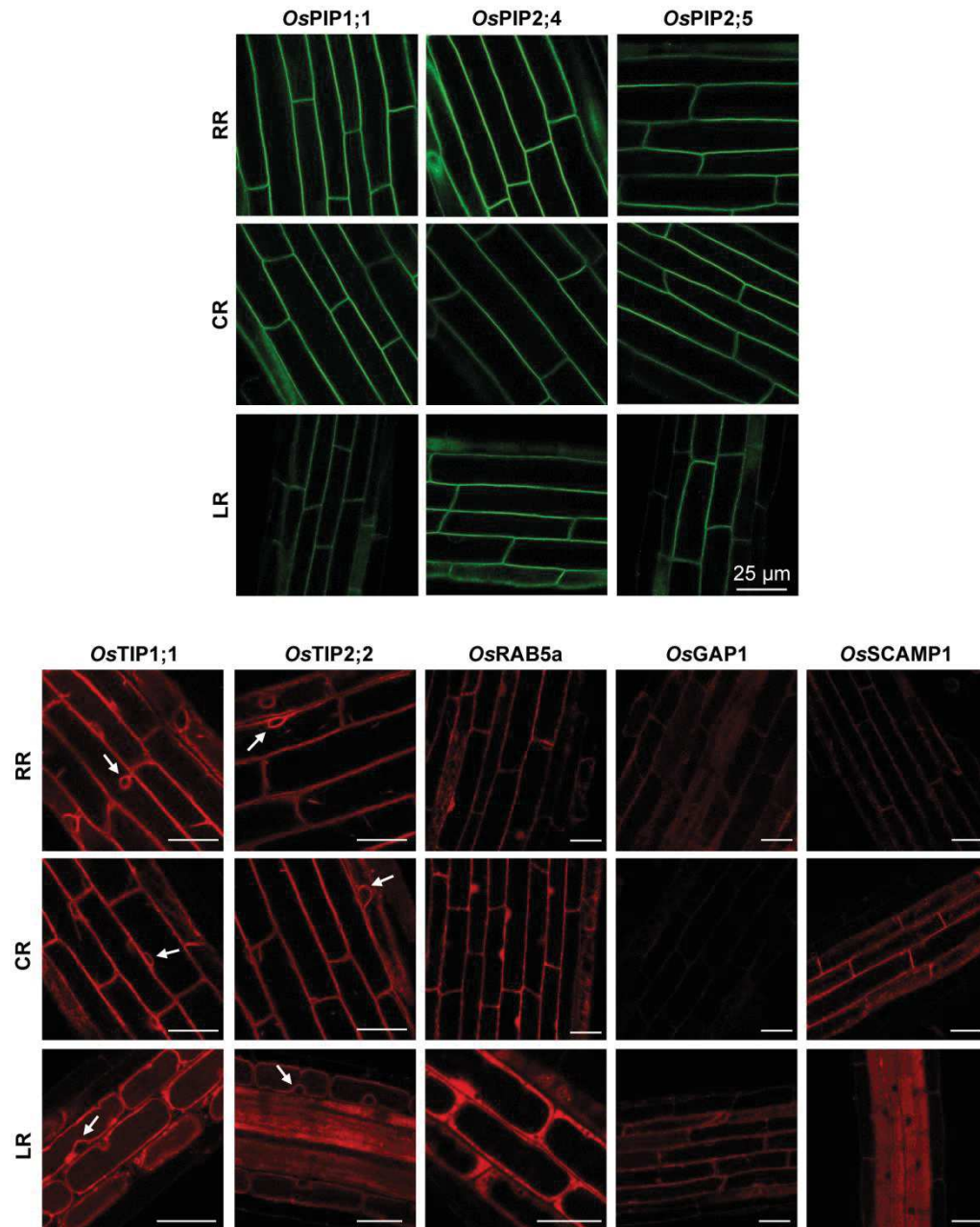
In plants, popularization of cell biology approaches, such as laser scanning confocal microscopy, was promoted by the use in particular of shared sets of transgenic lines expressing fluorescent-protein fusions to subcellular markers. This approach has been mostly developed in the plant model Arabidopsis (Cutler *et al.*, 2000). New sets of transgenic lines have also been developed in leading crop models such as maize (Krishnakumar *et al.*, 2015), and rice (Wu *et al.*, 2016). These sets of markers are interesting tools for highlighting subcellular compartments. Most interestingly, the use of protein markers with known biological functions allows one to uncover novel subcellular regulations. For instance, a set of multicolour markers of membrane compartments was used to study the intracellular dynamics of aquaporins in Arabidopsis (Wudick *et al.*, 2015). Here, we present a new set of transgenic rice lines stably-expressing individually fluorescent protein fusions with subcellular protein-markers. These include (i) plasma membrane (PM) and tonoplast aquaporins (Sakurai *et al.*, 2005), (ii) PM-marker low-temperature inducible protein 6A (LTi6a; (Cutler *et al.*, 2000)), (iii) *OsRab5a* known to be localized in a pre-vacuolar compartment (Shen *et al.*, 2011), (iv) *OsGAP1* which has a putative function for Golgi apparatus to PM and *trans*-Golgi network (TGN) trafficking and potentially localizes in endosomal compartments (Heo *et al.*, 2005), and (v) a rice secretory carrier membrane

protein (*OsSCAMP1*) which is localized in an early endosome compartment and may have a function in the early stage of membrane trafficking from the PM (Cai *et al.*, 2011). Apart from aquaporins and *LTi6a*, all of these proteins are identified components of key compartments en route towards the vacuole. Importantly, we challenged the aquaporin-expressing transgenic lines upon salt and osmotic stress to uncover their dynamic behaviour in rice roots.

## Results and discussion

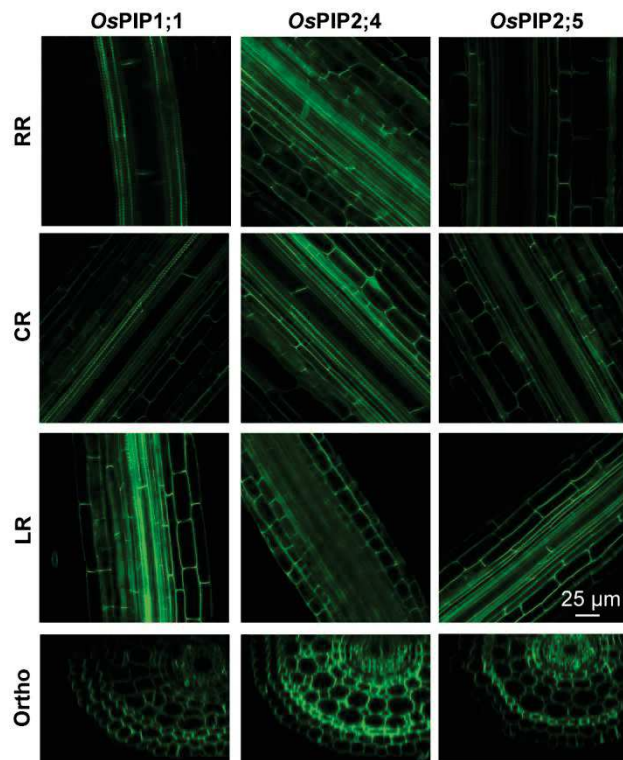
### *Rice transgenic line creation and subcellular localization visualization*

The genes of interest were cloned in fusion with the sequence of a fluorescent protein and under the transcriptional control of a constitutive promoter. Rice PM aquaporin (*OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*) sequences were fused with the green fluorescent protein (*GFP*) sequence to form *OsPIP-GFP* constructs, and PM *LTi6a* was fused with the cyan fluorescent protein (*CFP*) to form a *CFP-LTi6a* construct. The other proteins were fused with the *mCherry* sequence. The expression cassettes were cloned in the binary vector pGreenII 0179 (Hellens *et al.*, 2000) and transferred into either rice (*Oryza sativa* L. cv. Nipponbare) or *Arabidopsis thaliana* (Col-0 accession) by *Agrobacterium*-mediated transformation. When expressed in *Arabidopsis*, rice PM-aquaporin constructs were found to be located at their expected subcellular localization (**Figure S1**). The anatomical organization of rice roots is more complex than in *Arabidopsis*, as it comprises in particular more cell layers (Rebouillat *et al.*, 2009). In the present work, only the epidermis, exodermis, sclerenchyma and very first mesodermal cells could be visualized by confocal microscopy of rice root systems, and very weak autofluorescence background was detected there (**Figure S2**). The fluorescent signal of *OsPIPs* in epidermis was weak. In contrast, the small and flat sclerenchyma cells, the exodermal and mesodermal cells exhibited a strong and homogeneous signal, amenable for confocal microscopy. When expressed in rice, *OsPIP* constructs showed a typical homogeneous labelling of the PM (**Figure 1**), while tonoplast aquaporin (*OsTIP-mCherry*) constructs were associated with a labelling of intracellular invaginations that skirted the nucleus and are typical of the vacuolar membrane (**Figure 1**). In addition, we could observe a consistent labelling of intracellular compartments by using the *OsRab5a*, *OsGAP1* and *OsSCAMP1* markers (**Figure 1**). The thickness of rice roots is a limitation to the observation of deep tissues by laser scanning confocal microscopy. We overcome this limitation by means



**Figure 1. Subcellular localization of protein markers tagged with fluorescent proteins in rice root.**

*OsPIP*-GFP (**Upper**) and mCherry-constructions (*OsTIP1;1*, *OsTIP2;2*, *OsRab5a*, *OsGAP1* and *OsSCAMP1*) (**Lower**) labelling in exodermis of fresh roots in different types. Each row of photos refers to distinct types: RR, radicle root; CR, crown root; LR, lateral root. Root cells were observed by means of laser scanning confocal microscopy. Images were taken at a region ~0.5-1 cm from the root tip of plants, 7-8 days after germination. Scale bar: 25  $\mu$ m.



**Figure 2. In depth imaging of OsPIPs in rice root.**

ClearSee technique was applied prior to observation of OsPIP-GFP labelling in deep root tissues by multiphoton excitation microscopy. Root types: RR, radicle root; CR, crown root; LR, lateral root. Ortho stands for orthogonal section after reconstitution of Z-stack images. Scale bar: 25  $\mu$ m.

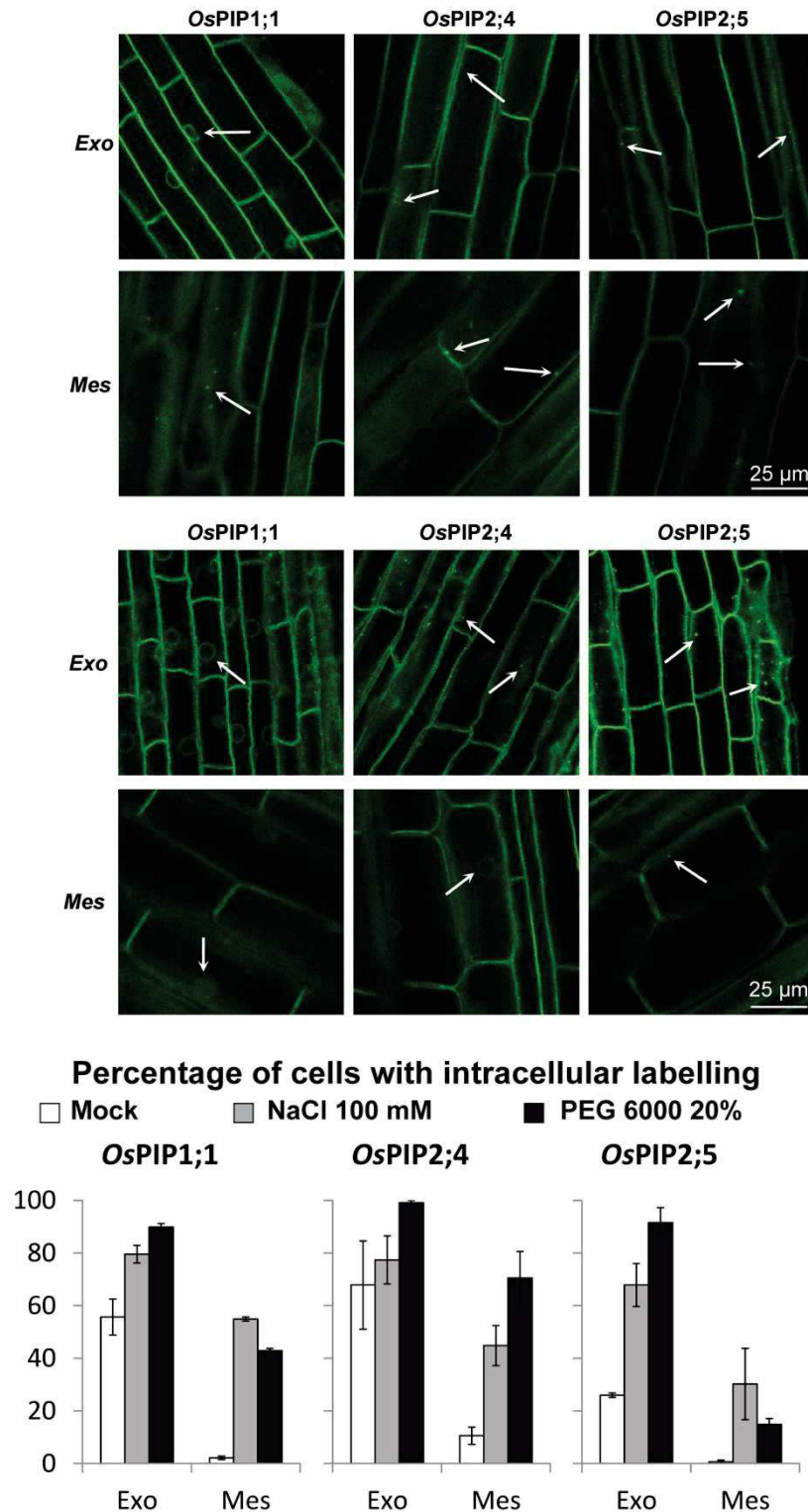
of the ClearSee technique which was initially developed to image in depth the morphology and gene expression of plant tissues (Kurihara *et al.*, 2015). This technique has a lot of advantages such as diminishing chlorophyll auto-fluorescence while preserving fluorescent protein stability. It is applicable to multicolour imaging of fluorescent proteins and compatible with chemical staining. It also allows long-term storage of sampled tissues. In addition, multiphoton excitation microscopy (MPEM) can provide a deeper penetration of infra-red light into plant tissues (Feijo and Moreno, 2004). Though the overall autofluorescence background is weak, caution should be taken since a higher signal is detected in the xylem vessels (**Figure S2**). By combining ClearSee and MPEM, the PM signal of OsPIP-GFP constructs could be visualized down to the central cylinder at a depth of  $\sim 150$   $\mu$ m (**Figure 2**). Although use of protein markers tagged with fluorescent protein should be done with caution, since sometimes the fusion could affect the subcellular localization, several reports addressed biological questions with this strategy. We have established here a new set of transgenic rice plants enlightening multiple cell compartments.

### ***Redistribution of PM aquaporins upon salt and drought stress***

This set of markers was also used for a dynamic survey of membrane compartments upon environmental challenges. To investigate the behaviours of aquaporins upon salt and osmotic stress, roots of transgenic lines expressing either OsPIP1;1, OsPIP2;4 or OsPIP2;5

constructs were challenged with 100 mM NaCl or 20% (w/v) PEG6000 for 30 min and observed by confocal microscopy. Firstly, we validated the behaviour of the *OsPIP* constructs in *Arabidopsis* and observed a specific relocalization of these PM aquaporins into intracellular compartments depending on the isoform, the cell type or the treatment (**Figure S3**). For instance, *OsPIP2* isoforms exhibited a stronger tendency to relocalize upon abiotic stress than *OsPIP1;1*. Secondly, when expressed in rice crown root cells, the *OsPIP* constructs exhibited a marked intracellular labelling (**Figure 3**). In any case, both the NaCl and PEG stresses resulted in a marked increase of intracellular labelling as compared to control conditions. For instance, in exodermal cells, we noticed that *OsPIP1;1* construct labelled a compartment surrounding the nucleus, tentatively identified as the endoplasmic reticulum. Redistributions of *OsPIP2;4* and *OsPIP2;5* were observed mainly in punctuated structures. In *Arabidopsis*, upon drought stress, a RING membrane-anchor E3 ubiquitin ligase has been reported to be involved in the ubiquitination of *AtPIP2;1* and the retention in the ER of this aquaporin (Lee *et al.*, 2009). In mesodermal cells, intracellular labelling with the *OsPIP1;1* construct was punctuated and detected in only 2% cells in control condition but in ~55% and 43% of cells, under salt and osmotic stress, respectively. The differences of subcellular redistribution of *OsPIP1;1* and *OsPIP2s*; upon stress; between exodermal and mesodermal cells suggested a isoform-specific and cell-specific response. We observed a similar phenotype in mesodermal cells of radicle root (**Figure S4**). The CFP-LTi6a construct showed a much lower tendency to relocalize in intracellular compartments upon salt or osmotic stress (**Figure S5**). Importantly, a specificity of *OsPIP* relocalization upon these stresses was observed in rice, whereas such phenomenon was not reported for *AtPIP* in *Arabidopsis* (Boursiac *et al.*, 2008). Following the description of *AtPIP* internalization upon salt and oxidative stress in *Arabidopsis* roots (Boursiac *et al.*, 2008), the present work extends this behaviour to their orthologues in rice. Since this phenotype can be observed in two representative dicot and monocot species, we propose that it represents a conserved adaptive mechanism upon abiotic environmental stress.





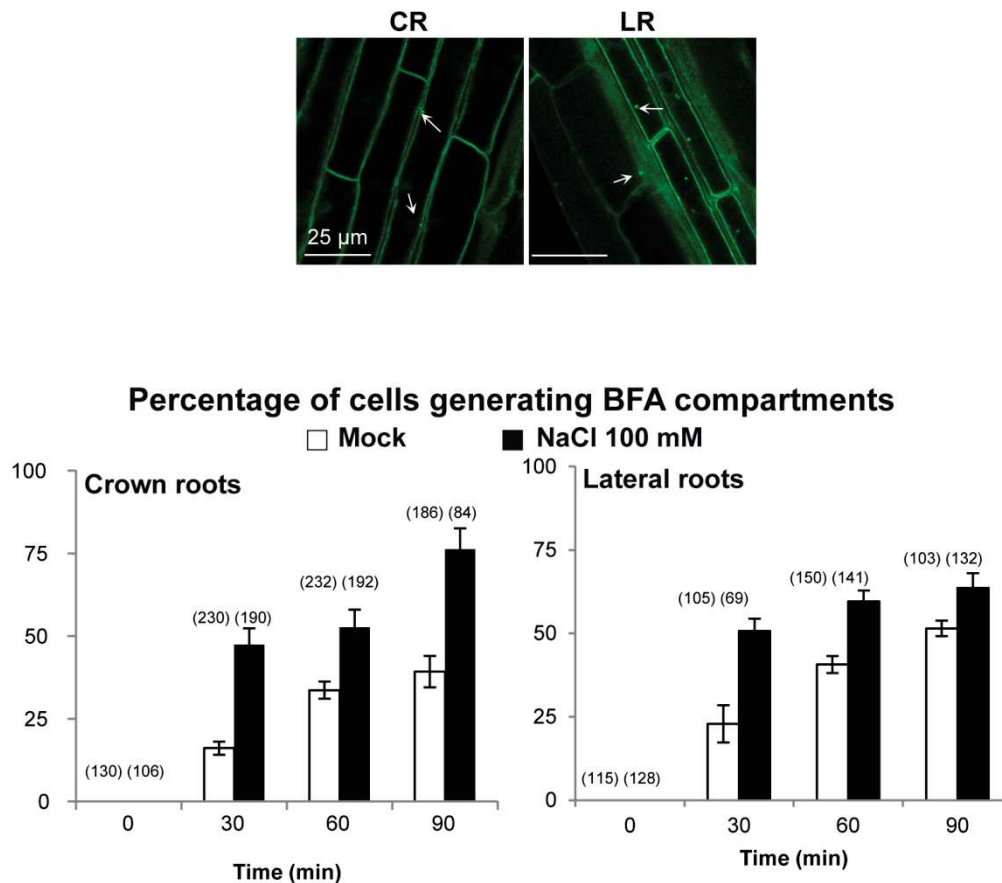
**Figure 3. Effects of salinity and osmotic stress on subcellular localization of rice PM aquaporins in root.**

Control, salt or PEG treatments were applied on whole root systems and crown root cells were observed by laser scanning confocal microscopy. Intracellular labelling observed upon salt (**Upper**) and osmotic stress (**Middle**) are indicated with arrows. Each column refers to an isoform and each line refers to a cell layer, either exodermis (Exo) or mesodermis (Mes). (**Lower**) Quantification of intracellular labelling upon either control treatment with water (opened bars), 100 mM NaCl (grey bars) or 20% (w/v) PEG6000 (closed bars) for 30 min, respectively. The mean values  $\pm$  SE are indicated. Scale bar: 25  $\mu$ m.

### ***Dynamic of endocytosis of PM aquaporins upon salt stress***

Brefeldin A (BFA) is a lactone antibiotic produced by fungal organisms which disturbs exocytosis by inhibiting the function of ADP-ribosylation factor-guanine-exchange factor. BFA thereby provokes the aggregation of endosomal vesicles including *trans*-Golgi network (TGN) compartment and induces the formation of large structures named as BFA compartments. We used *OsPIP2;5* as a prototypic PM aquaporin together with BFA treatments to address the membrane protein cycling dynamics in rice, as investigated earlier in *Arabidopsis* (Luu *et al.*, 2012). When *de novo* protein synthesis is prevented by a cycloheximide treatment (Jásik and Schmelzer, 2014), the kinetics of BFA compartment labelling by PM markers can be used to probe the dynamics of endocytosis. Rice roots were exposed to BFA (50  $\mu$ M) dissolved in either water or a 100 mM NaCl solution and corresponding to control or salt-stress conditions, respectively. In control conditions, *OsPIP2;5*-GFP labelled intracellular structures typical of BFA compartments, indicating that *OsPIP2;5* traffics through the TGN compartment. After a 90 min BFA treatment, ~39% and 51% of exodermal cells exhibited BFA compartments of 2  $\mu$ m and 1  $\mu$ m in diameter, in crown roots and lateral roots, respectively (**Figure 4**). In salt-stress conditions, we observed a higher percentage of root cells with a BFA compartment than in control conditions. For instance, exodermal cells of crown roots subjected for 30 min to a control or salt stress treatment showed ~16% and 47% of cells with a BFA compartment, respectively. These results suggest that salt treatment enhanced the endocytosis of *OsPIP2;5*. A similar result on *AtPIP* isoforms was obtained in *Arabidopsis* (Luu *et al.*, 2012). Effects of BFA might be concentration-dependent (Jásik and Schmelzer, 2014; Lam *et al.*, 2009). Therefore, we tested other concentrations (100  $\mu$ M) and found similar results (data not shown).

