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Research article

Morphological and metabolic responses to salt stress of rice (*Oryza sativa L.*) cultivars which differ in salinity tolerance

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ABSTRACT

Salinization is one of the most important abiotic stressors for crop growth and productivity. Rice (*Oryza sativa L.*), as the major food source around the world, is very sensitive to salt, especially at seedling stage. In order to examine how salt stress influences the metabolism of rice, we compared the levels of a range of sugars and organic acids in three rice cultivars with different tolerance under salt stress over time. According to the morphological result, the shoot length and root fresh weight were only affected by salinity in the salt sensitive cultivar (Nipponbare). The responses of metabolites to salinity were time-, tissue- and cultivar-dependent. Shikimate and quinate, involved in the shikimate pathway, were dramatically decreased in the leaves of all three cultivars, which was regarded as a response to salinity. Many sugars in the leaves of the salt tolerant cultivar (Dendang and Fatmawati) showed earlier increases to salt stress compared to Nipponbare leaves. Moreover, only in the leaves of tolerant cultivars (Dendang and Fatimawati), malate was significantly decreased while sucrose was significantly increased. In Dendang roots, mannitol levels were significantly higher than in Nipponbare roots after 14 days of salt treatment, which may be attributed to its higher salt tolerance. It is proposed that these responses in the more tolerant cultivars are involved in their resistance to high salt stress which may lay the foundation for breeding tolerant rice cultivars.

1. Introduction

Salinity is regarded as a major environmental constraint to crop productivity worldwide (Zhu, 2001). More than 6% of the world land area are either salinity or sodicity affected (FAO, 2008). Due to the human activities and natural causes, soil salinization is increasing. A saline soil is defined to have an electrical conductivity of the saturated paste extract above 4 dS/m (~40 mM NaCl) (Chinnusamy et al., 2005). The high concentration of salt in the soil makes it harder for roots to uptake water and nutrients, therefore inducing ion imbalances and water stress in plants (Hasegawa et al., 2000). Following a consequence of these primary effects, the secondary stresses, such as metabolic damage, growth arrest, and even death, can occur. For example, osmotic stress leads to cell dehydration and a subsequent decrease in shoot and root growth. The high accumulation of Na^+ in the leaf blades has been shown to be negatively correlated with plant growth in wheat (*Triticum aestivum*) (Munns et al., 2000), rice (*Oryza sativa L.*) (Zhu et al., 2001; Platten et al., 2013) and barley (*Hordeum vulgare L.*) (Garthwaite et al., 2005).

Metabolite changes are indicators of cellular regulatory processes. The osmotic adjustment in rice can be achieved by synthesis of compatible solutes, such as proline, glycine, GABA and sucrose (Ma et al., 2018; Banerjee et al., 2019; Gayen et al., 2019). Prolonged salt stress in wheat has showed progressive accumulation of sugars to avoid osmotic stress (Guo et al., 2015). Furthermore, cereals with different sensitivity to salt show different metabolite changes. For example, salt stress increased the levels of hexose phosphates and tricarboxylic acid (TCA) cycle intermediates in the salt tolerant barley (Sahara) while these solutes remained unchanged in the sensitive barley (Clipper) (Widodo et al., 2009). The metabolic pathways changes in cereals differing in salinity resistance may provide fundamental information to breed for tolerant cultivars.

Rice is the staple food for nearly half of the world's population. More than 50% of rice is produced and consumed in Asia (GRiSP, 2013). Compared with other crops, such as wheat and barley, rice is the most sensitive crop to salt stress (Munns and Tester, 2008). Rice grain yield can be reduced significantly by the addition of 50 mM NaCl (Yeo and Flowers, 1986), while barley, for instance, can withstand up to 450 mM

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NaCl (Garthwaite et al., 2005). Among the predominant abiotic stresses, such as drought and cold, salinity tolerance remains the main goal to breed stress-tolerant rice cultivars in order to assure food security (Jini and Joseph, 2017; Reddy et al., 2017).

Nipponbare, a *japonica* salt sensitive genotype has been used as a model rice cultivar in a variety of abiotic and biotic stress studies (Ahn et al., 2010; Wang et al., 2012). Dendang and Fatmawati are two *indica* genotypes with relatively high salt tolerance (Barus and Rauf, 2013). In general, *indica* rice has a higher level of salinity tolerance when compared with *japonica* (Lee et al., 2003). To our knowledge, there are no reports defining differences in metabolic responses to different salt treatments of these relatively high tolerant rice cultivars (Dendang and Fatmawati).

The growth stages, organ types and cultivars are important factors to select against the sensitivity of rice to salt. Many researchers have shown that salinity influences rice growth throughout its life cycle from germination to maturity, but the most sensitive growth stage is shown to be the seedling stage (Lutts et al., 1995; Khan et al., 1997; Nam, 2018). In this study, we investigated the salt stress response in the leaves and roots of three rice cultivars (Dendang, Fatmawati and Nipponbare) at seedling stage (four-leaf stage) at the metabolic level in order to understand their physiological responses of salt stress. Metabolic profiling may allow an initial functional insight into the metabolic pathways of tolerance acquisition without prior knowledge of genetic variation between these cultivars.

2. Materials and methods

2.1. Chemicals and reagents

Sugars and organic acids standards, internal standards (ISTD) and the derivatization reagent, methoxyamine hydrochloride (Meox), used for Gas chromatography-mass spectrometry (GC-MS) were acquired from Sigma Aldrich (Castle Hill, NSW, Australis). *N*,*O*-Bis (trimethylsilyl) trifluoroacetamide with 1% trimethylsilyl chloride (BSTFA + 1% TMCS) was from Thermo Scientific (Bellefonte, USA). All solvents used were High performance liquid chromatography (HPLC)-grade purchased from Merck (Australia).

2.2. Plant materials and stress treatment

The two *indica* rice cultivars selected from Indonesia were Fatmawati and Dendang. Nipponbare is a model *japonica* rice cultivar. These rice seeds were acquired from Associate Prof. Alexander Johnson (University of Melbourne).

