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The effect of genome duplication on seed germination and seedling growth of rice under salt stress

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Abstract

Polyploidy was widely believed to facilitate increased plant adaptability to environmental extremes. Reports of the effect of genome duplication on rice development under salt stress are rare. In this study, tetraploid rice cultivars and the diploid ancestors were subjected to six levels of salinity, and the effects of genome duplication at seed germination and at the seedling growth stage were investigated. The results suggest salt stress has a large negative impact at both stages, but genome duplication has important positive roles in modulating the response to salt stress in different rice cultivars. The germination rate of four tetraploid rice cultivars was generally higher than those of diploid cultivars under salt stress; in particular, cultivars Nipponbare-4x and HN2026-4x have >50% of the germination rate in 150 mM NaCl. The growth of all diploid and tetraploid cultivars studied was inhibited by salt stress. The mortality rates of tetraploids were all lower than those of diploids; the low mortality of HN2026-4x (12.3%) and Nipponbare-4x (12.6%) showed potentially better salt tolerance. The amount of free proline in different tetraploid rice cultivars varied greatly and was increased compared to that in the diploid cultivars. The difference of soluble sugar content under salt stress was not significant between tetraploid and diploid cultivars. Malonyldialdehyde accumulated in the leaves of tetraploid rice was significantly lower than that of diploid cultivars under salt stress. The content of MDA in Nipponbare-4x (55.3 μmol g^{-1}) under salt stress was the lowest among all cultivars, indicating membrane damage was the least.

Keywords: polyploidy rice; salt stress; seed germination; seedling growth.
Abbreviations: MDA, malonyldialdehyde; PMeS, polyploidy meiosis stability.

Introduction

Salt stress is one of the most important environmental constraints limiting plant growth and yield (Buchanan et al., 2005; Li et al., 2008). During evolution, plants developed a series of mechanisms to cope with salt stress at the biochemistry and molecular levels (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu, 2002; Shinji et al., 2001). Salt and drought stress signal transduction consists of ionic and osmotic homeostasis signaling pathways and detoxification response pathways, as well as pathways for growth regulation (Zhu, 2002). Plants produce a number of permeability substances in response to these stresses; e.g. a variety of osmotic regulatory materials, including proline, sugars and proteins that stabilize cellular structures (Kishor et al., 2005; Yamada et al., 2005; Jayasekaran et al., 2006; Munns and Tester, 2008). Proline acts as a signaling molecule that modulates mitochondrial functions, influences cell proliferation and triggers specific gene expression, which can be essential for plant recovery from stress (Szabados and Savouré, 2009). Measurement of malonyldialdehyde (MDA) has been regarded as an indirect quantification of lipid peroxidation, indicating the level of lipid peroxidation resulting from oxidative stress and the tolerance of plants under adverse conditions (Havaux et al., 2005; Moller et al., 2007; Ahmad et al., 2009). Many studies have examined the effects of salt stress on plants with the aim of understanding the mechanisms used by stress-tolerant plant species and the elements on the diploid genome that might confer tolerance to sensitive plants. During the response and adaptation to stress, many stress-related genes are induced to form signaling networks (Albrecht et al., 2003; Xiong and Yang, 2003). The 2D-PAGE results obtained in this study for diploid rice were used to examine changes in protein expression and specific protein in response to salt stress in rice seedlings, and the effect of salt stress on rice leaf lamina proteins has been analyzed (Abbasi and Komatsu, 2004; Kong-ngern et al., 2005; Parker et al., 2006). The rice gene OsBIRH1, which encodes a DEAD-box RNA helicase protein, was cloned and characterized, the results suggest that OsBIRH1 encodes a functional DEAD-box RNA helicase and has an important role in defense response against biotic and abiotic stresses (Li et al., 2008). Both transcriptional and translational machinery are important determinants in controlling salt stress response, and gene expression response in tolerant and susceptible rice plants differs mainly in quantitative terms (Nicolas et al., 2010). There are few reports concerning details of the regulation mechanism of polyploidy rice under salt stress. The discovery and application of polyploidy meiosis stability (PMeS) material for disrupting low seed set rate shows great potential for contributing to polyploid rice breeding in the future. Polyploidy rice might result in evolutionary dominance in terms of both yield and stress resistance (Cai et al., 2004; Cai et al., 2007; He et al., 2010; He et al., 2011). Polyploidy, which is widely believed to have an important role in plant evolution and breeding, can greatly improve the capacity of resistance genes and enrich the range of genetic variation in these genes, thus increasing the adaptability of plants to changes in the environment (Adams and Wendel, 2005; Chen and Tian, 2007; Soltis et al., 2009). At present, there is little information available about the effects of abiotic stress conditions on duplicate gene expression patterns in polyploidy plants (Shao et al., 2011). Some studies have focused on the effects of salt stress at the genome level. It is reported that citrus tetraploid genotypes are more tolerant of moderate saline stress than the diploid genotypes, and citrus tetraploid rootstocks are more tolerant to salt stress than the corresponding diploid rootstock genotypes (Saleh et al., 2008; Mouhaya et al., 2010). It was found that the effects of salinity on some growth parameters, including protein content and antioxidant enzymes, in hexaploid *Acanthophyllum* species were better protected than tetraploid species against saline-induced oxidative damage (Meratanl et al., 2008). An earlier study reported the application of PMeS alleviated low seed set rate, leading researchers to investigate adaptability under adverse conditions (Cai et al., 2004; Cai et al., 2007; He et al., 2010; He et al., 2011). Rice is more sensitive to salt during the early seedling stage than it is at the reproductive stage (Flowers and Yeo, 1981; Lutts et al., 1995). The present study examined the impact of polyploidy on rice seed germination and seedling growth during saline treatment in order to understand the adaptability of polyploidy rice to salt stress and to give some guidance to improving the production of rice under salt stress.