In conclusion, we have uncovered the relocalization and dynamics of PM aquaporins upon salt and osmotic stresses in rice. Importantly, our data support a model where relocalization of *OsPIPs* is concomitant with their high cycling dynamics. Altogether these data indicate that the rice research community has at its disposal a new set of subcellular markers amenable for cell biology approaches on a large array of topics.



**Figure 4.** Effects of salt stress on the dynamics of brefeldin A (BFA) compartment labelling by *OsPIP2;5* constructs in rice root cells.

**(Upper)** Typical BFA compartments observed by LSCM in exodermal cells of crown roots and lateral roots (pointed by arrows). CR, crown root; LR, lateral root. *Scale bar: 25 µm.* **(Lower)** Root systems of *OsPIP2;5*-GFP expressing line were incubated in either water (mock condition; open bars) or 100 mM NaCl (closed bars), both supplemented with 50 µM BFA. The number of cells with at least one labelled BFA compartment was counted from images acquired by confocal microscopy, from time 0 to the indicated times. This number was then normalized to the total cell number observed of each root type (crown and lateral roots). Numbers in parentheses indicate the number of cells observed in each assay. The mean values  $\pm$  SE are indicated.

## Materials and methods

### *Molecular cloning of membrane protein markers and plant transformation*

Molecular cloning information is summarized in supplementary **Table S1**. *OsPIP1;1* was subcloned into pDONR207 and transferred into the destination vector pGWB5 (Nakagawa *et al.*, 2007) using Gateway® Gene Cloning technology (Invitrogen, USA), according to the manufacturer's instruction. The whole set of the other protein markers were subcloned into pBluescript SK vector (Stratagene, USA) or pUC57 (**Table S1**), and then cloned into the pGreenII 0179 binary vector (Hellens *et al.*, 2000) under the transcriptional and translational control of a double enhanced cauliflower mosaic virus 35S promoter and

the 3' end of the pea ribulose-1,5-bisphosphate carboxylase small subunit *rbcS* gene. A molecular construct consisting of the maize ubiquitin-1 promoter controlling the expression of a fluorescent plasma-membrane-localized fusion protein (ECFP-LTI6a) (Cutler et al., 2000) was obtained by synthesis (Genscript) and cloned into the plasmid pUC57. The insert was released by a double digestion with *EcoRI* et *KpnI* and cloned into the binary vector pCAMBIA2300 linearized by *EcoRI* and *KpnI*. All constructs were confirmed by DNA sequencing by Eurofins Genomics (Germany). The recombinant DNA plasmids were electroporated into *Agrobacterium tumefaciens* strain EHA105 or GV3101 for rice or Arabidopsis transformation, respectively. *Japonica* rice Nipponbare cultivar was transformed according to a modified seed-embryo callus transformation procedure (Sallaud *et al.*, 2003). Arabidopsis transformation was performed according to flower dip protocol (Clough and Bent, 1998). Selection of transgenic plants was performed with medium supplemented with hygromycin.

### ***Plant materials and growth conditions***

Rice *Oryza sativa* L. cv. Nipponbare and *Arabidopsis thaliana* L. (Heyn.) accession Columbia 0 were used in this study. Rice seeds were dehusked then sterilized by dipping in 70% ethanol for 2 min, soaking in 3.6% (w/v) sodium hypochlorite solution for 30 min and rinsing several times with distilled water. After sterilization, the seeds were put in the petri dish containing moist filter paper. After emergence of the coleoptile and germination of the radicle, seeds were transferred onto a raft floating on deionised water. Conditions of the growth chamber were 14 h of day cycle ( $\sim 200 \mu\text{E m}^{-2} \text{sec}^{-1}$ ) and 10 h of night at 28/25°C and 70% relative humidity. Seven to eight days after germination (DAG) rice seedlings were used for cell biology approaches. Arabidopsis seeds were surface sterilized in a solution (50% ethanol, 4 g L<sup>-1</sup> Bayrochlore and 0.02% (w/v) Clean N for 10 min, thoroughly washed with 70% ethanol and air-dried under the sterile hood for 2 h. Sterilized seeds were sown on sucrose-added (10 g L<sup>-1</sup>) half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in clear polystyrene plates (12 x 12 cm) sealed with an air-permeable tape. After 48 h of stratification in 4°C dark room, plates were transferred vertically into a growth chamber with cycles of 16 h of light ( $\sim 150 \mu\text{E m}^{-2} \text{sec}^{-1}$ ) and 8 h of dark at 21°C and 65% relative humidity. Seven days after sowing (DAS), Arabidopsis seedlings were used for cell biology approaches.

### ***Confocal microscopy visualization***

A laser scanning confocal microscope (Leica TSC SP8 system, Germany) was used with the excitation wavelengths 405 nm, 488 nm and 561 nm for CFP, GFP and mCherry, respectively. The detection wavelengths were in the range of 450-500 nm for CFP, 500-535 nm for GFP and 580-630 nm for mCherry. Images were taken at a region ~0.5-1 cm from the root tips. Images were captured in a z-stack of 0.5  $\mu$ m intervals for subcellular localization and a time lapse for mobility of protein observation.

Multiphoton microscope (Zeiss LSM 7MP OPO, Germany) was used to observe ClearSee-prepared tissue with the excitation wavelength 836 nm and signals were collected in detection range of 500-550 nm.

### ***ClearSee tissue preparation***

ClearSee solution was prepared by mixing xylitol (10% w/v), sodium deoxycholate (15% w/v) and urea (25% w/v) in water (Kurihara et al., 2015). Briefly, rice seeds were dehusked and surface sterilized in 3.6% (w/v) sodium hypochlorite solution for 30 min, then washed carefully with sterile water. Next, seeds were sown on half-strength MS medium in clear polystyrene plates (24.5 x 24.5 cm), 25 seeds for 1 line at the middle per plate. Plates were kept vertically in culture room at 29°C for 12 h of day, 25°C for 12 h of night and relative humidity at 66%. Seven DAG, rice roots were collected and immediately immersed in a 4% (w/v) paraformaldehyde solution prepared with 1X PBS, subjected to vacuum for 30 min, washed again with 1X PBS, and then immersed in ClearSee solution under vacuum for 2 h, followed by 1 week at ambient conditions. When rice root became transparent, each root type was mounted in a 1% (w/v) agarose solution and visualized by means of a multiphoton microscope.

### ***Stress application and pharmacological approach***

Plants were stress challenged by incubating the roots in solutions of 100 mM NaCl or 20% (w/v) PEG6000. Brefeldine A was used at a concentration of 50  $\mu$ M, dissolved into water supplemented or not with 100 mM NaCl. Importantly, cycloheximide was added at a concentration of 50  $\mu$ M prior to and during BFA treatments to prevent new protein biosynthesis. All chemicals listed in this section were from Sigma-Aldrich (USA).

## **Declarations**

### **Acknowledgments**

We thank Xavier Dumont for assistance in Arabidopsis transformations, Cecile Fizames for assistance in bioinformatics sequence analysis and other staff members of the Institut de Biologie Intégrative des Plantes for technical assistance in biological material culture. We thank Thi Hue Nguyen and Van Lam Dinh from National key laboratory for Plant Cell Biotechnology for assistance in rice transformation. Confocal observations were performed at the Montpellier RIO Imaging facilities. TTHC was supported by a PhD scholarship from the Ministry of Education and Training (Vietnam) and the Ministry of Foreign Affairs (France).

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### **Authors’ contributions**

TTHC, DCT carried out the experiments, interpreted the results and wrote the paper. HTG and DTL designed experiments carried out the experiments, interpreted the results and wrote the paper. CB, DM, AV, MI carried out the experiments. NVD, CP, EM, PG, CM designed experiments, interpreted the results and wrote the paper.

### **Competing interests**

The authors declare that they have no competing interests.

### **Ethics approval and consent to participate**

Not applicable

## **Consent for publication**

Not applicable

## **Availability of data and material**

The datasets supporting the conclusions of this article are included within the article (and its additional files).

## **Authors' information**

(1) BPMP, CNRS, INRA, Montpellier SupAgro, Univ Montpellier, Montpellier, France

(2) National key laboratory for Plant Cell Biotechnology, Agricultural Genetics Institute, Pham Van Dong, Co Nhue, Tuliem, Hanoi, Vietnam

(3) CIRAD, UMR AGAP, 34398 Montpellier, France

(4) Université de Montpellier, CIRAD-INRA-Montpellier SupAgro, 34000 Montpellier France

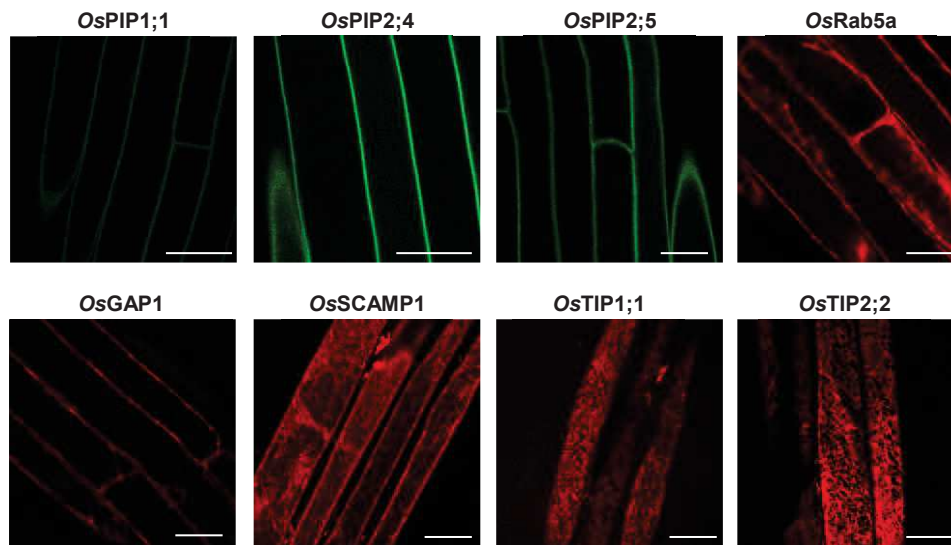
(5) University of Science and Technology of Hanoi, LMI RICE, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam

(6) IRD, LMI RICE, Agricultural Genetics Institute, Pham Van Dong road, Co Nhue, Tuliem, Hanoi, Vietnam

(7) Université de Montpellier, UMR DIADE, 911 Avenue Agropolis, 34394 Montpellier Cedex 5, France



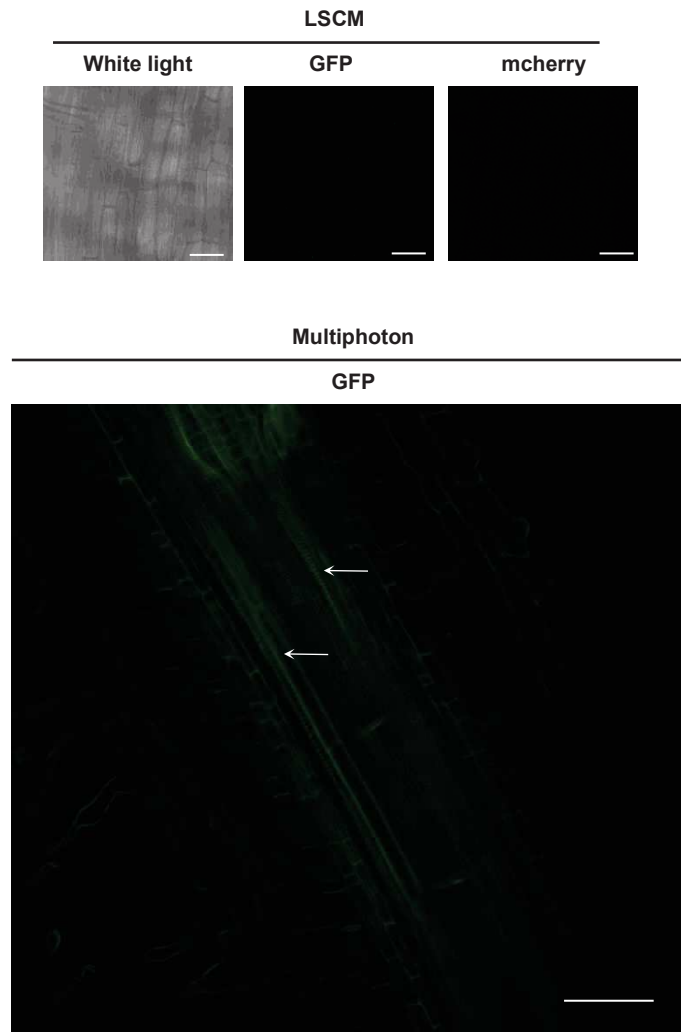
## Supplementary figures



**Figure S1. Subcellular localisation of rice proteins in Arabidopsis root cells.**

Fresh roots in half-strength MS medium were observed by laser scanning confocal microscopy (LSCM). In Arabidopsis, *OsPIPs* localized in the PM but *OsTIPs* and *OsSCAMP1* were slightly retained in endoplasmic reticulum, other markers distributed as expected in cytoplasm.

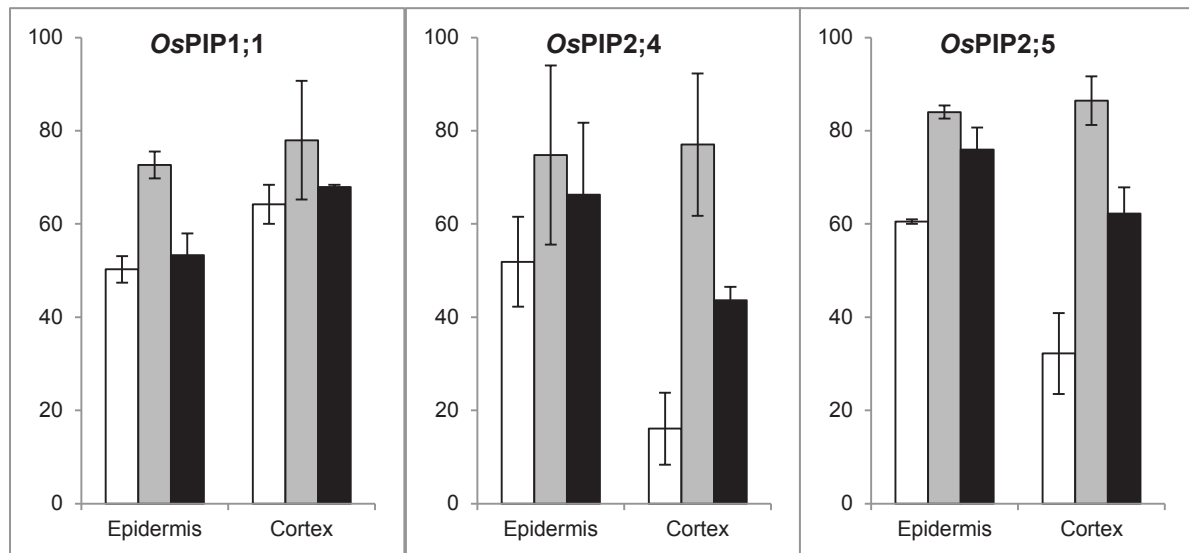
Scale bar: 20 μm.



**Figure S2. Autofluorescent background in rice crown root cells.**

**(Upper)** Fresh root of non-transgenic rice was observed by laser scanning confocal microscopy (LSCM) using either white light, GFP or mCherry channel. **(Lower)** Root system of non-transgenic rice was subjected to ClearSee technique and observed by means of multiphoton excitation microscopy; weak auto-fluorescent background was detected, especially in xylem vessels (arrows). *Scale bar: 25  $\mu$ m.*

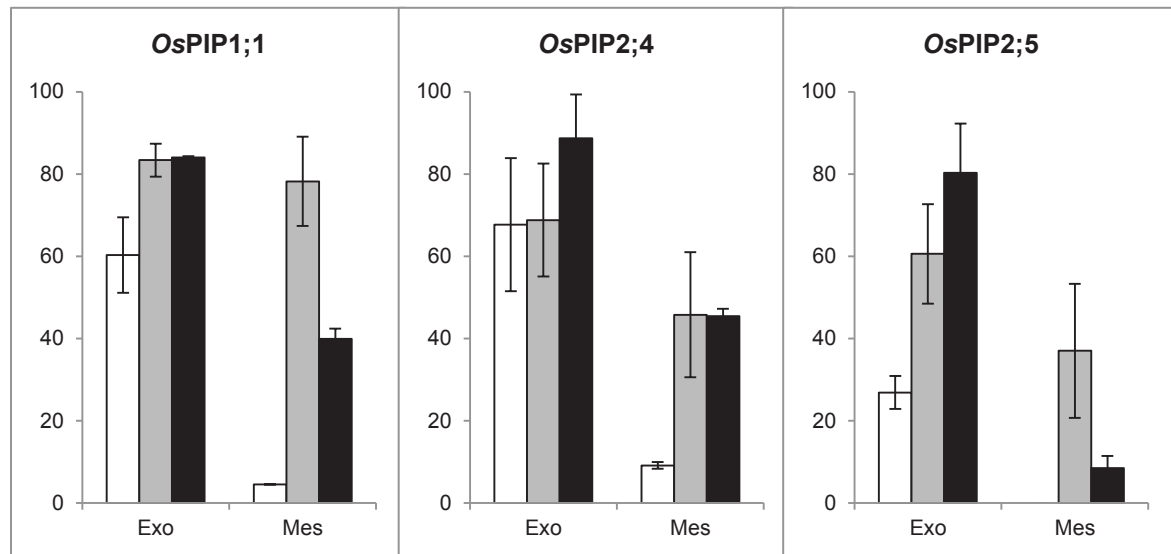
### Percentage of root cells with intracellular labelling (%)



**Figure S3. Re-localization of rice aquaporins in Arabidopsis root under salt and osmotic stress.**

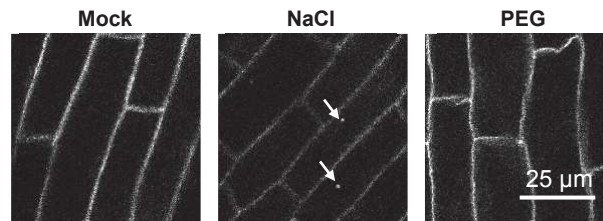
Charts show the percentage of root cells displaying intracellular labelling in either epidermis or cortex. Open, grey and closed bars correspond to mock (half-strength MS liquid medium), 100 mM NaCl and 20% (w/v) PEG6000, respectively. Experiments were done 2 times for each construct, ~10 plants were observed for each experiment. The mean values  $\pm$  SE are indicated.

### Percentage of root cells with intracellular labelling (%)

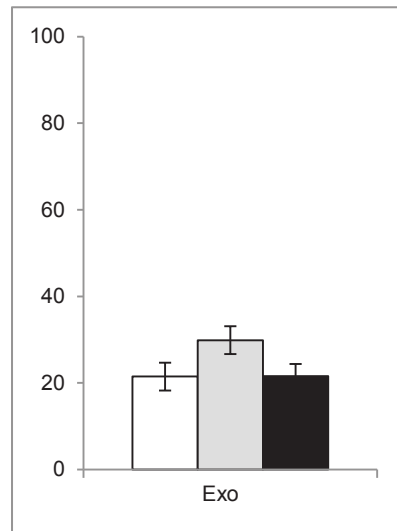


**Figure S4. Re-localization of plasma membrane aquaporins in rice radicle root under salt and osmotic stress.**

Charts show the percentage of root cells displaying intracellular labelling in either exodermis (Exo) or mesodermis (Mes). Open, grey and closed bars correspond to mock (half-strength MS liquid medium), 100 mM NaCl and 20% (w/v) PEG6000, respectively. *OsPIP*-GFP constructs were observed in radicle roots. Experiments were done 2 times for each construct, 4-6 plants were observed for each experiment. The mean values  $\pm$  SE are indicated.



**Percentage of root cells with intracellular labelling (%)**



**Figure S5. Subcellular localization of plasma-membrane protein-marker LTI6a-CFP in rice root under salt and osmotic stress.**

LTI6a-CFP construct was observed in crown roots. **(Upper )** Typical subcellular localization of LTI6a-CFP in exodermis observed by LSCM with intracellular labelling (pointed by arrows). *Scale bar: 25 µm.*

**(Lower)** Charts show the percentage of root cells displaying intracellular labelling. Open, grey and closed bars correspond to mock (half-strength MS liquid medium), 100 mM NaCl and 20% (w/v) PEG6000, respectively. Experiments were done 2 times, 5 plants were observed for each experiment. The mean values  $\pm$  SE are indicated.

## Supplementary table

**Table S1. Molecular cloning information on the sequences used as markers**

Gene name	RAP locus ID	Origin of cDNA clone*	ID of cDNA clone	Forward and reverse primer sequences used to subclone cDNA fragment if PCR was applied	Cloning strategy**	Binary vector***
<i>OsPIP1;1</i>	Os02g0666200	NIAS, Japan	001-039-C05	5'-GGAGATAGAACCATGGAGGGGAAGGAGGAGAC-3' 5'-CAAGAAAGCTGGGTCTAAAGACCTGCTCTTGAAT3'	G	pGWB5
<i>OsPIP2;4</i>	Os07g0448100	GenScript, USA	/	/	R	pGreen0179
<i>OsPIP2;5</i>	Os07g0448400	GenScript, USA	/	/	R	pGreen0179
<i>OsTIP1;1</i>	Os03g0146100	NIAS, Japan	001-014-B06	5'-TCTCTCTCTAAGCTTATGCCGATCCGCAACATCGC-3' 5'-GCCACCACCTCCTAAGTAGTCGGTGGTGGGGAGCT-3'	I	pGreen0179
<i>OsTIP2;2</i>	Os06g0336200	NIAS, Japan	J033044F19	5'-TCTCTCTCTAAGCTTATGTCGGGCAACATCGCCTT-3' 5'-GCCACCACCTCCTAAGAACTCGCTGCTGGCAACGG-3'	I	pGreen0179
<i>OsRab5a</i>	Os12g0631100	NIAS, Japan	J013078I11	5'- GAGAAAGCTTATGGCGGCCAACCCCGCAACAAGATCC-3' 5'- GACTCGAGCCACCACCTCCTGAGCAGCATGAAGAACTGCTC-3'	R	pGreen0179
<i>OsGAP1</i>	Os02g0709800	NIAS, Japan	J013162A17	5'-TCTCTCTCTAAGCTTATGCGTCGAGAAGAAGCCTC-3' 5'-GCCACCACCTCCTAACATGACCTCGTCTTCTTGTA-3'	I	pGreen0179
<i>OsSCAMP1</i>	Os07g0564600	NIAS, Japan	J033084E14	5'-TCTCTCTCTAAGCTTATGGCGGGGCGCTACGACAG-3' 5'-GCCACCACCTCCTAAAAAGCTGCCCCGATAGCAC-3'	I	pGreen0179

\*NIAS: National Institute of Agrobiological Sciences. \*\*G: Gateway® Gene Cloning (Invitrogen, USA), R: Restriction, I: In-Fusion® HD Cloning (Clontech, USA).

