Abstract: Salt stress is one of the key abiotic stresses causing huge productivity losses in rice. In addition, the differential sensitivity to salinity of different rice genotypes during different growth stages is a major issue in mitigating salt stress in rice. Further, information on quantitative proteomics in rice addressing such an issue is scarce. In the present study, an isobaric tags for relative and absolute quantitation (iTRAQ)-based comparative protein quantification was carried out to investigate the salinity-responsive proteins and related biochemical features of two contrasting rice genotypes—Nipponbare (NPBA, japonica) and Liangyoupeijiu (LYP9, indica), at the maximum tillering stage. The rice genotypes were exposed to four levels of salinity: 0 (control; CK), 1.5 (low salt stress; LS), 4.5 (moderate salt stress; MS), and 7.5 g of NaCl/kg dry soil (high salt stress, HS). The iTRAQ protein profiling under different salinity conditions identified a total of 5340 proteins with 1% FDR in both rice genotypes. In LYP9, comparisons of LS, MS, and HS compared with CK revealed the up-regulation of 28, 368, and 491 proteins, respectively. On the other hand, in NPBA, 239 and 337 proteins were differentially upregulated in LS and MS compared with CK, respectively. Functional characterization by KEGG and COG, along with the GO enrichment results, suggests that the differentially expressed proteins are mainly involved in regulation of salt stress responses, oxidation-reduction responses, photosynthesis, and carbohydrate metabolism. Biochemical analysis of the rice genotypes revealed that the Na\(^+\) and Cl\(^-\) uptake from soil to the leaves via the roots was increased with increasing salt stress levels in both rice genotypes. Further, increasing the salinity levels resulted in increased cell membrane injury in both rice cultivars, however more severely in NPBA. Moreover, the rice root activity was found to be higher in LYP9 roots compared with NPBA under salt stress conditions, suggesting the positive role of rice root activity in mitigating salinity. Overall, the results from the study add further insights into the differential proteome dynamics in two contrasting rice genotypes with respect to salt tolerance, and imply the candidature of LYP9 to be a greater salt tolerant genotype over NPBA.

Keywords: Salt stress; Oryza sativa; proteomics; iTRAQ quantification; cell membrane injury; root activity
1. Introduction

To satisfy the food demands of a population of more than nine billion people by 2050, the world’s food productivity needs to be increased by 50% above current production [1,2]. The current growth trends of the major food crops, including wheat, rice, maize, and soybean, suggest that crop production will not be sufficient to meet these ever-rising food demands [3]. Further, the occurrence of abiotic stresses owing to climate change is one of the major reasons for the productivity gap [4]. Soil salinity is considered to be a major problem in the productivity of rice (Oryza sativa L.) worldwide [4]. Rice is highly sensitive to salt stress; however, the range of sensitivity varies with rice ecotypes, genotypes, and growth stages [5,6]. Salt tolerance in rice is correlated with variations in the translocation of sodium (Na\(^+\)) and chloride (Cl\(^-\)) ions in the aboveground plant organs, including the shoot and panicles [7–12]. Salinity affects rice physiology and growth by causing osmotic stress, nutrient imbalance, ionic toxicity, oxidative damage, alteration of metabolic processes, reduced cell division, genotoxicity, decline of growth and yield, and even the death of the plant [8,9,13–17]. In rice, salinity tolerance is usually achieved as a result of a cocktail of physiological and genetic reprogramming, including selective ion uptake and exclusion, preferential compartmentation of Na\(^+\), alternation in stomatal closure, reactive oxygen species (ROS) signaling, and expression of salt-stress responsive genes and transcription factors [18–22].

Alterations in physiological and biochemical processes lead to changes in the protein pool in plants. In recent times, proteomic analysis has emerged as a significant molecular technique for the profiling and identification of proteins expressed in response to various abiotic stresses [23]. Isobaric tags for relative and absolute quantitation (iTRAQ)-based protein profiling and analysis has been performed in several crops, including rice [24], maize [25], wheat [26], tomatoes [27], and cotton [23], in response to abiotic stresses. Differential protein expressions in the areal tissues of rice subjected to salt stress have been reported by a few studies [28–30]. However, most of these studies have employed the 2D gel electrophoresis method to quantify the protein dynamics in rice. The 2D gel electrophoresis technique lacks efficiency in identifying the low abundant proteins, including extreme-acidic or basic proteins, proteins with molecular weights <15 kDa or >150 kDa, and hydrophobic proteins [23]. Furthermore, most of these works have been performed using the japonica rice genotype “Nipponbare” as the plant material. Therefore, in this study, we explored the proteomic dynamics of rice under salt stress in both japonica (Nipponbare, NPBA) and indica (Liangyoupeijiu, LYP9) rice genotypes by employing an iTRAQ-based proteomic study.

In the current study, the iTRAQ-based proteomic technique was used to identify the differentially expressed proteins in two rice genotypes of contrasting salt tolerance levels. The indica rice LYP9 has a higher salt tolerance level than the japonica rice NPBA [13]. Therefore, the proteomic analysis was performed with the aim of elucidating and comparing the effects of salt stress in these rice genotypes. Further, the physiological responses, such as cell membrane injury (CMI) and rice root activity of the NPBA and LYP9 genotypes, were assessed in response to various salt stress levels at the maximum tillering stage. Additionally, the Na\(^+\) and Cl\(^-\) uptake from soil to leaf via root under the subjected salt stress levels were determined in both rice genotypes. The results from this study will help us to achieve better insights into the salt stress resistance mechanisms in rice.

2. Results

2.1. Na\(^+\) and Cl\(^-\) in the Soil

The soil Na\(^+\) concentrations for LYP9 rice were recorded to be 0.17, 0.95, 1.7, and 2.0 mg g\(^{-1}\) for the control (no salt stress, CK), low salt stress (LS), moderate salt stress (MS), and high salt stress (HS) treatments, respectively. In NPBA, the soil Na\(^+\) was recorded to be 0.18, 1.0, 1.6, and 2.15 mg g\(^{-1}\) for the CK, LS, MS, and HS treatments, respectively. The Na\(^+\) concentration was found to be the highest in the HS treatment for NPBA rice, as most of the rice seedlings died under the HS condition before attaining the maximum tillering stage. Furthermore, the soil Na\(^+\) concentration was lower for the LYP9
rice than the NPBA rice (Table 1). On the other hand, the soil Cl\(^{-}\) concentrations were recorded to be 0.04, 0.59, 2.17, and 2.43 mg·g\(^{-1}\) for the CK, LS, MS, and HS treatments in LYP9 rice, respectively. In NPBA, the soil Cl\(^{-}\) was found to be 0.01, 0.66, 1.64, and 3.03 mg·g\(^{-1}\) for the CK, LS, MS, and HS treatments, respectively.

Table 1. Differential Na\(^{+}\) and Cl\(^{-}\) uptake from soil to leaf via root in LYP9 and NPBA under different salt stress levels at rice maximum tillering stage.

| Cultivars | Treatments | Na\(^{+}\) (mg/g) | Cl\(^{-}\) (mg/g) | Na\(^{+}\) (mg/g) | Cl\(^{-}\) (mg/g) |
|-----------|------------|------------------|-----------------|------------------|-----------------|
|           | Root       | Leaf             | Root            | Leaf             | Root            | Leaf            |
| LYP9      | CK         | 0.7 ± 0.05d      | 0.2 ± 0.03c     | 0.5 ± 0.3d       | 6.8 ± 0.4d      | 0.2 ± 0.01d     | 0.04 ± 0.01e    |
|           | LS          | 1.1 ± 0.03bc     | 0.5 ± 0.08b     | 1.6 ± 0.7cd      | 12.4 ± 1.7cd    | 1.0 ± 0.03c     | 0.6 ± 0.06de    |
|           | MS          | 1.5 ± 0.04b      | 0.8 ± 0.10a     | 7.9 ± 1.3ab      | 17.6 ± 2.4ab    | 1.7 ± 0.04b     | 2.2 ± 0.06bc    |
|           | HS          | 1.6 ± 0.09a      | 0.9 ± 0.11a     | 9.5 ± 1.6a       | 19.1 ± 2.9a     | 2.0 ± 0.07a     | 2.4 ± 0.33c     |
| NPBA      | CK          | 0.7 ± 0.03d      | 0.15 ± 0.01cd   | 1.0 ± 0.3d       | 9.9 ± 0.9cd     | 0.2 ± 0.01d     | 0.01 ± 0.01e    |
|           | LS          | 1.0 ± 0.07c      | 0.3 ± 0.01c     | 3.8 ± 0.4c       | 14.7 ± 2.8bc    | 1.0 ± 0.02c     | 0.7 ± 0.08c     |
|           | MS          | 1.3 ± 0.07b      | 0.9 ± 0.02a     | 6.3 ± 0.6b       | 18.7 ± 2.4ab    | 1.6 ± 0.12b     | 1.6 ± 0.035d    |
|           | HS          | -                | -               | -                | -               | 2.2 ± 0.07a     | 3.0 ± 0.23a     |

Values are denoted as mean ± SE (n = 3). Values followed by different letters denote significant difference (p ≤ 0.05) according to LSD test. Abbreviations: control (no salt stress, CK), low salt stress (LS), moderate salt stress (MS), and high salt stress (HS), Liangyoupeijiu (LYP9), Nipponbare (NPBA). The similar lettering within rice genotype shows the significant and different lettering mean non-significance within treatment levels.

