# IMPROVEMENT OF RICE THROUGH "-OMICS" APPROACHES

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# IMPROVEMENT OF RICE THROUGH "-OMICS" APPROACHES

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EDITORIAL
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# Editorial: Improvement of Rice Through "-omics" Approaches

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**Keywords: rice, omics, yield, food security, stress**

**Editorial on the Research Topic**

**Improvement of Rice Through "-omics" Approaches**

Rice is one of the most consumed food worldwide and is the first crop to have a complete genome sequence (Goff et al., 2002; Yu et al., 2002). However, rice productivity is at risk during this climate-changing era and thus improvement of rice is of utmost importance to address the issue of global food security. Advancements in omics technologies provided a platform for systemic dissection of regulatory pathways underlying rice development, growth, and productivity during optimal or stressful growth conditions. Consequently, tremendous genomics and transcriptomics studies were performed in the last few decades that identified hundreds of desirable genes and QTLs related to yield and stress tolerance (Song et al., 2018). More recently, this has been supported by multi-omics studies to further identify related genes, proteins, and metabolites (Khan et al., 2021).

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## STRATEGIES TO IMPROVE PROTEOME COVERAGE

Membrane proteomics has allowed the investigation of cell signaling pathways despite the associated challenges of working with highly hydrophobic proteins. To overcome such limitations, and increase the identification of key signaling pathways that may lead to crop improvement in terms of yield and tolerance to environmental stresses, Van Nguyen et al. developed an azo chemical to improve the solubilization of rice microsomal membrane proteins. Results obtained suggested a higher efficacy of azo in solubilizing the microsomal proteins than the well-known anionic surfactant SDS and suggested that the azo can be successfully utilized for the large-scale identification of membrane proteins.

## TRAIT IMPROVEMENT AND YIELD ENHANCEMENT

Improvement of grain quality and yield is a key goal governing rice research as exemplified by the following papers published in the present issue. A mini-review by Ding et al. provides a short overview on cis-regulator elements (CREs) regulating gene expression in rice and how their targeting *via* genome editing techniques can be exploited to improve rice grain quality while increasing our understanding of gene expression regulatory networks. In the present issue,

several studies focused on embryo development, and its impact on endosperm quality as these are determining features for size and grain quality in rice. The study by Hu et al. identified genes involved in the regulation of embryo size, such as the *GIANT EMBRYO* (*GE*) gene. The authors showed that a "giant embryo" phenotype was due to a single nucleotide deletion in the first exon of the *GE*. Furthermore, a comparison of the gene expression and metabolite accumulation in two cultivars differing in the size of their embryo suggested an increase in biosynthesis of L-Aspartic acid and L-Tryptophan in the giant embryo cultivar. The study by Tao et al. emphasized the role played by the embryo in the formation of the chalky endosperm, which is a key desired quality trait of the rice grain. Non-targeted metabolomics and NGS RNAseq were performed in a notched-belly (NB) mutant which is exhibiting a distinct separation between the translucent and chalky regions of the endosperm. Data showed that the embryo negatively regulates the accumulation of sucrose, amino acid, starch, and storage proteins in the chalky region of the endosperm by reducing the expression of starch, prolamin, and glutelin synthesis-related genes, suggesting that the embryo induces a metabolic shift in the endosperm which is important in developing high-quality rice by balancing embryo-endosperm interaction.

In another study, Andrew-Peter-Leon et al. focused on the identification of gene loci responsible for controlling yield, following the mutagenization of an Improved White Ponni (IWP) variety. One of the mutant lines, WP-22-2, exhibited semi-dwarfism, early flowering, and higher yield without alteration of grain quality. Whole-genome sequencing identified large-scale deletions and single nucleotide polymorphism (SNP), including mutations that lead to the functional disruption of *OsGA2oox2* and *OsFBX267*. These results suggest that both of these genes can be exploited for the development of early maturing and semi-dwarf varieties of rice. Bhatta et al. used and showed the efficacy of an image-based geometric traits phenotyping method for selecting rice genotypes tolerant to low phosphorus conditions.

