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Comparative Study on Growth Performance of Transgenic (Over-Expressed *OsNHX1*) and Wild-Type Nipponbare under Different Salinity Regimes

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Abstract: Transgenic Nipponbare which over-expressed a Na^+/H^+ antiporter gene *OsNHX1* was used to compare its growth performance, water status and photosynthetic efficiency with its wild type under varying salinity regimes. Chlorophyll content, quantum yield and photosynthetic rate were measured to assess the impact of salinity stress on photosynthetic efficiency for transgenic and wild-type Nipponbare. Effects of salinity on water status and gas exchange to both lines were studied by measuring water use efficiency, instantaneous transpiration rate and stomatal conductance. Dry shoot weight and leaf area were determined after three months of growth to assess the impacts of salinity on the growth of those two lines. Our study showed that both lines were affected by salinity stress, however, the transgenic line showed higher photosynthetic efficiency, better utilization of water, and better growth due to low transpiration rate and stomatal conductance. Reduction of photosynthetic efficiency exhibited by the wild-type Nipponbare was correlated to its poor growth under salinity stress.

Key words: growth performance; salinity stress; Na^+/H^+ antiporter gene *OsNHX1*; transgenic rice; photosynthetic efficiency; water status

Salinity is one of the major environmental stress that can affect photosynthetic performance, growth and yield of rice. Changes in physiology in any plants caused by salt stress may affect overall growth of the plant which eventually cause low yield and thus reduce rice production as a whole (Aslam et al, 1993; Chowdhury et al, 1995; Sohn et al, 2005; Khan and Panda, 2008; Cha-um et al, 2009).

Increasing sea levels caused by global warming and frequent flooding which lead to high tide would cause an increase in soil salinity as happened in Mekong Delta (Ozaki et al, 2014). In Tanjong Maya (Tutong District, 4°45′50″ N, 114°39′6″ E), due to seepage of saline water, the whole paddy cultivation area is not suitable for growing rice and the land has been now converted to Rumbia (*Metroxylon sago*) tree cultivation (Yunos, 2010).

One possible way to make those lands viable for rice cultivation is introduction of rice varieties with tolerance to salinity. Salt-tolerant rice can be produced using biotechnology by expressing a Na^+/H^+ antiporter gene *OsNHX1* into salt-sensitive rice varieties (Fukuda et al, 2004). Vacuolar Na^+/H^+ antiporters are thought to be responsible for salt tolerance in plants (Blumwald et al, 2000; Fukuda et al, 2004). Blumwald et al (2000) confirmed that the transfer of Na^+ from cytosol or cytoplasm into vacuoles is driven by Na^+/H^+ antiporter using electrochemical gradient of protons generated by vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-PPiase. Salt-tolerant species tend to accumulate large amount of Na^+ in the vacuoles (Bjorkman and Demmig, 1987; Maxwell and Johnson, 2000). This eventually allows more water to be driven into cell, thus increasing salinity tolerance in rice. By

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expressing this antiporter gene into salt-sensitive rice variety, the transgenic rice will show better tolerance to salinity resembling the salt-tolerant rice variety.

According to Fukuda et al (2004) and Islam and Seraj (2009), the expression of *OsNHX1* is controlled by salt stress and exposing rice plants with high salt level will increase transcript level of *OsNHX1* in rice roots and shoots. With frequent transcription of this gene, the expression of Na^+/H^+ antiporter gene will increase and eventually improve rice salt tolerance.

Oryza sativa L. cv. Nipponbare is a temperate japonica rice variety which is known to be sensitive to salt stress. Growth of this variety is inhibited when exposed to high salinity stress. However, the salinity tolerance of the transgenic Nipponbare is improved after introduction of *OsNHX1* into its genome (Fukuda et al, 2004). The transgenic Bangladeshi rice variety Binnatoa with *OsNHX1* produced by Islam et al (2009) also showed significantly higher yield compared to its wild type.

Chen et al (2007) and Faiyue (2011) reported that the transgenic rice line of *O. sativa* L. cv. IRAT109 showed improvement in salinity tolerance after introducing *OsNHX1*. Damage or death appearance caused by salinity stress was delayed in this line. The control plants of the wild type gradually wilted in 4 d when exposed to 200 mmol/L NaCl and eventually died after one week. In comparison, the transgenic line wilted 3–4 d after the control wilted and was able to survive for another two weeks. The osmotic potentials of transgenic plants were lower than those of the control, inferring that the transgenic lines had absorbed more Na^+ in their vacuoles, which would allow the transgenic lines to absorb more water from its surrounding.