2.2. Na\(^{+}\) and Cl\(^{-}\) in the Rice Plants

The concentration of Na\(^{+}\) was found to increase in rice in proportion to rice growth. At the time of rice transplanting, the Na\(^{+}\) concentration in the LYP9 and NPBA roots was 0.44 and 0.37 mg·g\(^{-1}\), respectively. However, at the maximum tillering stage, Na\(^{+}\) concentrations in rice roots was increased in both rice genotypes, with the increase in subjected salt stress levels. In LYP9 rice, LS, MS, and HS levels of salt stress resulted in the increase of Na\(^{+}\) concentrations in rice roots amounting to 67.2%, 126.9%, and 138.8%, respectively, as compared with the CK treatment. Similarly, in NPBA rice, Na\(^{+}\) concentration in the roots was increased by 42.9% for LS and 128.6% for MS as compared with the CK treatment. However, the NPBA rice could not survive under HS salinity conditions. These results indicated that the uptake of Na\(^{+}\) is higher in rice in the maximum tillering stage as compared to the seedling stage (Table 1). Similar proportions were observed for Na\(^{+}\) concentration in rice leaves, where the Na\(^{+}\) concentrations were found to be increased by 163.2%, 305.3%, and 357.9% under LS, MS, and HS conditions, respectively, as compared with the CK condition in LYP9 rice, and by 86.7% and 480% under LS and MS conditions, respectively, as compared with the CK condition in NPBA rice (Table 1). The Na\(^{+}\) uptake from root to shoot was found to be higher in LYP9 than NPBA. These results suggest that LYP9 has an enhanced ability to uptake Na\(^{+}\) in the plant parts than compared to NPBA, which might aid in improved salt tolerance in LYP9 compared with NPBA. Likewise, at the maximum tillering stage, the Cl\(^{-}\) uptake by the rice roots and leaves was increased with the increase in the salt stress levels (Table 1). Moreover, these increases in the Cl\(^{-}\) ion uptakes were found to be higher in LYP9 leaves and roots than those of NPBA.

2.3. Cell Membrane Injury (CMI) in Rice Flag Leaves

Evaluations of cell membrane injury (CMI) in both LYP9 and NPBA rice revealed that salt concentrations and CMI are directly proportional, where higher salt concentrations cause severe cell membrane damage. The CMI was found to be higher in the HS condition as compared with MS, LS, and CK conditions in both rice cultivars (Figure 1). CMI was recorded as 5% for CK, 6.7% for LS, 7% for MS, and 15.2% for HS in LYP9. However, CMI in NPBA was recorded as 9.8% for CK, 10.6% for LS, and 11.9% for MS. Compared with the control (CK), the CMI in the LYP9 rice cultivar was increased by 34%, 40%, and 204% under LS, MS, and HS, respectively. On the other hand, CMI was increased by 8.1% (LS), and 21.4% (MS) in the NPBA rice, whilst rice seedlings died under HS conditions before
reaching the maximum tillering stage in this genotype of rice (Figure 2). These results strongly suggest that salt stress negatively affects the cell membrane stability, and cell membrane integrity was found to be higher in LYP9 as compared with NPBA. Collectively, these results indicated that LYP9 is more tolerant to salt stress than NPBA.

**Figure 1.** Evaluation of cell membrane injury under the subjected salt stress in LYP9 and NPBA. Bars denoted mean values ± SE (n = 3). Values followed by different letters denote significant difference (p ≤ 0.05) according to LSD test. The similar lettering within rice genotype shows the significant and different lettering mean non-significance within treatment levels.

**Figure 2.** Effects of different levels of salt stress on the rice growth at the early stage in both LYP9 and NPBA.

### 2.4. Rice Root Activity

High root activity is an indicator of resistance against stress [31]. Rice root activity was increased by 2.1% for LS, 50.2% for MS, and 173.7% for HS as compared with CK in LYP9. In the case of NPBA, the rice root activity was decreased by 3.3% for LS, while it increased by 111.4% for MS, as compared to CK. In this study, the rice root activity was higher in LYP9 compared with NPBA under various salt stress levels, inferring the role of root activity in salt tolerance (Figure 3).
2.5. iTRAQ-Based Protein Identification at the Rice Maximum Tillering Stage

Quantitative proteomic analysis of three leaf samples (CK, LS, and MS) from NPBA rice and four leaf samples (CK, LS, MS, and HS) from LYP9 rice were performed using the iTRAQ method. In total, 5340 proteins were identified with 1% FDR (Table 2). In LYP9, 28, 368, and 491 proteins were found to be up-regulated under LS, MS, and HS treatments, respectively, as compared with the CK treatment. On the other hand, in NPBA, 239 and 337 up-regulated proteins were detected under the LS and MS treatments as compared with the CK treatment (Table 3). The longest length of enriched peptides was 7 to 18, with the mass error below 0.025 to 1.00 and with a high performing Pearson correlation coefficient with repeated samples, showing a high quality of the mass spectroscopy data and sample preparation. Proteins with a 1.2 fold change and Q-value of >0.05 were considered as differentially expressed proteins.

Table 2. Overview of the total protein identification in both rice genotypes.

| Total Spectra | Spectra | Unique Spectra | Peptides | Unique Peptide |
|---------------|---------|----------------|----------|----------------|
| 402,823       | 71,146  | 53,833         | 21,741   | 18,899         |
Table 3. Differentially expressed proteins in NPBA and LYP9 rice under different salt levels with 1.2 fold change and Q-value > 0.05.