Once the husk was removed, rice seeds (24 per cultivar) were surface sterilized with 70% (v/v) ethanol for 1 min and incubated in 1% (v/v) sodium hypochlorite for 10 min. The seeds were then rinsed with distilled water (10 times) to remove all traces of sodium hypochlorite. Surface sterilized seeds were imbibed in deionized water with aeration for 16 h at room temperature and then placed in petri dishes (5 seeds for each dish) on moist Whatman filter paper to germinate. The dishes were sealed completely with parafilm to prevent evaporation and contamination. The petri dishes were placed in a growth chamber (Fitotron, Weiss Gallenkamp, UK) at 12 h light/12 h dark (light intensity was 200 μmol m^{-2} S^{-1}), 29 °C light/26 °C dark, 70% humidity. The seedlings were then sowed on a styrofoam seedling float after the height of shoots was 4–6 cm (around 10 days after germination). The seedlings were transferred to 12 L tanks filled with Yoshida solution (91.4 g L^{-1} NH_4NO_3, 35.6 g L^{-1} NaH_2PO_4, 71.4 g L^{-1} K_2SO_4, 117.4 g L^{-1} $CaCl_2{\cdot}H_2O$, 324 g L^{-1} $MgSO_4$ and the following micronutrients: 1.500 g L^{-1} $MnCl_2{\cdot}4H_2O$, 0.074 g L^{-1} $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$, 0.035 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 2.515 g L^{-1} H_3BO_3, 0.031 g L^{-1} $CuSO_4$, 7.700 g L^{-1} $FeCl_3{\cdot}6H_2O$, 11.900 g L^{-1} $C_6H_8O_7{\cdot}H_2O$) (Yoshida et al., 1976) with pH adjusted to 5.0–6.0 with pH meter and separated randomly into control and salt stress groups with four replicates per treatment. The hydroponic solution was replaced once a week. Salt treatment was applied when rice plants reached the four-leaf stage and was performed in a gradient with 20 mM NaCl per day. Briefly, 20 mM NaCl was applied on the first day and the plants were allowed to acclimatize for 24 h. Next, 40 mM NaCl was added by replacing the existing solution and incubated for 24 h. The final 50 mM NaCl was applied on the third day.

Plants grown under salt and control conditions were harvested at three time points: 0 day (T0, before the addition of NaCl), 7 days (T1) and 14 days (T2) after salt treatment. Plant shoot and root length were measured. Tissues from roots and the two youngest leaves from each individual plant were harvested and immediately snap frozen in liquid nitrogen for metabolomics analysis.

2.3. Metabolite extraction and derivatization

For the extraction of metabolites (sugars and organic acids) from rice leaves and roots, 30 ± 3 mg fresh frozen tissue was weighed into a cryo-mill tube and the accurate weight was recorded. The frozen tissue was extracted with either 400 μL (in the case of leaves) or 250 μL (in the case of roots) of 100% methanol containing 4% ^{13}C-Sorbitol/$^{13}C^{15}N$-Valine. Samples were homogenised using a cryo-mill (Bertin Technologies, program: 6100 rpm-3 x 45 s × 45 s at −10 °C). The mixture was extracted for 15 min at 30 °C in a thermomixer at 1400 rpm and then centrifuged for 10 min at 15,000 rpm at room temperature. The supernatant was transferred into a new Eppendorf tube. The remaining pellets were re-extracted with 400 μL (leaves) or 250 μL (roots) water by vortexing for 1 min, prior to centrifugation. The supernatants were combined. As the extract contained low and high abundant metabolites, aliquots of 80 μL and 5 μL (leaves) or 160 μL and 8 μL (roots) were analysed, respectively, to detect as many metabolites as possible within the dynamic range of the instrument. For sucrose analysis, the extract from leaves was diluted 160-fold, while the extract from roots was diluted 50-fold. The sample aliquots were kept frozen at −20 °C prior to analysis.

For sugars and organic acids derivatization, all resulting aliquots were dried under speed vacuum. The dried sample was redissolved and derivatized for 2 h at 37 °C in Meox (20 μL of 30 mg mL^{-1} in pyridine) followed by trimethylsilylation (TMS) for 30 min at 37 °C with 20 μL BSTFA + 1% TMCS. Samples (1 μL) were then injected onto a GC column (Dias et al., 2015).

2.4. Preparation of calibration standards and quality control samples

The calibration standards for sugars and organic acids quantification were prepared by diluting mixed standard stock solution into concentrations of 9 levels (0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 μM) and derivatized as described in section 2.3. Pooled biological quality control (PBQC) samples were prepared by pooling equal aliquots of each individual sample and used for quality control as described in Hill et al. (2014).

2.5. GC-MS analysis

The sugars and organic acids in rice leaf and root tissues were determined using GC-QqQ-MS system consisting of a Gerstel 2.5.2 autosampler, a 7890A Agilent gas chromatograph and a 7000 Agilent triple quadrupole MS (Agilent Technologies Santa Clara, CA, USA) (Dias et al., 2015). Gas chromatography was performed on a 30 m VF-5MS column with 0.25 μm film thickness (Varian, Inc, Victoria, Australia). The injection temperature was set at 250 °C, the MS transfer line at 290 °C, the ion source was adjusted to 230 °C and the quadrupole at 150 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The analysis of TMS-derivatized samples was performed under the following temperature program: start at injection 50 °C, a hold for 1 min, followed by a 15 °C·min^{-1} oven temperature ramp to 325 °C and

a final 5 min heating at 325 °C. The analytes were detected by multiple reaction monitoring (MRM) mode using electron ionization mass spectrometry (EI-MS) (Dias et al., 2015). The transitions used for quantification and qualification of target metabolites are listed in Table S1. Retention time locking using the chromatographic peak of $^{13}C_1$-mannitol prior to the sample run was performed to ensure repeatable retention times across systems regardless of operator, detector type, and column maintenance (Hill et al., 2013).

2.6. Statistical analysis

Raw data was evaluated using MassHunter MS Quantitative Analysis software (ver. B.08.00, Agilent Technologies). The concentration of each metabolite was determined according to the calibration curve of its respective standard and then normalized to fresh weight of the tissue and is presented as picomoles per mg fresh weight (Tables S2 and S4).

The final concentration of metabolites was calculated according to the formula below:

$$Final\ conc. = \frac{Cal\ conc.\ \ x\ derivatization\ vol.\ \ x\ extraction\ vol.}{Dried\ aliquot\ vol.\ \ x\ ISTD\ correction\ x\ sample\ weight}$$

The fold changes of metabolites between control and salt treatment groups at the same time point was calculated according to the formula below:

$$Fold\ changes = \frac{Final\ conc.\ \ in\ salt\ treatment\ group}{Final\ conc.\ \ in\ control\ group}$$

Statistical significance between the control and treatment groups was performed by Student's *t*-test after $\log_{10}$ transformation. False discovery rate (FDR) was used to reduce type I errors in multiple comparison (Hochberg and Benjamini, 1990). FDR-adjusted p value < 0.1 and FDR-adjusted p value < 0.05 were regarded as significant and highly significant. Analysis of variance (ANOVA) was performed in SPSS to determine the significance of shoot or root length changes between control and salt groups at different sampling time points. The heatmap analysis was performed using MetaboAnalyst (https://www.metaboanalyst.ca/).