Overall, transgenic rice which over-expressed Na^+/H^+ antiporter gene shows improvements in salinity tolerance than its wild type. This may help to increase yield of salt-sensitive rice varieties even when experiencing salinity stress. In this investigation, we compared growth performance, photosynthetic efficiency and water status between transgenic (over-expressed antiporter gene *OsNHX1*) and wild-type Nipponbare grown under different salinity regimes to evaluate the best conditions under which the variety could be cultivated in Brunei Darussalam.

MATERIALS AND METHODS

Salinity treatments of plants in soil

Transgenic (over-expressed *OsNHX1*) and wild-type Nipponbare plants were grown in pots (16 cm in height and 20 cm in diameter) for three months at greenhouse (Universiti Brunei Darussalam) with an average of 286 μmol/(m^2·s) light intensity, 66% relative humidity and 32.6 °C environmental temperature. Each pot comprised of five plants (transgenic or wild-type Nipponbare) was subjected to salinity treatments. Three pots were prepared for each salinity regimes of 0 (control), 50, 150 and 300 mmol/L. Randomized block design described by Cha-um et al (2009) was employed (uniform microhabitat conditions were provided for all pots). Twice a week, 500 mL the saline solution (0, 50, 150 or 300 mmol/L) and 50 mL half strength Hoagland's solution (Taiz and Zeiger, 2002) were supplied to respective pot. The seedlings were planted till the maturity stage starting from October to December 2013. Gathering of physiological measurements (photosynthetic efficiency, leaf gas exchange and water status) and growth data were commenced 3 d after the first salinity treatment and continued for two months at a 4 d interval (Zhao et al, 2006a).

Chlorophyll fluorescence and chlorophyll content measurements

Chlorophyll content was measured twice a week for two months using a chlorophyll content meter-200 (Opti-Sciences, Tyngsboro, Massachusetts, USA). Measurement was taken from the same youngest fully extended leaf following the method described by Wankhade et al (2013). This parameter was measured six times from the leaf tip to base, and the average was used for assessing the chlorophyll content in the leaf sample (Jamil et al, 2014).

Quantum yield (Fv/Fm) was measured using Fluorpen FP100 (Photon Systems Instruments, Brno, Czech Republic). Measurements were taken from the same leaf as for chlorophyll content measurement at three different locations (at the leaf base, middle and near the leaf tip). Prior to the measurement, the leaf was dark-adapted with aluminium foil for 30 min (Wankhade et al, 2013).

Leaf gas exchange and water status

Stomatal conductance, transpiration rate and photosynthetic rate were measured using a LI-6400XT Portable Photosynthesis System with light emitting diode chamber (LI-COR Inc, Lincoln, Nebraska, USA) in three plants per pot for each line and salinity regime. These measurements were taken in the morning till midday (8:00 am to 2:00 pm) from the youngest fully

extended leaf. Initial carbon dioxide concentration within leaf chamber was adjusted to 350 μmol/L before each measurement. Light intensity was maintained during measurement at approximately 500 μmol/(m^2·s) photosynthetically active radiation following Wankhade et al (2013). All measurements were taken at a constant air flow rate of 500 μmol/s and the temperature was maintained at (25 ± 2) °C as described by Yu et al (2013). Water use efficiency (WUE) was calculated as photosynthesis/transpiration ratio by referring to Yu et al (2013).

Growth measurement and yield performance

After three months of experiment, dry shoot weight and leaf area of the whole plant were measured. Leaf area was measured using a LiCor-3000A portable area meter and a LiCor-3050 belt conveyer (LI-COR Inc, Lincoln, Nebraska, USA).

After harvesting, topsoil of the extracted roots was flushed away using tap water and later dried with tissue paper. Dry weights were taken after plants were dried at 70 °C up to a constant weight (Chen et al, 2014).

Analysis of transgenic line

Transgenic status was confirmed by performing PCR reaction using primer 5′-GCTGGATTGCTCAGTGC ATA-3′ (forward-679) and 5′-AAGGCTCAGAGGT GACAGGA-3′ (reverse-679) following the method described by Islam et al (2009). The PCR condition was as followed: Initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2.3 min and final extension at 72 °C for 7 min. Agarose gel electrophoresis (1.5%) was then followed to observe the expected band at 679 bp.

The PCR product was then sent to FirstBase for sequencing. PCR sequences were analyzed, edited and similarity searched using Sequencher 5.1 Demo (Gene Codes Corporation, Ann Arbor, MI) and NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) according to da Silva et al (2004) and Wang et al (2006).