| Protein ID | NCBI Accession | Protein Name | NPBA | LYP9 |
|------------|----------------|--------------|------|------|
|            |                | LS vs. CK | MS vs. CK | LS vs. CK | MS vs. CK | HS vs. CK |
| Salt responsive |     |            |      |      |      |      |
| tr|B9FWE4|B9FWE4_ORYSJ                 | gi 1222636749 | Uncharacterized protein | 1.516 | 1.415 | 0.906 | 1.234 | 1.255 |
| tr|A2Y7R4|A2Y7R4_ORYSI                 | gi 1115465579 | Malate dehydrogenase | 1.393 | 2 | 1.014 | 1.488 | 1.573 |
| tr|B8BBS3|B8BBS3_ORYSI                 | gi 1115476908 | Os08g0478200 protein | 1.389 | 1.593 | 0.951 | 1.402 | 2.706 |
| tr|A2WT84|A2WT84_ORYSI                 | gi 1115438875 | Malate dehydrogenase | 1.897 | 2.835 | 1.027 | 1.871 | 2.006 |
| tr|A0A0P0VS15|A0A0P0VS15_ORYSJ | gi 1115450217 | Nascent polypeptide-associated complex subunit β (Fragment) | 2.523 | 2.558 | 1.017 | 1.594 | 1.384 |
| tr|A2X1A0|A2X1A0_ORYSI                 | gi 124605452 | Os02g0768600 protein | 1.506 | 2.225 | 1.071 | 2.213 | 2.212 |
| tr|A0A190X658|A0A190X658_ORYSI | gi 1115477769 | L-isospartate methyltransferase | 1.575 | 2.403 | 0.901 | 1.591 | 1.69 |
| sp|Q43008|SODM_ORYSJ                  | gi 1115463191 | Superoxide dismutase | 1.775 | 2.06 | 1.071 | 1.534 | 1.828 |
| sp|Q9F6E1|APX2_ORYSJ                  | gi 1115474285 | Ascorbate peroxidase | 1.308 | 2.26 | 0.966 | 1.227 | 1.119 |
| sp|Q07661|NDK1_ORYSJ                  | gi 1116179762 | Nucleoside diphosphate kinase 1 | 1.295 | 1.816 | 0.909 | 1.068 | 1.435 |
| sp|Q5N7251|ALFC3_ORYSJ                | gi 11297598143 | Fructose-bisphosphate aldolase 3 | 1.399 | 1.639 | 1.023 | 1.089 | 1.532 |
| sp|Q7XDC8|MDHC_ORYSJ                  | gi 1115482534 | Malate dehydrogenase | 1.37 | 1.749 | 1.004 | 1.284 | 1.523 |
| tr|A2X753|A2X753_ORYSI                 | gi 1115447273 | Os02g0612900 protein | 1.441 | 1.552 | 1.036 | 1.506 | 1.597 |
| tr|A2X7X9|A2X7X9_ORYSI                 | gi 1125540544 | Putative uncharacterized protein | 1.152 | 1.502 | 0.882 | 1.378 | 1.25 |
| tr|A0A0P0V8X8|A0A0P0V8X8_ORYSJ | gi 108706531 | Os03g0182600 protein | 0.852 | 1.876 | 0.908 | 0.949 | 1.367 |
| tr|E0X6V4|E0X6V4_ORYSJ                 | gi 1306415973 | Triosephosphate isomerase | 1.003 | 2.132 | 1.027 | 1.107 | 1.256 |
| tr|A2ZA7A1|A2ZA7A1_ORYSI              | gi 1115438466 | Nucleoside diphosphate kinase | 1.197 | 2.406 | 0.882 | 1.168 | 1.768 |
| sp|Q9ATR3|APX1_ORYSI                  | gi 1132491410 | Glucanase | 1.063 | 2.09 | 0.876 | 0.912 | 1.39 |
| tr|A2ZXH2|A2ZXH2_ORYSI                 | gi 1115477556 | Expressed protein | 1.048 | 1.561 | 0.957 | 1.204 | 1.424 |
| tr|B9FWE4|B9FWE4_ORYSJ                 | gi 1222636335 | Peroxidase | 0.888 | 1.812 | 0.966 | 1.298 | 1.814 |
| tr|B8B893|B8B893_ORYSI                 | gi 218199240 | Plasma membrane ATPase | 1.404 | 1.435 | 0.906 | 0.716 | 0.86 |
| tr|A2X2A0|A2X2A0_ORYSI                 | gi 111448935 | Proteasome subunit β type | 0.862 | 1.109 | 0.946 | 1.067 | 1.059 |
| tr|A2Y628|A2Y628_ORYSI                 | gi 1255528289 | Cysteine proteinase inhibitor | 0.96 | 1.775 | 1.056 | 1.438 | 2.205 |
| tr|Q5GZ11|Q5GZ11_ORYSA               | gi 14097938 | Beta-1,3-glucanase | 0.795 | 1.711 | 1.003 | 0.932 | 1.619 |
| tr|A2ZCK1|A2ZCK1_ORYSI               | gi 1148762354 | Nucleoside diphosphate kinase 2 | 0.63 | 0.866 | 0.975 | 1.019 | 1.019 |
| sp|A2ZCF7|APX1_ORYSI                  | gi 1158512874 | L-aspartate peroxidase 1 | 1.216 | 1.343 | 0.942 | 1.21 | 1.327 |
| tr|A2X822|A2X822_ORYSI                  | gi 125540087 | Glutathione peroxidase | 0.717 | 0.617 | 0.924 | 1.77 | 1.567 |
| tr|A2XFD1|A2XFD1_ORYSI                 | gi 125543402 | Putative uncharacterized protein | 1.1 | 1.543 | 0.943 | 1.23 | 1.554 |
| tr|A2YL13|A2YL13_ORYSI                 | gi 1115472191 | Os07g0495200 protein | 1.159 | 1.821 | 0.971 | 1.505 | 1.818 |
| tr|B8AD11|B8AD11_ORYSI                 | gi 218187601 | NADH-cytochrome b5 reductase | 0.771 | 0.72 | 1.081 | 2.182 | 2.316 |
| tr|A2YSB2|A2YSB2_ORYSI                 | gi 1115473275 | Os08g0205400 protein | 1.587 | 2.217 | 0.908 | 1.597 | 1.194 |
| tr|B8B893|B8B893_ORYSI                 | gi 2181967721 | Fructose-bisphosphate aldolase | 0.808 | 1.047 | 0.74 | 1.074 | 1.605 |
| tr|B8AY17|B8AY17_ORYSI                | gi 218196757 | Putative uncharacterized protein | 0.725 | 0.849 | 0.996 | 1.174 | 1.649 |
| tr|Q5NZ11|Q5NZ11_ORYSA                | gi 14097938 | Beta-1,3-glucanase | 0.795 | 1.711 | 1.003 | 0.932 | 1.619 |
| sp|Q941Z0|NQR1_ORYSJ                   | gi 115442299 | Putative uncharacterized protein | 0.686 | 0.766 | 0.984 | 0.931 | 1.369 |
| tr|A2WWV4|A2WWV4_ORYSI                 | gi 125528336 | Putative uncharacterized protein | 0.518 | 0.55 | 1.031 | 1.159 | 1.304 |
| Protein ID | NCBI Accession | Protein Name                                      | NPBA | LYP9 |
|------------|----------------|--------------------------------------------------|------|------|
| sp|P93438|METK2_ORYSJ|S-adenosylmethionine synthase|1.282|1.017 |
| tr|A2XU9B1|A2XU9B1_ORY I|B0812A04.3 protein|1.074|1.225 |
| tr|A2ZZZ0|A2ZZZ0_ORYSI|Putative uncharacterized protein|1.01|0.776 |
| tr|B8AEU4|B8AEU4_ORYSI|Putative uncharacterized protein|0.954|1.212 |
| tr|A0A0PVTX8|A0A0PVTX8_ORYS|Os3gp0162600 protein|0.852|1.876 |
| tr|Q6B8N9|Q6B8N9_ORYRS|putative endo-1,3,4-β-D-glucanase|1.16|1.14 |
| tr|B8AT7W|B8AT7W_ORYS|Os4g0602100 protein|1.386|1.494 |
| sp|Q7FAH2|G3PC2_ORYS|Glyceraldehyde-3-phosphate dehydrogenase 2|0.887|1.03 |
| tr|Q0JC30|Q0JC30_ORYSJ|Os01g0946300 protein|0.95|0.844 |
| tr|Q6L3H4|Q6L3H4_ORYS|Putative aldolase|0.735|0.737 |
| sp|A2XW22|DHE2_ORYS|Amylopectin|1.357|1.021 |
| sp|Q259G4|PMM_ORYS|Phosphomannomutase|0.836|1.19 |
| tr|A2YWS7|A2YWS7_ORYS|Photosynthesis related|0.95|1.611 |
| tr|Q3QWM7|Q3QWM7_ORYS|Photosystem I 9K protein|1.77|2.373 |
| tr|B8BCC6|B8BCC6_ORYS|Photosystem II oxygen-evolving complex protein 2|2.24|2.885 |
| tr|B8AY24|B8AY24_ORYS|Peroxidase|0.956|1.173 |
| sp|Q0D6V8|Q0D6V8_ORYSA|Photosystem I 9K protein|1.77|2.373 |
| tr|A2XZK1|A2XZK1_ORYS|Putative uncharacterized protein|1.246|1.015 |
| tr|Q8D6V8|Q8D6V8_ORYS|Putative uncharacterized protein|1.418|2.178 |
| tr|Q7XH15|Q7XH15_ORYS|2Fe-2S iron-sulfur cluster protein-like|1.016|1.414 |
| tr|A2X7M2|A2X7M2_ORYS|Photosystem II oxygen-evolving complex protein 2|1.77|2.373 |
| tr|B0FFP0|B0FFP0_ORYS|Chloroplast polysaccharide polysaccharide: PS II (Fragment)|1.319|1.705 |
| tr|Q7MIU9|Q7MIU9_ORYSA|Photosystem I 9K protein|1.832|3.172 |
| tr|A0A0PFX80|A0A0PFX80_ORYS|Photosystem II reaction center protein H|0.795|0.694 |
| tr|Q7MIY7|Q7MIY7_ORYS|Photosystem II oxygen-evolving complex protein 2|1.77|2.373 |
| tr|B8AJX7|B8AJX7_ORYS|Serine hydroxymethyltransferase|2.24|2.885 |
| tr|B8AY24|B8AY24_ORYS|Putative uncharacterized protein|1.288|1.56 |
| sp|Q6Z2Z6|Q6Z2Z6_ORYS|Geranylgeranyl reductase|0.956|1.173 |
| sp|P0CA201|P0CA201_ORYS|Photosystem II reaction center protein H|0.795|0.694 |
| Oxidation reduction responsive|tr|A3BVS6|A3BVS6_ORYS|Superoxide dismutase|1.512|1.903 |
| sp|Q8D6V8|Q8D6V8_ORYS|Putative uncharacterized protein|0.956|1.681 |
| sp|Q95D6X|Q95D6X_ORYS|Peroxiredoxin-2F, mitochondrial|1.363|1.772 |
| tr|B7FAE9|B7FAE9_ORYS|Glutathione peroxidase|0.98|1.347 |
| tr|A2Y043|A2Y043_ORYS|Peroxidase|1.232|2.046 |
| tr|Q9FTN6|Q9FTN6_ORYS|Os01g0106300 protein|0.732|1.977 |
Table 3. Cont.