3. Results

3.1. Morphological responses to salt stress

The three rice cultivars with different tolerance to salt (Dendang, Fatmawati, and Nipponbare) were evaluated for growth parameters after the time-series salt treatment. There were no obvious symptoms at the beginning of salt stress, but the reduction in shoot and root growth, and faster senescence of leaves occurred as exposure time increased. Rice plants began to develop leaf symptoms such as yellowing and necrotic lesions of old leaf tips after 3–4 days of exposure. After 2 weeks of stress, the senescence of older leaves was obvious in all cultivars compared to leaves of rice grown under control conditions. Nipponbare roots also showed significant less lateral branching roots after salt treatment compared to the control roots at 7 days (T1) and 14 days (T2) (Fig. S1).

The shoot length increased with time at either fast or low rate depending of the cultivar (Fig. 1). No significant difference was observed on shoot length of Dendang and Fatmawati between control and salt stress groups during 14 days of salt stress. However, Nipponbare showed significant decrease in shoot length at T2 between control and exposure groups (one-way ANOVA, $p = 0.002$).

No significant difference was observed on root length of Nipponbare, Fatmawati, and Dendang between control and treatment groups during two weeks of salt treatment (Fig. 1). The salt stress had relatively fewer effects on root length whereas the root fresh weight decreased 29.3% at T1 and 34.2% at T2 in Nipponbare cultivar (Fig. 1).

3.2. Differential changed metabolites in leaves of salt treated rice cultivars

We compared the concentrations of each metabolite in the stressed plants to the levels in the control plants at each time point in order to determine the responses of each rice cultivar to salinity stress and the influence of the duration of treatment. A total of 43 compounds of known structure, comprising 19 organic acids and 24 sugars, sugar alcohols and sugar phosphates, were quantified in leaf extracts of rice (Table S2). The fold changes and significance of each metabolite in rice leaves are shown in Fig. 2 and Table S3.

No significant differences in sugar and organic acid concentrations were observed between the samples before salt treatment (T0) in the leaves of Dendang, Fatmawati and Nipponbare cultivars.

After 7 days of salt exposure (T1), most organic acids involved in the TCA cycle and shikimate pathway significantly decreased in the three cultivars: quinate and shikimate significantly decreased in both Dendang (-8.4-fold, $p < 0.05$; -3.1-fold, $p < 0.05$) and Nipponbare (-5.2-fold, $p < 0.05$; -3.7-fold, $p < 0.1$) leaves. The concentrations of quinate and shikimate decreased -1.9-fold and -1.7-fold in Fatmawati leaves without significance. Malate decreased significantly in Dendang (-3.4-fold, $p < 0.05$) and Fatmawati (-7.8-fold, $p < 0.1$) leaves. Levels of malonate (-3.8-fold) and pipecolate (-2.5-fold) decreased strongly only in Dendang leaves ($p < 0.05$). 2-oxoglutarate (-2.9-fold) and citrate (-2.7-fold) only show significant difference between control and salt treated leaves in Fatmawati leaves ($p < 0.1$). Among the sugars and derivatives, erythritol ($+3.7$-fold, $p < 0.05$), ribose ($+2.0$-fold, $p < 0.05$), rhamnose ($+1.6$-fold, $p < 0.05$), fucose ($+1.7$-fold, $p < 0.05$), mannitol ($+1.8$-fold, $p < 0.05$), galactitol ($+1.5$-fold, $p < 0.05$), sucrose ($+2.1$-fold, $p < 0.05$), melibiose ($+3.6$-fold, $p < 0.05$), raffinose ($+3.8$-fold, $p < 0.05$) and xylose ($+1.8$-fold, $p < 0.1$) increased significantly in Dendang leaves at T1. In the leaves of Fatmawati, ribose ($+1.8$-fold, $p < 0.1$), mannose ($+1.3$-fold, $p < 0.1$), galactose ($+1.7$-fold, $p < 0.1$), sucrose ($+1.8$-fold, $p < 0.1$), fructose ($+6.6$-fold, $p < 0.05$), glucose ($+5.8$-fold, $p < 0.05$), and raffinose ($+2.0$-fold, $p < 0.05$) showed significant increase while turanose (-6.0-fold, $p < 0.05$) significantly decreased. In contrast, only fructose ($+12.2$-fold, $p < 0.05$) and glucose ($+8.6$-fold, $p < 0.05$) significantly increased in Nipponbare leaves. The concentrations of ribose ($+2.5$-fold), galactose ($+2.4$-fold), turanose ($+2.5$-fold), and raffinose ($+2.8$-fold) were also observed to be higher in the salt treated group than the control in Nipponbare leaves at T1, although the changes were not significant.

After 14 days of salt treatment (T2), organic acids, comprising quinate (-5.1-fold, $p < 0.05$), shikimate (-3.0-fold, $p < 0.05$), and malate (-3.5-fold, $p < 0.05$) in Dendang leaves showed similar response compared with the changes at 7 days (T1). However, citrate (-2.2-fold) showed significant lower level in salt-treated Dendang leaves and malonate was 2.2-fold higher ($p < 0.05$). In Fatmawati leaves, only malate (-8.3-fold, $p < 0.1$) kept decreasing in salt treated group at T2. Shikimate (-3.0-fold), isocitrate (-2.5-fold), citrate (-4.8-fold), and quinate (-3.2-fold) levels were also lower in the salt exposure group than control group although there was no significant difference. In Nipponbare leaves, the decreases of shikimate (-3.6-fold, $p < 0.1$) and quinate (-6.2-fold, $p < 0.05$) levels were similar with that at T1. However, succinate ($+1.6$-fold), isocitrate ($+2.8$-fold), and citrate ($+3.0$-fold) levels were significantly higher after salt treatment at T2 ($p < 0.05$), which is unique compared with other two cultivars. Compared with 7 days (T1) of salt stress, fewer sugars in Dendang leaves were affected at 14 days (T2) while more sugars were influenced in Nipponbare leaves. In Dendang leaves, two sugars increased, sucrose ($+1.7$-fold, $p < 0.1$) and raffinose ($+2.9$-fold, $p < 0.05$). Similar with this result, sucrose ($+2.3$-fold, $p < 0.1$) and raffinose ($+3.3$-fold, $p < 0.05$) in Fatmawati leaves also showed significantly increase after 14 days of salt treatment. Moreover, fructose ($+9.4$-fold, $p < 0.1$) and glucose ($+8.2$-fold, $p < 0.1$) exhibited