Confirmation of transgenic Nipponbare (over-expressed *OsNHX1*)

After PCR analysis, band at about 700 bp was observed in all transgenic lines. No band at 700 bp was found in wild-type Nipponbare (lanes N1 and N2, for purified and unpurified, respectively) (Fig. 1). By performing BLAST to the sequence result for the purified PCR sample, it was further confirmed that the transgenic Nipponbare used did possess antiporter gene *OsNHX1*.

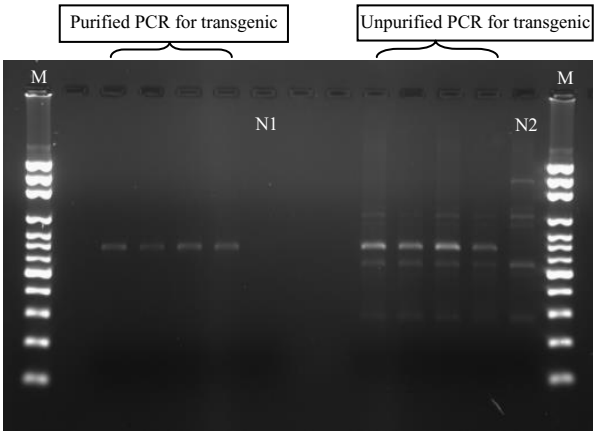

Fig. 1. PCR amplification of *OsNHX1* in transgenic and wild-type Nipponbare.

M, Marker; N1, Purified wild-type Nipponbare; N2, Unpurified wild-type Nipponbare.

Statistical analysis

Bar graph was used to present the data of growth performance parameters against different salinity regimes at reproductive stage for both transgenic rice and its wild type. The Tukey test of two-way analysis of variance (if data was normally distributed) was used to test the following hypothesis:

H_0: No significant difference in parameter studied (quantum yield for instance) between both transgenic and wild-type Nipponbare;

H_1: There is significant difference in parameter studied (quantum yield for instance) between both transgenic and wild-type Nipponbare;

H_0': There is no significant effect of salinity to parameter for transgenic or wild-type Nipponbare;

H_1': There is significant effect of salinity to parameter for transgenic or wild-type Nipponbare.

RESULTS

Effect of salinity stress on photosynthetic efficiency of transgenic and wild-type Nipponbare

Quantum yield values for wild-type Nipponbare (N) and transgenic Nipponbare (TN) are significantly reduced as salinity regime increased from 50 to 300 mmol/L and from 150 to 300 mmol/L, respectively (Fig. 2). For all salinity levels studied, the transgenic Nipponbare showed higher quantum yields when

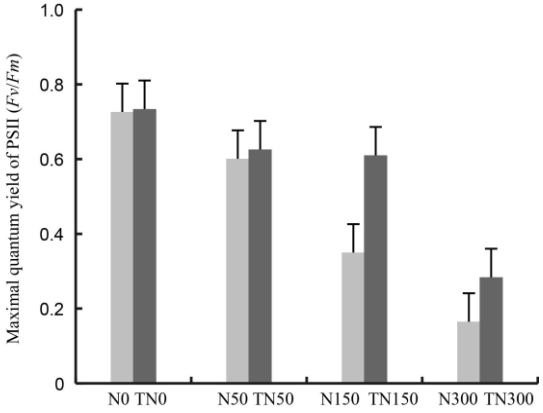

Fig. 2. Effect of salinity stress on maximal quantum yield of PSII (Photosystem II) for both transgenic and wild-type Nipponbare.

N, Wild-type Nipponbare; TN, Transgenic Nipponbare; 0, 50, 150 and 300 refer to 0, 50, 150 and 300 mmol/L salinity regimes, respectively.

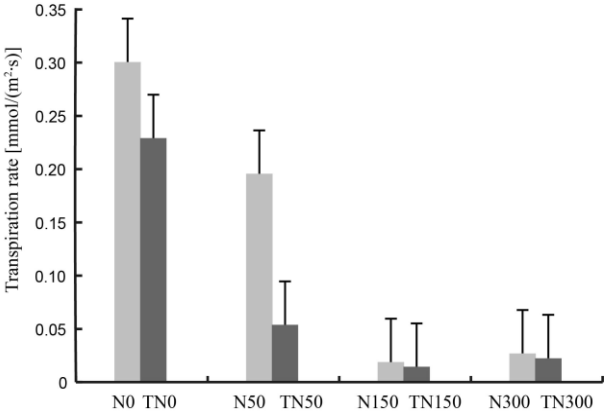

Fig. 4. Transpiration rate for both transgenic and wild-type Nipponbare under different salinity regimes.