Results

Analysis of the impact of genome duplication on rice seed germination under salt stress

The seed germination rate of polyploidy and diploid rice was recorded for 7 days during treatment with different concentrations of NaCl. The results showed salt stress has a large negative impact on the seed germination rate, and genome duplication has important positive roles in modulating salt stress adjustability in different rice cultivars (Fig. 1).The seed germination rate of four tetraploid species was generally higher than that of diploid cultivars in 50–200 mM NaCl, but the seed germination rate of the four diploid rice cultivars used in this study under normal culture conditions all exceeded those of tetraploid rice (Fig. 1, A–D). Generally, treatment with low concentrations of salt (0–50mM) have no significant influence on the seed germination rate, but a marked difference was found at concentrations of salt >100 mM. Nipponbare-4x and HN2026-4x have germination rate >50% in 150 mM NaCl. In 200 mM NaCl, a lower germination rate was recorded for Nipponbare-2x (14.2%), HN2026-2x (16.5%) and NJ11-2x (14.2%), Nipponbare-4x still maintained the germination rate >30%, ~20% of HN2026-4x and NJ11-4x seeds germinated successfully (Fig. 1A, B, D), but all seeds of Balilla-2x and -4x failed to germinate (Fig. 1 C).

The effect of genome duplication on the growth of rice seedlings under salt stress