\*\*\*pGWB5 (Nakagawa et al., 2007), pGreen0179 (Hellens et al., 2000).

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## **Chapter II:**

### **Genetic manipulations of rice to improve drought and salt tolerance**

# Genetic manipulations of rice to improve drought and salt tolerance

## Introduction

Rice is one of the most important cereal crops in the world and is ranked the second of production just after maize with a world paddy production of ~756 million tons in 2017 (FAO, 2017). The tremendous augmentation of human population worldwide has increased the demand for rice production. However, the global climate changes caused a lot of stresses for agriculture, especially drought and salinity, which extremely reduce rice productivity. Therefore, studying genes and mechanisms for adaptation of rice to drought and salinity is quite imperative.

Being a water-loving crop, rice can be severely affected by water deficit leading to 15-50% yield loss depending on the vigour and period of stress (Kumar *et al.*, 2008). To cope with drought stress, at whole plant level, rice exhibits 3 major mechanisms: (1) drought escape, (2) drought avoidance and (3) drought tolerance (Basu *et al.*, 2016; Kumar *et al.*, 2017). Drought escape indicates the ability of rice to complete its life cycles before the development of severe stress. Drought avoidance indicates the strategy of rice to maintain relatively high tissue water potential despite low soil water content. Rice plants can either minimize the water loss by reducing transpiration or optimize water uptake by increasing the root system or root hydraulic conductance, etc. Drought tolerance is the ability of rice to survive under low tissue water content by maintaining the cell turgor through osmotic adjustment, cell elasticity enhancement, cell size decrease and protoplasmic resistance.

Rice is also one of the most sensitive species to salt stress (Munns and Tester, 2008). Suffering from high salinity, plants have to cope with two major stresses, osmotic and ionic stress (Horie *et al.*, 2012). Osmotic stress occurs immediately with an excess level of salt outside the root, leading to inhibitions of water uptake (even a water efflux can occur under severe stress), cell expansion and lateral bud development (Munns and Tester, 2008). Ionic stress comes afterwards when Na<sup>+</sup> is over-accumulated in plant, especially in leaves, causing leaf chlorosis and necrosis, reducing essential cellular metabolism activities such as protein synthesis, enzyme activity, and photosynthesis. As a consequence, crop yield is highly

affected by salinity (Yeo and Flowers, 1986). Several mechanisms are developed by plants to withstand salt stress (Munns and Tester, 2008; Horie *et al.*, 2012): (1) Water uptake control by regulating aquaporin function and localization or ions/solutes/organic compound accumulation. (2) Restricting Na<sup>+</sup> accumulation in shoots at whole plant level and in cytosol at cellular levels by increasing root barriers (casparian strips and suberin lamellae), Na<sup>+</sup> extrusion at the plasma membrane of soil-root interface cells, Na<sup>+</sup> sequestration into the vacuole and Na<sup>+</sup> reabsorption from the xylem vessels by means of Na<sup>+</sup> transporters.

As molecular players involved into the water transport through biological membranes, aquaporin regulation and their roles in stress responses were deeply investigated in plants (Afzal *et al.*, 2016) and particularly in rice (**See General introduction**). Aquaporins were identified in rice with 33 different isoforms including 11 PIPs (plasma membrane intrinsic proteins), 10 TIPs (tonoplast intrinsic proteins), 10 NIPs (nodulin26-like intrinsic proteins) and 2 SIPs (small basic intrinsic proteins) (Sakurai *et al.*, 2005). Many studies have proved that aquaporins are multifunctional channels, which can transport not only water but also various small neutral molecules / physiological substrates such as carbon dioxide, ammonia, urea, glycerol, metalloids like boron and silicon, reactive oxygen species (Maurel *et al.*, 2015), but it is widely accepted that PIPs and TIPs are the two most abundant aquaporins in plant cells and the major contributors of water transport in plant roots.

Studying the contribution of a single aquaporin by genetic engineering is a new approach. Aquaporin over-expressing transgenes were generated in some species (Afzal *et al.*, 2016) but contrasting results did not clearly establish a causal link. For instance, transgenic tobacco plants overexpressing foreign aquaporins such as *BnPIP1* from *Brassica napus*, *TaAQP7* (an PIP2 isoform) from wheat enhanced the drought tolerance (Yu *et al.*, 2005; Zhou *et al.*, 2012). Over-expressing in *Arabidopsis* either a *Vicia faba* PIP1 (*VfPIP1*), a banana PIP (*MaPIP1;1*) or a *Festuca arundinacea* PIP (*FaPIP2;1*) also gave the plant beneficial effect under water stress (Cui *et al.*, 2008; Xu *et al.*, 2014; Zhuang *et al.*, 2015). Overexpressing *PgTIP1* from *Panax ginseng* enhanced salt and drought tolerance in transgenic *Arabidopsis* (Peng *et al.*, 2007). Constitutive expression of *SlTIP2;2* in tomato showed significant increases in fruit yield under both normal and water-stress conditions (Sade *et al.*, 2009). For rice aquaporins, overexpressing *OsPIP1;3* made transgenic plants with higher osmotic root hydraulic conductivity (*L<sub>p</sub>*), leaf water potential and relative

cumulative transpiration under drought stress compared to wild-type plants; this suggests a role of this aquaporin in drought avoidance (Lian *et al.*, 2004). Moreover, overexpressing, either *OsPIP1;1* or *OsPIP2;2*, in *Arabidopsis* gave transgenic plants a better elongation of roots compared to the control plants in both drought and salt stress (Guo *et al.*, 2006). The role of the level of expression of *OsPIP1;1* in salt tolerance was investigated by analyzing transgenic rice with high or lower expression of a transgenic *OsPIP1;1* (Liu *et al.*, 2013). High expression level ( $\geq 4 \times 10^5$  copies/mg total RNA) promoted vegetative growth of rice in control, but not in salt stress condition (150 mM NaCl), and even made the plants more sensitive when exposed to more intense stress (200 mM NaCl). Transgenic seedlings with middle to low levels of transgene expression ( $\leq 4 \times 10^5$  copies/mg total RNA) exhibited tolerance to salt (100 mM NaCl), and showed longer roots and shoots compared to wild-type plants. However, some contrasting results have also been obtained. Over-expression of *HvPIP2;1* from barley induced salt hypersensitivity phenotypes in transgenic rice plants (Katsuhara *et al.*, 2003). Over-expressing *GoPIP1* from *Galega orientalis* enhanced drought sensitivity in transgenic *Arabidopsis*, and showed no effect in response to salt stress (Li *et al.*, 2015). Tobacco plants over-expressing *AtPIP1b* showed remarkably increase of plant growth rate, transpiration rate, stomatal density, and photosynthetic efficiency under favorable growth condition, but showed no beneficial effect under salt stress and even exhibited deleterious effect under water stress (Aharon *et al.*, 2003). Therefore, more research is necessary to elucidate the roles of aquaporins in drought and salinity stress response.

Beside of the role of aquaporins in osmotic adjustment, sequestration of  $\text{Na}^+$  into vacuoles to prevent cellular toxicity by tonoplast transporters has been well described as a key mechanism for salt tolerance (Munns and Tester, 2008). Moreover, recent studies pointed to the involvement of endosomal compartments and the trafficking between these compartments in the sequestration of  $\text{Na}^+$ . Loss of functions of two transporters localized at endosomal compartments associated with Golgi apparatus and the trans-Golgi network (TGN) rendered the *Arabidopsis atnhx5/atnhx6* double mutant plants more salt sensitive (Bassil *et al.*, 2011). These findings suggest that endosomal trafficking may be crucial to control the salt tolerance and potentially drought tolerance in rice plants.

There is a fact that most of studies using transgenic rice under drought or salinity were performed at early growth stage without reporting grain yield data, which is the most

relevant parameter for rice breeding under stress conditions (Gaudin *et al.*, 2013; Basu *et al.*, 2016).

The present study used a strategy of individual over-expression of 5 rice aquaporins including *OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;1* and *OsTIP2;2* for a better understanding of their respective contribution to drought and salinity response at reproductive stage of rice (*Oryza sativa* L.) cv. Nipponbare (NB). Moreover, to get some clues in the involvement of endosomal trafficking proteins in abiotic stress response, we used rice transgenic plants over-expressing individually *OsRab5a*, *OsGAP1* and *OsSCAMP1* to test their stress tolerance. Rice Rab5a (*OsRab5a*) has been shown to localize in a pre-vacuolar compartment (PVC) also named the late endosome or multivesicular body (LE/MVB) (Wang *et al.*, 2010). *OsGAP1*, a Rab-specific rice GTPase-activating protein has a putative function for Golgi apparatus to plasma membrane (PM) and TGN trafficking and potentially localizes in endosomal compartments (Heo *et al.*, 2005). Rice secretory carrier membrane protein (*OsSCAMP1*) is localized in an early endosome compartment and may have a function in the early stage of membrane trafficking from the PM (Lam *et al.*, 2007, 2008; Cai *et al.*, 2011). All of these proteins are identified components of key compartments en route towards the vacuole (Müller *et al.*, 2007). This role in the trafficking towards the vacuole may be essential for osmotic adjustment and for the optimal sequestration of Na<sup>+</sup> in this compartment.

## Results

We used the set of rice transgenic lines introduced in **Chapter I**. Homozygote and heterozygote T2 transgenic seeds were used for experiments. Based on the availability of seeds and the capacity to carry out the experiments, one or two lines for each transgene were used in three independent experiments (**Table 1**). Experiment A was performed in the greenhouse of Centre de coopération internationale en recherche agronomique pour le développement (CIRAD), located in Montpellier (France), during the fall 2016. Experiment B was performed in the net-house of Agricultural Genetics Institute (AGI), Hanoi (Vietnam), during the fall 2017. Experiment C was performed in the net-house of International Rice Research Institute (IRRI), Los Banos (The Philippines), during the fall 2017. The moments of sampling or types of measuring was also modified to fit each experiment (**Tables 2 and 3**).

### ***Characteristics of transgenic plants compared to wild-type in control condition***

NB wild-type grown in control condition (without stress application) had the plant height about 73.2 to 86.5 cm in all experiments. The number of tillers was counted in experiment B and C with a low value in experiment B (~3.4 tillers) and higher value in experiment C (~17 tillers or ~15 tillers when growing in pots or paddy field, respectively); this suggested a sensitivity of NB cv. to growing conditions (**Figure 1**). In non-stress condition, transgenic rice seemed to have the same morphology as the wild-type, no difference in plant height, number of tillers or flag leaf area, with the exception for *OsPIP2;4* (B) plants which were significantly shorter, whereas *OsPIP2;5* (A) plants were significantly higher than the NB wild-type (LSD test,  $p < 0.05$ ) (**Figure 1**). Leaf criteria of transgenic rice including water content, chlorophyll content, reactive oxygen species (ROS) production, stomatal conductance and  $\text{Na}^+/\text{K}^+$  ratio were also similar to NB wild-type. Days to flower (DTF) indices were evaluated in experiment B and C. It took about 72 days or only 50 days for NB wild-type to reach anthesis in experiment B and C, respectively. Most of transgenic plants flowered about the same time as wild-type with only few exceptions (**Figure 2A**). In our hands, percentage of fertility of the first panicle and grain yield were measured as productive indices. These parameters varied among experiments (**Figure 2B and 2C**). NB wild-type reached over 90% fertility in experiment B but only 53-60% in experiment A and C (control plants in pots). In general, transgenic rice showed no significant higher percentage of fertility compared to wild-type, though *OsPIP2;5* (A) and *OsTIP1;1* (A) plants showed higher values in experiment A and C. Surprisingly, *OsSCAMP1* (A) line was significantly more sterile compared to NB wild-type. In experiment A, NB wild-type could produce up to 6.7 g seeds per plant while only 1.9 g in experiment B; this is consistent with the difference in tiller numbers in growing conditions. Overexpressing aquaporin or endosomal trafficking proteins in rice seemed not to exhibit enhancement in grain yield in control condition. Grain yield was reduced remarkably in some lines such as *OsPIP2;4* (B), *OsRab5a* (A) and *OsSCAMP1* (A) (**Figure 2C**). **Table 4** summarizes the data.

### ***Characteristics of transgenic plants compared to wild-type upon drought stress***

Drought stress experiments were carried out either in pots (experiment A and B) or paddy field (experiment C), and the level of stress was estimated by measurements of fraction of transpirable soil water (FTSW) index (Ben Saad *et al.*, 2012) or tensiometer-



estimated soil moisture, respectively. The smaller FTSW index, the more serious stressed the plants are. Suffering from the same period of water withholding, FTSW index for NB wild-type was smaller than that for *OsRab5a* (A) line, but higher than that for *OsPIP1;1* (A) and *OsGAP1* (A) lines in experiment A. This indicated that the wild-type rice plants were not exposed to the exact same stress level as the transgenic plants, in experiment A. Moreover, *OsPIP2;4* (B) line exhibited a higher FTSW value compared to NB wild-type in experiment B, indicating a lower severity of stress (**Figure 3**). In response to water deficit, transgene overexpression did not make the plants higher. We even observed a significant reduced plant height for some lines (**Figure 4A**). Drought stress reduced the number of tillers compared to control condition in experiment C, but not experiment B. We observed opposite results, for instance, for *OsTIP1;1* (B) line which produced more or less tillers compared to wild-type in experiment B or C, respectively (**Figure 4B**). The chlorophyll content index was found similar between transgenic and wild-type rice in experiment A and C. However, in experiment B, chlorophyll contents of *OsPIP1;1* (A), *OsPIP2;4* (B), *OsRab5a* (A), *OsGAP1* (A and B) and *OsSCAMP1* (A and B) lines were found significantly higher than NB wild-type. The other morphological parameters showed significant differences between transgenic lines and NB wild-type, but only in several cases (see **Table 5** which summarizes the data). In experiment B, we noticed that *OsPIP2;4* (B) line had a smaller size, and this logically resulted in less transpiration, therefore, less water loss, less ROS production and more water content, more chlorophyll in leaves in comparison with NB wild-type. We observed the same behavior in experiment C, for *OsTIP1;1* (B) line which showed less tiller number, less ROS production and higher leaf water potential compared to wild-type (**Table 5, Figure 5**). Different from the similarity of DTF in experiment C, almost all transgenic plants flowered later than NB wild-type in experiment B. Suffering from water deficit, almost all transgenic rice showed more failure in panicle fertility; consequently, they produced less filled grains than NB wild-type, and especially rice plants over-expressing endosomal trafficking proteins (**Figure 6**). Interestingly, we observed that *OsTIP1;1* (A) line exhibited higher grain yield upon drought stress than NB but the difference was not significant.

### ***Characteristics of transgenic plants compared to wild-type upon salt stress***

Dealing with salt stress, almost all transgenic rice showed the same or less growth compared to wild-type which were shorter and produced less tillers (**Figure 7**). Other

vegetative characteristics did not point out a remarkable advantage for transgenic plants compared to wild-type (**Table 6**). Salinity led to an increase of  $\text{Na}^+$  accumulation in rice leaves. Fourteen days after stress application (DASA), wild-type rice accumulated  $\text{Na}^+$  in leaves 3 times more than in normal condition, but no difference was observed between NB and transgenic rice in experiment A. However, in experiment B, leaves collected at 20 DASA showed the highest  $\text{Na}^+$  content in wild-type plant compared to almost all transgenic plants. Surprisingly, measurements at 27 DASA exhibited a reverse result; which is higher  $\text{Na}^+$  accumulation in leaves of transgenic plants compared to NB, especially for *OsPIP2;5* (A), *OsRab5a* (A), *OsGAP1* (B) and *OsTIP1;1* (B) (**Figure 8A**). Only *OsTIP1;1* (B) leaves accumulated significantly higher  $\text{K}^+$  compared to wild-type, in experiment B. As a consequence,  $\text{Na}^+/\text{K}^+$  ratio showed nearly the same trend as  $\text{Na}^+$  content (**Figure 8B**). In response to salt stress, transgenic rice seemed to flower later than wild-type (**Figure 9A**). No transgenic rice showed better fertility than wild-type, salinity dramatically decreased the rate of fertility in *OsPIP1;1* (A), *OsPIP2;4* (B), *OsRab5a* (A) and *OsSCAMP1* (A and B) lines (**Figure 9B**). These lines also showed the least grain yield upon salt stress (**Figure 9C**). Transgenic lines exhibited lower maximum root length, shoot dry weight or root dry weight compared to the wild-type (**Table 6**).

## Discussion

Studies of the role of a single aquaporin in drought and salt tolerance by overexpressing strategy were published in some species (Afzal *et al.*, 2016). As far as we known, only three rice aquaporins were studied by this way, in which, two transformed in rice (Lian *et al.*, 2004; Liu *et al.*, 2013) and one transformed in Arabidopsis (Guo *et al.*, 2006). This is the first time that a set of aquaporin and endosomal trafficking proteins individually overexpressed was studied simultaneously. The stress application at reproductive stage is also a big challenge because it is time consuming and needs a lot of efforts, therefore, in the literature, a small percentage of studies included the grain yield as the key component for stress resistance (Gaudin *et al.*, 2013; Basu *et al.*, 2016). The present study focused on both grain yield and secondary traits such as plant height, flag leaf area, tiller number, leaf water content, leaf chlorophyll content, leaf water potential, stomatal conductance, leaf  $\text{Na}^+/\text{K}^+$  content, time to flower, maximum root length, root dry weight, shoot dry weight to address the behavior of transgenic rice in response to drought and salt stress. However, the

secondary traits seemed not highly correlated with grain yield (**Table 7**). Those weak correlation were also reported in literature (Pantuwan *et al.*, 2002). Therefore, we believe that yield parameter should be the crucial components for stress resistance studies.

Though the grain yield data from experiment C is still missing, results from experiment A and B did not show beneficial effects of over-expressing either aquaporin or endosomal trafficking protein in both non-stress and stress conditions. Effects of over-expressing *OsPIP1;1* in rice on grain yield was reported dosage-dependent, in which low level of expression stimulated seed production but high level of expression made the plant produce less seeds (Liu *et al.*, 2013). Over-expressing *OsPIP1;3* under the control of SWPA2 promoter, which has moderate induction capability were reported to enhance drought avoidance in rice (Lian *et al.*, 2004). One possibility to explain the non-beneficial effects of over-expressing aquaporins in the present study can be the strong induction capability of double-enhanced *CaMV35S* promoter which is ‘harmful’ for the plants. This ‘negative’ effect was somehow noticed in literature (Aharon *et al.*, 2003; Katsuhara *et al.*, 2003).

## **Materials and methods**

### ***Experimental design***

T2 transgenic seeds (generated as described in chapter I) were used for experiments. Experiments were carried out in 3 different places. The pre-trial was carried out in the net-house of AGI (Hanoi, Vietnam) in 2016. The first official experiment was carried out in the greenhouse of CIRAD, Montpellier, France in 2016, (assigned as experiment A). The second experiment was in the net-house of AGI, Hanoi, Vietnam (2017), (assigned as experiment B). The third one was organized in the net-house of IRRI, Los Banos, Philippines (2017) (assigned as experiment C). The experimental design was adjusted to be appropriated for each institute.

In general, seeds were soaked in sufficient water or wet papers in the dark for 2 days to germinate, then seedlings were selected by checking fluorescence. Afterwards, one-week-old seedlings were transplanted into pots (16 cm diameter x 16 cm height) filled with 1.4 kg substrate (GO M2, Jiffy) or pots (19 cm diameter x 15.5 cm height) filled with 1.5 kg substrate (GT 05, Research Center for Fertilizers and Plant Nutrients, Vietnam) in experiment

A and B, respectively. In experiment C, seedlings were sown in soil trays 2 weeks before pulling and transplanting into pots (16 cm diameter x 18 cm height) filled with 2.3 kg soil for salinity stress experiment or paddy field for drought experiment. Fertilizers were applied as common use in each institute.

Stresses were applied when 50% of plants started booting.

For salinity stress, pots were drained out overnight then filled with salt solution (NaCl 100 mM). The solution volume and water electrical conductivity (EC) were maintained every day by adding water and/or NaCl until harvesting. When at least one plant showed typical symptoms of salinity stress (dry and yellow color occurred at the tip of the leaf), some parameters were recorded including plant height, tiller number, leaf water content, chlorophyll content, stomatal conductance, ROS production, leaf  $\text{Na}^+/\text{K}^+$  content. When all plants were ripe, they were collected and evaluated the grain yield, percentage of fertility, shoot and root dry weight, maximum root length.

For drought stress, in experiments A and B, all pots were fully watered then drained out overnight. Subsequently, pots were covered with opaque plastic bags to control the water loss only by plant transpiration. When at least one plant showed the typical symptom of drought stress (leaf rolling), some parameters were investigated including FTSW or soil water potential index, plant height, tiller number, leaf water content, leaf water potential, chlorophyll content, stomatal conductance, ROS production. Subsequently, pots were uncovered and re-watered until harvesting. When all plants were ripe, they were collected and evaluated the grain yield, percentage of fertility, shoot and root dry weight, maximum root length. In experiment C, sampling was done as described in pot experiments but plants were not re-watered.

The control plants were sampled the same day as their respective stressed plants.

### ***Fraction of transpirable soil water***

Fraction of transpirable soil water (FTSW) index was performed as described by Ben Saad *et al.* (Ben Saad *et al.*, 2012). The pots were weighted every day at the same hour, water-saturated pot weights were considered as pot capacity values (PC). Wilting point (WT) was known by pre-test (the point that constant weight was observed for 3 days, indicating

that the transpirational extraction of soil water had stopped). The FTSW index was calculated as the proportion of actual transpirable soil water (ATSW) to maximal transpirable soil water (MTSW). ATSW was the difference between actual weight and WT. MTSW was the difference between PC and WT.

### ***Leaf water content***

A piece of 7 cm of leaf from the middle of the leaf was collected in pre-weighted plastic bag to measure the fresh weight (FW) then dried at 60<sup>0</sup>C for 4 days to estimate the dry weight (DW). The leaf water content (WC) was calculated as the equation:  $WC = (FW - DW) / FW \times 100$ . WC measurements were done in experiment A and B. Leaf piece was collected from either the flag leaf or the leaf just below the flag leaf of the first panicle from each plant in experiment A or B, respectively.

### ***Chlorophyll content***

Chlorophyll contents were measured by either invasive method in experiment B or non-invasive method in experiments A and C.