N, Wild-type Nipponbare; TN, Transgenic Nipponbare; 0, 50, 150 and 300 refer to 0, 50, 150 and 300 mmol/L salinity regimes, respectively.

compared to the wild-type. For the transgenic line, there was no significant difference ($P > 0.05$) between TN0-TN150 and N0-N150.

There was no significant different in chlorophyll content between transgenic or wild-type ($P > 0.05$), and both transgenic and wild-type Nipponbare showed reduction in chlorophyll content as stress increased (Fig. 3). However, the transgenic Nipponbare showed slightly higher chlorophyll content compared to the wild-type Nipponbare.

Photosynthetic rates of both transgenic and wild-type Nipponbare showed significant reduction at very high salinity regime in 150 and 300 mmol/L ($P < 0.05$). There were no significant differences in photosynthetic rate between transgenic or wild-type as both showed almost similar values in all salinity regimes investigated. Nonetheless, the photosynthetic rate of the transgenic line at 300 mmol/L was slightly higher [9.52 μmol/(m^2·s)] than that of the wild-type [9.05 μmol/(m^2·s)].

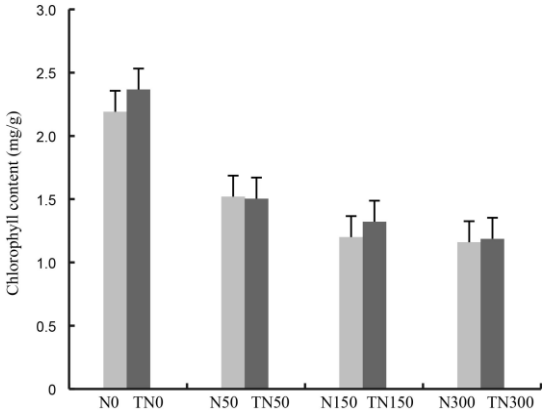

Fig. 3. Chlorophyll content meter of transgenic and wild-type Nipponbare under varying salinity regimes.

N, Wild-type Nipponbare; TN, Transgenic Nipponbare; 0, 50, 150 and 300 refer to 0, 50, 150 and 300 mmol/L salinity regimes, respectively.

Effect of salinity stress on transpiration rate, WUE and stomatal conductance of transgenic and wild-type Nipponbare

Fig. 4 shows that the transpiration rate significantly decreased ($P < 0.05$) in both transgenic and wild-type Nippobare with increasing salinity regimes. Transpiration rate for wild-type Nipponbare in all salinity regimes were higher when compared to the transgenic.

Transgenic lines showed lower stomatal conductance when compared to the wild-type in all salinity regimes except 150 mmol/L. The transgenic line showed less percentage reduction in conductance (34.8%) while its wild type showed 51.4% reduction as stress increased.

The transgenic lines maintained almost equal rates of carbon dioxide uptake, 0.024 mmol/(m^2·s) at control and 0.015 mmol/(m^2·s) at 300 mmol/L salinity regimes, respectively. The stomatal conductance of wild-type Nipponbare between control [0.037 mmol/(m^2·s)] and 300 mmol/L salinity regime [0.020 mmol/(m^2·s)] showed higher reduction. WUE in transgenic line showed more improvements than its wild-type.

Effect of salinity stress on growth performance of transgenic and wild-type Nipponbare

Dry weight of transgenic Nipponbare was higher than the wild-type in almost all salinity regimes, except 50 mmol/L (Fig. 5). Transgenic Nipponbare at 150 and 300 mmol/L (high salinity stress) showed higher dry shoot weight than wild-type Nipponbare at 0 mmol/L. The average leaf area for both transgenic and wild-type Nipponbare reduced as salinity stress increased.

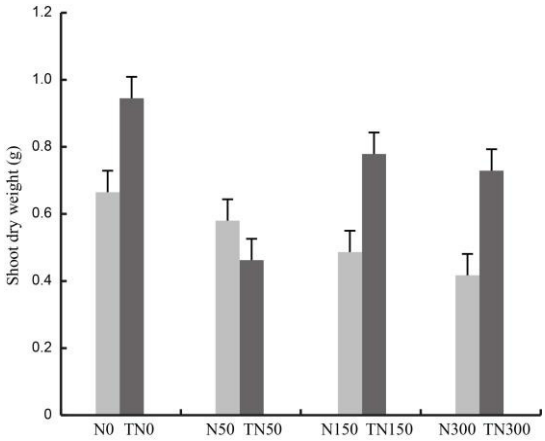

Fig. 5. Shoot dry weight for both transgenic and wild-type Nipponbare under different salinity regimes.