Seedlings (10 days old) were cultured for 5 days in half-strength Murashige and Skoog (1/2MS) medium containing 150 mM NaCl and without NaCl as a control. The mortality rate, shoot height, dry weight and chlorophyll *a*/*b* ratio were recorded as part of an effort to understand the adaptability of polyploid rice seedlings grown under salt stress (Table 1). The mortality rates of diploids and tetraploids were all increased under salt stress, but those of tetraploids were all lower than those of diploids. The low mortality rate of HN2026-4x (12.3%), Nipponbare-4x (12.6%), NJ11-4x (17.3%) and Balilla-4x (21.3%) showed potentially greater salt tolerance than that of the corresponding diploid rice. The growth of most rice cultivars was inhibited in the treatment with 150 mM NaCl. The dry weight of tetraploid rice seedlings was significantly different from that of the diploid seedlings. The Nipponbare-4x (10.1mg), HN2026-4x (8.2mg) and NJ11-4x (5.3mg) strains had greater dry weight than the corresponding diploid rice, and the dry weight of Nipponbare-2x, HN2026-2x and NJ11-2x (5.2, 4.2 and 2.5mg, respectively) is only half the weight of the corresponding tetraploid rice. There was no significant difference in seedling height. The chlorophyll *a*/*b* ratio, which was decreased in response to treatment with salt in all rice cultivars, was greater in tetraploid than that in the diploids cultivars (Table 1). When Nipponbare-2x, Nipponbare-4x, HN2026-2x and HN2026-4x were grown in 200 mM NaCl for 5 days and for 10 days, a lower mortality rate was found at two stages in the tetraploid rice cultivars (Fig. 2 A–F1). After salt stress for 5 days, the mortality rate in Nipponbare-4x and HN2026-4x was significantly lower than that in the diploids (Fig. 2 B, B1, E and E1). After treatment with salt for 10 days, many Nipponbare-4x seedlings were green but all Nipponbare-2x seedlings died (Fig. 2 F and F1). The difference of mortality rate between HN2026-2x and HN2026-4x was not significant, indicating Nipponbare-4x had better adaptability to salt stress.

Effect of genome duplication on accumulation of free proline in diploid and tetraploid rice under salt stress

The amount of free proline in diploid and polyploid rice seedlings exposed to 150 mM NaCl for 5 days was measured (Fig.3). The amount of free proline in tetraploid rice cultivars under salt stress varied greatly and was increased compared to that in the diploid cultivars. The amount of free proline of Nipponbare-2x, Balilla-2x, NJ11-2x and HN2026-2x was 60.1, 80.5, 90.5 and 270.4 μg g^{-1}, respectively. The amount of free proline was highest in HN2026-4x (320.8 μg g^{-1}); however, the amount of free proline in the Balilla-4x, NJ11-4x and Nipponbare-4x leaves under salt stress was only half of that in HN2026-4x.

The content Malonyldialdehyde (MDA) in diploid and tetraploid rice cultivars under salt stress

MDA accumulated in similar amounts in all of the rice cultivars tested and there was no difference between diploid and tetraploid rice cultivars without salt stress. However, the amount of MDA in the leaves of various rice cultivars under salt stress was significantly greater than that in the control. By contrast, the amount of MDA in the leaves of rice under salt stress was significantly lower in the tetraploid cultivars than that in the diploid cultivars (Fig. 4).

Table 1 The effect of genome duplication on mortality rate, height, dry weight and chlorophyll *a*/*b* ratio of rice seedlings under salt stress. Data represent mean ±SD (n = 30×3 independent biological replicates). Means followed by common letters are not significantly different at P＝0.05 using a protected least significant difference.

Cultivar	Control				Salt stress			
	Mortality rate (%)	Height (cm)	Dry weight (mg)	Chlorophyll *a*/*b*	Mortality rate (%)	Height (cm)	Dry weight (mg)	Chlorophyll *a*/*b*
HN2026-2x	−	7.1±0.2（b）	10.2±1（b）	3.25±0.2（ab）	18.3±0.2（b）	6.3±0.1（ab）	4.3±0.5（de）	2.56±0.2（b）
HN2026-4x	−	7.9±0.1（ab）	12.5±1（a）	3.80±0.3（a）	12.3±0.1（c）	7.6±0.3（a）	6.9±0.7（b）	3.50±0.4（a）
Nipponbare-2x	−	8.6±0.3（a）	11.2±0.5（ab）	3.18±0.1（b）	16.7±0.1（bc）	7.4±0.3（a）	5.6±1.0（c）	2.75±0.1（b）
Nipponbare-4x	−	8.2±0.1（a）	12.8±0.7（a）	3.95±0.3（a）	12.6±0.2（c）	7.3±0.2（a）	7.9±0.4（a）	3.69±0.4（a）
NJ11-2x	−	7.1±0.2（b）	10.1±1（b）	2.94±0.1（c）	20.3±0.1（ab）	6.2±0.1（ab）	3.6±0.5（e）	2.10±0.1（bc）
NJ11-4x	−	8.0±0.2（a）	12.7±1（a）	3.11±0.1（b）	18.1±0.2（b）	7.3±0.3（a）	4.7±0.4（d）	2.50±0.2（b）
Balilla-2x	−	6.1±0.1（c）	9.5±0.5（c）	3.21±0.2（ab）	27.3±0.4（a）	5.2±0.1（b）	3.6±0.1（e）	1.59±0.1（c）
Balilla-4x	−	5.9±0.2（c）	9.8±0.5（b）	3.28±0.4（ab）	23.2±0.2（a）	5.3±0.1（b）	3.8±0.1（e）	2.04±0.2（bc）