In experiment B, chlorophyll was extracted and dosed following the protocol reported by Kaur *et al.* (Kaur *et al.*, 2016). A 2 cm piece of the leaf just below the flag leaf of the first panicle in each plant was collected and immediately ground in liquid nitrogen. Afterwards, chlorophyll was extracted by 2 mL of 85% acetone solution in Tris pH 8 buffer then centrifuged at 12000 *g* at 4<sup>0</sup>C for 15 minutes. The supernatant was withdrawn and measured the absorbance spectrophotometrically at 645 and 663 nm. The chlorophyll content was determined as equations below:

$$\text{Total chlorophyll } (\mu\text{g/mL}) = 20.2 (A_{645}) + 8.02 (A_{663})$$

$$\text{Chlorophyll a } (\mu\text{g/mL}) = 12.7 (A_{663}) - 2.69 (A_{645})$$

$$\text{Chlorophyll b } (\mu\text{g/mL}) = 22.9 (A_{645}) - 4.68 (A_{663})$$

In experiments A and C, chlorophyll content indices were determined using a hand-held chlorophyll content meter (Handy-PEA, Hansatech Instruments, King's Lynn, Norfolk, UK and CCM-200 plus, Apogee Instrument Inc., respectively). Two flag leaves were measured in each plant. For each leaf, 3 points of readings were made then averaged.

### ***Leaf water potential***

Leaf water potential (LWP) was measured only in experiment C by inserting upside down one leaf into a pressure chamber (300HGBL Plant Water Status Console, Soilmoisture Equipment Corp., CA, USA) with the cut point at stem out of the chamber. The leaf was pressurized using N<sub>2</sub> gas until the first drop of sap was visible at the base of the stem. All the measurements were carried out in the morning in shiny condition.

### ***Stomatal conductance***

Stomatal conductances of the flag leaves of the 2 first panicles were measured only in experiment C by Porometer (AP4, Delta-T Devices-Cambridge-U.K.)

### ***DAB staining***

Production of H<sub>2</sub>O<sub>2</sub> was *in situ* detected by diaminobenzidine (DAB) staining according to Le Deunff *et al.* (Le Deunff *et al.*, 2004). One 1.5-2 cm piece of leaf was cut then immediately covered with aluminum foil and stored in ice box to avoid tissue oxidation and brought to the lab as soon as possible for staining step. Leaf pieces were incubated overnight in 5ml DAB solution (1mg/ml citrate buffer, pH 6) and kept shaking gently at 90 rpm. Then, chlorophyll was washed by hot 96% ethanol. Leaf pieces were scanned at 600 dpi with white background and measured the grey scale with ImageJ 1.50i software. In experiment A, samples were collected from the first flag leaf, whereas samples were collected from the leaf just below the flag leaves in experiment B and C.

### ***Na<sup>+</sup> and K<sup>+</sup> content measurements***

Na<sup>+</sup> and K<sup>+</sup> contents of rice leaves were measured by means of atomic absorption spectrometer. In experiment A, 2 cm piece of the first flag leaf in each plant was collected and extracted with 10% HNO<sub>3</sub> solution then the Na<sup>+</sup> and K<sup>+</sup> contents were determined by microwave plasma atomic emission spectroscopy (MP-AES 4100, Agilent Technologies, US). In experiment B, 7 cm pieces of the 2 youngest leaves below the flag leaf of the first panicle in each plant was collected and extracted with 0.1N HCl solution. Afterwards, Na<sup>+</sup> and K<sup>+</sup> contents were measured by SpectrAa 220FS atomic absorption spectrometer (Varian, US). In experiment C, whole flag leaves were extracted with 0.1N CH<sub>3</sub>COOH solution then Na<sup>+</sup> and K<sup>+</sup> contents were analyzed by AAnalyst 400 atomic absorption spectrometer (PerkinElmer, US).

### ***Statistical analysis***

Data are presented as the means  $\pm$  SE. Significantly different levels were performed by one way ANOVA test followed by Least Significant Difference (LSD) test at confident level of 0.95 using R version 3.3.2 software.

### **Acknowledgments**

We thank staff members of the Institut de Biologie Intégrative des Plantes, Centre de coopération internationale en recherche agronomique pour le développement, Agricultural Genetics Institute and International Rice Research Institute for technical assistance in biological material culture. We thank the Ministry of Education and Training (Vietnam) and the Ministry of Foreign Affairs (France) for their funding supports. This project is also supported by Agropolis Fondation under the reference ID 1502-001 through the «Investissements d'avenir» programme (Labex Agro: ANR-10-LABX-0001-01).



**Table 1. List of transgenic lines used in experiments.**

Promoter	Gene of interest	FM	Lines used in:		
			Experiment A	Experiment B	Experiment C
<i>CaMV35S</i>	<i>OsPIP1;1</i>	<i>GFP</i>	A	A, B	A, B
<i>CaMV35Sx2</i>	<i>OsPIP2;4</i>	<i>GFP</i>	A	A, B	A, B
<i>CaMV35Sx2</i>	<i>OsPIP2;5</i>	<i>GFP</i>	A	A, B	A, B
<i>CaMV35Sx2</i>	<i>OsTIP1;1</i>	<i>mCherry</i>	A	A, B	A, B
<i>CaMV35Sx2</i>	<i>OsTIP2;2</i>	<i>mCherry</i>	-	A, B	C
<i>CaMV35Sx2</i>	<i>OsRab5a</i>	<i>mCherry</i>	A	A, B	-
<i>CaMV35Sx2</i>	<i>OsGAP1</i>	<i>mCherry</i>	A	A, B	-
<i>CaMV35Sx2</i>	<i>OsSCAMP1</i>	<i>mCherry</i>	A	A, B	-

FM, fluorescent marker. (-) indicates no line was tested.

**Table 2. Timetable and sampling/measuring methods in drought experiments.**

Parameter	Experiment	DASA	Sampling/measuring method
Plant height	A	9	Measure from the soil surface to the tip of the longest leaf or panicle
	B	19	
	C	21	
Tiller number	A	-	Count the total number of tillers in each plant
	B	19	
	C	21	
Flag leaf area	A	9	Measure the length and width (at the largest part) then multiply to have leaf area
	B	19	
	C	21	
Leaf water content	A	11	Collect 7 cm of the flag leaf of the 1 <sup>st</sup> panicle
	B	32	Collect 7 cm of the 1 <sup>st</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
	C	-	-
Leaf chlorophyll content	A	11	Measure the flag leaf the 1 <sup>st</sup> panicle with handheld-equipment
	B	32	Collect 1.5 cm of the 1 <sup>st</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle to extract
	C	22/26/31	Measure on 2 flag leaves with handheld-equipment than average
ROS production (DAB staining)	A	11	Collect 1.5 cm of the flag leaf of the 1 <sup>st</sup> panicle
	B	32	Collect 1.5 cm of the 1 <sup>st</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
	C	24	Collect 1.5 cm of the 1 <sup>st</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
Stomatal conductance	A	-	-
	B	-	-
	C	21/28	Measure the flag leaf of the two first panicle than average
Leaf water potential	A	-	-
	B	-	-
	C	24	Measure one mature leaf

(-) indicates no measurement. DASA, days after stress application.

**Table 3. Timetable and sampling/measuring methods in salinity experiments.**

Parameter	Experiment	DASA	Sampling/measuring method
Plant height	A	13	Measure from the soil surface to the tip of the longest leaf or panicle
	B	19	
	C	21	
Tiller number	A	-	Count the total number of tillers in each plant
	B	19	
	C	21	
Flag leaf area	A	13	Measure the length and width (at the largest part) then multiply to have leaf area
	B	19	
	C	21	
Leaf water content	A	14	Collect 7 cm of the flag leaf of the 1 <sup>st</sup> panicle
	B	20/27	Collect 7 cm of the 1 <sup>st</sup> /2 <sup>nd</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
	C	-	
Leaf chlorophyll content	A	14	Measure the flag leaf the 1 <sup>st</sup> panicle with handheld-equipment
	B	20/27	Collect 1.5 cm of the 1 <sup>st</sup> /2 <sup>nd</sup> leaf below the 1 <sup>st</sup> flag leaf to extract chlorophyll
	C	21/26/31	Measure on 2 flag leaves with handheld-equipment than average
ROS production (DAB staining)	A	-	-
	B	20/27	Collect 1.5 cm of the 1 <sup>st</sup> /2 <sup>nd</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
	C	24	Collect 1.5 cm of the 1 <sup>st</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
Stomatal conductance	A	-	-
	B	-	-
	C	26	Measure the flag leaf of the two first panicle than average
Na <sup>+</sup> /K <sup>+</sup> content	A	14	Collect 2 cm of the flag leaf of the 1 <sup>st</sup> panicle
	B	20/27	Collect 7 cm of the 1 <sup>st</sup> /2 <sup>nd</sup> leaf below the flag leaf of the 1 <sup>st</sup> panicle
	C	24	Collect the 1 <sup>st</sup> leaf below the flag leaf of the first panicle

(-) indicates no measurement. DASA, days after stress application.

**Table 4. Characterization of transgenic and wild-type rice in control condition.**

<i>Parameter</i>	<i>Experiment A</i>	<i>Experiment B</i>	<i>Experiment C-S</i>	<i>Experiment C-D</i>
Plant height	ns	NB > OsPIP2;4 (B)	OsPIP1;1 (A) , OsPIP2;5 (A) , OsTIP1;1 (B) > NB > OsPIP2;4 (B)	OsPIP2;5 (A) > NB > OsPIP2;4 (B)
Tiller number	-	OsTIP2;2 (B) > NB	NB > OsPIP2;5 (B), OsTIP1;1 (A)	ns
Flag leaf area	ns	NB > OsPIP2;4 (B)	ns	OsPIP1;1 (A), OsPIP2;5 (B) > NB
Leaf water potential	-	-	-	NB > OsTIP2;2 (C)
Leaf water content	ns	ns	-	-
Leaf chlorophyll content	ns	ns	ns	ns
Stomatal conductance	-	-	ns	ns
ROS production	-	ns	ns	ns
Na <sup>+</sup> content	OsRab5a (A), OsSCAMP1 (A) > NB	ns	On going	-
K <sup>+</sup> content	OsPIP1;1 (A) , OsRab5a (A) > NB	ns	On going	-
Na <sup>+</sup> /K <sup>+</sup> ratio	ns	ns	On going	-
Days to flower	-	OsPIP2;4 (B) > NB > OsTIP1;1 (B)	OsPIP2;4 (B), OsPIP2;5 (A+B), OsTIP1;1 (A+B) > NB	ns
Fertility of the 1 <sup>st</sup> panicle	NB > OsSCAMP1 (A)	NB > OsPIP2;4 (B), OsRab5a (B), OsSCAMP1 (A), OsTIP1;1 (B)	ns	-
Yield	NB > OsRab5a (A), OsSCAMP1 (A)	NB > OsPIP2;4 (B), OsSCAMP1 (A), OsTIP1;1 (B)	On going	On going
Maximum root length	-	-	ns	-
Root dry weight	-	-	ns	-
Shoot dry weight	-	-	NB > OsTIP1;1 (A)	-

(-) indicates no measurement. NB indicates Nipponbare wild-type.

ns, no significant difference between transgenic rice and wild-type, (>) indicates significantly higher value (LSD test,  $\alpha = 0.05$ ).

Experiments C-S and C-D indicate the control plants in salinity (pots) and drought (paddy field) assay in experiment C, respectively.

**Table 5. Characterization of transgenic and wild-type rice in response to drought stress.**

<i>Parameter</i>	<i>Experiment A (pot)</i>	<i>Experiment B (pot)</i>	<i>Experiment C (field)</i>
FTSW	<i>OsRab5a</i> (A) > NB > <i>OsPIP1;1</i> (A) <i>OsGAP1</i> (A)	<i>OsPIP2;4</i> (B) > NB	
Plant height	NB > <i>OsRab5a</i> (a), <i>OsPIP2;4</i> (A)	NB > <i>OsTIP2;2</i> (B), <i>OsSCAMP1</i> (B), <i>OsPIP2;4</i> (B)	ns
Tiller number	-	<i>OsTIP1;1</i> (B), <i>OsSCAMP1</i> (A+B) > NB	NB > <i>OsTIP1;1</i> (B)
Flag leaf area	NB > <i>OsTIP1;1</i> (A)	<i>OsTIP1;1</i> (A) > NB > <i>OsPIP2;4</i> (B)	<i>OsPIP1;1</i> (A) > NB
Leaf water potential	-	-	NB > <i>OsPIP1;1</i> (B), <i>OsPIP2;5</i> (B), <i>OsTIP1;1</i> (B)
Leaf water content	NB > <i>OsGAP1</i> (A)	ns	-
Leaf chlorophyll content	ns	<i>OsPIP1;1</i> (A), <i>OsPIP2;4</i> (B), <i>OsRab5a</i> (A), <i>OsGAP1</i> (A+B), <i>OsSCAMP1</i> (A+B) > NB	ns
Stomatal conductance	-	-	ns
ROS production	<i>OsTIP1;1</i> (A) > NB	NB > <i>OsPIP2;4</i> (B), <i>OsRab5a</i> (A)	NB > <i>OsTIP1;1</i> (B) , <i>OsTIP2;2</i> (C)
Days to flower	-	<i>OsPIP1;1</i> (A), <i>OsPIP2;4</i> (A+B), <i>OsPIP2;5</i> (A+B), <i>OsRab5a</i> (A+B), <i>OsGAP1</i> (B), <i>OsSCAMP1</i> (A+B), <i>OsTIP1;1</i> (A) > NB	ns
Fertility of the 1 <sup>st</sup> panicle	ns	NB > <i>OsPIP2;4</i> (B), <i>OsRab5a</i> (A), <i>OsGAP1</i> (A+B), <i>OsSCAMP1</i> (A)	-
Yield	ns	NB > <i>OsPIP2;4</i> (B), <i>OsRab5a</i> (A), <i>OsGAP1</i> (B), <i>OsSCAMP1</i> (B)	On going

(-) indicates no measurement. NB indicates Nipponbare wild-type.

ns, no significant difference between transgenic rice and wild-type, (>) indicates significantly higher value (LSD test,  $\alpha = 0.05$ ).

**Table 6. Characterization of transgenic and wild-type rice in response to salt stress.**

<i>Parameter</i>	<i>Experiment A</i>	<i>Experiment B</i>	<i>Experiment C</i>
Plant height	ns	NB > OsPIP2;4 (B), OsRab5a (A), OsSCAMP1 (A)	OsPIP1;1 (A) > NB > OsPIP2;4 (A+B), OsPIP2;5 (B), OsTIP1;1 (A+B)
Tiller number	-	NB > OsTIP1;1 (A)	NB > OsPIP1;1 (A+B), OsPIP2;4 (A+B), OsPIP2;5 (B), OsTIP1;1 (A+B)
Flag leaf area	ns	OsPIP2;4 (A) > NB > OsPIP2;4 (B), OsSCAMP1 (B)	OsPIP1;1 (A), OsPIP2;4 (A) > NB
Leaf water content	ns	ns	
Leaf chlorophyll content	ns	ns	OsPIP1;1 (A) > NB
Stomatal conductance	-	-	ns
ROS production	-	ns	ns
Na <sup>+</sup> content	ns	<b>(20 DASA):</b> NB > OsPIP1;1 (A+B), OsPIP2;4 (A+B), OsPIP2;5 (B), OsGAP1 (A+B), OsSCAMP1 (A+B), OsTIP1;1 (A+B), OsTIP2;2 (A+B) <b>(27 DASA):</b> OsPIP2;5 (A), OsRab5a (A), OsGAP1 (B), OsTIP1;1 (B) > NB	On going
K <sup>+</sup> content	ns	OsTIP1;1 (B) > NB	On going
Na <sup>+</sup> /K <sup>+</sup> ratio	ns	<b>(20 DASA):</b> NB > OsPIP1;1 (B), OsPIP2;4 (A+B), OsPIP2;5 (B), OsGAP1 (A+B), OsSCAMP1 (A+B), OsTIP1;1 (A+B) <b>(27 DASA):</b> OsRab5a (A), OsPIP2;5 (A), OsGAP1 (B) > NB	Ongoing
Days to flower	-	OsPIP1;1 (A), OsPIP2;4 (B), OsPIP2;5 (A+B), OsRab5a (A), OsGAP1 (B), OsSCAMP1 (A+B), OsTIP1;1 (A) > NB	OsPIP1;1 (A), OsPIP2;4 (A+B), OsPIP2;5 (A+B), OsTIP1;1 (A+B), OsTIP2;2 (C) > NB
Fertility of the 1 <sup>st</sup> panicle	NB > OsRab5a (A), OsSCAMP1 (A)	NB > OsPIP1;1 (A), OsPIP2;4 (B), OsRab5a (A), OsSCAMP1 (A+B)	NB > OsPIP1;1 (A)
Yield	ns	NB > OsPIP1;1 (A), OsPIP2;4 (B), OsRab5a (A), OsSCAMP1 (A+B)	On going
Maximum root length	-	-	NB > OsPIP1;1 (A), OsPIP2;4 (B), OsPIP2;5 (A), OsTIP1;1 (A+B)
Root dry weight	-	-	NB > OsPIP2;4 (B), OsPIP2;5 (B), OsTIP1;1 (A+B)
Shoot dry weight	-	-	NB > OsPIP2;4 (B), OsPIP2;5 (B), OsTIP1;1 (A+B)

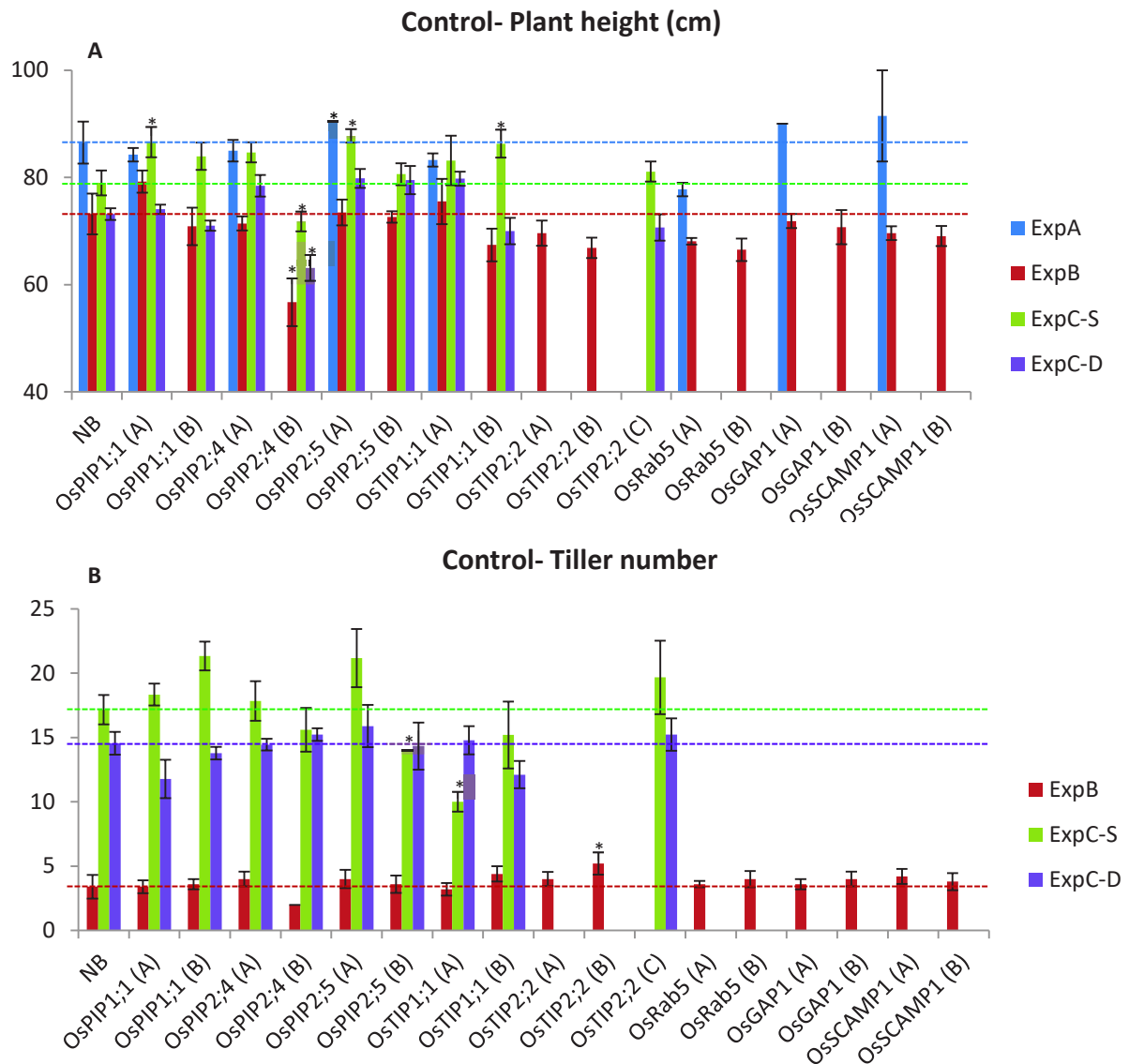
(-) indicates no measurement. NB indicates Nipponbare wild-type. DASA, days after stress application.

ns, no significant difference between transgenic rice and wild-type, (>) indicates significantly higher value (LSD test,  $\alpha = 0.05$ ).