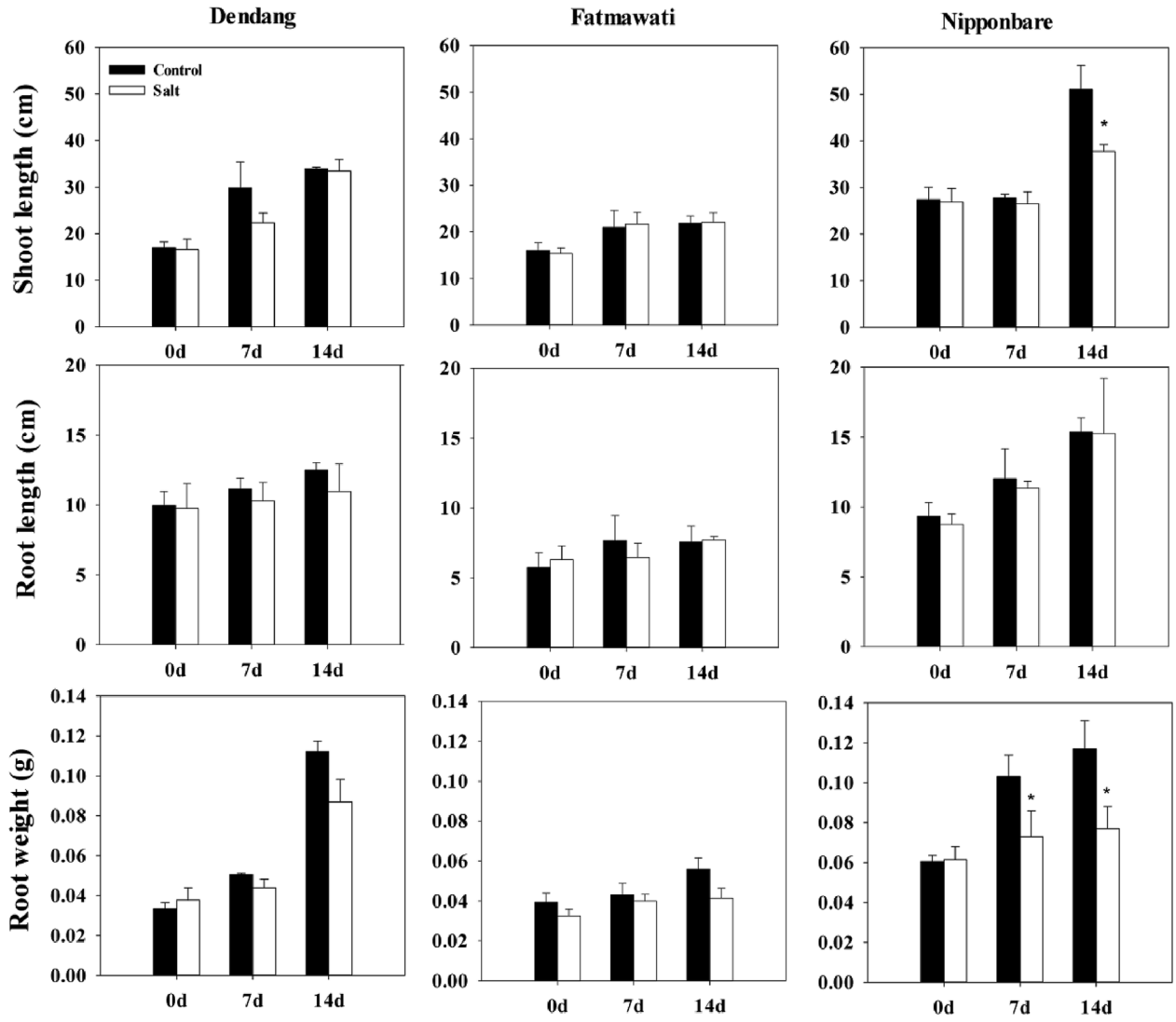

Fig. 1. Shoot and root length, and root fresh weight in control and salt (50 mM NaCl) treatment groups at 0, 7 and 14 days of three rice cultivars. * represents significant difference between control and salt treatment groups at the same time point, n = 4 (one-way ANOVA, $p < 0.05$). Data are presented as mean ± SD.

longer term responses on salinity in Fatmawati leaves. In Nipponbare leaves, the levels of beta-gentibiose (+1.6-fold) and raffinose (+16.4-fold) were increased while mannose (−1.4-fold) and trehalose (−1.7-fold) were decreased significantly under salinity stress ($p < 0.05$).

3.3. Differential changed metabolites in roots of salt treated rice cultivars

In Dendang, Fatmawati, Nipponbare roots, a total of 44 compounds were identified and quantified including 20 organic acids and 24 sugars and derivatives (Table S4).

There were no significant differences in metabolite concentrations in the roots of each cultivar between the two tubs just before salt treatment was started (T0).

After 7 days of salt treatment (T1), more metabolites in Nipponbare and Fatmawati roots showed significant changes than in Dendang roots (Fig. 3 and Table S5). For the changes of organic acids, only quinate (−3.1-fold, $p < 0.1$) was significantly decreased in Dendang roots after 7 days of salinity stress and shikimate level decreased −2.0-fold without significance. As the result of salt stress, succinate (−1.8-fold, $p < 0.05$; −1.7-fold, $p < 0.05$), malate (−2.8-fold, $p < 0.05$; −2.8-fold, $p < 0.05$), 2-oxoglutarate (−2.5-fold, $p < 0.05$; −1.6-fold, $p < 0.05$), and shikimate (−1.4-fold, $p < 0.1$; −1.4-fold, $p < 0.05$) were at significantly lower concentrations in Fatmawati and Nipponbare roots, respectively. In addition, fumarate levels were −1.4-fold lower and 2-keto gluconic acid levels were 1.3-fold higher compared with the concentrations in control Nipponbare roots ($p < 0.1$). Most sugars were significantly decreased in rice roots after 7 days of salt exposure, which was opposite compared to the changes in leaves. The concentrations of fructose were −7.2-fold ($p < 0.1$), −11.9-fold ($p < 0.05$) and −14.7-fold ($p < 0.05$) lower in Dendang, Fatmawati, and Nipponbare roots compared with their respective control. In Fatmawati and Nipponbare roots, mannose (−1.8-fold, $p < 0.05$; −2.0-fold, $p < 0.05$), galactose (−1.3-fold, $p < 0.05$; −1.6-fold, $p < 0.1$), glucose (−1.8-fold, $p < 0.05$; −4.3-fold, $p < 0.1$), fructose-6-phosphate (−1.3-fold, $p < 0.05$; −1.5-fold, $p < 0.1$), glucose-6-phosphate (−1.9-fold, $p < 0.1$; −1.8-fold, $p < 0.1$), and sucrose (−2.3-fold, $p < 0.1$; −2.0-fold, $p < 0.1$) also showed a dramatic decrease. Furthermore, galactinol levels were −2.9-fold decreased in Nippobare roots ($p < 0.05$). At the same time, erythritol (5.0-fold), xylitol (7.6-fold), and trehalose (2.6-fold) in Fatmawati roots and mannitol (4.2-fold), raffinose (2.0-fold) in Nipponbare roots were dramatically increased after salt treatment ($p < 0.05$). In Dendang roots, mannose (−2.0-fold) and raffinose (3.9-fold) also show alterations without significance.