N, Wild-type Nipponbare; TN, Transgenic Nipponbare; 0, 50, 150 and 300 refer to 0, 50, 150 and 300 mmol/L salinity regimes, respectively.

DISCUSSION

Moradi and Ismail (2007) revealed that increase in salt stress can cause an indirect effect on the photosynthetic apparatus, thus leading to reduction in quantum yield of rice varieties. For all salinity regimes studied, the transgenic Nipponbare showed higher quantum yield when compared to the wild-type, which was in agreement to the result obtained by Zhao et al (2006a, b) and Oh et al (2005) for *O. sativa* L. cv. Nakdong and Zhonghua 11. This result can be attributed to the increasing accumulation of salt in leaves after prolong exposure to salt stress (Xu et al, 2015). No significant difference in quantum yield between TN0-TN150 and N0-N150 has proven that the transgenic line has managed to resist salinity stress better than the wild-type. This might indicate that the gene *OsNHX1* in the transgenic line helped to sequest Na^+ ions from the cytoplasm into vacuoles, thus protecting the photosynthesis apparatus located in the cytoplasm from Na^+ toxicity (Bao et al, 2014) that can lead to a higher quantum yield.

The highest quantum yield value recorded was 0.72 in the study, which is lower when compared to the suggested optimal value (0.83) reported by Bresson et al (2015). The lower value might have been caused due to the microhabitat conditions of our investigation, where the mean average temperature was 32.5 °C. According to Maxwell and Johnson (2000), high temperature alone can reduce quantum yield of any crops.

Decrease in chlorophyll content in leaves of both transgenic and wild-type Nippobare as salinity regime increase might be caused by increasing activity of the chlorophyll degrading enzyme or disruption of chloroplast structure and instability in pigment formation (Djanaguiraman and Ramadass, 2004; Senguttuvel et al, 2014).

Slightly higher chlorophyll content recorded for the transgenic Nipponbare compared to the wild-type was similar to transgenic Binnatoa reported by Islam and Seraj (2009). The transgenic Nipponbare might have experianced less chlorophyll degradation than the wild-type, meaning that the photosynthetic pigments in transgenic line were less affected by salinity stress and consequently showed a higher photosynthetic rate than the wild-type.

Significant reduction in photosynthetic rate at high salinity regime at 150 and 300 mmol/L for both transgenic and wild-type Nipponbare was in agreement to the results obtained by Zhao et al (2006a, b) for *O. sativa* L. cv. Zhonghua 11. Reduction in photosynthetic rate under salt stress may have been caused by direct effect of salt on stomatal resistance via reduction in guard cell turgor (Dionisio-Sese and Tobita, 2000; Moradi and Ismail, 2007), stomatal closure and decreased efficiency of Rubisco (Moradi and Ismail, 2007). Delfine et al (1998) and Senguttuvel et al (2014) reported that salinity affects photosynthesis by lowering carbon dioxide supply due to the closing of stomata or changes in the mesophyll cell structure.

The almost similar values in photosynthetic rate between transgenic and wild-type lines in all salinity regimes were contradicted with the findings of Zhao et al (2006a, b) and Liu et al (2010), which indicated that the wild-type lines showed more significant reduction of photosynthetic rate after exposure to high stress when compared to the transgenic. Nonetheless, the photosynthetic rate of the transgenic line at 300 mmol/L was slightly higher than the wild-type, which indicated salinity cause less impact on the photosynthetic apparatus of transgenic Nipponbare than the wild-type.

The significant decreased in the transpiration rate of both lines with increasing salinity regimes were in agreement with the results reported by Yu et al (2013). Reduced stomatal conductance might have caused low water diffusion from leaf, thus leading to low transpiration rates in transgenic and wild-type Nippobare. Decrease in transpiration rates of both lines along with increasing salinity stress can be related to lower water potential in roots and the transport of abscissic acid from root to shoot that induce stomatal closure (Zheng

et al, 2001; Moradi and Ismail, 2007). Higher reduction of water loss in transgenic Nipponbare might have caused by increasing stomatal closure in transgenic leaves that eventually prevent more water loss from the plant (Ueda et al, 2013).