**Table 7. Correlation between morphological / productive characteristics and grain yield.**

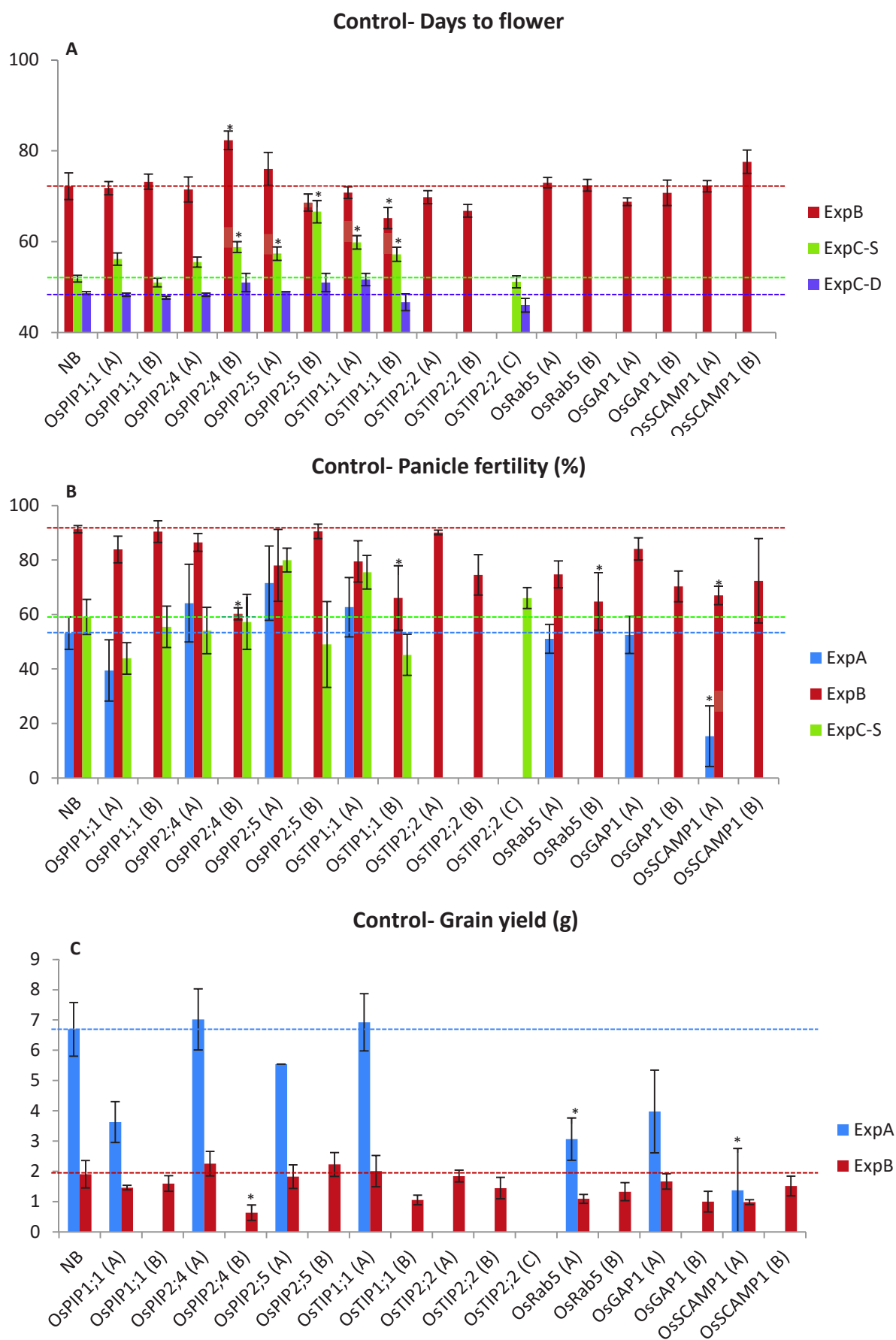
	<i>Control</i>		<i>Drought</i>		<i>Salinity</i>	
	<i>ExpA</i>	<i>ExpB</i>	<i>ExpA</i>	<i>ExpB</i>	<i>ExpA</i>	<i>ExpB</i>
<i>Plant height</i>	-0.07	0.54*	0.36	0.54*	0.48*	0.57*
<i>Tiller number</i>	-	0.19	-	-0.44*	-	0.07
<i>Flag leaf area</i>	-0.12	0.49*	0.11	0.31*	0.51*	0.35*
<i>Leaf water content</i>	0.34	0.32*/0.52*	0.3	-0.5*	0.63*	-0.45*/-0.08
<i>Leaf chlorophyll content</i>	-0.4	0.00/-0.35*	-0.01	-0.56*	-0.08	-0.32*/0.39*
<i>ROS production</i>	-	0.12/0.1	0.41*	0.55*	-	0.14/0.27*
<i>K<sup>+</sup> content</i>	-0.24	0.37*/0.25*	-	-	-0.19	0.22*/0.23*
<i>Na<sup>+</sup> content</i>	-0.41	-0.05/-0.1	-	-	0.62*	0.25*/-0.03
<i>Na<sup>+</sup>/K<sup>+</sup> ratio</i>	-0.42	-0.13/-0.18	-	-	0.54*	0.2/-0.12
<i>Panicle fertility</i>	0.6*	0.67*	0.73*	0.82*	0.64*	0.69*

Values indicate the Pearson's coefficient of correlation. \*:  $p < 0.05$ . (-) not available, cell with 2 numbers corresponds to 2 different sampling times.



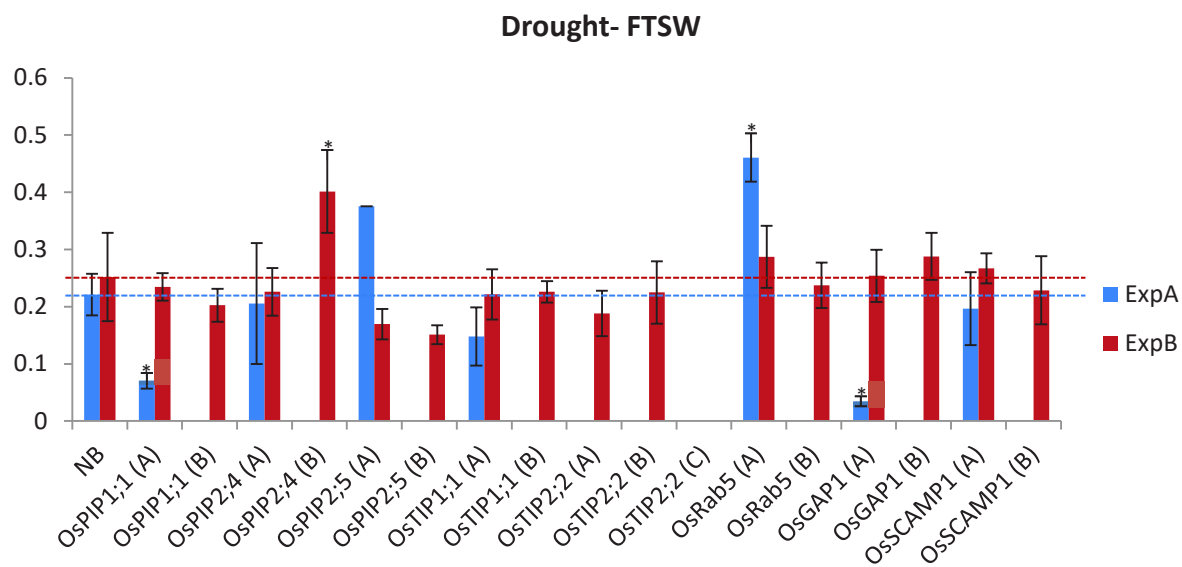
**Figure 1. Plant height (A) and tiller number (B) of transgenic and wild-type rice in control condition.**  
 NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ).  
 Experiments C-S and C-D indicate the control plants in salinity (pots) and drought (paddy field) assay in experiment C, respectively.  
 Dotted lines indicate base values of wild-type.





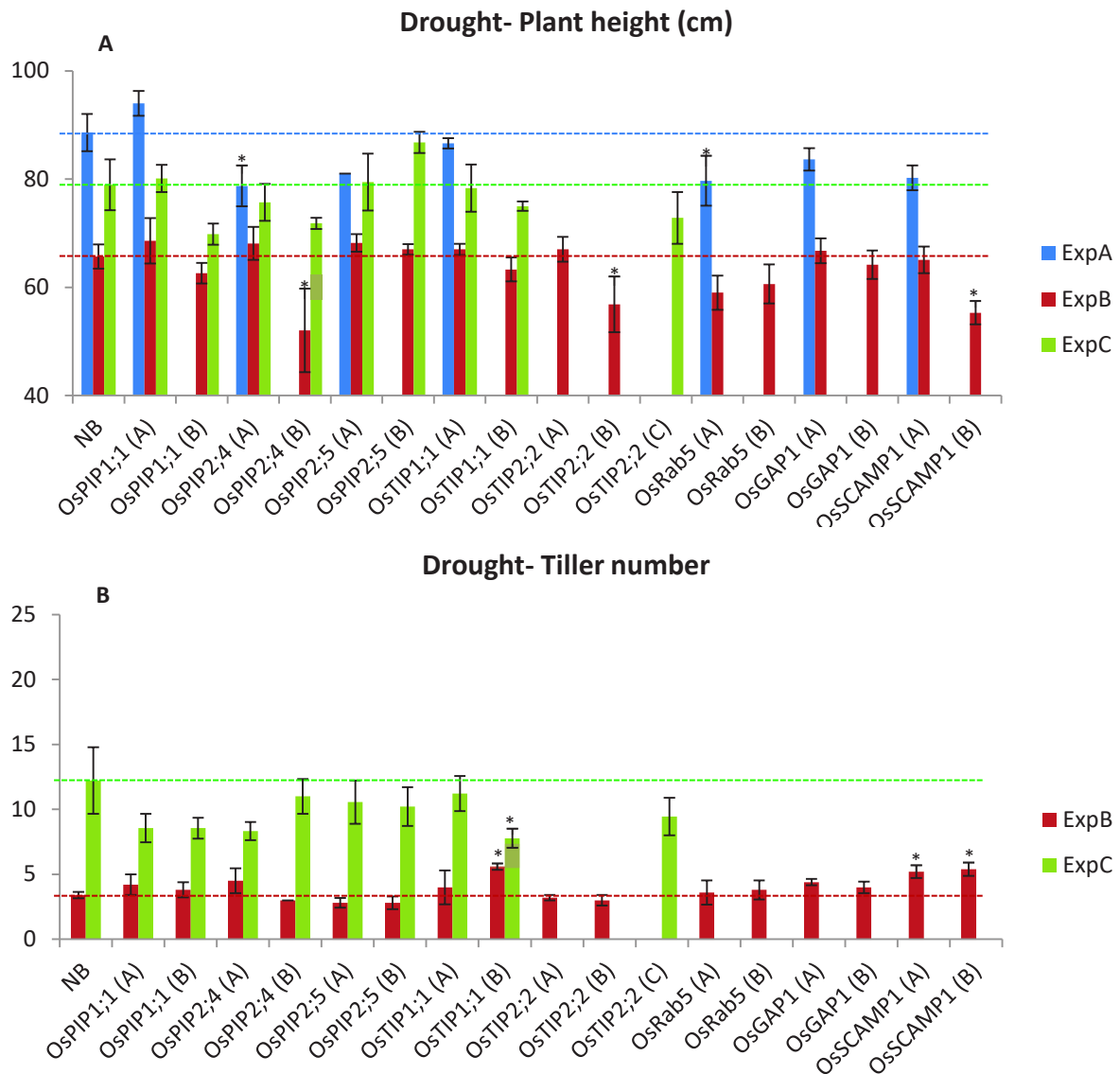
**Figure 2. Days to flower (A), panicle fertility (B) and grain yield (C) of transgenic and wild-type rice in control condition.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Experiments C-S and C-D indicate the control plants in salinity (pots) and drought (paddy field) assay in experiment C, respectively. Dotted lines indicate base values of wild-type.



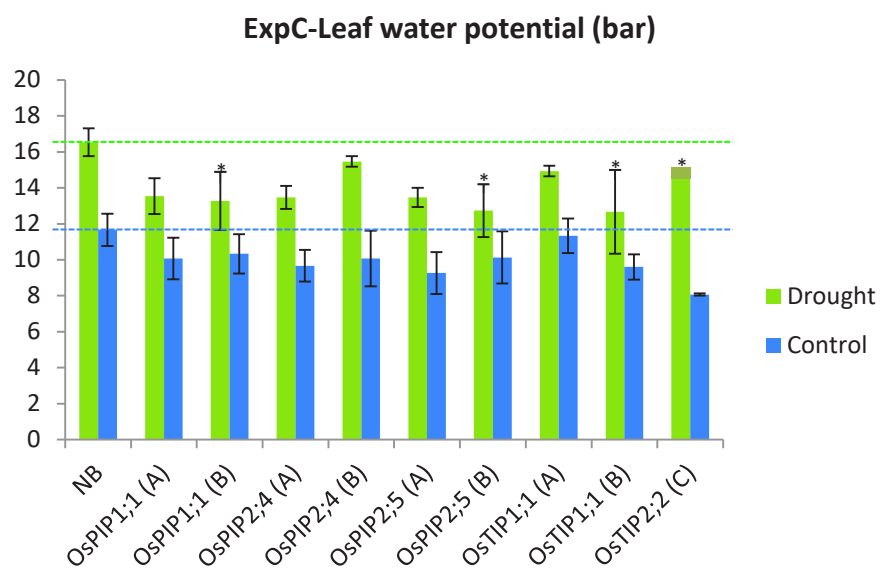
**Figure 3. FTSW indices of transgenic and wild-type rice in drought experiments.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.



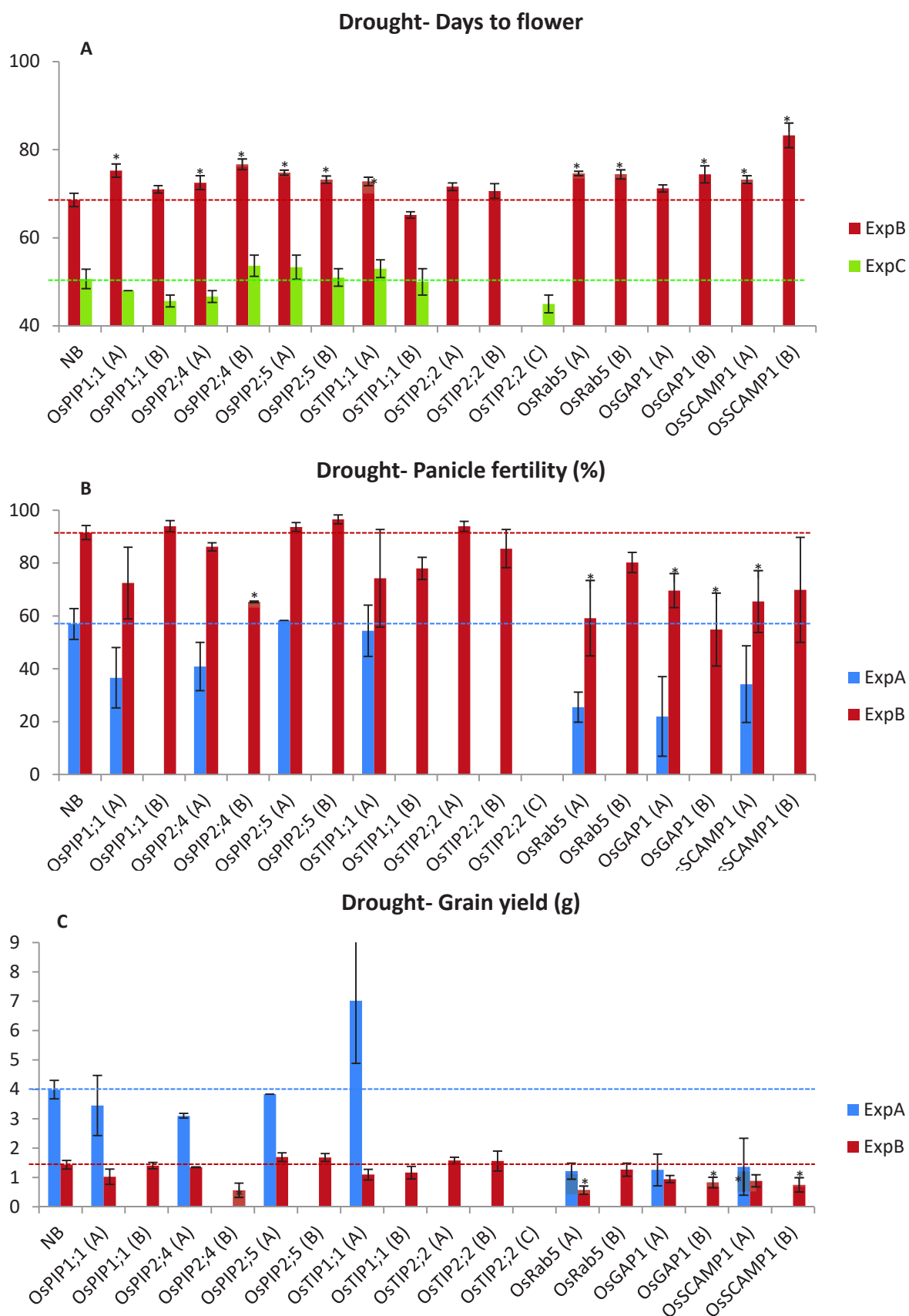
**Figure 4. Plant height (A) and tiller number (B) of transgenic and wild-type rice in response to drought stress.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.



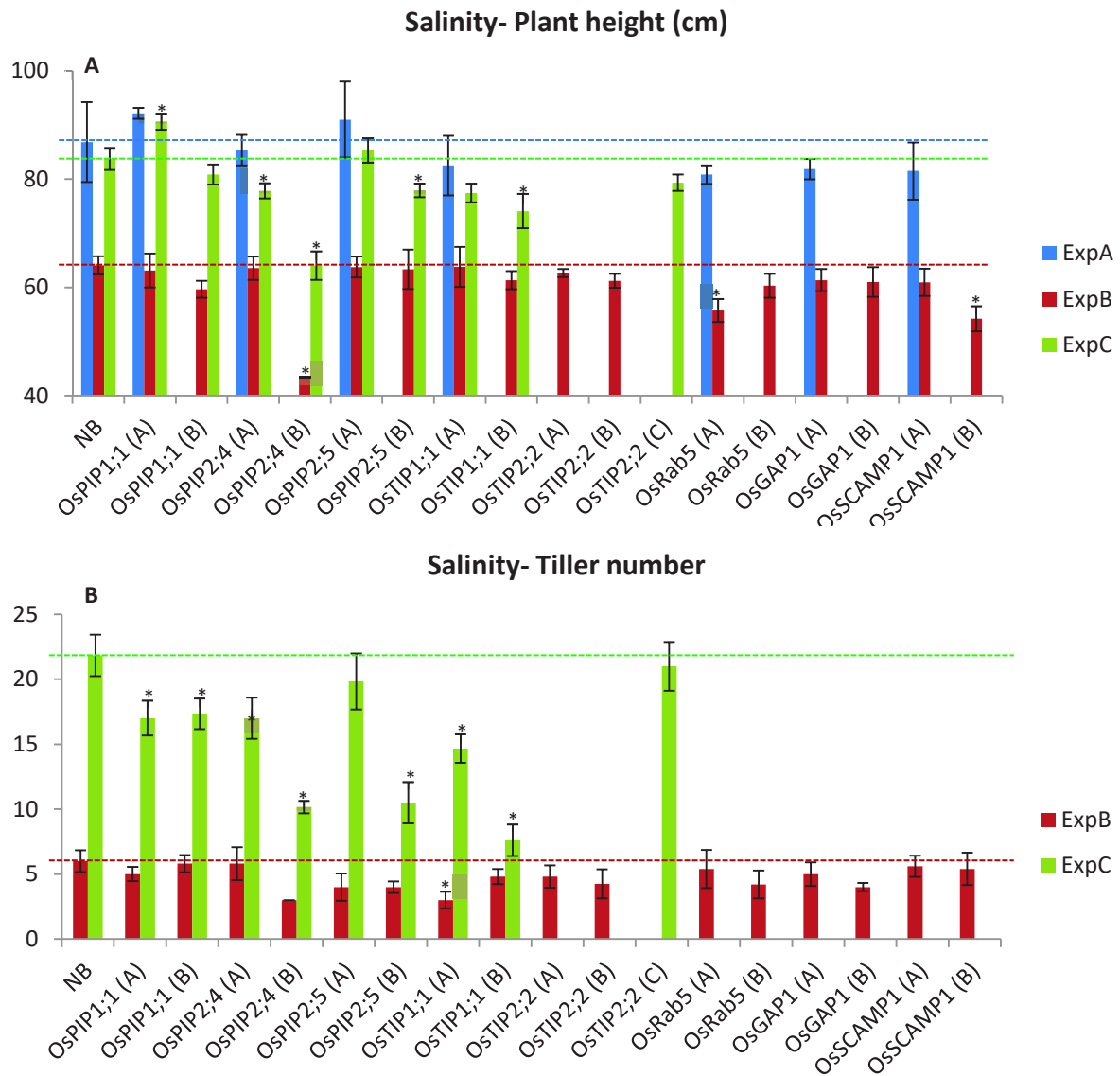
**Figure 5. Leaf water potential indices of transgenic and wild-type rice in response to drought stress in experiment C**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.

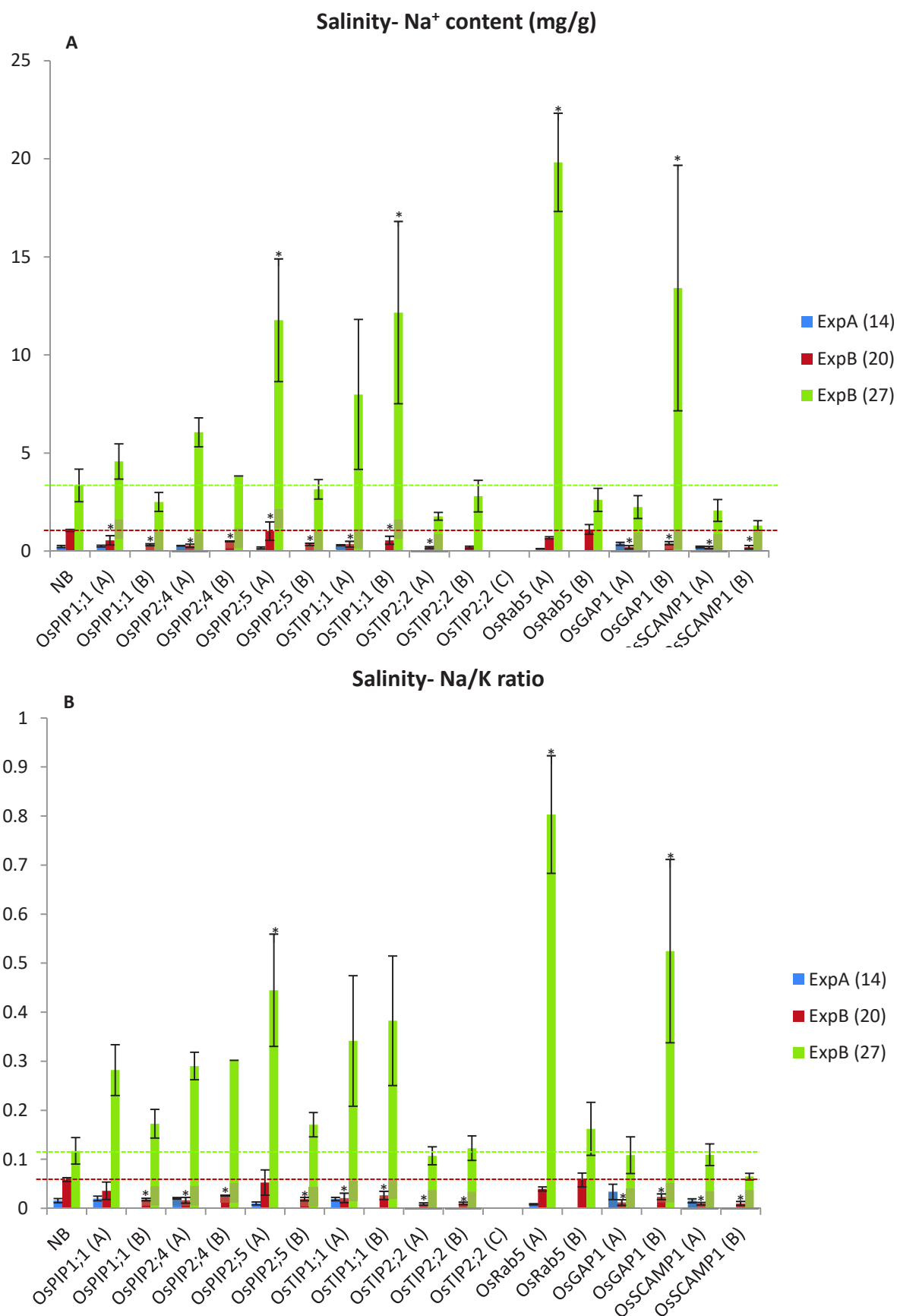


**Figure 6. Days to flower (A), panicle fertility (B) and grain yield (C) of transgenic and wild-type rice in response to drought stress.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.