After 14 days of salinity stress (T2), more metabolites were affected in Dendang roots than in Nipponbare and Fatmawati roots (Fig. 3 and

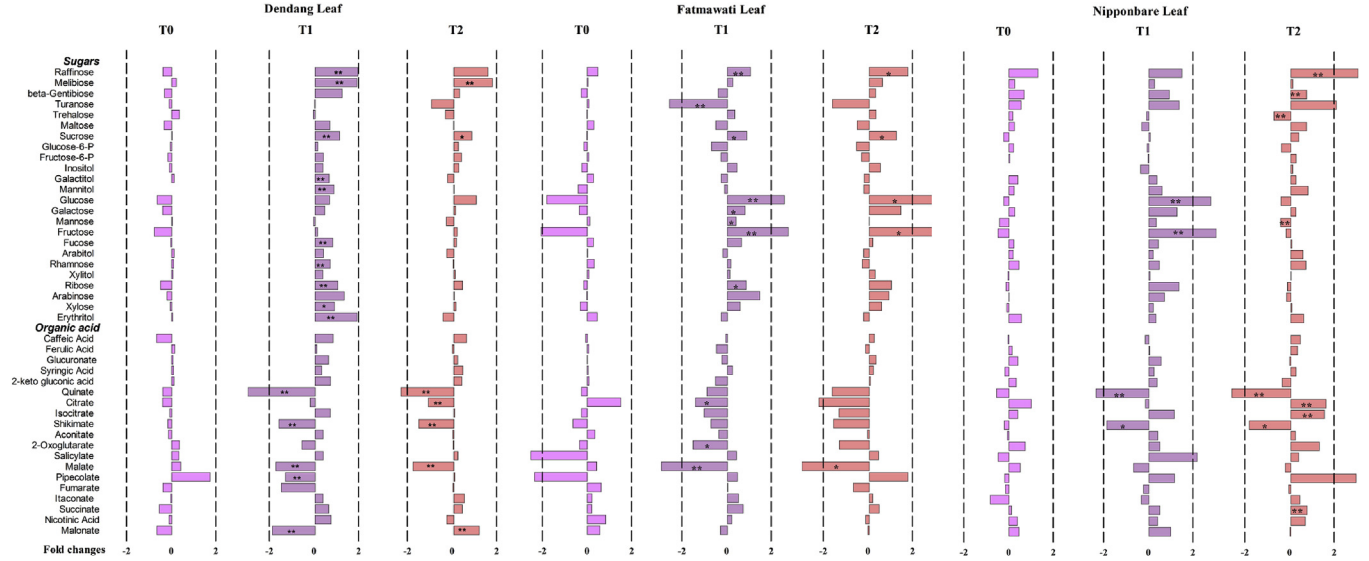

Fig. 2. Log_2 fold changes of primary metabolites in leaves of Dendang, Fatmawati, and Nipponbare cultivars at 0 (T0), 7 (T1) and 14 (T2) days after salt treatment. Fold changes were calculated by dividing the concentrations of control by the concentrations of salt treated at the same time point. * and ** represent significant differences (FDR-adjusted p value < 0.1) and highly significant differences (FDR-adjusted p value < 0.05) compared with control. A threshold of ± 2-fold change is indicated as a dashed line.

Table S5). For the changes of organic acids, in Dendang roots, succinate (−2.6-fold), malate (−8.8-fold), and quinate (−2.6-fold) levels showed a sustained and significant decrease at T2 ($p < 0.05$). However, the concentrations of malonate (3.0-fold, $p < 0.1$), pipecolate (2.6-fold, $p < 0.05$) were significantly up-regulated. Only two metabolites, including shikimate (−2.5-fold) and mannitol (5.3-fold) were significantly influenced by salinity in Fatmawati roots ($p < 0.05$). In Nipponbare roots, succinate (−1.6-fold), 2-oxoglutarate (−1.8-fold), shikimate (−1.6-fold) levels continued to decrease significantly ($p < 0.05$) at 14 days except citrate (1.9-fold, $p < 0.1$) levels which were increased. For the changes of sugars, fructose decreased −33.3-fold, −1.7, −4.8-fold in Dendang, Fatmawati and Nipponbare root respectively. Glucose (−4.4-fold, $p < 0.05$) in Dendang roots and galactitol (−2.0-fold, $p < 0.05$) in Nipponbare roots significantly decreased in their concentrations after salt treatment. At the same time, raffinose in both Dendang (7.3-fold) and Nipponbare (2.0-fold) roots was significantly increased ($p < 0.05$). Dendang was the only cultivar that showed significantly increase of trehalose (4.4-fold) in roots after 14 days of treatment ($p < 0.05$). Furthermore, mannitol level was the highest increased sugar in Dendang roots (70.7-fold), followed by 5.3-fold increase in Fatmawati roots ($p < 0.05$), while mannitol was not significantly changed in Nipponbare roots at T2. At T2, the fold changes of mannitol in Dendang roots were 26.9-fold higher than in Nipponbare roots. Although no significant differences were observed in Fatmawati and Nipponbare roots at 14 days, the concentrations of sucrose were −2.2-fold and −1.5-fold lower compared with that in the control group.