| Protein ID | NCBI Accession | Protein Name                  | NPBA  | LYP9  |
|------------|----------------|------------------------------|-------|-------|
| tr|A2X2T0|A2X2T0_ORYSI   |gi 155700921 Peroxidase     |0.775 |0.85  |1.697 |
| tr|O22440|O22440_ORYSA   |gi 115474063 Peroxidase     |1.763 |2.05  |1.612 |
| tr|A3AY73|A3AY73_ORYSJ   |gi 125582491 Uncharacterized protein |1.099 |1.101 |2.4   |
| tr|B9FL20|B9FL20_ORYRS   |gi 115464801 Uncharacterized protein |1.175 |1.159 |1.356 |
| tr|Q4AS12|Q4AS12_ORYSJ   |gi 115436300 Peroxidase     |4.654 |1.948 |2.334 |
| tr|B8ATW7|B8ATW7_ORYSI   |gi 115460338 Os04g0602100 protein |1.386 |1.115 |1.448 |
| tr|B9FCM4|B9FCM4_ORYSJ   |gi 116309795 OSIGBu148A10.12 protein |2.208 |1.365 |1.123 |
| tr|Q0JB49|Q0JB49_ORYRS   |gi 115459848 Glutathione peroxidase |1.449 |1.537 |1.271 |
| tr|Q4306|Q43006_ORYSA   |gi 20286|emb Peroxidase |4.58  |1.421 |1.233 |
| tr|Q5ZT7|Q5ZT7_ORYRS    |gi 155701041 Peroxidase     |5.025 |2.469 |2.659 |
| tr|Q25AK7|Q25AK7_ORYSA   |gi 190265065 H0510A06.15 protein |1.326 |1.209 |1.022 |
| tr|Q6K414|Q6K414_ORYRS   |gi 115479691 Peroxidase     |1.23  |1.16  |0.919 |
| tr|A2WQ7|A2WQ7_ORYSI    |gi 115434036 Os01g0106400 protein |0.884 |0.973 |2.057 |
| sp|P41095|RLA0_ORYRS     |gi 115474653 60S acidic ribosomal protein |1.312 |1.082 |0.83  |
| sp|B8AUI3|GLO3_ORYSI     |gi 115460650 Peroxosial (S)-2-hydroxy-acid oxidase GLO3 |0.627 |0.833 |0.972 |
| tr|A0A0N7K36|A0A0N7K36_ORYSJ |gi 155700967 Peroxidase |0.895 |1.403 |1.041 |
| tr|B85W71|B85W71_ORYSI   |gi 1218200254 Peroxidase     |1.11  |2.708 |1.966 |
| tr|A2WPA1|A2WPA1_ORYSI   |gi 125525692 Peroxidase     |1.258 |2.133 |3.577 |
| tr|A2ZAA6|A2ZAA6_ORYSI   |gi 115487964 Putative peptide methionine sulfoxide reductase |1.121 |1.739 |1.32  |
| tr|A2XVK6|A2XVK6_ORYSI   |gi 125549044 Putative uncharacterized protein |0.844 |1.321 |1.19  |
| tr|B9F688|B9F688_ORYRS   |gi 1222642472 Uncharacterized protein |2.063 |2.407 |2.351 |
| tr|B8AU10|B8AU10_ORYRS   |gi 121894884 Putative uncharacterized protein |1.206 |1.386 |1.226 |
| tr|Q7FI19|Q7FI19_ORYYS   |gi 115477368 Os08g0522400 protein |1.225 |1.309 |1.121 |
| sp|Q6K471|Q6K471_ORYRS   |gi 175125055 Ferrodoxin-thioredoxin reductase |1.28  |1.598 |1.668 |
| sp|B8BAM3|B8BAM3_ORYSI   |gi 115467518 Os05g0601000 protein |1.178 |1.016 |1.233 |
| sp|Q6AV34|ARGC_ORYRS     |gi 1218193315 Os05g0601000 protein |1.046 |0.966 |1.237 |
| tr|A0A0B4U1V7|A0A0B4U1V7_ORYSA |gi 115467518 Aldehyde dehydrogenase ALDH2b |1.178 |1.016 |1.233 |
| sp|Q7F229|Q7F229_ORYYS   |gi 115471449 Os07g0260300 protein |0.924 |1.375 |2.104 |
| tr|A6NOB2|A6NOB2_ORYRS   |gi 114939139 Mitochondrial formate dehydrogenase 1 (Fragment) |0.993 |0.931 |0.99  |
| Protein ID | NCBI Accession | Protein Name | NPBA LS vs. CK | MS vs. CK | LYP9 MS vs. CK | HS vs. CK |
|------------|----------------|--------------|----------------|-----------|----------------|----------|
| sp|Q10L32|MSRB5_ORYSJ|Putative uncharacterized protein|1.116|1.471|0.84|1.272|1.479|
| tr|Q41T6|Q41T6_ORYSJ|Os01g0847700 protein|1.028|0.871|1.18|1.416|1.504|
| sp|B8BF2|B8BF2_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| sp|Q7XPL2|HEM6_ORYS|OSIGBa01512.9 protein|0.993|1.224|0.873|0.963|1.253|
| sp|P0C5D4|P0C5D4_ORYS|Peroxisidoxin Q, chloroplastic protein|1.215|1.691|0.909|1.71|2.077|
| tr|A0A0P0W9R9|A0A0P0W9R9_ORYSJ|Os06g0472000 protein|1.308|1.269|1.032|1.532|1.593|
| tr|A2W7L9|A2W7L9_ORYS|Peroxisidoxin|0.826|0.852|1.047|1.182|1.233|
| sp|P37834|PER1_ORYS|Peroxidase|0.702|0.999|0.819|0.742|1.764|
| tr|Q01LB1|Q01LB1_ORYSA|Os01g0847700 protein|1.028|0.871|1.18|1.416|1.504|
| sp|P0CUL1|APX6_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| sp|Q7X5R5|TRX2M_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| tr|B7E4H4|B7E4H4_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| sp|Q7X5R8|Q7X5R8_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| tr|Q7X5R8|Q7X5R8_ORYS|Formate dehydrogenase|1.014|1.19|0.97|0.898|1.278|
| Carbohydrate metabolism |
| sp|Q8L7J2|BCL6_ORYS|Beta-glucosidase 6|0.177|0.383|1.004|0.424|1.741|
| sp|Q76B5|XTH8_ORYS|Xyloglucan endotransglycosylase/hydrolase protein 8|0.953|2.101|0.939|1.074|1.369|
| tr|Q01JC3|Q01JC3_ORYSA|Malate dehydrogenase|0.795|0.74|0.995|0.591|0.889|
| tr|Q0DCB1|Q0DCB1_ORYS|Os06g0356700 protein|0.849|1.073|0.912|1.227|2.764|
| tr|Q10CU4|Q10CU4_ORYS|GH family 3 N terminal domain containing protein, expressed|0.72|2.799|0.66|0.663|2.234|
| tr|Q9ZNZ1|Q9ZNZ1_ORYS|Beta-1,3-glucanase|0.795|1.711|1.003|0.932|1.619|
| tr|H2KWT0|H2KWT0_ORYS|HIPI1 protein, putative, expressed|1.106|2.099|0.908|1.231|2.014|
| tr|B8A52|B8A52_ORYS|Putative uncharacterized protein|0.773|0.837|0.875|1.437|1.452|
| sp|Q0INM3|BGA15_ORYS|Beta-galactosidase 15|1.348|1.602|0.924|1.187|1.56|
| tr|Q6Z8F4|Q6Z8F4_ORYS|Phosphoribulokinase|1.143|1.318|1.066|1.144|1.249|

**Table 3. Cont.**

| Protein ID | NCBI Accession | Protein Name | NPBA LS vs. CK | MS vs. CK | LYP9 MS vs. CK | HS vs. CK |
|------------|----------------|--------------|----------------|-----------|----------------|----------|
| tr|A2XM08|A2XM08_ORYS|GH family 3 N terminal domain containing protein, expressed|0.859|1.124|0.866|0.78|1.387|
| sp|Q10NX8|BGAL6_ORYS|Beta-galactosidase 6|1.063|1.684|0.938|1.413|1.814|
| tr|B8AI11|B8AI11_ORYS|Putative uncharacterized protein|0.904|1.55|0.954|1.167|1.478|
| tr|Q01HI0|Q01HI0_ORYS|H502G05.3 protein|0.728|0.783|0.894|1.001|1.193|
| tr|Q01JK3|Q01JK3_ORYS|Aldose 1-epimerase|0.823|1.226|0.939|1.515|1.515|
| tr|B8BF97|B8BF97_ORYS|Alpha-galactosidase|0.723|1.32|1.006|1.231|1.544|
| tr|A2Z9V6|A2Z9V6_ORYS|Uncharacterized protein|0.723|1.32|1.006|1.231|1.544|
| tr|Q0DT59|Q0DT59_ORYS|Os03g0227400 protein (Fragment)|1.101|1.226|0.804|1.06|1.306|
| tr|A2XME9|A2XME9_ORYS|Malate dehydrogenase|1.049|1.261|1.151|1.502|1.548|
| tr|Q6Z8F4|Q6Z8F4_ORYS|Phosphoribulokinase|1.143|1.318|1.066|1.144|1.249|
### Table 3. Cont.