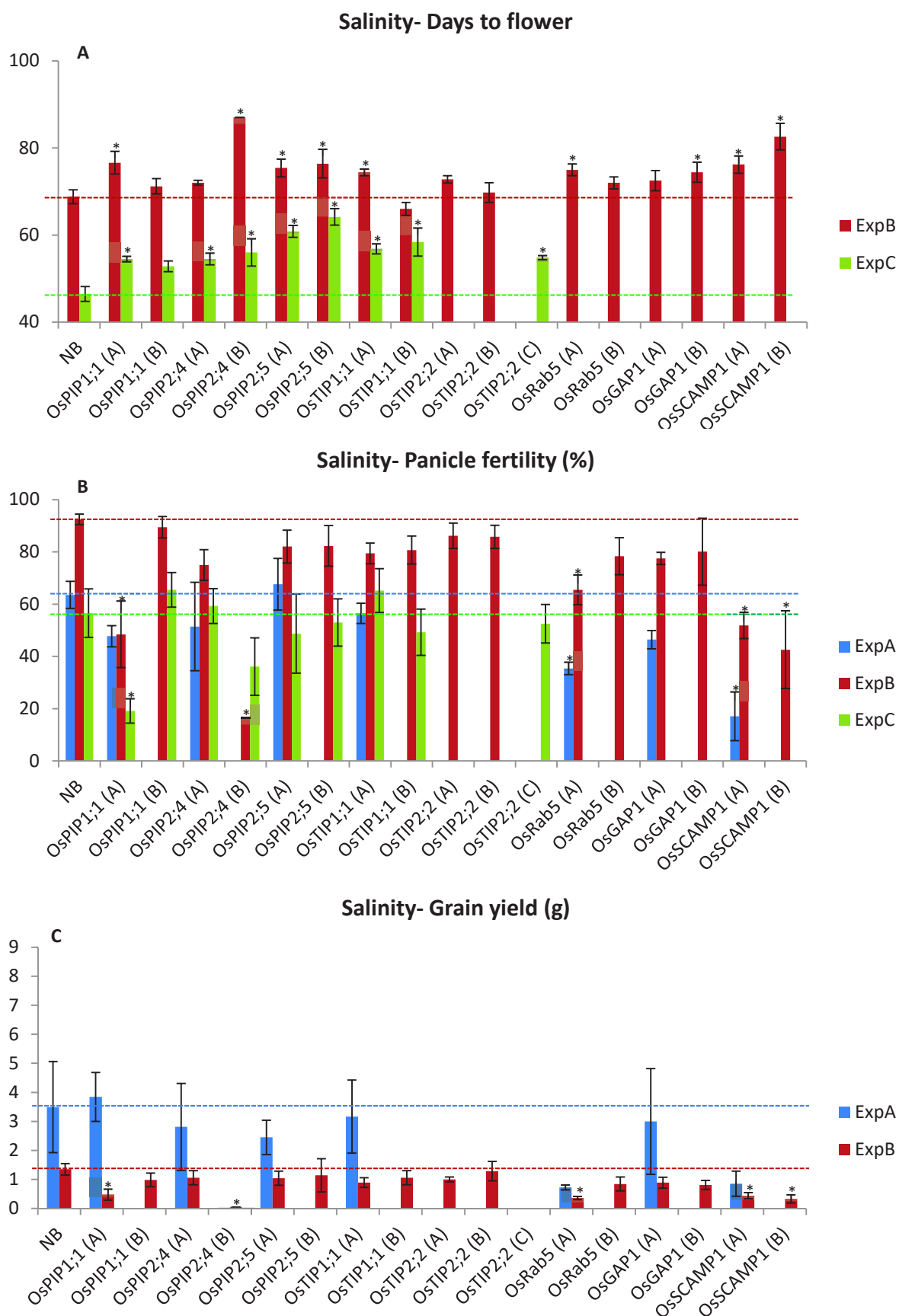
**Figure 7. Plant height (A) and tiller number (B) of transgenic and wild-type in response to salt stress.**  
*NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.*



**Figure 8. Leaf Na<sup>+</sup> content (A) and Na<sup>+</sup>/K<sup>+</sup> ratio (B) of transgenic and wild-type rice in response to salt stress.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type. Numbers in parenthesis indicate days after stress application (DASA).





**Figure 9. Days to flower (A), panicle fertility (B) and grain yield (C) of transgenic and wild-type rice in response to salt stress.**

NB indicates Nipponbare wild-type. (\*) indicates significant difference between transgenic line and wild-type (LSD test,  $\alpha = 0.05$ ). Dotted lines indicate base values of wild-type.

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### **Chapter III:**

## **Control of water uptake by root system architecture in rice (*Oryza sativa* L.)**

**Title:**

**Control of water uptake by root system architecture in rice (*Oryza sativa* L.)**

**Running title:**

Water uptake in rice root system

**Key words:**

*Oryza sativa*, water transport, apoplastic, cell-to-cell, aquaporins, salt stress.

**Names of authors:**

Thi Thu Huyen Chu<sup>1,2</sup>, Duy Chi Trinh<sup>1,2</sup>, Christophe Maurel<sup>1</sup>, and Doan-Trung Luu<sup>1</sup>

**Foot notes:**

<sup>1</sup> BPMP, CNRS, INRA, Montpellier SupAgro, Univ Montpellier, Montpellier, France

<sup>2</sup> University of Science and Technology of Hanoi, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Vietnam

**Corresponding author:**

Dr. Doan-Trung Luu

Biochimie et Physiologie Moléculaire des Plantes

Institut de Biologie Intégrative des Plantes

UMR 5004 CNRS/UMR 0386 INRA/Montpellier SupAgro/Université de Montpellier

2, Place Viala

F-34060 Montpellier Cedex 2

France

E-mail : luu@supagro.fr

## Abstract

As a plant model, rice has been subjected to an intensive research. However, its hydraulics is still a matter of debate. How does rice root system function as a water uptake organ is an important open question in order to improve rice adaptation to global change for sustainable agricultural practices. Root hydraulic traits have been previously reported to be dependent on aquaporin activity, apoplastic barriers such as Casparian strip development and suberin lamellae deposition, and root anatomy. In the present study, we characterized hydraulic properties of two mutants altered in root system architecture. We reported that crown roots had higher contribution to the overall transport capacity of the root system (so-called conductance,  $L_0$ ) than radicle root. Intrinsic water transport capacity (so-called conductivity,  $L_{pr}$ ) of primary roots has been found higher than lateral roots (LRs). Use of aquaporin inhibitor and apoplastic pathway (so-called bypass flow) blocker led to the interpretation of a higher contribution of cell-to-cell pathway in primary roots than in LRs, and conversely, a higher contribution of apoplastic pathway in LRs than in primary roots. Based on these data, we estimated that the cell-to-cell (aquaporin-dependent) pathway contributes ~3.5 fold more than apoplastic pathway in the primary root water transport capacity. These results therefore underline the importance of root system architecture and root anatomy, also, aquaporin activities in the overall root hydraulics, and indicate a potential for manipulation of root hydraulics for improvement of rice not only in well-watered conditions but also under abiotic stresses such as soil salinization.

## Introduction

Water is essential for every living cell. In plants, water is captured from the soil by the root system, and then transported into the shoot by the driving force of transpiration. How does the root system control the water uptake in normal and stress conditions is still an open question. This question is of high interest for crops such as rice (*Oryza sativa* L.). Rice is an important crop with a world paddy production of ~756 million tons in 2017 and also a model of monocotyledon plants. Having ability of growth in both flooded and rain-fed soils, associated with a high genetic diversity, rice offers unique potential of improvement of physiological functions such as water uptake by manipulating root system architecture (Courtois *et al.*, 2009; Coudert *et al.*, 2010).



Like in other cereals, rice has a root system architecture consisting of different root types (Rebouillat *et al.*, 2009). The radicle (so-called seminal root) is the first root which emerges from the coleorhiza. The crown roots emerge from nodes. In general, radicle and crown roots are called as primary roots to distinguish with lateral roots (LRs), which emerge from the formers. LR can be classified into two different types. Large LR is thinner than primary roots, and are able to produce small LR. Small LR display determinate elongation, grow laterally and never bear LR. Moreover, primary roots and large LR show indeterminate growth down-ward and produce LR.

Though having various sizes or numbers of cell layers in each tissue, in general, primary roots and large LR include epidermis, exodermis, schlerenchyma, mesodermis, endodermis and central cylinder from the outer to the inner. At mature zone of roots, mesodermis differentiates into spokes and aerenchyma. This radial anatomy is typical in many aquatic or semiaquatic plants. Small LR display much simpler internal structure with no mesodermis and aerenchyma (Rebouillat *et al.*, 2009). Exodermal and endodermal cells exhibit Casparian strips and suberin lamellae deposition (Rebouillat *et al.*, 2009; Krishnamurthy *et al.*, 2009). Casparian strips deposit on the radial and transverse walls of the cells, whereas, suberin lamellae develop in inner tangential walls (Clark and Harris, 1981).

In root vascular tissues, water moves axially inside xylem vessels. In non-vascular tissues, water flows radially through a series of cell layers. In 2000, Ernst Steudle introduced a composite transport model to explain root water uptake in non-vascular tissues (Steudle, 2000). In the model, three parallel pathways are involved in radial water transport. The first one is apoplastic pathway around protoplasts or between cell walls. The second one is symplastic pathway mediated by plasmodesmatas which link cell walls of adjacent cells. The third one is transcellular pathway, where water and solutes have to cross cell membranes. This latter pathway is now considered as dependent on the aquaporin function (Maurel *et al.*, 2015). The symplastic and the transcellular pathways cannot be separated experimentally and are considered as cell-to-cell water flow (Steudle, 2000).

Aquaporins are classified into the ancient superfamily of major intrinsic proteins and divided into 5 subfamilies based on sequence homology and subcellular localization. They include: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and uncategorized (X) intrinsic proteins (XIPs) (Maurel *et al.*, 2015). The genome of rice (cv. Nipponbare)

comprises 33 aquaporin sequences, among them 11 PIPs, 10 TIPs, 10 NIPs and 2 SIPs based on the phylogenetic relationship with *Arabidopsis thaliana* and *Zea mays* (Sakurai *et al.*, 2005). Many studies have proved that aquaporins are multifunctional channels, which can transport not only water but also various small neutral molecules / physiological substrates such as carbon dioxide, ammonia, urea, glycerol, metalloids like boron and silicon, and reactive oxygen species (Maurel *et al.*, 2015), but it is widely accepted that PIPs and TIPs are the two most abundant aquaporins in plant cells and the major contributors of water transport in plant roots.

Water transport properties of rice root system were examined by physiological approach with hydrostatic or osmotic driving forces and using pressure chamber at the level of whole root system, or root pressure probe at the level of single root, or cell pressure probe at the level of cortical cells. Quantification of suberin deposition or treatment with China ink, a supposedly blocker of apoplast (Ranathunge *et al.*, 2004), allowed an estimation of apoplastic pathway. Mercury ( $\text{HgCl}_2$ ) and azide ( $\text{NaN}_3$ ), the most commonly-used aquaporin inhibitors, allowed an estimation of the contribution of these channels in the cell-to-cell pathway.

Root hydraulic conductivity ( $L_{pr}$ ) of rice tends to be in the same order of magnitude as in *Arabidopsis thaliana* (Sutka *et al.*, 2011), but lower than other herbaceous species such as maize (*Zea mays*) or common bean (*Phaseolus vulgaris*) (Miyamoto *et al.*, 2001). Relationship between this low root hydraulic conductivity and exceptional apoplastic barriers in root anatomy has been hypothesised (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003, 2004; Grondin *et al.*, 2016a). The presence of Casparian strips and suberin lamellae on the wall of exodermal and endodermal cells has been reported to restrict the flow of water and solutes (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003, 2004; Krishnamurthy *et al.*, 2009). In rice, these barriers are extremely well-developed with 6- and 34-fold more suberin in the cell wall of endodermis and exodermis, respectively, compared to maize (Schreiber *et al.*, 2005). China ink exhibited a higher inhibition level of  $L_{pr}$  than  $\text{HgCl}_2$  did in the outer part of the root (OPR) (Ranathunge *et al.*, 2004). OPR comprises rhizodermis, exodermis, sclerenchyma and cortical cell layer. Importantly, this result suggests a relatively larger apoplastic flow in this part of the root. Also, the contribution of aquaporins (monitored by inhibition of  $L_{pr}$  with azide) to the overall root hydraulics is relatively high (up to 79%) in rice (Grondin *et al.*, 2016a). Thus, the larger contribution of apoplastic- compared to aquaporin-dependent water flow in the OPR may be explained by a differential functional activity of

aquaporins at different cell layers. In rice, a weak negative correlation between PIP transcript abundance and  $L_{pr}$  was found (Grondin *et al.*, 2016a); a similar relationship was also found in Arabidopsis (Sutka *et al.*, 2011). These surprising results might be the indication of regulations of the aquaporins at post-translational level.

$L_{pr}$  of the OPR was found 30 fold higher compared to the values of overall single root or the overall root system (Ranathunge *et al.*, 2003). Furthermore,  $L_{pr}$  was found positively correlated with the percentage of root cortical aerenchyma (Grondin *et al.*, 2016a), confirming the importance of the OPR in rice root hydraulics. However, in Arabidopsis  $L_{pr}$  was found not linked with the number of endodermal and cortical cell files (Sutka *et al.*, 2011). Additionally, in lupin (*Lupinus albus*), the  $L_{pr}$  was found higher in endodermal compared to cortical cells in younger part of the LR and with the same order of magnitude in older parts (Zarebanadkouki *et al.*, 2013). The whole set of studies suggests that pattern of hydraulics at the cell-layer level is plant species-dependent.

Differentiation of rice root types suggests different contribution in root hydraulics (Ranathunge *et al.*, 2003). However, only data in barley (*Hordeum vulgare* L.) are available. Root system architecture of this species at the stage of plantlet (14- to 17-day-old) exhibits six to seven seminal (radicle) roots and about two adventitious (crown) roots, with LR only on radicle roots (none on crown roots) (Knipfer and Fricke, 2011). LR surface area represents 58% of the total radicle root. Radicle and crown roots exhibited similar  $L_{pr}$ , but due to a higher surface area in radicle roots, the hydraulic conductance ( $L_0$ ) of these latter roots were found four times higher, indicating a contribution of 92% to the overall plant water uptake (Knipfer and Fricke, 2011). Cortical cell hydraulic conductivity in LR was found 5- to 8-fold higher compared to radicle primary roots and subjected to 90% of inhibition upon  $HgCl_2$  treatment (Knipfer *et al.*, 2011). These data suggest that LR and aquaporins are both major contributors of hydraulics in barley roots.

Mutants exhibiting root system architecture alteration are available in rice. For instance, *crown rootless1* (*cr1*) mutant was successfully isolated from Taichung 65 cultivar population (Inukai *et al.*, 2001). *CROWN ROOTLESS1* (*CRL1*) encodes an AS2/LOB-domain transcription factor which expression is induced by auxin, and that acts upstream of the gene regulatory network controlling crown root development. Mutant *cr1* plants show a defect in crown root formation and reduction in the number of LR in the radicle compared to the wild-type but no significant difference was observed for other morphological

properties such as number of central conducting vessel or suberin development (Inukai *et al.*, 2001, 2005). Other mutants altered in the LR initiation are available, such as *lateral-rootless* (*lrt2*) which mutated gene encodes a cyclophilin protein involved in auxin signalling pathway. Mutant *lrt2* plants exhibit very few LRs per plant (99-100% reduction) and have longer radicle roots, less root gravitropism, more central vessels but less suberin development than their wild-type Nipponbare (NB) cultivar (Wang *et al.*, 2006; Faiyue *et al.*, 2010b).

Rice is a salt sensitive crop (Munns and Tester, 2008). Using two rice cultivars which differ in root morphology, stomatal regulation and aquaporin expression, it was reported that change in root hydraulic properties after one-week of osmotic or salt treatment was mainly contributed by root morphological characteristics, such as the number of primary and lateral roots, surface area ratio of root to shoot and plant transpiration rate, whereas accumulation of aquaporin transcripts exhibited a poor correlation (Meng and Fricke, 2017).

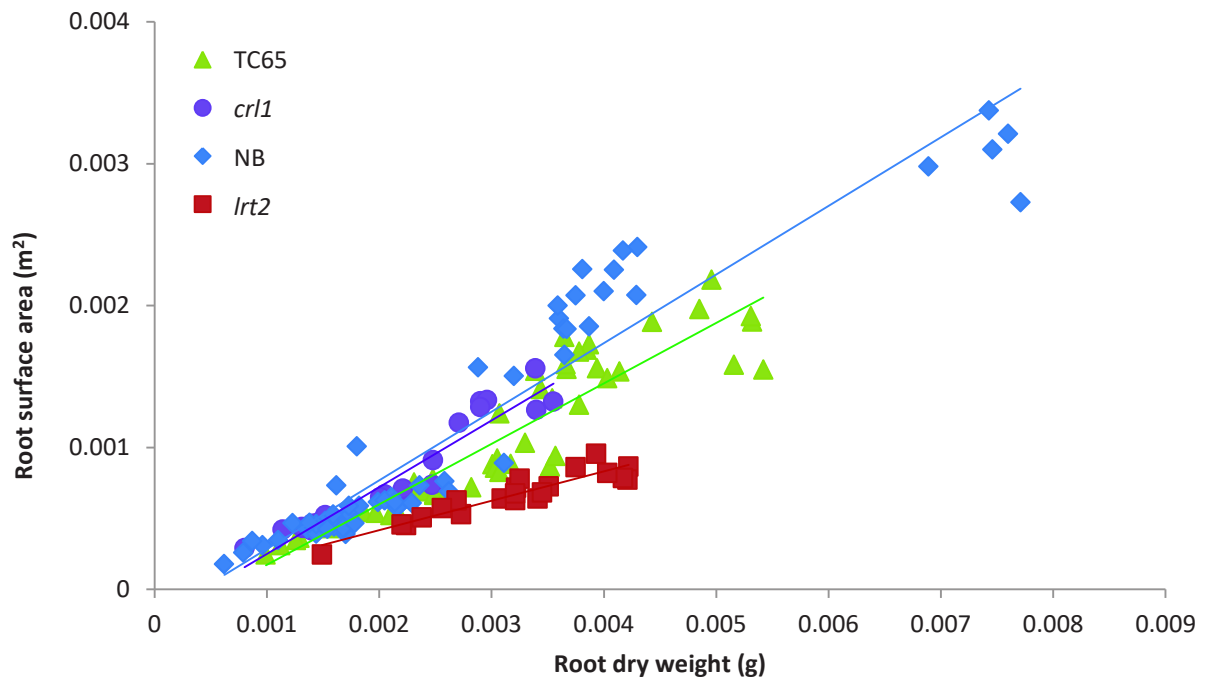
In the present study, we used pressure chamber with hydrostatic driving force to determine  $L_{pr}$  and  $L_0$  values of *crl1* and *lrt2* mutant plants and their respective wild-types Taichung 65 (TC65) and NB. This allowed us to have indications of the respective contributions of radicle, crown and lateral roots to the overall root hydraulics. Root systems were treated with China ink or azide to estimate the respective contributions of apoplastic and cell-to-cell pathways, respectively. Treatment of root systems with salt (NaCl) solution for 30 min and  $L_{pr}$  measurements allowed us to know the early effects of salinity on each root type. By integrating these hydraulics data with the variability of root system architecture, our aim was to better understand water fluxes in rice roots.

## Results

### ***Root morphological characteristics***

First, we determined a relationship between the overall root surface area (RSA) and the root dry weight (RDW), with seedlings of genotypes grown hydroponically. We collected root systems at 7, 10, 13 and 21 days after germination (DAG). Thanks to hydroponic culture we were able to obtain intact root systems, a prerequisite for root hydraulics characterization. A linear regression was used to establish a relationship between RSA ( $m^2$ ) and RDW (g) for each genotype:  $y = 4.835 \times 10^{-1}x - 2 \times 10^{-4}$  for NB ( $R^2 = 0.9038$ );  $y = 2.069 \times 10^{-1}x + 4 \times 10^{-6}$  for *lrt2* ( $R^2 = 0.8622$ ),  $y = 4.258 \times 10^{-1}x - 3 \times 10^{-4}$  for TC65 ( $R^2 = 0.858$ ) and  $y =$

$4.706 \times 10^{-1} x - 2 \times 10^{-4}$  for *crl1* ( $R^2 = 0.9125$ ), where y and x stand for RSA and RDW, respectively. We found a strong relationship for the whole set of genotypes ( $R^2 > 0.85$ ). Interestingly, NB, TC65 and *crl1* exhibited the same relationship, while *lrt2* had a smaller slope, which is consistent with the lack of LR in this mutant (**Figure 1**).