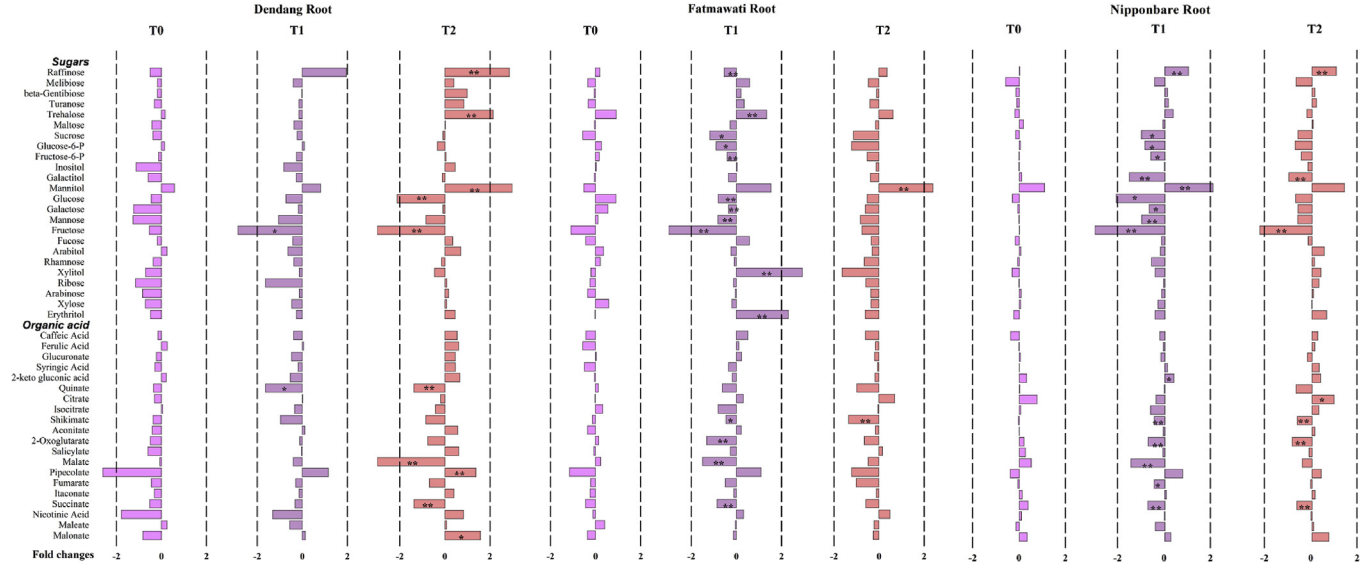

Fig. 3. Log_2 fold changes of primary metabolites in roots of Dendang, Fatmawati, and Nipponbare cultivars at 0 (T0), 7 (T1) and 14 (T2) days after salt treatment. Fold changes were calculated by dividing the concentrations of control by the concentrations of salt treated at the same time point. * and ** represent significant differences (FDR-adjusted p value < 0.1) and highly significant differences (FDR-adjusted p value < 0.05) compared with control. A threshold of ± 2-fold change is indicated as a dashed line.

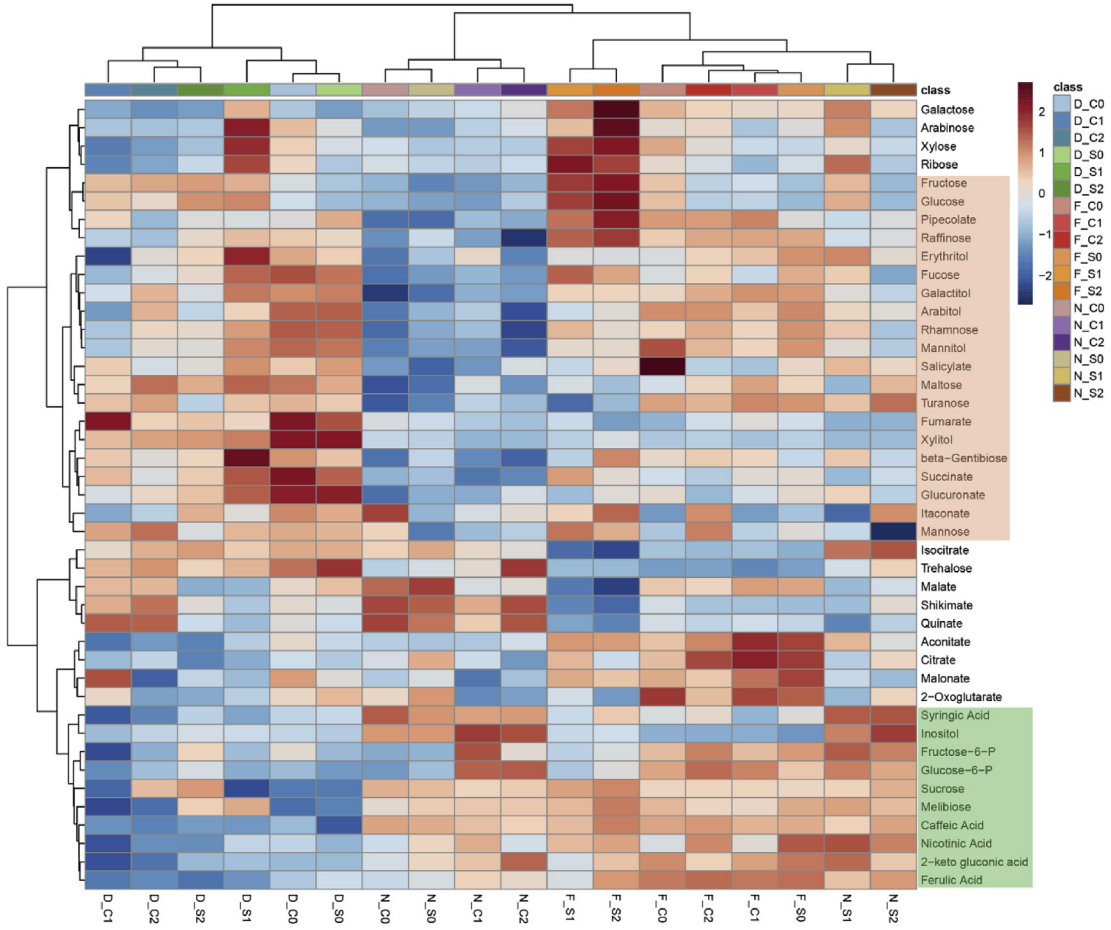

Fig. 4. Clustered heatmap of metabolite concentrations (normalized by log10) in Dendang, Fatmawati and Nipponbare leaves. Each coloured cell represents metabolites average concentrations (n = 4). Samples are colour coded as the legend on the figure. D, Dendang; F, Fatmawati; N, Nipponbare; C, control group; S, salt stress; 0, 0 days; 1, 7days; 2, 14 days. The highlighted metabolites with light red colour indicated higher levels in tolerant cultivars while the highlighted metabolites with light green colour indicated lower levels in tolerant cultivars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. An overview of metabolite changes in rice roots and leaves