| Protein ID | NCBI Accession | Protein Name | NPBA | LYP9 |
|------------|----------------|--------------|------|------|
| tr|A2YJ5|gi|50509727|Os07g0168600 protein |0.779|0.93|0.952|1.118|1.317|
| sp|Q75F93|gi|115454825|Beta-glucosidase |1.201|1.066|0.95|1.276|1.18|
| tr|Q7XIV4|gi|115474081|Alpha-galactosidase |0.786|1.367|0.919|1.115|1.491|
| tr|A3A285|gi|115443693|Uncharacterized protein |0.83|1.101|0.843|1.151|1.262|
| tr|A1AIPX7Y51|gi|297610712|Alpha-galactosidase (Fragment) |0.72|1.115|0.848|1.16|1.321|
| tr|BTF946|gi|1297603789|Os06g0356800 protein|0.681|1.016|0.7|1.159|2.984|
| **Stress responsive** | | | | |
| tr|Q9AQU0|gi|1348673|Peptidyl-prolyl cis-trans isomerase |1.249|1.825|0.965|1.578|1.772|
| sp|Q8GT80|gi|27476086|Putative heat shock 70 KD protein, mitochondrial |1.294|1.354|0.92|1.027|1.208|
| tr|Q84Z20|gi|28979168|CHP-rich zinc finger protein-like |2.605|2.416|0.869|1.374|1.439|
| sp|Q73H4Q1|gi|115464027|Heat shock 70 kDa protein BIP4 |10|10|1.058|0.8|0.72|
| tr|Q53NM0|gi|115468793|DnaK-type molecular chaperone hsp70-rice |1.87|1.487|1.009|0.821|0.793|
| sp|Q5VRY1|gi|11545223|70 kDa heat shock protein |2.198|1.668|1.086|0.907|0.816|
| tr|Q5Y511|gi|115434946|17.5 kDa heat shock protein |1.413|3.508|1.045|1.084|1.043|
| sp|Q6UA7|gi|11547692|Os08g0464000 protein |1.323|1.3|1.041|0.866|1.034|
| tr|A2YK26|gi|115471453|NADH-dehydrogenase |1.096|1.252|0.994|0.995|1.314|
| sp|Q5Y511|gi|11545223|Uncharacterized protein |1.028|1.106|1.031|1.314|1.25|
| sp|Q5VRY1|gi|115434946|DnaK-type molecular chaperone hsp70-rice |1.1|1.218|0.999|1.144|1.342|
| tr|Q5AV43|gi|115458153|26S proteasome regulatory particle |1.138|1.146|0.981|1.293|1.427|
| sp|Q5VRY1|gi|11545223|26S proteasome regulatory particle |1.066|1.434|1.03|1.71|2.189|
| tr|A2Y826|gi|125552829|Cysteine protease inhibitor |0.96|1.775|1.056|1.438|2.205|
| **Osmotic stress responsive** | | | | |
| tr|A2XH11|gi|125544232|Sucrose synthase |0.82|1.102|0.545|0.366|1.005|
| sp|Q88355|gi|115473055|NADH-dehydrogenase |0.992|1.182|0.879|1.282|1.538|
| tr|Q2RBD1|gi|115483847|Non-specific lipid-transfer protein |0.988|1.244|0.894|1.274|2.093|
| sp|Q1O234|gi|29762544|Non-specific lipid-transfer protein |1.226|2.979|0.78|1.023|2.235|
| sp|Q88355|gi|115473055|Non-specific lipid-transfer protein |0.988|1.244|0.894|1.274|2.093|
| **Ethylene responsive** | | | | |
| tr|B9G3V3|gi|122641669|Uncharacterized protein |1.837|2.313|1.837|1.825|1.982|
| sp|Q8W3D9|gi|17524671|Putrophorphyline reductase B |0.881|1.621|0.891|1.192|2.065|
| sp|Q8W3D9|gi|115452897|Uroporphyrinogen decarboxylase 2 |1.265|1.835|0.902|0.95|1.425|
| tr|A2X877|gi|242062934|2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase |1.362|1.399|0.746|1.202|1.538|
| tr|Q2RBD1|gi|115483847|Non-specific lipid-transfer protein |0.988|1.244|0.894|1.274|2.093|
Table 3. Cont.

| Protein ID | NCBI Accession | Protein Name                      | NPBA  | LYP9  |
|------------|----------------|-----------------------------------|-------|-------|
|            |                |                                   | LS vs. CK | MS vs. CK | LS vs. CK | MS vs. CK | HS vs. CK |
| Metabolic responsive |              |                                   |       |       |
| tr|Q0D572|Q0D572_ORYSJ | gi|297607511 | Os07g0577300 protein | 1.28 | 1.719 | 1.105 | 0.899 | 2.422 |
| tr|A2YIJ5|A2YIJ5_ORYSI | gi|50509727 | Os07g0168600 protein | 0.779 | 0.93 | 0.952 | 1.118 | 1.317 |
| tr|B9F240|B9F240_ORYSJ | gi|222622048 | Uncharacterized protein | 0.739 | 1.149 | 1.372 | 1.24 | 1.422 |
| tr|B9F7T1|B9F7T1_ORYSJ | gi|222624734 | Uncharacterized protein | 1.389 | 1.083 | 1.317 | 0.854 | 0.954 |
2.6. Identification of Differential Expressive Proteins in LYP9 and NPBA Subjected to Different Salt Stress Levels

From the iTRAQ-based identified proteins in both rice genotypes, the proteins that showed a relative abundance of >1.2 fold or <0.8 fold in the salt stressed plants, as compared to the control, were considered to be differential expressive proteins (DEPs). In LYP9 rice, 1927 DEPs were identified under various salt levels. For instance, 93 (28 up-regulated, 65 down-regulated) DEPs were identified in the LS condition, 782 (368 up-regulated, 414 down-regulated) DEPs were identified in the MS condition, and 1052 (561 up-regulated, 491 down-regulated) DEPs were identified in the HS plants, as compared to the control (Figure 4A). On the other hand, 1154 DEPs were identified in the NPBA rice under the applied salt stress levels. Briefly, 432 (239 up-regulated, 193 down-regulated) DEPs were identified in the LS condition and 722 (385 up-regulated, 337 down-regulated) DEPs were identified in the MS plants, as compared with the control (Figure 4B). Identification of the DEPs in both rice genotypes indicated that, with an increase in the salt levels, the number of DEPs was also increased in both rice types. Further, under LS stress levels, the number of DEPs was significantly less in the salt tolerant LYP9 genotype than in the salt sensitive NBPA rice.

![Figure 4](image)

**Figure 4.** Identification of the differential expressive proteins (DEPs). (A) DEPs in LYP9 rice under various salt stress levels as compared with the control plants. (B) DEPs in NPBA rice under various salt stress levels as compared with the control plants. CK: control, LS: low salt, MS: medium salt, HS: high salt.

2.7. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Enrichment of the DEPs

To deduce the functionality and biological processes associated with the identified DEPs in the rice genotypes, GO analysis, Clusters of Orthologous Group (COG) annotations, and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichments were performed. The GO analysis revealed that the identified DEPs were associated with different molecular and biological processes (Figure 5A). Most of the identified DEPs in both rice genotypes were involved in cellular and metabolic processes (biological process). At the molecular level, most of the identified DEPs were involved in catalytic activity, binding, transporter and carrier activity, and structural molecule activity. Similarly, at the cellular component level, the identified DEPs were linked to the cell (membrane and cytoplasm) and organelles. In addition to that, COG analysis of the DEPs grouped them into 24 specific categories on the basis of their functional annotations (Figure 5B). Most of the DEPs were clustered in the “general functional prediction only” category, whereas the post-translational modifications, translation, energy production, carbohydrate metabolism, and amino acid metabolism clusters were found to be the other abundant ones. Altogether, these results suggest that, under salt stress in the rice, salt-responsive proteins might be involved in different metabolic and cellular processes and localize in different cell parts and organelles.
Figure 5. Gene ontology (GO) and Clusters of Orthologous Group (COG) analysis of the differentially responsive proteins in response to salt stress. (A) The distribution of number of differentially responsive proteins alongside their corresponding GO terms. Different colors represent different GO categories. (B) The distribution of number of differentially responsive proteins alongside their different functions as annotated by COG analysis.

In addition, the KEGG enrichment of the identified DEPs in both rice genotypes revealed their functionality as per the associated pathways. The KEGG pathways, including the metabolic
pathway, oxidative phosphorylation, photosynthesis, lysine degradation, glyoxylate metabolism, carbon fixation, photosynthesis-antenna proteins, chlorophyll metabolism, pyruvate metabolism, and ribosomes were found to be the top 10 annotated pathways for the DEPs (Figure 6). From these, the metabolic pathways were found to be the primary enriched pathways in both the rice genotypes. Moreover, analysis of the detail of the KEGG enrichments and associated GO terms revealed that DEPs involved in the salt stress response, redox reactions, photosynthesis, and osmotic stress response were the most abundant in the rice genotypes (Figure 7). For instance, in LYP9, 41 salt-responsive proteins were found to be upregulated under various salt levels, whereas 26 upregulated DEPs were found in the NPBA rice. Similarly, 24 DEPs associated with carbohydrate metabolism were found to be upregulated in LYP9 rice, while 16 DEPs involved with carbohydrate metabolism were found to be upregulated in NPBA. The DEPs from both rice genotypes, with their corresponding fold changes as compared to the controls and their associated physiological pathways, are listed in Table 3. In addition, prediction of the subcellular localizations of the identified DEPs in both the rice genotypes revealed that most of the DEPs localize in the cytoplasm and chloroplasts (Figure 8).
3. Discussion

3.1. Biochemical Responses of Rice Plants to Salt Stress

Salt stress is a major concern in agriculture, affecting crop productivity across the world. Nutrient imbalance, due to the competition of Na\(^+\) and Cl\(^-\) with other nutrients, including potassium (K\(^+\)), calcium (Ca\(^{2+}\)), and nitrate (NO\(_3^-\)) ions, is a result of salt stress that compromises normal plant growth.
and development [8–12,32]. In addition, salt stress induces early leaf-senescence and a decrease in photosynthesis area [33]. Moreover, osmotic imbalance, poor leaf growth, high CMI, and decreased root activity are associated with the typical salt stress responses in plants [31]. In the current study, the subjection of salt stress negatively affected rice growth in the early stages. All four levels of applied salt stress to both rice cultivars resulted in compromised growth parameters along with CMI. The degree of CMI was found to be higher in NPBA as compared with LYP9, suggesting LYP9 has a higher salt tolerance capacity than NPBA (Figure 1). Further, high rice root activity is usually associated with the interaction of the root with rhizosphere soil and the microbial environment [34], changes in physico-chemical status [35], and plant growth [36]. Further, by enhancing the root activity, plants cope better under an unfavorable environment [34] (Figure 3). In this study, the salt tolerance levels of LYP9 were found to be much higher than those of NPBA at high salt conditions (HS). LYP9 plants could survive by significantly increasing their root activities, whereas none of NPBA plants could survive at the same salt concentrations (Figure 2).