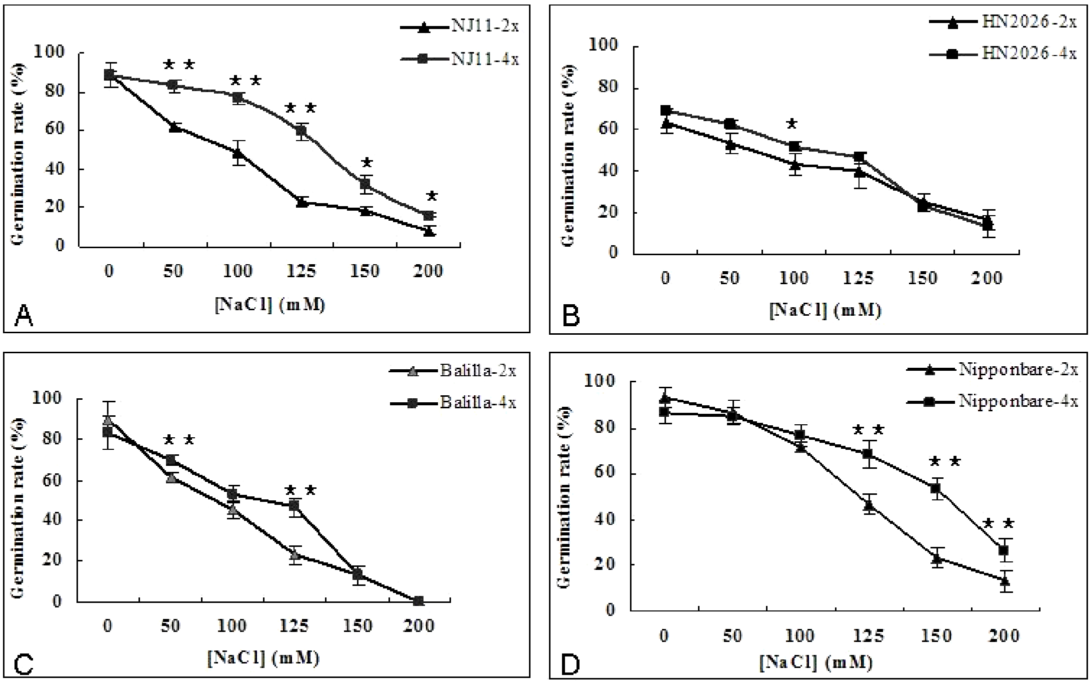

Fig 1. The impact of genome duplication on rice seed germination under salt stress.
A: Seed germination rate of NJ11-2x and NJ11-4x; B: Seed germination rate of HN2026-2x and HN2026-4x; C: Seed germination rate of Balilla-2x and Balilla-4x; D: Seed germination rate of Nipponbare-2x and Nipponbare-4x. Data represent mean ±SD (n = 30 ×3 independent biological replicates).The significance of the difference between diploid and tetraploid groups are indicated by asterisks: * P ≤0.05, ** P ≤0.01.