**Figure 1. Linear relationship between root surface area (RSA) and root dry weight (RDW).**

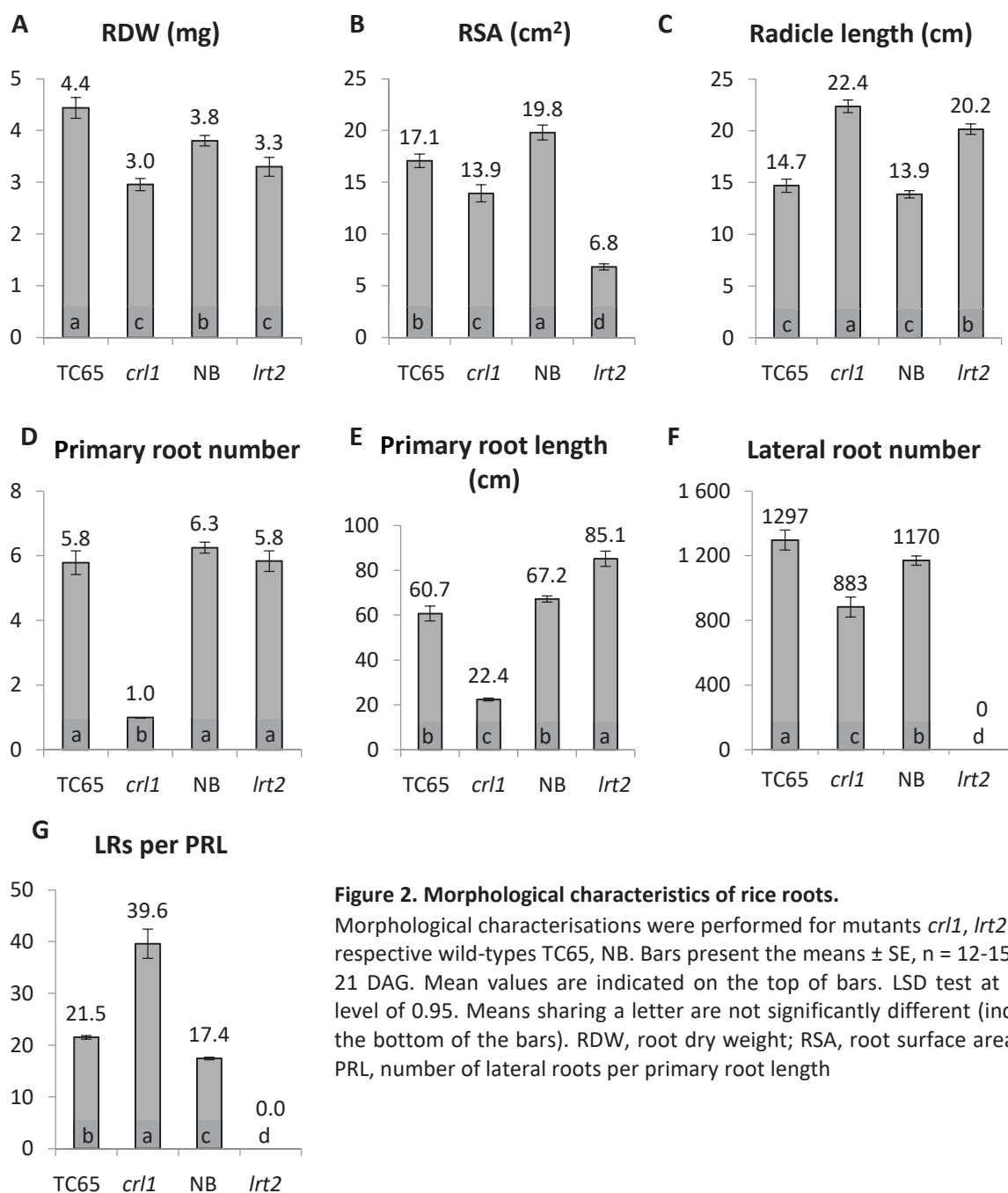
Linear relationships between RSA and RDW were obtained in all 4 genotypes, *crown rootless* mutant (*crl1*), *lateral rootless* mutant (*lrt2*) and their respective wild-types Taichung 65 (TC65), Nipponbare (NB). Rice root systems were collected from seedlings grown hydroponically of 7, 10, 13 and 21 days after germination.

Root systems of *crl1* and *lrt2* mutant plants and their respective wild-types TC65 and NB aged of 21 DAG were characterized in detail to better understand the hydraulics in these tissues. As shown in **Table 1** and **Figure 2**, wild-type genotypes TC65 and NB exhibit similar radicle root length, primary root number, and total primary root length (PRL), whereas their LR number, number of LRs per PRL, RDW and RSA are slightly different. Compared to its wild-type, *crl1* had remarkably longer radicle root length but lower PRL and LR number. These morphological alterations were associated with a lower RDW and RSA compared to TC65. The mutant *lrt2* exhibited no LRs at the age of 21 DAG. Though compared to its wild-type it showed higher radicle root length and PRL, and similar number of primary roots, its root system still had lower RDW and RSA ( $p < 0.05$ ). Importantly, our data showed a marked variability in these morphological characteristics.

**Table 1. Morphological characteristics of mutants *crl1*, *lrt2* and their respective wild-types TC65, NB.**

	TC65	<i>crl1</i>	NB	<i>lrt2</i>
Root dry weight (mg)	4.4 ± 0.20 a	3.0 ± 0.12 c	3.8 ± 0.10 b	3.3 ± 0.18 c
Root surface area (cm <sup>2</sup> )	17.1 ± 0.66 b	13.9 ± 0.83 c	19.8 ± 0.73 a	6.8 ± 0.30 d
Radicle length (cm)	14.7 ± 0.86 c	22.4 ± 0.62 a	13.9 ± 0.37 c	20.2 ± 0.50 b
Number of primary roots	5.8 ± 0.37 a	1.0 ± 0.00 b	6.3 ± 0.17 a	5.8 ± 0.32 a
Primary root length (cm)	60.7 ± 3.31 b	22.4 ± 0.62 c	67.2 ± 1.35 b	85.1 ± 3.40 a
Number of lateral roots	1297 ± 61.65 a	883 ± 0.37 c	1170 ± 0.00 b	0 ± 0.00 d
Number of lateral roots per primary root length	21.5 ± 0.35 b	39.6 ± 2.85 a	17.4 ± 0.25 c	0.0 ± 0.00 d

Data presented are means ± SE, n = 12-15 plants at 21 DAG. Means sharing a letter are not significantly different (LSD test at confident level of 0.95).



**Figure 2. Morphological characteristics of rice roots.**

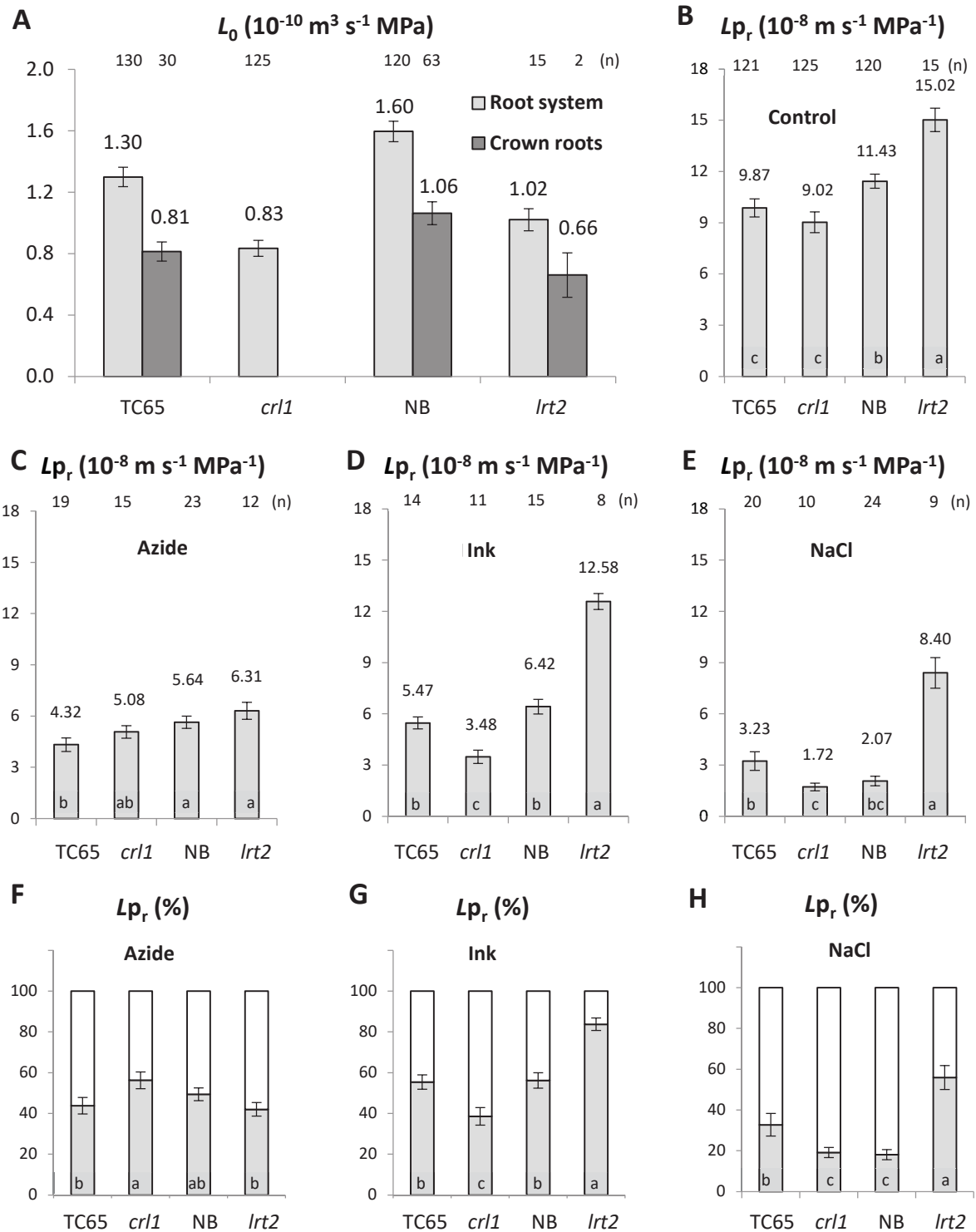
Morphological characterisations were performed for mutants *crl1*, *lrt2* and their respective wild-types TC65, NB. Bars present the means ± SE, n = 12-15 plants at 21 DAG. Mean values are indicated on the top of bars. LSD test at confident level of 0.95. Means sharing a letter are not significantly different (indicated at the bottom of the bars). RDW, root dry weight; RSA, root surface area; LRs per PRL, number of lateral roots per primary root length

### **Root hydraulic conductance and conductivity**

$L_0$  and  $L_{pr}$  of *crl1* and *lrt2* mutant plants and their respective wild-types TC65 and NB aged of 21 DAG were measured by means of pressure chambers.  $L_0$  value represents the total capacity of water uptake of the root system. NB had a slightly significant higher  $L_0$  compared to TC65 ( $1.60 \pm 0.066 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$  and  $1.30 \pm 0.063 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ , respectively) and *crl1* and *lrt2* mutants showed significant lower  $L_0$  compared to their respective wild-types ( $0.83 \pm 0.052 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$  and  $1.02 \pm 0.071 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ , respectively) (**Figure 3A**). These measurements allowed an estimation of a similar reduction of  $L_0$  by 36.15% and 36.25% for *crl1* and *lrt2* compared to their wild-types, respectively.

To get more insight in the contribution of the crown roots, we measured their conductance independently. Interestingly, we found that crown roots contributed 63-67% of total conductance of a root system, in 3 genotypes TC65, NB and *lrt2* (**Figure 3A**).

$L_0$  is a function of both RSA and intrinsic water transport capacity of the root system ( $L_{pr}$ ). To measure this latter parameter, we took into account the RAS and found that NB had a significantly higher  $L_{pr}$  compared to TC65 ( $11.43 \pm 0.41 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$  and  $9.87 \pm 0.53 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , respectively), but its  $L_{pr}$  was significantly lower than *lrt2* ( $15.02 \pm 0.68 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ) (**Figure 3B**). Also, *crl1* ( $L_{pr} = 9.02 \pm 0.60 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ) and its wild-type had not significantly different  $L_{pr}$  values.



**Figure 3. Root hydraulic properties of rice root system**

Measurements were performed with mutant *cr1*, *lrt2* and their respective wild-types TC65, NB. Roots of 21 DAG plants were subjected for conductance ( $L_0$ ) (A) or root hydraulic conductivity ( $L_{pr}$ ) measurements (B-E). In A, light-grey and darker bars indicate measurements for whole root systems and crown roots, respectively. Light-grey bars present the means  $\pm$  SE in absolute values (A-E) or relative values (%) (F-H). Open bars indicate the percentage of relative reduction of  $L_{pr}$  by azide (F), China ink (G) and NaCl (H). Mean values are indicated on the top of bars in A-E. (n), number of measurements. LSD test at confident level of 0.95. Means sharing a letter are not significantly different (indicated at the bottom of the bars).



### **Effects of azide treatment on $L_p$**

To investigate the contribution of aquaporins to hydrostatic  $L_p$ , we used 4 mM azide as an aquaporin inhibitor.  $L_p$  values for TC65, *cr1*, NB and *lrt2* were  $4.32 \pm 0.54 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $5.08 \pm 0.22 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $5.64 \pm 0.28 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , and  $6.31 \pm 0.89 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , respectively (**Figure 3C**). Though  $L_p$  of TC65 and *cr1* in control conditions were not significantly different (**Figure 3B**), under azide treatment *cr1* had a slightly higher  $L_p$ . In opposite, though  $L_p$  of NB and *lrt2* in control conditions were significantly different (**Figure 3C**), under azide treatment their  $L_p$  were not significantly different. The percentage of  $L_p$  inhibition by azide (*Azide\_inh*) indicates the relative contribution of aquaporin-dependent pathway to  $L_p$ . Azide treatment consistently reduced the  $L_p$  of TC65, *cr1*, NB and *lrt2* by  $56.18 \pm 4.02\%$ ,  $43.76 \pm 4.06\%$ ,  $50.66 \pm 3.12\%$ ,  $58.00 \pm 3.35\%$ , respectively (**Figure 3F**). Inhibition of  $L_p$  by azide in *cr1* was weaker than in its wild-type TC65 ( $p < 0.05$ ), whereas slightly stronger in *lrt2* compared to its wild-type NB ( $p > 0.05$ ).

### **Effects of China ink treatment on $L_p$**

Here, we tested China ink suspension in the same condition as previous investigations (Ranathunge *et al.*, 2004). The particle size was measured at  $85.23 \pm 8.42 \text{ nm}$  of diameter.  $L_p$  values for TC65, *cr1*, NB and *lrt2* were  $5.47 \pm 0.35 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $3.48 \pm 0.39 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $6.42 \pm 0.43 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$  and  $12.58 \pm 0.46 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , respectively (**Figure 3D**). Though  $L_p$  of TC65 and *cr1* in control conditions were not significantly different (**Figure 3B**), under China ink treatment *cr1* had a lower  $L_p$ . In contrary,  $L_p$  of NB and *lrt2* kept the same tendency in both control and China ink treatment conditions with a highly significant value for the mutant (**Figure 3D**). The percentage of  $L_p$  reduction by China ink (*Ink\_inh*) indicates the relative contribution of apoplastic pathway to  $L_p$ . Whereas China ink treatment consistently reduced the  $L_p$  of TC65, *cr1*, and NB, by  $44.61 \pm 3.52\%$ ,  $61.44 \pm 4.34\%$ , and  $43.84 \pm 3.77\%$ , respectively, a weaker effect was observed in the mutant *lrt2* ( $16.28 \pm 3.06\%$ ) (**Figure 3G**).

### **Effects of salt stress on the root hydraulics**

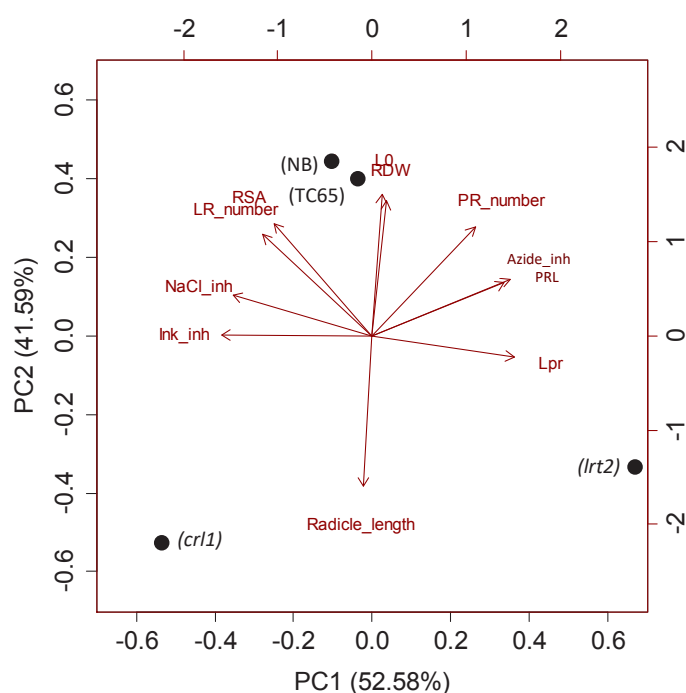
TC65, *cr1*, NB and *lrt2* roots of plants at 21 DAG were exposed to 100 mM NaCl for 30 min, and afterwards, they were subjected for  $L_p$  measurement.  $L_p$  values for TC65, *cr1*, NB and *lrt2* were  $3.23 \pm 0.54 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $1.72 \pm 0.22 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ,  $2.07 \pm 0.28 \times$

$10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , and  $8.40 \pm 0.89 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ , respectively (**Figure 3E**). We determined the percentage of inhibition (*NaCl\_inh*) and found that its value for *lrt2* was only  $44.10 \pm 5.81\%$  as compared to much higher values for NB, *cr1* and TC65 ( $81.88 \pm 2.49\%$ ,  $80.93 \pm 2.48\%$  and  $67.25 \pm 5.51\%$ , respectively) (**Figure 3H**). The inhibition of  $L_p_r$  by salt stress in the wild-type NB was 1.85 higher compared to its lateral rootless mutant. Such a difference was not observed between *cr1* and its wild-type.

### ***Covariation of root hydraulic properties and root morphology***

To interpret the relationships between root hydraulic properties and root morphology, we performed a principal component analysis (PCA) for the four genotypes. Mean values of parameters obtained in each genotype were used, including  $L_0$ ,  $L_p_r$ , *Azide\_inh*, *Ink\_inh*, *NaCl\_inh* and morphological data (**Figure 4**). The first principal component (PC1), which can account for approximately 53% of the total variation in the data set, is contributed positively by  $L_p_r$ , *Azide\_inh*, PRL, primary root number, and negatively by RSA, LR number, *Ink\_inh*, and *NaCl\_inh*. The second principal component (PC2; approximately 42% of total variation) is contributed positively by RDW and by  $L_0$ , and negatively by radicle length. Interestingly, PCA suggested no correlation between  $L_p_r$  with radicle length or RDW. It was suggested a positive correlation between *Azide\_inh* with primary root characteristics, as shown by their clustering, but no correlation with LR number. Conversely, it was suggested a positive correlation between *Ink\_inh* with the LR number and a negative correlation with primary root characteristics. PCA suggested a positive correlation between *NaCl\_inh* with LR number, and a negative correlation with primary root characteristics.

Pearson's correlation tests confirmed no correlation between  $L_p_r$  with radicle length or RDW with Pearson's correlation coefficient (PCC) values of 0.07 or -0.16, respectively (**Table 2**). It was confirmed a strong positive correlation between *Azide\_inh* with primary root number or PRL (PCC = 0.83 or 0.9, respectively), and no correlation with LR number (PCC = -0.35). Conversely, it was confirmed a strong negative correlation between *Ink\_inh* and PRL (PCC = -0.92). *NaCl\_inh* was confirmed weakly positively correlated with LR number (PCC = 0.81).



**Figure 4. Covariation of root hydraulics and root architecture in 4 genotypes.**

Mean values of root hydraulics ( $L_p$ ,  $L_0$ ,  $Azide\_inh$ ,  $Ink\_inh$ ,  $NaCl\_inh$ ) and root architecture (RDW, RSA,  $Radicle\_length$ , PRL,  $LR\_number$ ) obtained in 4 genotypes were used for Principal Component Analysis (PCA). The percentage in parentheses indicates the contribution of each component. The black points represent genotypes in parentheses. TC65, Taichung 65; *cr1*, crown rootless 1; NB, Nipponbare; *lrt2*, lateral rootless 2.

**Table 2. Correlation between root morphology and root hydraulics of wild-types and mutants.**

	RDW	RSA	$Radicle\_length$	$PR\_number$	PRL	$LR\_number$	$L_0$	$L_{pr}$	$Azide\_inh$	$Ink\_inh$	$NaCl\_inh$
RDW	1										
RSA	0.55	1									
$Radicle\_length$	-0.88	-0.71	1								
$PR\_number$	0.68	0.11	-0.77	1							
PRL	0.37	-0.30	-0.44	0.91	1						
$LR\_number$	0.60	0.95*	-0.62	-0.02	-0.43	1					
$L_0$	0.71	0.70	-0.96*	0.75	0.46	0.53	1				
$L_{pr}$	-0.16	-0.68	0.07	0.57	0.85	-0.82	0.03	1			
$Azide\_inh$	0.54	-0.35	-0.39	0.83	0.90	-0.35	0.29	0.68	1		
$Ink\_inh$	-0.06	0.65	0.05	-0.68	-0.92	0.74	-0.08	-0.96*	-0.85	1	
$NaCl\_inh$	0.03	0.84	-0.23	-0.41	-0.71	0.81	0.29	-0.82	-0.80	0.90	1

Values indicate the Pearson's coefficient of correlation. \*:  $p < 0.05$ .

RDW, Root dry weight ; RSA, root surface area ;  $Radicle\_length$ , radicle length ;  $PR\_number$ , number of primary roots ; PRL, primary root length ;  $LR\_number$ , number of lateral roots ;  $L_0$ , root hydraulic conductance ;  $L_{pr}$ , root hydraulic conductivity ;  $Azide\_inh$ , percentage of  $L_{pr}$  inhibition by azide ;  $Ink\_inh$ , percentage of  $L_{pr}$  reduction by China ink ;  $NaCl\_inh$ , percentage of  $L_{pr}$  reduction by NaCl.

Projection of the four genotypes on the first factorial (PC1/PC2) plane resulted in the clustering of the two wild-type genotypes, and the scattering of the two mutants. This and the analyses above allowed the identification of genotypes with marked differences. Both wild-type genotypes exhibit highest  $L_0$ , associated with equivalent contribution of aquaporin-dependent and apoplastic-dependent pathways. The mutant *lrt2* exhibited the highest  $L_{pr}$ , associated with a higher contribution of aquaporin-dependent pathway, whereas *cr1* was associated with a higher apoplastic-dependent pathway.

## Discussion

### *Root morphological characteristics*

Interestingly, a linear relationship between RSA and RDW was conserved in all 4 rice genotypes. The slope coefficient for NB, TC65, and *cr1* were similar and slightly distinct from *lrt2*, which led us to normalize  $L_{pr}$  values with RSA rather than RDW. Also, thanks to these calibration experiments providing a robust relationship between RDW and RSA, after each flow measurement, we measured RDW to extrapolate RSA for each individual root system and calculated  $L_{pr}$  value as  $L_0$  per surface unit. This prevented measuring individual RSA which is laborious. However caution should be aware when normalization by the entire root size is applied, since the hydraulic property may vary according to parts of the root system.

Although grown not exactly in the same conditions and not analysed at the same age, morphological characteristics of TC65 and *cr1* observed in this study are slightly similar to a previous study (Faiyue *et al.*, 2010b). For instance, using the number of LRs per PRL reported in the latter study, we calculated that *cr1* exhibited ~2.4-fold more LRs per PRL than its wild-type, a value not very different to the value of ~1.8-fold obtained in the present study. Considering NB and its mutant *lrt2*, we calculated the ratio between radicle root length and PRL and found ~0.2 and ~0.5 for both genotypes in the present study and in the previous study, respectively. Difference in the age of the plants might explain this difference, but most importantly, we found very similar ratio values for both genotypes in both studies, suggesting again consistent morphological characteristics between the present study and the previous one.