The metabolite concentrations in roots and leaves of all cultivars are summarized in a heat map as shown in Fig. 4 and Fig. 5 in order to provide an overview of metabolite changes under salt stress. In the leaves, 30 metabolites were significantly different between Dendang and Nipponbare samples. Among these metabolites, 14 sugars mainly involved in fructose metabolism and 6 organic acids involved in the TCA cycle showed higher levels in Dendang leaves, which may be attributed to its higher salt tolerance. In the roots, noteworthy was that 12 metabolites involved in shikimate pathway and sucrose metabolism grouped together in Dendang and Fatmawati roots and showed significant lower levels compared with that in Nipponbare roots at T1 and T2. In addition, 8 sugars and 3 organic acids levels were lower in Dendang roots than in Nipponbare roots either before or after salt treatment. Metabolites in Dendang or Nipponbare roots grouped together at different exposure time points while many metabolites in Fatmawati roots showed significant differences at T1 and T2 compared with T0. Metabolites in Fatmawati leaves or roots showed similarity with both Dendang and Nipponbare, which indicated the moderate tolerance to salt stress.

4. Discussion

4.1. Shoot length and root fresh weight in Nipponbare were affected by salt stress

Under salinity, the three cultivars performed differently during the 14 days of treatment. Compared with Nipponbare, Dendang and Fatmawati were less affected as indicated by a smaller reduction in shoot length, root length and root fresh weight. As morphological parameters are used for screening salt tolerant cultivars (Munns, 2002; Shahzad et al., 2012; Yildirim et al., 2015), our results demonstrate that Dendang and Fatmawati showed higher salt tolerance than Nipponbare.

The shoot and root growth of Nipponbare was affected by salinity in different ways. The shoot length was significantly decreased while the root fresh weight, rather than root length, was significantly reduced in Nipponbare after salt treatment. The reduction of shoot growth in a plant occurs in two phases: a rapid response to the increase in external osmotic pressure, and a slower response due to the accumulation of Na^+ in leaves (Munns and Tester, 2008). Thus, the decreased shoot length after longer term salt treatment may be due to gradual Na^+ accumulation. As the root length did not show significant differences and root lateral branches decreased compared to control roots (Fig. S1), the decreased root fresh weight may be a result of inhibited root lateral growth after salt stress. According to the morphological results, it can be concluded that growth of the more salt tolerant rice cultivars was less affected than the salt sensitive one under salt stress at the seedling

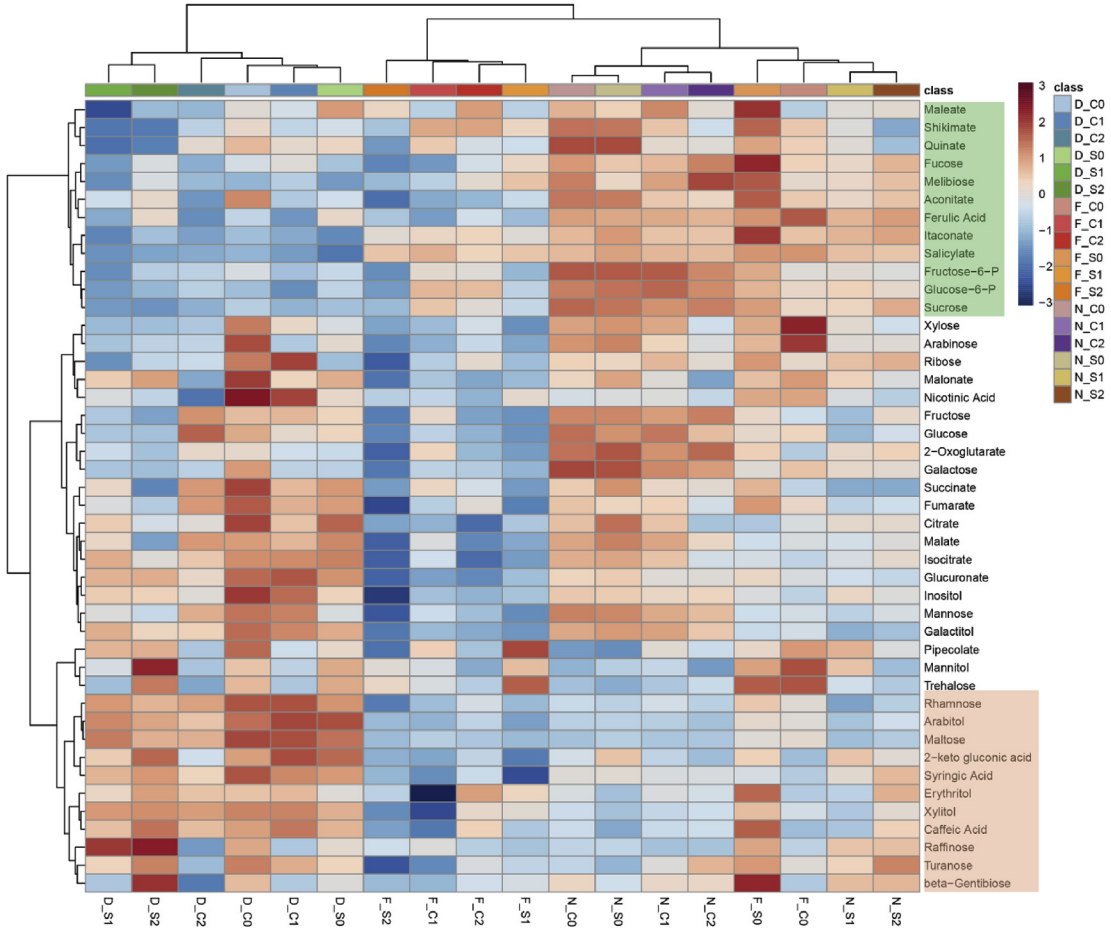

Fig. 5. Clustered heatmap of metabolite concentrations (normalized by log10) in Dendang, Fatmawati and Nipponbare roots. Each coloured cell represents metabolites average concentrations (n = 4). Samples are colour coded as the legend on the figure. D, Dendang; F, Fatmawati; N, Nipponbare; C, control group; S, salt stress; 0, 0 days; 1, 7days; 2, 14 days. The highlighted metabolites with light red colour indicated higher levels in tolerant cultivars while the highlighted metabolites with light green colour indicated lower levels in tolerant cultivars. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stage. Similar with our results, the growth of salt sensitive barley (Clipper) was more severely affected than Sahara, which is salt tolerant (Widodo et al., 2009).