Genotypes Nipponbare-4x (55.3 μmol g^{-1}), HN2026-4x (70.5 μmol g^{-1}), NJ11-4x (85.6 μmol g^{-1}) and Balilla-4x (135.4 μmol g^{-1}) accumulated less MDA in their leaves compared to that in the corresponding diploid cultivars .The amount of MDA in Nipponbare-4x under salt stress (55.3 μmol g^{-1} μmol g^{-1}) was the lowest among all cultivars, which is lower than that in Nipponbare-2x (Fig. 4).

The effect of salt stress on the accumulation of soluble sugar in diploid and tetraploid rice cultivars

Genome duplication led to different outcomes in different rice cultivars in terms of the accumulation of soluble sugar under salt stress; the difference was not significant between tetraploid and diploid, except for HN2026 (Fig. 5). Without salt stress, the amount of soluble sugar was similar among the rice cultivars investigated but >30% greater compared to the control. The amount of soluble sugar in the tetraploid rice was slightly greater than that in the corresponding diploid cultivars, except for NJ11-4x and HN2026-4x, and was greatest in HN2026-2x (450.3 mmol L^{-1}).

Discussion

Salt stress is one of the most serious environmental hazards to crop productivity worldwide (Sahi et al., 2006). This adverse environmental condition impairs plant growth via water deficiency andionic toxicity (Munns and Tester, 2008; Apse and Blumward, 2007). Salt stress affected the germination and early seedling growth significantly, so these are regarded as indicators of salt stress (Shahid and Pervez, 2012). High seed germination rate and vigorous seedling growth contribute to the plant's potential to resist salt stress, indirectly having a vital role for better growth and productivity (Carpici et al., 2009). Various reports indicate that salt tolerance characteristics are specific to the

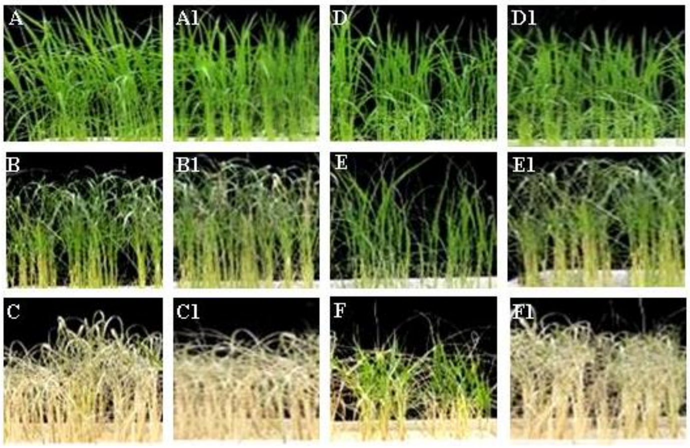

Fig 2. Genome duplication affects the growth of HN2026 and Nipponbare rice seedlings under salt stress
A: HN2026-4x grown in normal conditions (Con); B:HN2026-4x grown in 200 mM NaCl for 5 days, the mortality rate was significantly lower than that in HN2026-2x (B1); C:HN2026-4x grown in 200 mM NaCl for 10 days, the mortality rate was similar to diploids (C1) ; A1: HN2026-2x grown under normal conditions (Con); B1:HN2026-2x grown in 200 mM NaCl for 5 days;C1: HN2026-2x grown in 200 mM NaCl for 10 days; D: Nipponbare-4x grown under normal conditions (Con); E: Nipponbare-4x grown in 200 mM NaCl for 5 days, the mortality rate was significantly lower than that in Nipponbare-2x (E1); F: Nipponbare-4x grown in 200 mM NaCl for 10 days, the mortality rate was also significantly lower than that in the diploids (F1); D1:Nipponbare-2x grown under normal conditions (Con); E1: Nipponbare-2x grown in 200 mM NaCl for 5 days; F1:Nipponbare-2x grown in 200 mM NaCl for 10 days.