Higher transpiration rate for wild-type Nipponbare in all salinity regimes than the transgenic lines indicated that the wild-type Nipponbare tend to lose more water than the transgenic. High transpiration rate can lead to plant wilting and eventual death. However, low transpiration rate of transgenic line is also disadvantageous as nutrient uptake will be limited. Nonetheless, high loss of water is more critical as nutrient uptake can still be happening via leaf (Voogt et al, 2013). According to Yu et al (2013), low transpiration rates lead to enhanced photosynthesis and improved WUE in transgenic line, which inturn cause the plant grow well when compared to plants that show high transpiration rates similar to wild-type Nipponbare.

Lower stomatal conductance in transgenic lines when compared to the wild-type in all salinity levels except 150 mmol/L was also similar to the findings of Yu et al (2013). Low stomatal conductance in transgenic Nipponbare caused by salt stress may have been caused by increasing stomatal closure thus lower transpiration rate in this line as reported by Moradi and Ismail (2007). This inturn causes an improvement in WUE which eventually leads to better plant growth (Dhashnamurthi and Chenniappan, 2013). Over-expressed gene *OsNHX1* may have triggered rapid stomatal closure, which led to low stomatal conductance and transpiration rate, thus resulting in an improvement in WUE of transgenic Nipponbare.

Hasthanasombut et al (2011) reported that transgenic japonica rice Sasanishiki shows less reduction in stomatal conductance compared to the wild-type. This result is in agreement with transgenic Nipponbare. High reduction of stomatal conductance shown in wild-type Nipponbare may have been caused by the accumulation of salt in mesophyll cells, which can lead to low carbon dioxide intake and reduced carbon dioxide concentration in leaves (Maxwell and Johnson, 2000; Moradi and Ismail, 2007) and consequently lead to the inhibition of photosynthesis.

Lower dry weight in wild-type Nipponbare may have been caused by higher reduction in carbohydrate supply, which is needed for shoot growth as photosynthesis is reduced. Lower water potential attributed to reduce turgor, limited mineral supply and reduction in cell size and cell production might have also caused low shoot dry weight in the wild-type in line with the results showed by a salt-sensitive rice variety BRRI dhan reported by Hakim et al (2014).

Increasing dry weight for transgenic Nipponbare from 50 to 300 mmol/L indicated that the carbon assimilation in leaves and nutrient uptake by roots are not affected by salinity stress (Andriolo et al, 2005; Turhan et al, 2014). Even though the instantaneous quantum yield of PSII and chlorophyll content of transgenic Nipponbare were high under 50 mmol/L NaCl level, high rates of transpiration exhibited under this treatment suggest a low integrated WUE that leads to a relatively lower cumulative dry weight when compared to those plants grown under 150 mmol/L salinity regime.

Even though shoot dry weight was positively affected by salinity stress, leaf area was negatively affected by salinity. According to Andriolo et al (2005), this can attribute to its influence on water uptake by plants. Decreasing leaf area for both lines parallel to increasing salinity stress might caused by suppression in leaf elongation and formation of new leaf (Gregorio et al, 1997; Haq et al, 2014). Transgenic japonica rice Sasanishiki shows no reduction in leaf area while the wild-type shows a significant reduction (Hasthanasombut et al, 2011), and it is somewhat different from our results, in which both transgenic and wild-type Nipponbare showed reductions. However, in almost all salinity regimes, transgenic Nipponbare showed slightly higher leaf area than its wild type.

Reduction of photosynthetic rates of both transgenic and wild-type Nippobare with increasing salinity regimes can be attributed to the reduction in leaf area. O'Toole and Detta (1986) and Davatgar et al (2009) have reported that reduction in leaf area may lead to low nutrient uptake levels as overall transpiration rates reduce. This can inturn affect photosynthestic rates. Our investigation revealed that transgenic Nipponbare that showed high leaf area exhibited higher photosynthetic rates than the wild-type when exposed to high salinity stress. This confirmed that *OsNHX1* may have helped to increase salinity tolerance in Nipponbare.

Our results confirm that the transgenic Nipponbare can be cultivated in 150 mmol/L salinity regime or less, however it is probably best to be cultivated at 50 mmol/L or less, while its wild type should also be grown in areas showing less than 50 mmol/L salinity stress. This is because transgenic Nipponbare successfully produced paddy harvest only at 0 and 50 mmol/L

salinity regimes while its wild type did not produce any paddy harvest at 50 mmol/L or higher regimes.

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