3.2. Proteomic Analysis in the Rice Genotypes Under Salt Stress

Both transcriptomic and proteomic dynamics occurring when subjected to salt stress have already been reported in several plants [37]. Further, the availability of substantial sequential information on rice has paved the way for the use of analytical proteomic studies, including iTRAQ analysis. In this study, iTRAQ-based protein identifications in LYP9 and NPBA cultivars revealed their proteome dynamics in response to salt stress. The comparative analysis of the total of identified proteins (5340) revealed that 93, 782, and 1052 proteins were differentially regulated in LYP9 as compared to the control (CK) under LS, MS, and HS salt stress conditions, respectively. On the other hand, in NPBA, 432 and 722 differentially expressed proteins were found as compared to CK under LS and MS salt stress conditions, respectively (Table 3). These results suggest that the numbers of identified proteins are in direct proportion to the increasing salt stress levels. In addition, the finding of increased numbers of differentially expressed proteins in between LS and MS in both cultivars, and in between LS and MS, and MS and HS in LYP9, further strengthens the proposed proportional relationship between differential protein expression and salt stress levels. Moreover, using the iTRAQ identified protein information, we compared the proteins expressed in LYP9 and NPBA, and thereby the biochemical pathways were identified, including salt stress-responsive protein synthesis, redox responses, photosynthesis, and other metabolic processes. Some of these pathways in response to salt stress have been confirmed in some of the previous studies [38,39]; therefore, the functions of the identified DEPs in this study are discussed further below.

The proteome dynamics and the DEPs in NPBA and LYP9 rice genotypes under different salt stress levels were determined by using iTRAQ analysis. Further, to detect and quantify the proteins in the rice genotypes, the high-resolution LC–MS/MS technique was employed. The identified proteins were quantified on automated software called IQuant [40]. Sequences of the identified DEPs were retrieved from the rice protein database based on the GI numbers, and a blastp algorithm was performed against the GO and KEGG databases. GO annotations of the DEPs were performed over three domains—cellular component, molecular function, and biological process—by using R software packages. Likewise, the COGs were delineated by using a PERL scripted pipeline. The pipeline of the iTRAQ-based protein identification and the subsequent bioinformatic characterizations are represented in Figure 9.
3.2.1. Proteins Related to Salt Stress

The comparative proteomics study of both rice genotypes (LYP9 and NPBA) under salt stress revealed new insights into the salt resistance or sensitive mechanisms in rice. In both the rice genotypes, some of the major salt stress-responsive proteins exhibited differential up regulations as compared to the control, including malate dehydrogenase (gi|115482534), glucanase (gi|13249140), nascent polypeptide-associated complex (NAC) subunit (gi|115450217), methyltransferase (gi|115477769), and chloroplast inorganic pyrophosphatase (gi|46805452) (Table 3). Plant malate dehydrogenase (MDH) (EC 1.1.1.37) is a member of the oxidoreductase group that catalyzes the inter-conversion of malate and oxaloacetate in a redox reaction [24]. Further, MDH has been shown to play a vital role in regulating the salt stress response in plants [41,42]. Likewise, glucanase and inorganic pyrophosphatases have been associated with salt resistance properties in plants [43,44]. NAC has been reported to be involved in the translocation of newly synthesized proteins from the ribosomes to the endoplasmic reticulum during various physiological conditions, by directly interacting with the signal recognition particles. Further, overexpression of SαβNAC from Spartina alterniflora has been reported to enhance the salt tolerance in Arabidopsis [45]. In addition, methylation is often utilized by plants under unfavorable conditions as a strategy for gene regulation, protein sorting, and repairs [46]. IbSIMT1, a methyltransferase gene, has been observed to be activated by salt stress, and confers salinity resistance in sweet potato [47]. On the contrary, DEPs associated with salt stress responses, including glutathione peroxidase (GP) (gi|125540587), fructose-bisphosphate aldolase (FBA) (gi|1218196772), pyruvate dehydrogenase (gi|125564321), and triosephosphate isomerase (TPI) (gi|125528336) were found to be significantly upregulated in LYP9, but down regulated in NPBA. Recently, the rice GP gene (OsGPX3) has been reported to play a vital role in regulating the salt stress response [48]. Rice plants with silenced OsGPX3 were found to be highly salt sensitive, confirming the positive role of GP in salinity tolerance. FBA is involved in plant glucose pathways, including glycolysis and gluconeogenesis, and also plays a role in the Calvin cycle [49]. However, the FBA gene has been reported to exhibit induced expressions under salt stress in plants, indicating its role in salt stress. The FBA genes in Arabidopsis and Camellia oleifera were found to be strongly upregulated under salt stress, conferring salinity tolerance [48,50]. Likewise, the transcription of TPI genes has been reported
to become active in rice in response to salt stress [51,52]. The upregulated expression of these salt related proteins in the salt-tolerant genotype LYP9, and their down regulation in the salt-sensitive NPBA, suggests that these genotypes possess a different protein pool in response to salinity. Moreover, the difference in salt tolerance between these two rice genotypes might have resulted due to the differential expression of these key proteins. A functional validation study, such as the Western blot or protein interactions, will add further insights to this hypothesis.

3.2.2. Proteins Related to Redox Reactions

Salt stress in plants induces osmotic imbalances, disrupts ion-homeostasis, and triggers oxidative damage, including the generation of reactive oxygen species (ROS) [53,54]. A fitting response to these adversities caused by salinity stress includes physiological and developmental changes, reprogramming of salt-induced gene or proteins, and activation of ROS scavenging pathways [55]. In the current study, the proteomic analysis of LYP9 and NPBA revealed that redox reactions and ROS signaling are involved in the salt stress response in rice. Major enzymes involved in ROS signaling and redox reactions, including peroxidases (POD) (gi|125525683), superoxide dismutase (SOD) (gi|125604340), and glutathione s-transferase (GST) (gi|115459582), were found to be highly expressive in LYP9 and NPBA genotypes under the multiple salt stress levels we investigated (Table 3). Under salt stress, the cell membrane-bound peroxidases like NADPH oxidase and the diamine oxidases present in apoplast are activated, leading to generation of ROS [56,57]. In addition, SOD act as the first line of antioxidant defense in plants under multiple stress responses, and confer enhanced tolerance levels to oxidative stress [54]. Similarly, increased levels of GSTs in response to multiple stimuli have been reported in plants to mitigate oxidative stress [58]. Induced expressions and differential regulation of antioxidant enzymes, including PODs, SODs, and GSTs, have been reported by several studies in rice in response to salt stress [59,60]. Furthermore, comparative proteome analysis has confirmed the involvement of ROS and redox related protein in salt stress in plants, including alfalfa [61], searocket [62], maize [63], barley [64], and wheat [65]. Moreover, as many as 56 DEPs annotated with redox reaction functions were identified in both the rice genotypes under the various salt stress levels, suggesting oxidation and reduction reactions might be the key biochemical changes taking place in rice under salinity.

3.2.3. Proteins Related to Photosynthesis

Photosynthesis is a major physiological process accounting for sustainability and energy production in plants. However, salt stress has adverse effects on the plant photosynthesis process by causing a decrease in the leaf cellular CO$_2$ levels [7,66]. Additionally, salinity affects the Rubisco activity, retards chlorophyll synthesis, and destabilizes photosynthetic electron transport [66]. The findings from our study revealed that salt stress in rice affects the expression of the proteins involved in the photosynthesis process. These proteins, including the thylakoid luminal protein (gi|115477166), psbP domain-containing protein 6 (gi|115440559), psbP-like protein 1 (gi|38636895), ferredoxin-thioredoxin reductase (gi|115447507), photosystem I 9K protein (gi|218186547), photosystem II oxygen-evolving complex protein 2 (gi|164375543), and protochlorophyllide reductase B (gi|75248671), were found to be highly expressed under salt stress conditions (Table 3). Thylakoid luminal protein is required for the functioning of photosystem II (PsP), whereas ferredoxin reductase is a key enzyme that facilitates the conversion of ferredoxin to NADPH in the photosystem I (PSI) complex, and these are also affected by salt stress [67,68]. Moreover, the psbP proteins, thylakoid luminal proteins, and ferredoxin reductase have been reported to be differentially expressed under salt stress [68]. Likewise, differential expression of photosynthesis proteins was reported in tomatoes in response to salt stress [69]. Similarly, the differential protein expression of protochlorophyllide reductase between the salt stress-induced and control, and its effects on chlorophyll biosynthesis, has been reported in rice [70]. Usually, in salt sensitive plants, salinity causes the down-regulation of photosynthesis proteins, compromising plant sustainability [2,71]. However, the analysis of iTRAQ-based proteomics revealed that the proteins
involved in photosynthesis were upregulated in both rice genotypes, which might have aided the rice types to withstand salinity pressures.