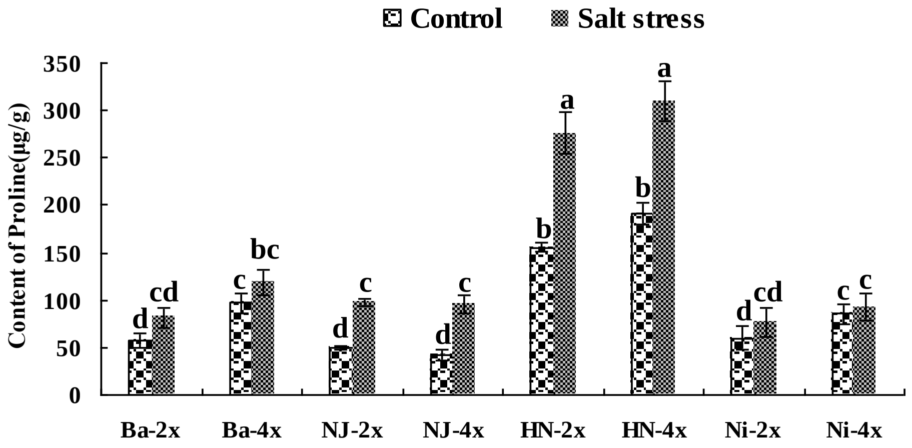

Fig 3. Amount of free proline in diploid and tetraploid rice cultivars under salt stress
Ba=Balilla; HN=HN2026; NJ=NJ11; Ni=Nipponbare. Data represent mean ±SD (n = 30×3 independent biological replicates). Means followed by common letters are not significantly different at P＝0.05 using a protected least significant difference.

developmental stage; one might be affected drastically whereas another could exhibit tolerance to salts (Lauchli and Epstein, 1990). Therefore, the screening for salt tolerance in various plant genotypes should be done at the initial growth stages as well as at the germination stage (Alian et al.,2000; Al-Karaki,2000). Although several studies have shown that salt stress has a great negative impact on seed germination and seedling growth rates, little is known about its role in polyploidy rice. In this study, it was found salt stress had a great negative impact on seed germination rate and seedling growth, and genome duplication had important roles in modulating salt stress tolerance in different rice cultivars. Plants produce various osmotic regulation materials, such as proline, sugars and proteins, that stabilize cellular constituents. Proline is considered to be a signaling molecule that can be essential for plant recovery from stress (Szabados and Sovouré, 2009; Al-Khayri and Al-Bahrany, 2002). Under salt stress, the amount of free proline in the leaves of tetraploid rice varies greatly among cultivars and is increased compared to that in the diploid cultivars. The soluble sugar acts as an osmotic regulation substance and dehydrator, enhancing the ability of cells to retain water (Chyzhykova and Palladina, 2006). The amount of soluble sugar in leaves was significantly greater than that in the control in various rice cultivars under NaCl stress, but the difference between tetraploids and diploids was not significant. Salt-induced oxidative stress might disrupt membrane structure because overproduction of reactive oxygen species triggers lipid and protein peroxidation (Dionisio-Sese and Tobita, 2000; Azevedo et al., 2009; Radic et al., 2006). Accumulation of MDA, which has been regarded as a marker of oxidative damage (Moller et al., 2007; Ahmad et al., 2009), without salt stress was similar in all rice cultivars tested. However, the amount of MDA in all tetraploid rice cultivars tested was less than that in the diploid cultivars. In summary, the results of this work suggest that tetraploid rice has better protection