4.2. The changes of metabolites depend on different exposure time points

In the leaves, compared with Nipponbare, 11 sugars in Dendang and 7 sugars in Fatamawati, such as raffinose, fructose, ribose and sucrose were significantly increased at T1. Sugars in the leaves of tolerant cultivars showed earlier responses to salt stress than the sensitive cultivars.

Nine sugars and six organic acids were only affected in Nipponbare roots at early stress time (T1) compared to that in Dendang roots. This result is consistent with a previous study that more strongly and exclusively decreased organic acids were observed at 7 days in IR64 (moderate tolerant rice cultivar) than FL478 (tolerant rice cultivar) roots (Zhao et al., 2014). The growth of the root system depends on the availability of carbon to the roots (Muller et al., 1998). The significant decreases of sugars in Nipponbare roots may explain the significant decrease of root fresh weight after 7 days of salt treatment.

4.3. Potential stress markers in the tricarboxylic acid (TCA) cycle and shikimate pathway

In our study, shikimate and quinate, both involved in shikimate pathway, were decreased in all rice leaves after salt treatment, which should be important indicators for salinity stress. Generally, the shikimate pathway is involved in biosynthesis of aromatic amino acids, including tryptophan, tyrosine, and phenylalanine (Shelden et al., 2016). Thus, the decrease of shikimate and quinate may have been increasingly used as precursors for amino acid synthesis to provide more energy under salt stress. Another interesting finding is that metabolites involved in shikimate pathway were significantly lower in Dendang roots (Fig. 5) either before or after salt stress compared with Nipponbare, which may also indicate a distinct tolerance mechanism.

Malate, which is involved in the TCA cycle, was only significantly decreased in Dendang and Fatmawati leaves following salt treatment. In consistency with this result, the decreases of malate were also evident in the drought tolerant wheat cultivars (Bowne et al., 2012). It is proposed that salt stress may have induced a depletion of malate which may be a key factor to enhance salt tolerance.

As the degree of cation-anion imbalance is one of the key factors to determine organic acid levels in plants, the excessive Na^+ uptake would require more organic acids (Yang et al., 2010). In our study, citrate, isocitrate and succinate involved in TCA cycle only significantly increased in Nipponbare leaves at T2, which may act to compensate the charge imbalance. Furthermore, more organic acids in Nipponbare roots were affected at T1 than in leaves. This phenomenon may indicate that Nipponbare roots showed more quickly response to salinity than leaves which was opposite compared with Dendang.

4.4. Raffinose and sucrose in leaves are potential markers for salt stress

The accumulation of sugars is regarded as a common response to different abiotic stresses (Gupta and Kaur, 2005). Raffinose is synthesized from sucrose and galactinol condensation, which can be used for carbon storage and as compatible solute (Hannah et al., 2006). Thus, we propose that the increases of raffinose levels in Dendang, Fatmawati and Nipponbare leaves may be a response of leaves to the increased energy requirements and osmotic stress. Sucrose levels in Dendang and Fatmawati leaves were significantly higher after salt stress compared with the respective control group at T1 and T2 while not changed in Nipponbare leaves. Previous studies have demonstrated that sucrose metabolism is among the key regulatory systems conferring tolerance to abiotic stress (Ruan et al., 2010). Thus, the sucrose metabolism pathway in the leaves of tolerant cultivars may act to resistant salinity, which needs further research.

4.5. Mannitol and trehalose in roots are potential markers for salt stress

Mannitol and trehalose have been suggested as regulatory components in a variety of stress survival strategies (Stoop et al., 1996; Goddijn and van Dun, 1999). Physiologically, these metabolites can act as compatible solutes in the cytoplasm under salt stress, increase osmotic potential of the cytoplasma in shoot/root cells to avoid cell dehydration (Bogdan and Zagdanska, 2006), and protect proteins from salt-induced dissociation (Wang et al., 2016). In our study on rice, mannitol showed a high level of increase in rice roots after salt treatment for 2 weeks. The fold changes of mannitol in the different cultivars from high to low were Dendang > Fatmawati > Nipponbare. The mannitol levels in Dendang roots were 26.9-fold higher than in Nipponbare roots at T2. This result strongly indicated that the high levels of mannitol in Dendang roots may act as compatible solute and attribute to its higher salt tolerance. Similar with our results, transgenic peanut with overexpressing mannitol-1-phosphate dehydrogenase (*mltD*) gene could enhance salinity tolerance via mannitol accumulation (Patel et al., 2016). As *mltD* gene plays an important role in synthesis of mannitol (Conde et al., 2011), we predict that the modification of *mltD* gene would improve the tolerance of rice to salinity. Trehalose also showed higher levels in Dendang roots than Nipponbare, but not as dramatically as mannitol. Wang et al. (2016) found that trehalose was also the only sugar with a relatively high accumulation in the roots of two salt tolerant rice genotypes (PL177 and IR64) after abiotic stress. Above all, the accumulation of mannitol and trehalose must be considered as important salt stress reaction markers in rice roots.

4.6. Tolerance screening of Dendang, Fatmawati and Nipponbare

According to the morphological results, the shoot and root growth was only affected by salt in Nipponbare, which indicated higher salt tolerance of Dendang and Fatmawati than Nipponbare. Based on the metabolite profiles, sugars and organic acids patterns showed high similarities between Dendang and Fatmawati leaves. In contrast, many sugars and organic acids showed similar changes between Fatmawati and Nipponbare roots especially at T1. In addition, metabolites in Fatmawati clustered together with either Dendang or Nipponbare in the heatmaps. These results demonstrated that Fatmawati is a moderate tolerant cultivar and the tolerance order can be concluded as: Dendang > Fatmawati > Nipponbare.

In conclusion, our study investigated the different metabolite responses to salinity in three rice cultivars. The shoot length, root length and root fresh weight in salt tolerant cultivars (Dendang and Fatmawati) were not affected by salinity. Eleven sugars in Dendang leaves showed earlier responses to salt stress compared with these in roots, which may be related with sodium concentration in tissues, however this requires further study. Organic acids, such as shikimate, quinate and malate, and non-polar sugars, such as trehalose, raffinose, sucrose and mannitol are important stress markers after salt treatment. Moreover, mannitol is a potential target metabolite in tolerant cultivars that needs further research to elucidate its role in tolerance mechanisms. Our study provides insight into the tolerant responses of Indonesian rice cultivars at the metabolite level.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Ute Roessner and Jing Chang developed the experiment. Siria Natera and Ute Roessner provided assistance with metabolite analysis. Jing Chang and Ute Roessner analysed data. Jing Chang drafted the manuscript which has been revised and reviewed by all other authors. Bo Eng Cheong provided advice and suggestions on plant growth and data analysis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.017.

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