3.2.4. Proteins Related to Carbohydrate Metabolism

Apart from being the building blocks in plants, soluble carbohydrates act as osmolytes, and thereby participate in salt tolerance in plants [72]. Besides, the onset of salt stress affects the protein dynamics in plants, resulting in differential protein accumulations [73]. In this study, several carbohydrate metabolism related proteins, including xylolgenic endotransglycosylase/hydrolase protein (XTH) (gi|115475445), β-glucosidase (gi|115454825), and polygalacturonase (gi|115479865), were found to be upregulated in both rice genotypes under various salt stress levels. XTH is known as a cell wall-modifying enzyme, however it also plays a role in salinity resistance responses in plants (Table 3). For instance, the constitutive and heterologous expression of CaXTH3 resulted in increased salt tolerance levels in Arabidopsis and tomato plants [74,75]. Similarly, β-glucosidase is a key enzyme in the cellulose hydrolysis process, and has been reported to be involved in the salt stress response. In barley, the activity of an extracellular β-glucosidase was reported to be highly induced in response to salt stress, and cause abscisic acid-glucose conjugate hydrolysis [76]. Further, the overexpression of Thkel1, a fungal gene that modulates β-glucosidase activity, improved the salt tolerance levels in transgenic Arabidopsis plants [77]. Polygalacturonase, another enzyme capable of hydrolyzing the α-1,4 glycosidic bonds, participates in the salt stress responses in plants. Characterization of the salt stress responses and the associated signal transduction pathways in Arabidopsis revealed the elevated transcript accumulation of a polygalacturonase gene (At1g48100) under salt stress [78]. However, several proteins related to carbohydrate metabolism, including xylanase inhibitor protein (XIP) (gi|297605789, gi|115467998) and MDH (gi|116310134), were found to be downregulated in the NPBA rice, while being upregulated in the LYP9 rice. MDH is a key enzyme in stress responses and actively participate in the tricarboxylic acid (TCA) cycle [74]. In the current study, upregulated expression of MDH was found in LYP9, however down-regulation in NPBA suggests the inhibition of the TCA cycle in the salt sensitive NPBA, but not in the tolerant LYP9 genotype. Further, OsXIP was reported to be induced under various abiotic stresses, including salt stress, and to take part in the rice defense mechanisms against several biotic and abiotic stresses [79]. Moreover, the induced many-fold expression of the carbohydrate metabolism related proteins in LYP9, but their down regulation in NPBA, indicates that carbohydrate metabolism might be a major physiological process that is affected under salinity in rice, and can show the dynamic changes in protein expression depending on the salt tolerance capacity of a genotype.

3.2.5. Proteins Related to Osmotic Stress

Often, salt stress induces the reduction of cellular water potential, causing osmotic stress to the plant. Osmotic stress responses in plants can be very complex in higher plants, including rice [80]. In this study, 11 osmotic stress related proteins were differentially expressed in both rice genotypes under various salt levels, suggesting salt stress in rice leads to the onset of osmotic stress. For instance, a putative lipid transferase protein (gi|297612544) identified as a DEP in both the rice genotypes was found to be upregulated under salt stress. The induced expression of TSW12 and SilTP, coding the lipid transferase proteins in tomato and foxtail millet plants, has been reported under salt stress [80,81]. Conversely, osmotic stress responsive proteins such as sucrose synthase (gi|125544232) and NADH dehydrogenase (gi|115473055) were found to exhibit an induced response in LYP9 rice under salt stress, but were not significantly induced in the NPBA rice. Sucrose synthase (Sus) is the major enzyme in sucrose metabolism, however it also plays a part in osmotic stress responses in plants. In Arabidopsis, up-regulation of Sus1 has been reported in response to osmotic stresses and water deficit conditions [82]. In addition, involvement of Sus in the osmotic stress response has been reported in Beta vulgaris [83]. On the other hand, NADH dehydrogenase facilitates electron transfer from NADH to the mitochondrial respiratory chain [84]. The up-regulation of NADH dehydrogenase under salt
stress indicates an increase in the ATP pool in the LYP9 rice, subsequently aiding in sustainable plant growth and salinity tolerance. However, no induced expression of the same in NPBA suggests that, under salt stress, the ATP pool might decrease, resulting in declining plant growth (Table 3).

3.2.6. Proteins Related to Other Metabolic Processes

Salt stress alters the protein pool that contributes to many metabolic mechanisms, such as stress responses, energy metabolism, and phytohormone synthesis [23,85]. In this study, several DEPs have been identified in the rice genotypes under salt stress, with various physiological and metabolic functions. For instance, putative glucan endo-1,3-β-glucosidase 4 (gi |297607511|) was found to be up-regulated in both rice types under salt stress conditions. Similar findings were reported in cotton plants, where the subjected salt stress caused an increased accumulation of glucan endo-1,3-β-glucosidase [23]. Further, the strong induced response of a putative zinc finger protein (gi |28971968|) was found under salt stress in both rice genotypes. Induced expression of gene finger proteins has been associated with several stresses, including salt stress. Overexpression of a rice zinc-finger protein OsISAP1 in transgenic tobacco resulted in enhanced abiotic stress tolerance levels, including salinity, dehydration, and cold [86]. Recently, OsZFP213 was reported to interact with OsMPK3, conferring salinity tolerance in rice [87]. In addition, many other proteins with annotated functions or which are uncharacterized were found to be differentially regulated at various salt levels in the rice genotypes. Moreover, these results collectively suggest that salinity affects many physiological processes in rice, irrespective of their salt tolerance levels. Furthermore, the protein pool of a salt tolerant and a salt sensitive rice genotype might differ at a specific point of time, which could be the basic reason of their differential salt tolerance responses (Table 3).

4. Materials and Methods

4.1. Plant Material and Growth Conditions

A pot culture experiment was conducted in a greenhouse at China National Rice Research Institute (39°4′49″ N, 119°56′11″ E), Zhejiang Province, China, during the rice growing season (May–November, 2017). Two rice cultivars (origin, China and Japan), Liangyoupiejiu (LYP9, Hybrid, indica) and Nipponbare (NPBA, japonica) were used as the planting materials. Thirty-day old seedlings were transplanted in pots (45 × 30 cm) with different salt stress levels and 23 kg air-dried soil. The experimental soil was loamy clay with an average bulk density of 1.12 g/cm, 4.7% organic matter, 0.086 dS/m EC, and 5.95 pH. Each pot contained six rice seedlings with three replications.

Sodium chloride (NaCl) was used in each pot to develop artificial salinity in soil until the maximum tillering stage of the rice seeding was reached (about 45 days). The treatments were comprised of four NaCl levels: 0 (control, CK), 1.5 g NaCl/kg dry soil (low salt stress, LS), 4.5 g NaCl/kg dry soil (moderate salt stress, MS), and 7.5 g NaCl/kg dry soil (high salt stress, HS). After salinity development, the corresponding EC for these levels was 0.086 dS/m (CK), 1.089 dS/m (LS), 3.20 dS/m (MS), and 4.64 dS/m (HS).

Nitrogen was applied in the form of urea (N: 46%), phosphorous as superphosphate (P₂O₅: 12%), and potassium as potassium sulfate (K₂O: 54%). Urea was used at the rate of 4.02 g/pot in two splits: 50% was applied as the basal dose, and 50% was applied at the tillering stage. Potassium sulfate (3.08 g/pot) was applied in two equal splits, as a basal dose and at the tillering stage, while the whole amount of superphosphate (6.93 g/pot) was applied as a basal dose.

4.2. Soil and Plant Sampling

Rice flag leaves were collected at the maximum tillering stage and stored at −80 °C after being frozen in liquid nitrogen. Plants were collected for measurement of Na⁺ and Cl⁻ contents in the roots and leaves at the maximum tillering stage. Soil samples were collected at the transplanting stage and at the maximum tillering stage to check the Na⁺ and Cl⁻ contents in the soil. Five flag leaves with three...
replicates were collected to measure the cell membrane injury in rice leaves at the maximum tillering stage, while root samples were collected to measure the rice root activity. All these experiments were performed with three independent biological replicates.

4.3. Leaf Proteomics Analysis Pipeline

4.3.1. Protein Extraction

A total of 1–2 g of plant leaves with 10% PVPP were ground in liquid nitrogen and then sonicated on ice for 5 min in Lysis buffer 3 (8M Urea and 40 mM Tris-HCl containing 1 mM PMSF, 2 mM EDTA, 10 mM DTT, and pH 8.5) with 5 mL of samples. After centrifugation, 5 mL of 10% TCA/acetone with 10 mM DTT were added to the supernatant to precipitate the proteins. The precipitation step was repeated with acetone alone until the supernatant became colorless. The proteins were air dried and re-suspended in Lysis buffer 3. Ultra-sonication on ice for 5 min was used to improve protein dissolution with the help of Lysis buffer 3. After centrifugation, the supernatant was incubated at 56 °C for 1 h for reduction, and then alkylated by 55 mM iodoacetamide (IAM) in the dark at room temperature for 45 min. Acetone (5 mL) were used to precipitate the proteins and stored at –80 °C.

The quality and quantity of the isolated proteins were estimated by performing Bradford assay and SDS-PAGE [88].

4.3.2. Digestion of Proteins and Peptide Labeling

About 100 µg of the protein solution with 8 M urea was diluted four times with 100 mM TEAB. For the digestion of the proteins, Trypsin Gold (Promega, Madison, WI, USA) was used at a ratio of trypsin: protein of 40:1, at 37 °C, and was put into the samples overnight. After the digestion with trypsin, Strata X C18 column (Phenomenex, Torrance, CA, USA) were used to desalt the peptides and vacuum-dry them according to the manufacturer’s protocol. For peptide labeling, the peptides were dissolved in 30 µL 0.5 M TEAB. Then, the peptide labeling was performed by an iTRAQ reagent 8-plex kit. The labeled peptides with different reagents were combined and desalted with a Strata X C18 column (Phenomenex), and vacuum-dried.