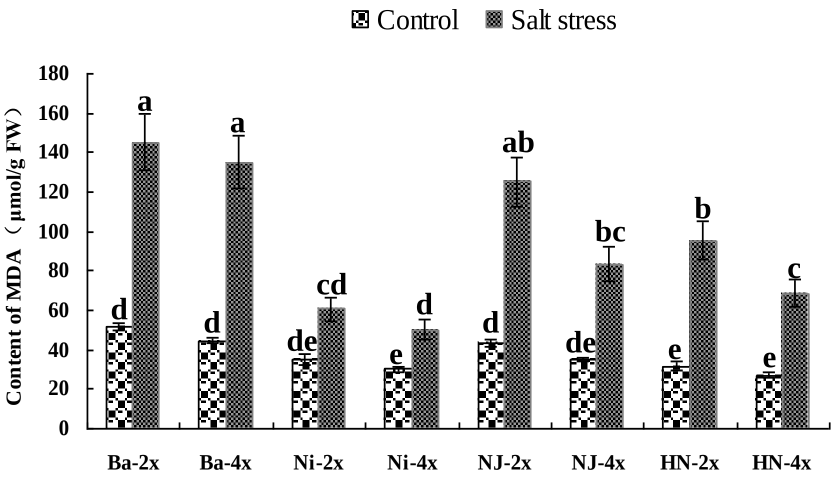

Fig 4. The accumulation of MDA in diploid and tetraploid rice under salt stress.
Ba=Balilla; HN=HN2026; NJ=NJ11; Ni=Nipponbare. Data represent mean ±SD (n = 30×3 independent biological replicates). Means followed by common letters are not significantly different at P＝0.05 using a protected least significant difference.

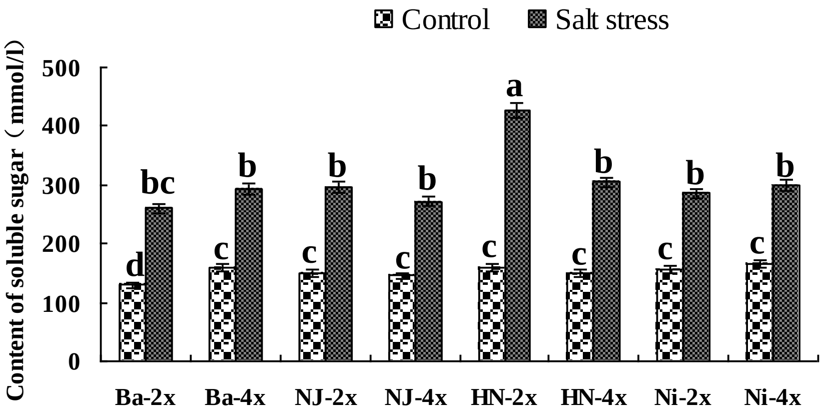

Fig 5. The amount of soluble sugar in diploid and tetraploid rice under salt stress.
Ba=Balilla; HN=HN2026; NJ=NJ11; Ni=Nipponbare. Data represent mean ±SD (n = 30×3 independent biological replicates). Means followed by common letters are not significantly different at P＝0.05 using a protected least significant difference.

than diploid rice against salinity, in agreement with the results of earlier studies on the genome duplication effect in citrus and *Acanthophyllum* spp. under salt stress (Saleh et al., 2008; Mouhaya et al., 2010). Several studies indicate that the response of plant cells to high salt is controlled by multiple genes (Bartels and Sunkar, 2005; Chinnusamy et al., 2005; Sahi et al., 2006). Salt stress response has been analyzed in barley (*Hordeum vulgare*) and in wheat (*Triticum aestivum*) (Forster et al., 2000; Francki and Appels, 2002; Colmer et al., 2005; Kawaura et al., 2006; Walia et al., 2006). Model plants like *Arabidopsis thaliana* and *Nicotiana tabacum* have been used in a transgenic approach to test the roles of candidate genes in governing tolerance of salt stress (Zhang et al., 2001; Zhang et al., 2011). Polyploidy was widely believed to have an important role in plant evolution and breeding (Adams and Wendel, 2005; Chen and Tian, 2007; Soltis et al., 2009). Several categories of regulatory function and transporter activity were over-duplicated, and the complexity of regulatory networks and adaptability to changing environmental conditions would be increased in polyploidy (Osborn et al., 2003; Blanc and Wolfe, 2004; Basel et al., 2008). Research indicates that abiotic stress conditions can have considerable effects on duplicate gene expression in a polyploidy, with the effects varying in relation to gene, stress and organ. Differential expression in response to environmental stress might be a factor in the preservation of some duplicated genes in polyploidy (Blanc and Wolfe, 2004).The regulation mechanism is complicated in polyploidy rice and understanding how duplicated genes can affect rice development under salt stress could be very important for biological and agricultural applications.