The mutant *cr1* had remarkably longer radicle root length but lower PRL and LR number, compared to its wild-type. The lower PRL in *cr1* may explain the significant higher number of LRs per PRL ( $p < 0.05$ ), and this suggested that the deleterious effects of the mutation were compensated by morphological alteration on the radicle. The mutant *cr1*

exhibited also a lower RDW and RSA compared to TC65, indicating that in this mutant, the lack of crown roots was not compensated by a sufficient development of the radicle and the lateral roots, in order to maintain a comparable root system. The mutant *lrt2* exhibited lower RDW and RSA compared to its wild-type whereas higher radicle root length and PRL, and similar number of primary roots. This suggested that the lack of LRs was not compensated by a sufficient development of the radicle and the crown roots to maintain a comparable root system.

### ***Root hydraulic properties***

$L_0$  of 4 genotypes varied by a factor of two-fold, from 0.83 to  $1.6 \times 10^{-10} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ . A similar reduction of  $L_0$  by ~36% for both mutants compared to their wild-types was observed. Transpiration rates of *crl1* and *lrt2* was reported to be 50%- and 61%-reduced compared to their respective wild-types, respectively (Faiyue *et al.*, 2010b). Under these detrimental hydraulics conditions reported in the previous and present studies, *crl1* and *lrt2* succeeded in surviving and could grow. An explanation might be that the mutants exhibited a greater water use efficiency compared to their wild-types (Faiyue *et al.*, 2010b). A major characteristic of *crl1* was a higher density of LRs on radicle; whereas *lrt2* had longer primary roots. These observations were interpreted as compensation by LR density if crown roots are lacking, and as compensation by PRL when LRs are lacking.

Comparison of the respective contributions of radicle and the crown roots on the overall  $L_0$  showed that crown roots contributed 63-67% of total conductance of a root system, and consistently,  $L_0$  of crown roots were found 1.8-2.7 higher than the radicle root. These results suggested a higher contribution of the crown roots compared to the radicle root to the overall transport capacity of the root system. In barley, at a stage of 14- to 17-day-old plants, the authors reported that radicle roots contributed 92% and the crown roots contributed only 8% to plant water uptake (Knipfer and Fricke, 2011). A marked difference in root architecture system could account for this difference between rice and barley, since barley exhibits six to seven radicle roots and about two crown roots, with LRs only on radicle roots, whereas 21 DAG rice exhibits a single, 5-6 crown roots and LRs on all primary roots

$L_{pr}$  of 4 genotypes varied from about 9 to  $15 \times 10^{-8} \text{ m}^3 \text{ s}^{-1} \text{ MPa}^{-1}$ . These observations were in the same range as many other publications on rice (**Table 3**). Indeed,  $L_{pr}$  were similar between rice varieties and between growth conditions (hydroponic, aeroponic, soil culture). Compared to other plant species,  $L_{pr}$  in rice is in the same order of magnitude as in wheat

( $\sim 6 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Bramley *et al.*, 2009)), *Arabidopsis thaliana* ( $\sim 5 \times 10^{-8} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Sutka *et al.*, 2011)), lupin ( $\sim 1 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Bramley *et al.*, 2009)), and lower than maize or common bean ( $\sim 2\text{-}3 \times 10^{-7} \text{ m s}^{-1} \text{ MPa}^{-1}$ ; (Miyamoto *et al.*, 2001)).

**Table 3. Root hydraulic conductivities ( $L_p$ ) of rice varieties**

Rice genotype	$L_p$ ( $\times 10^{-8} \text{ m}^3 \text{ m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ )	Age	Growth condition	Reference
Azucena	$5.95 \pm 0.9$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
Azucena	$2.8 \pm 1.3$	31-40 D	Hydroponic	(Ranathunge <i>et al.</i> , 2003)
Azucena	$6.3 \pm 3.1$	31-40 D	Hydroponic	(Miyamoto <i>et al.</i> , 2001)
Moroberekan	$5.23 \pm 0.6$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
FR13A	$6.03 \pm 0.8$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
Dular	$3.16 \pm 0.3$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
IR64	$3.78 \pm 0.4$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
IR64	$4.0 \pm 1.7$	31-40 D	Hydroponic	(Ranathunge <i>et al.</i> , 2003)
IR64	$5.6 \pm 2.7$	31-40 D	Hydroponic	(Miyamoto <i>et al.</i> , 2001)
Swarna	$3.28 \pm 0.6$	29 DAS	Soil	(Grondin <i>et al.</i> , 2016b)
Akitachomachi	$20.6 \pm 1.6$	18-22 D	Hydroponic	(Murai-Hatano <i>et al.</i> , 2008)
Pokkali	$29.8 \pm 7.4$	30-31 D	Hydroponic	(Krishnamurthy <i>et al.</i> , 2011)
IR20	$42.5 \pm 9.2$	30-31 D	Hydroponic	(Krishnamurthy <i>et al.</i> , 2011)
Taichung 65	$9.87 \pm 0.53$	21 DAG	Hydroponic	Present study
<i>crl1</i>	$9.02 \pm 0.60$	21 DAG	Hydroponic	Present study
Nipponbare	$11.43 \pm 0.41$	21 DAG	Hydroponic	Present study
<i>lrt2</i>	$15.02 \pm 0.68$	21 DAG	Hydroponic	Present study

D, Days; DAS, Days after sowing; DAG, Days after germination.

We observed that *crl1* and its wild-type had not significantly different  $L_p$  values. Importantly, since  $L_p$  in *lrt2* reflects the intrinsic hydraulics of primary roots, its higher value compared to the wild-type NB strongly suggested that absence of LR development in the *lrt2* mutant stimulated  $L_p$ . A complementary explanation would be that primary roots have a higher intrinsic water transport capacity compared to LRs. The consequence of this assumption is a lower aquaporin activity and/or a lower apoplastic pathway in LRs.

Azide treatment reduced  $L_p$  by  $\sim 40\text{-}60\%$ . A similar range of inhibition was observed for other rice cultivars (Grondin *et al.*, 2016a). *Azide\_inh* was higher in *lrt2* compared with NB. This suggested a higher contribution of cell-to-cell pathway in primary roots than in LRs, and conversely, a lower contribution of cell-to-cell pathway in LRs than in primary roots. However, it is difficult to explain their marked difference in  $L_p$  values by the only contribution of aquaporin activity. This prompted us to consider apoplastic pathway in the overall root system intrinsic water transport capacity.

Insoluble salt precipitates of copper-ferrocyanide were used to block apoplastic pathway, and provoked a three- to four-fold reduction of  $L_p$  in rice root system (Ranathunge *et al.*, 2005). However, copper has an inhibitory effect on water transport activity of Aquaporin-3 expressed in mammalian tissues (Zelenina *et al.*, 2004), and potentially has the

same effect on plant PIPs. Interestingly, water uptake in rice root was reduced by ~30% using China ink particles as an apoplastic blocker (Ranathunge *et al.*, 2004). Here, we tested China ink suspension in the same conditions as (Ranathunge *et al.*, 2004) and not salt precipitates of copper-ferrocyanide to avoid any inhibitory effect on aquaporins. To make sure of removing any traces of undesired molecules which could have an inhibitory effect on aquaporins, China ink suspension was dialysed against nutrient solution.

Our results suggested that the contribution of the apoplastic pathway is the highest in *crl1*, similar between the wild-types TC65 and NB, and the lowest in *lrt2*, since *Ink\_inh* was ~60%, ~44% and ~16%, respectively. The lower value observed in *lrt2* mutant was surprising due to the lesser development of suberin lamellae in exodermis and endodermis reported previously (Faiyue *et al.*, 2010b). However, as discussed in the same study, the authors stressed in the nature of suberin (aliphatic vs aromatic) rather than on its abundance to explain its function as an apoplastic barrier. In the mutant *lrt2*, we observed that the *Azide\_inh* was ~3.5 fold higher than *Ink\_inh*. At variance to the three other genotypes where *Azide\_inh* and *Ink\_inh* could account for the overall inhibition of the root system, in *lrt2* the treatments with the aquaporin inhibitor and the apoplastic blocker could account for ~75% of the overall inhibition. Although a clear explanation is missing, the absence of LR development in *lrt2* might explain this observation. The lowest contribution of apoplastic pathway in *lrt2* led to the interpretation of a lower contribution of apoplastic pathway in primary roots than in LRs, and conversely a higher contribution of apoplastic pathway in LRs than in primary roots. Based on these data, we estimated that the cell-to-cell pathway (aquaporins) contributes ~3.5 fold more than apoplastic pathway in the primary root intrinsic water transport capacity. Importantly, this result is consistent with *Azide\_inh* and support the hydraulic model that primary roots exhibited higher aquaporin activity (lower apoplastic pathway) than LRs, and conversely that LRs exhibited higher apoplastic pathway (lower aquaporin activity) than primary roots.

Proteins of several aquaporin isoforms were reported to be highly accumulated at 4 mm from the root apex (Sakurai *et al.*, 2008). Although much lower accumulation was observed in a more mature region, *OsPIP2;1* and *OsPIP2;5* exhibited an expression in the endodermis (Sakurai-Ishikawa *et al.*, 2011). Moreover, a larger contribution of apoplastic compared to cell-to-cell pathway was previously reported in the outer part of the primary roots (Ranathunge *et al.*, 2004). Our results suggesting that aquaporins are contributing more than apoplastic pathway in primary roots led us to the interpretation that *OsPIP2;1* and *OsPIP2;5* isoforms have a predominant role in the overall water transport capacity



within tissues of the inner part of the root (endodermis and stele). Fine analysis of expression at root tissue level is needed to identify clearly the whole set of PIPs expressed in the inner part of the root. Nevertheless, the whole set of data identified *OsPIP2;1* and *OsPIP2;5* as key players of root hydraulics and hypothesised for this isoform a regulatory role when root is challenged by environmental constraints. Indeed, several post-translational regulations of aquaporins have been uncovered in plant species except for rice (Maurel *et al.*, 2015). For instance, redistribution of PIPs from plasma membrane to intracellular compartments upon salt or oxidative stress is a common mechanism (Boursiac *et al.*, 2005a; Wudick *et al.*, 2015). The existence of such a regulatory mechanism for *OsPIP2;1* and *OsPIP2;5* in the endodermis is an open question.

### ***Effects of salt stress on the root hydraulics***

Under salinity, plants have to cope with not only osmotic stress but also ionic stress. Osmotic stress comes immediately with a high level of salt outside the roots, which causes an inhibition of water uptake, even a water efflux can occur under severe stress (Horie *et al.*, 2012). Reduction of  $L_{pr}$  under salt stress was also reported in other plant species, such as *Arabidopsis* (Boursiac *et al.*, 2005a), and barley (Horie *et al.*, 2011). The  $L_{pr}$  reduction assumed a shutdown of water transport to minimize water loss (Horie *et al.*, 2011).

In the present study, *NaCl\_inh* was ~2-fold higher in the wild-type NB compared with its *lrt2* mutant. A possible explanation is presented as the following. Ionic toxicity develops afterwards by excess accumulation of  $Na^+$  in plant cells, leading to a decrease of cell metabolisms (Horie *et al.*, 2012). By which way  $Na^+$  enters the cells of plant in general and of rice in particular is still a controversy issue. It is believed that abundant amount of  $Na^+$  was uptaken in rice by apoplastic pathway (Yeo *et al.*, 1987; Krishnamurthy *et al.*, 2009, 2011). The precise entry of  $Na^+$  was suggested through LR emerging sites and root tip regions where the Casparian strips and suberin lamellae are disrupted or immature, respectively (Miyamoto *et al.*, 2001; Ranathunge *et al.*, 2003, 2004, Krishnamurthy *et al.*, 2009, 2011). However, experiments using silicate dramatically decreased transpirational apoplastic flow in rice roots; deposition of silicon as insoluble silica in the cell wall in the regions of the endodermis and in the outer parts of the rice root system prevented  $Na^+$  uptake (Gong *et al.*, 2006). Moreover, by using *crl1* and *lrt* rice mutants and their respective wild-types, it was interpreted that apoplastic uptake of  $Na^+$  in rice is not at the sites of LR emergence, but rather through the cortical layers of LRs ((Faiyue *et al.*, 2010b,a). Moreover, higher



apoplastic pathway was suggested in a rice cultivar with higher LR number (Meng and Fricke, 2017). In conclusion, we believed that the higher inhibition of NaCl in NB compared to its *lrt* mutant is explained by a lower Na<sup>+</sup> (apoplastic) uptake capacity of the latter genotype, provoking a lower inhibitory effect on aquaporin activity.

### ***Towards a global understanding of root hydraulics strategies in rice***

Several authors successfully used PCA as a tool to have a global view of the root hydraulic strategies developed by various genotypes (Sutka *et al.*, 2011; Grondin *et al.*, 2016a). The present analysis revealed that  $L_{pr}$  showed no correlation with radicle length or RDW or  $L_0$  showed, confirming for instance that the intrinsic water transport capacity is independent of a morphological parameter such as RDW. On the contrary,  $L_{pr}$  showed a positive correlation with *Azide\_inh*, as previously reported, suggesting increasing contribution of aquaporins when intrinsic water transport capacity increases. Conversely and consistently, a negative correlation between  $L_{pr}$  and *Ink\_inh* was found, suggesting a decreasing contribution of apoplastic pathway when intrinsic water transport capacity increases.

*Azide\_inh* and *Ink\_inh* showed high positive correlation with primary root and LR characteristics, respectively. These observations led us to the interpretation that primary roots exhibited a higher aquaporin activity (cell-to-cell pathway), whereas in the LRs the apoplastic pathway exhibited a major contribution, confirming by a statistical-analysis way our previous assumption.

LR and primary root characteristics were positively and negatively correlated with *NaCl\_inh*, respectively. This led us to the interpretation that salt stress inhibitory effect on water transport activity is stronger when LR characteristics increase. Here again, the statistical-analysis tool confirm our former assumption on the role of LRs in the uptake of Na<sup>+</sup>.

## **Conclusion**

Root system is responsible for water uptake and thus controls the whole physiology of the plant. Root hydraulics for each part of the root system has been shown not uniform and, in the opposite, exhibits high contrasting behaviour. Indeed, our results suggest a higher contribution of cell-to-cell pathway in primary roots than in LRs, and conversely, a lower contribution of cell-to-cell pathway in LRs than in primary roots. LR development could

also contribute to root hydraulics. This underlines the importance of root system architecture with root anatomy, and aquaporin activity in the overall root hydraulics. The large genetic diversity of rice varieties implies a variability in root system architecture, anatomy, aquaporin activity, and consequently in root hydraulics. This indicates a potential for manipulation of root hydraulics for improvement of rice not only in well-watered conditions but also under abiotic stresses such as soil salinization.

## **Materials and methods**

### ***Plant materials and growth conditions***

Seeds of *crl*, *lrt2* and their respective wild-types TC65 and NB were soaked in sufficient deionized water in the dark, for two days to germinate, then seedlings were continued to grow in water for a week. Then, uniform seedlings were cultured hydroponically in modified Yoshida's solution (Yoshida *et al.*, 1971) containing 0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.6 mM MgSO<sub>4</sub>, 1.2 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.7 mM KNO<sub>3</sub>, 60 μM FeSO<sub>4</sub>, 20 μM MnSO<sub>4</sub>, 0.32 μM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 1.4 μM ZnSO<sub>4</sub>, 1.6 μM CuSO<sub>4</sub>, 45.2 μM H<sub>3</sub>BO<sub>3</sub> and 0.8 mM KH<sub>2</sub>PO<sub>4</sub>; solution was adjusted at pH 5-5.5. Chemicals are from Sigma-Aldrich (MO, US). About twenty seedlings were hold in a Styrofoam plate with root system bathed in 2.5 L of modified Yoshida's solution. Conditions of the growth chamber were 14 h of day cycle (~ 200 μE m<sup>-2</sup> sec<sup>-1</sup>) and 10 h of night at 28/25°C and 70% relative humidity.

### ***Root phenotyping***

Root systems of plantlets at the age of 7, 10, 13 and 21 or 23 days after germination (DAG) were collected for root phenotyping. Root systems were laid out on a tray and submerged with water, contrasted with a black background, scanned using Epson Perfection V700 scanner (Suwa, Japan) at 600 dpi. The root surface areas (RSA) were quantified using ImageJ software (Rasband W., NIH, USA) from the projected areas of roots that were assumed to be cylindrical in shape. Afterwards, root systems were dried in an oven at 60°C for 4 days and weighted to obtain the root dry weight (RDW). Scanning root systems of 21 DAG were analyzed in deeper details to obtain the number of primary roots and number of lateral roots, total primary root length and radicle length.

### ***Root hydraulic property measurements***

Root hydraulic conductance ( $L_0$ ) and conductivity ( $L_{pr}$ ) of rice root systems were measured by means of pressure chambers as described elsewhere (Javot *et al.*, 2003; Boursiac *et al.*, 2005b). Briefly, the primary roots of 21 DAG rice plants were cross-sectioned at their base, and then inserted into a hermetic pressure chamber with the roots bathing into modified Yoshida's solution, while the cross sections were connected to a flow detector by an adapter and silicon tube. Hydrostatic pressure was applied to the chamber by nitrogen gas. At first, chambers were pressurized at 360 kPa for 10 min to equilibrate. Next, three different pressures of 320, 160 and 240 kPa were applied.  $L_0$  was determined according to the flow which was measured and the hydrostatic pressure applied.  $L_{pr}$  was calculated as  $L_0/RSA$ . For azide treatments, modified Yoshida's solution was supplemented with 4 mM  $NaN_3$  and a pressure of 320 kPa was applied until stable flow occurred. For China ink treatment, we followed indications of a previous study (Ranathunge *et al.*, 2004). Briefly, China ink (Rotring, Germany) was diluted 1:1 with modified Yoshida's solution then dialyzed against the nutrient solution (ratio 1:20) through a dialysis membrane with a molecular weight cut-off of 6-8000 (Spectrum Laboratories, CA, US) for 2 days, and with solution replacement twice a day. Afterwards, the dialyzed China ink solution was used to bath root systems and a pressure of 320 kPa was applied until stable flow occurred. For salt treatments, root systems of entire plants were exposed to modified Yoshida's solution supplemented with 100 mM NaCl for 30 min. Primary roots were cross-sectioned at their base, and inserted in the pressure chambers. Hydrostatic pressures at 700, 400, 600 kPa were applied and the rate of sap flow were determined.

### **Statistical analysis**

Data presented indicate the means  $\pm$  SE. Significantly different levels were performed by one way ANOVA test followed by Least Significant Difference (LSD) test at confident level of 0.95 using R version 3.3.2 software (R Development Core Team, 2005). Principal component analysis (PCA) and correlation analyses were also performed using R software.

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## **Authors' contributions**

TTHC and DTL designed experiments, carried out the experiments, interpreted the results, and wrote the paper. DCT designed experiments, carried out the experiments, and interpreted the results. CM designed experiments, interpreted the results and wrote the paper.

## **Competing interests**

The authors declare that they have no competing interests.

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## General conclusion and perspectives

So far, we successfully generated 8 transgenic rice lines based on Nipponbare cultivar overexpressing a single protein in fusion with a fluorescent marker; among them, 3 plasma membrane AQPs (*OsPIP1;1*, *OsPIP2;4*, *OsPIP2;5*) fused with GFP, 2 tonoplast AQPs (*OsTIP1;1* and *OsTIP2;2*) fused with mCherry, and 3 endosomal trafficking proteins (*OsRab5a*, *OsGAP1*, *OsSCAMP1*) fused with mCherry. Stable transformations of these constructs were also carried out in Arabidopsis. The subcellular localization of each transgene was observed by means of laser scanning confocal microscope (LSCM). *OsPIP* isoforms showed typical homogeneous labelling of the plasma membrane in both rice and Arabidopsis. *OsTIP* isoforms were observed localized in the tonoplast with a typical labelling of intracellular invaginations that skirted the nucleus in rice but remained in the endoplasmic reticulum in Arabidopsis. Three endosomal trafficking proteins were observed in cytoplasm as expected in both rice and Arabidopsis, except *OsSCAMP1* which remained in the endoplasmic reticulum of Arabidopsis root cells. Furthermore, localizations of plasma membrane AQPs were visualized in deeper rice root tissues by combining ClearSee as clearing solution and Multiphoton as visualizing equipment. The behaviors of plasma membrane AQPs were tested in salt and drought stress-mimicked-conditions. The results suggested a relocation of plasma membrane rice AQPs upon abiotic stress; therefore, a contribution of AQPs in stress response was hypothesized. We also found that salinity enhanced endocytosis process of plasma membrane AQP (*OsPIP2;5*). Since these phenotypes were also observed in Arabidopsis, a dicot model plant, we propose that they represent conserved adaptive mechanisms upon abiotic environmental stress. These results were formatted into a manuscript and submitted for publication in Rice journal. Since these observations were made on over-expressing transgenic plants, an interesting perspective would be to address the relocation with constructs under native-promoter-driven expression. In this perspective, we had already generated a set of transgenic rice expressing either *OsPIP1;1*, *OsPIP2;4* or *OsPIP2;5* in a fusion with GFP under the control of their native promoters.

Next, we tested the effects of the overexpression of these AQPs and endosomal trafficking proteins upon drought and salt stress conditions at reproductive stage. Overexpressing such transgenes seemed not to affect the plant morphology and showed no beneficial effect for grain yield in control condition and even made rice transgenic plants more sensitive to stresses. Because overexpressing AQPs at a low level was reported to



increase seed production, studying regulatory factors which control AQP expression and functional activity could be more relevant.

Though our set of subcellular markers may not positively contribute to breeding projects, they are still a worthy resource for cell biology approaches on a large array of topics.

Because of the root system is the organ in charge of water uptake for the whole plant, we pushed more focus on the contribution of AQPs in rice root water transport in link with root morphology. We characterized morphological and hydraulic properties of two mutants altered in root system architecture (*crl1*, *crown rootless 1* and *lrt2*, *lateral rootless 2*) in comparison with their wild-types (Taichung 65 and Nipponbare, respectively). Intrinsic water transport capacity (so-called conductivity,  $L_p$ ) of primary roots has been found higher than lateral roots (LRs). Use of AQP inhibitor and apoplastic pathway (so-called bypass flow) blocker led to the interpretation of a higher contribution of cell-to-cell pathway in primary roots than in LRs, and conversely, a higher contribution of apoplastic pathway in LRs than in primary roots. Based on these data, we estimated that the cell-to-cell (AQP-dependent) pathway contributes ~3.5 fold more than apoplastic pathway in water transport capacity of the primary root. In all cases, AQPs always contribute a relative high percentage of water transport (44-58%). Quantifying the level of expression of AQPs at protein level in each genotype and root type by western blot or ELISA techniques is a perspective in order to interpret the contribution of these proteins. On the other hand, the set of transgenic rice expressing either *OsPIP1;1*, *OsPIP2;4* or *OsPIP2;5* in a fusion with GFP under the control of their native promoters would be of interest, since acquiring the localization and level of expression of AQPs by observation of fluorescent signals in these transgenic lines will be a means to firmly understand the involvement of AQP in rice root water transport. We are preparing to format these results into a manuscript and submit for a publication.