4.3.3. Peptide Fractionation and HPLC

The peptide fractionations were performed by using a Shimadzu LC-20AB HPLC pump attached to a high pH RP column. About 2 mL of the reassembled peptides with buffer A (5% ACN, 95% H2O, pH 9.8) was loaded on a 5 µm particulate column (Phenomenex). The flow rate was adjusted to 1 mL/min with a 5% buffer B (5% H2O, 95% ACN, pH 9.8) gradient for 10 min, with 5–35% buffer B for 40 min, and with 35–95% buffer B for 1 min, to separate the peptides. An incubation of 3 min in 95% buffer B, and for 1 min in 5% buffer B, followed this, before the final equilibration with 5% buffer B. Each peptide fraction was collected at 1 min time intervals, and OD of the eluted fractions were measured at 214 nm. Twenty fractions were pooled together and vacuum dried. Post drying, the fractions were re-suspended in buffer A solution (2% CAN; 0.1% FA in water) individually and centrifuged. Then, the supernatant was collected and loaded onto a C18 trap column with a rate of 5 µL/min by using a LC-20AD nano-HPLC device (Shimadzu, Kyoto, Japan). Peptide elutions were performed afterwands and separated by using an analytical C18 column with an inner diameter of 75 µm. The gradients were run at 300 nL/min starting from 8 to 35% of buffer B (2% H2O; 0.1% FA in ACN) for 35 min, with an increase up to 60% in 5 min, then were maintained at 80% buffer B for 5 min before returning to 5% in 6 s, with a final equilibration period of 10 min.

4.3.4. Mass Spectrometer Detection

The spectrometric data were acquired using a TripleTOF 5600 System (SCIEX, Framingham, MA, USA) fit to a Nano-Spray III source (SCIEX, Framingham, MA, USA) and a pulled quartz tip-type emitter (New Objectives, Woburn, MA, USA), which was controlled with the franchise software
Analyst v1.6 (AB-SCIEX, Concord, ON, Canada). The MS data procurements were undertaken as per the following conditions: the ion spray voltage was set to 2300 V, the curtain gas was set to 30, the nebulizer gas was set to 15, and the interface heater temperature was 150 °C. High sensitivity mode was used for the whole data acquisition process. The MS1 accumulation time was set to 250 ms, while 350–1500 Da was the allowed mass range. At least 30 product ion scans were collected based on the MS1 survey intensity, exceeding a threshold of 120 counts/s and a 2+ to 5+ charge-state. A value of 1/2 peak width was set for the dynamic exclusion. The collision energy was adjusted to all precursor ions for the collision-induced dissociation for the iTRAQ data acquisition, and the Q2 transmission window for 100 Da was at 100%. Three independent biological replicates were included for each sample in the experiment.

4.4. Cell Membrane Injury

Cell membrane injury (CMI) was determined by using flag leaves. Twenty pieces (1 cm diameter) were cut from these flag leaves, and were submerged into 20 mL distilled water (DI) contained in test tubes. The test tubes were kept at 10 °C in an incubator for 24 h. After 24 h, the samples were kept at 25 °C to warm the samples, and the electrical conductivity (C1) of the samples was measured. These samples were then autoclaved for 20 min at 120 °C and the electrical conductivity (C2) was determined again. Cellular injury was determined by using the following formula [89]:

\[
\text{Cellmembraneinjury} = \frac{C_1}{C_2} \times 100
\]

where, ‘C’ refers to EC 1 and 2. The experiment was performed with three independent biological replicates.

4.5. Rice Root Activity

Rice root activity was analyzed by the triphenyl tetrazolium chloride (TTC) method [90]. Briefly, rice root samples (0.5 g, root tips) were taken, and 5 mL of the phosphate buffer (pH 7) and 5 mL 0.4% TTC (Vitastain, C₁₉H₁₅N₄Cl) were added to keep the root activity alive. The samples were kept in an incubator in the dark at 37 °C for 3 h. After 3 h, the samples were taken out and 1 mL 1 mol/L H₂SO₄ was added to stop the reaction. The rice roots were then removed from the test tubes. These root samples were ground by adding a pinch of silica sand, and mixed with 8 mL ethyle acetate. The extract was transferred to test tubes and a 10 mL final volume was reached by adding ethylene acetate. These samples were analyzed using a spectrophotometer (UV-2600, UV-VIS Spectrophotometer Shimadzu) at 485 nm. The formula used for calculation of root activity is as follows:

\[
\text{RootActivity} = \frac{C}{W/3}
\]

where C is the concentration of the samples calculated from a standard curve. W is the weight of the root samples. The experiment was performed with three independent biological replicates.

4.6. Na⁺ Concentration in the Soil and Plants

Na⁺ was extracted from the soil by ammonium acetate solution using Rihards (1954) method [91]. About 5 g ground (particle size ≤ 2mm) air dried soil was placed in 250 mL plastic bottles and 50 mL ammonium acetate (NH₄OAc, 1 mol/L) was added. These bottles were kept on a shaker for 30 min at 120 rpm. After that, the samples were filtered by using filter paper to obtain the soil solution.

Na⁺ in the plants’ parts was extracted by digestion with sulfuric acid (H₂SO₄) by following Rihards (1954) method [91] with the necessary modifications. About 0.3 g ground (particle size ≤ 2 mm) root and leaf were taken in 50 mL glass tubes and mixed with 5 mL H₂SO₄. These glass bottles were kept overnight. The samples were put into the fume hood and were incubated at 320 °C for 2 h. After 2 h,
hydrogen peroxide solution (H₂O₂) was added drop by drop and the samples were mixed until a whitish or transparent color appeared. Then, the samples were cooled at room temperature before being filtered by using filter paper to get the plant part extracts.

The soil and plant extracts were used to measure the sodium ions (Na⁺) by using a flame photometer. The standards used were 0, 2, 4, 6, 8, 10, 15, and 20 mL NaCl. The final soluble sodium (Na) in soil was measured by using the formula:

\[ \text{Na} \left( \frac{\mu g}{g} \right) = \frac{A \times C}{W} \]  

where A is the total volume of the extract (mL), C is the sodium concentration values given by the flame-photometer (µg/mL), and W is the weight of the air dried soil (g). The experiment was performed with three independent biological replicates.

4.7. Cl⁻ Concentration in the Soil and Plants

About 10 g air dried soil (particle size ≤ 2 mm) was placed in 250 mL plastic bottles and mixed with 50 mL deionized water. These bottles were transferred onto a shaker and were shaken for 5 min at 180 rpm. The samples were then filtered by using filter paper to obtain the soil solution extract for Cl⁻.

Plant samples weighing approximately 0.1 g were placed in 50 mL glass tubes and mixed with 15 mL deionized water. The tubes were transferred into a hot water bath and kept for 1.5 h. The samples were then diluted with 25 mL deionized water after cooling at room temperature.

The soil and plant extracts were used to measure the chloride (Cl⁻) by using a chloride assay kit (QuantiChrom™ Chloride Assay Kit, 3191 Corporate Place Hayward, CA 94545, USA) following the manufacturer’s instructions. The standards used were 0, 10, 20, 30, 40, 60, 80, and 100 mL. The final chloride concentration in the solution was measured by the formula:

\[ \text{Chloride} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{Slop}} \times n \left( \frac{\text{mg}}{\text{dL}} \right) \]  

where ODsample is the OD 610 nm values of the samples, and ODblank is the OD 610 nm values of the blanks (water). The experiment was performed with three independent biological replicates.

4.8. Statistical Analysis

The statistical software package IBS SPSS Statistics 19.0 was used for the analyses of data. For evaluating the statistical significance of the biochemical parameters, a one-way ANOVA was employed with LSD at the level of \( p = 0.05 \). For the iTRAQ-based protein quantification, all identified DEPs were required to satisfy the \( t \)-test at \( p \leq 0.05 \), and with a fold change ratio of >1.2 or <0.8.

5. Conclusions

Using comparative iTRAQ-based protein quantification, the proteome dynamics of LYP9 and NPBA rice were explored in this study. The results from the study suggest that rice cell membrane integrity was inversely correlated and root activity was positively correlated with the concentration of salinity. Furthermore, the physiological processes, including carbohydrate metabolism, redox reactions, and photosynthesis, made significant contributions towards the salt tolerance in rice. The number of differentially expressed proteins—salt responsive proteins in particular—suggested that the protein pool in response to salt stress is different in a salt tolerant compared to a susceptible rice genotype. Finally, the indica rice LYP9 showed promising results under the subjected salt stress levels, and can be selected over the japonica NPBA for salt tolerance. Further works deciphering the functions of some particular proteins of interest will add new insights into their roles in salt tolerance in rice.

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the data. S.H. (Sajid Hussain), S.N., and C.Z. wrote the manuscript. S.N., S.H. (Saddam Hussain), and A.R. revised the manuscript. Q.L., L.W., and Y.L. help in formal analysis. The manuscript has been read and approved by all authors.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| LYP9 | Liangyoupeijiu |
| NPBA | Nipponbare |
| iTRAQ | Isobaric tags for relative and absolute quantitation |
| CMI | Cell membrane injury |
| RRA | Rice root activity |
| DEPs | Differentially expressed proteins |
| GO | Gene ontology |
| KEGG | Kyoto encyclopedia of genes and genomes |
| PSI | Photosystem I |
| LS | Low salt stress |
| MS | Moderate salt stress |
| HS | High salt stress |
| COG | Cluster of orthologous groups |

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