Materials and Methods

Plant materials

Balilla-2x (commonly used in Japan) and Balilla-4x; HN2026-2x and -4x; Nipponbare-2x and -4x; and NJ11-2x and -4x were used in this study. The tetraploid rice cultivars were cultured according to our patent and as described (Cai et al., 2004).

Analysis of seed germination under salt stress

Seeds of eight species were surface-sterilized and incubated at 35ºC for 7 days with different concentrations of NaCl (0, 50, 100, 125, 150 and 200 mM). The seed germination frequency with or without salt stress was compared. Each experiment was done at least 3 times and the average value was calculated.

Seedling growth under salt stress

Seedlings (10 days old) were cultured for 5 days in half-strength Murashige and Skoog medium (1/2MS)(Murashige and Skoog, 1962) with 150 mM NaCl or without NaCl as a control. The mortality rate, shoot height, dry weight, chlorophyll *a*/*b* ratio and the content of proline, MDA and soluble sugar in leaves after treatment with salt for 5 days was investigated. Each experiment was done at less 3 times and the average value was calculated.

Extraction of free *proline*

The acid ninhydrin method was used to extract and quantify the free proline content as described (Bates et al., 1973). The content of proline was calculated from the standard curve. All samples were tested in three independent experiments with three replicates each.

Malondialdehyde (MDA) content

The malondialdehyde **(**MDA**)** content was measured as described (Hodges et al., 1999). Briefly, 0.5g fresh leaves was homogenized with 5 ml 5% (v/v) 2,4,6-trichloroanisole, and then centrifuged at 3000 rpm for 10 min. The supernatant was mixed with 2 ml 0.67% (v/v) 2,4,6-tribromoanisole, boiled for 30 min, cooled and centrifuged again. The absorbance (A) of the supernatant at 532 nm, 600 nm and 450 nm was measured and the MDA content (C) was calculated as:

C (μmol L^{-1}) = $6.45\ (A_{532} - A_{600}) - 0.56\ A_{450}$

Soluble reducing sugar content

The soluble reducing sugar content was measured as described (Ranney et al., 1991). Briefly, 0.1 g fresh leaves was homogenized with 5 ml distilled water then centrifuged at 8000 rpm for 10 min. The soluble reducing sugar content was measured as follows: 1.0 ml supernatant and 0.5 ml 3,5-dinitrosalicylic acid were mixed, immersed in boiling water for 5 min and cooled. The absorbance was measured at 520 nm and the amount of reducing sugar was calculated from the standard curve.

Statistical analysis

All values are given in this study as the mean of three replicates and then the average is calculated. In Fig. 1 and Table1, analysis was done using *t*-tests and statistically significant difference was set at $P \leq 0.05$, the level of significance of the difference between diploid and tetraploid rice is indicated by asterisks: * $P \leq 0.05$, ** $P \leq 0.01$. The results in Fig3, Fig4 and Fig5 were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA) to determine the significant differences. Means followed by common letters are not significantly different at P=0.05 using a protected least significant difference.

Conclusion

In this study, tetraploid rice and the diploid ancestors were investigated at germination and seedling stages in order to understand the regulation mechanism of genome duplication under salt stress. The germination rate of four tetraploid rice cultivars was generally higher than those of diploid cultivars with different concentrations of NaCl, but which is contrary under normal conditions. The growth of all rice cultivars studied was inhibited by salt stress, the height, dry weight and chlorophyll *a*/*b* ratio of tetraploid rice were higher than those of diploid rice, however, the mortality rate for tetraploid rice was less than that for diploid rice. Under salt stress, the synthesis of permeable substances, such as free proline and soluble sugar in tetraploid rice was greater than that in diploid rice. The content of malonyldiadelhyde in tetraploid rice was lower than that in diploid rice, indicating membrane damage in the tetraploid rice was slightly less than that in diploid rice. In a word, genome duplication has important positive roles in modulating salt stress adjustability in different rice cultivars.

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