## MOLECULAR SIGNALING OF RICE UNDER STRESS CONDITIONS

Plant growth and crop yield are severely constrained under stress conditions. Thus, understanding plant responses to stress are instrumental for engineering stress-tolerant rice cultivars. In this issue, two reviews describe multi-omics studies describing rice responses to drought (Zargar et al.) and other abiotic and biotic stresses (Iqbal et al.), to catalog the key components involved in trait management and yield enhancement under stress conditions. In an original, study, Chutimanukul et al. utilized an integrated genome and gene co-expression network analysis to identify candidate genes potentially involved in the salinity stress response in rice, such as *OsNodulin, OsBTBZ1, OsPSB28, OsERD, OsSub34, peroxidase precursor* genes, as well as investigating gene co-expression network (GCN) involved in the regulation of salinity stress response in rice. Likewise, in the study by Sonsungsan et al., transcriptome profiling of a salt-tolerant rice variety identified 107 salinity responsive genes related to the sensing of salt stress, signaling, hormone response, and gene regulation. Lee et al. investigated the effects of salinity stress on developing rice seeds using a RNASeq-based study comparing the transcriptome of the Samgwang rice cultivar cultivated in reclaimed and normal land. It was shown that this rice cultivar "Samgwang" showed higher sensitivity to salinity stress when cultivated in the reclaimed land, showing adverse effects such as decreased yield, grain weight, palatability, and amylose content, and this was associated with upregulation of genes related to the biosynthesis of ABA, trehalose, raffinose, and maltose under salinity stress conditions. Interestingly, Khan et al. reported that the impact of salinity stress in rice can be significantly reversed by the application of sugarcane press-mud. It was shown that the application of press mud induced the activity of antioxidant enzymes and promoted the growth, yield, total soluble proteins, free amino acids, and soluble sugars, thus mitigating the negative effects of salinity stress. Similarly, Huang et al. reported that biochar application can protect rice against heat-induced damage possibly *via* alteration of the soil properties and its microbiome in the root zone environment. Decreased heat damage following biochar treatment was associated with decreased abundance of heat stress markers such as heat-shock proteins.

Altogether, the review articles and original studies reported in this Research Topic are contributing to the general understanding of the molecular mechanisms regulating rice development and responses to environmental stresses, in particular for their impact on grain quality and crop yield and such information will be instrumental for engineering new rice varieties, with improved grain quality, yield, and stress tolerance.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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# Biochar Application Mitigates the Effect of Heat Stress on Rice (*Oryza sativa* L.) by Regulating the Root-Zone Environment

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Coping with global warming by developing effective agricultural strategies is critical to global rice (*Oryza sativa* L.) production and food security. In 2020, we observed that the effect of heat stress on rice plants was mitigated by biochar application (40 g $kg^{-1}$ soil) in a pot experiment with six consecutive days (6–11 days after transplanting) of daily mean temperatures beyond the critical high temperature (33°C) for tillering in rice. To further determine the eco-physiological processes underlying the effect of biochar on resistance to heat stress in rice plants, we compared root-zone soil properties as well as some plant growth and physiological traits related to nitrogen (N) utilization between rice plants grown with and without biochar in the pot experiment. The results showed that the application of biochar improved the root-zone environment of rice plants by reducing soil bulk density, increasing soil organic matter content, and altering soil bacterial community structure by increasing the ratio of Proteobacteria to Acidobacteria, for example. As a consequence, root morphology, architecture, and physiological traits, such as N assimilation and transport proteins, as well as shoot N uptake and utilization (e.g., photosystems I and II proteins), were improved or up-modulated, while the heat-shock and related proteins in roots and leaves were down-modulated in rice plants grown with biochar compared to those without biochar. These results not only expand our understanding of the basic eco-physiological mechanisms controlling increased heat-stress tolerance in rice plants by the application of biochar, but also imply that improving the root-zone environment by optimizing management practices is an effective strategy to mitigate heat stress effects on rice production.

**Keywords: biochar, heat stress, nitrogen utilization, rice, root zone**

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## INTRODUCTION

Rice (*Oryza sativa* L.) feeds approximately 50% of the world's human population, and rice yield must increase by about 1% annually to meet the growing food demand that will result from population growth and economic development (Normile, 2008). However, this task is not easy to achieve due to changes in socioeconomic and physical environments related to rice production (Peng et al., 2009). Among these changes, global climate change is expected

to be a particularly serious challenge for global rice production in the near future (Wassmann et al., 2009).

Global warming, the phenomenon of rising average air temperatures near earth's surface over the past 100–200 years, is a major aspect of climate change that threats rice production (Horie, 2019). Peng et al. (2004) reported that rice yield decreased by 10% when night temperatures during the growing season increased by 1°C, which is associated with global warming. Moreover, global warming has and will continue to increase the frequency and severity of extreme weather events, such as droughts and heatwaves (Stott, 2016; Perkins-Kirkpatrick and Lewis, 2020), which can partially or completely damage crop production (Lesk et al., 2016). Therefore, it is crucial to find effective agricultural strategies to cope with global warming in order to ensure global food security (Wassmann et al., 2009; Horie, 2019).

In 2020, we conducted a pot experiment to determine the effect of biochar application on rice growth (**Figure 1A**; see the Materials and Methods section for details). Because the pots were placed on concrete-covered ground exposed to the sun, high temperatures were induced during the experimental period. Average daily maximum and minimum air temperatures around the rice plants reached 37.9 and 25.4°C, respectively, during the period from transplanting to 18 days after transplanting (**Figure 1B**). Even more remarkably, there were six consecutive days (6–11 days after transplanting) with daily mean air temperatures (the average of daily maximum and minimum air temperatures) beyond the critical high air temperature (33°C) for tillering in rice (Krishnan et al., 2011). In addition, high soil temperatures also occurred during the experimental period. For example, maximum and minimum soil temperatures were 42.1 and 27.6°C, respectively, for the rice plants without biochar application (C0 treatment) and 43.0 and 27.5°C for the rice plants with biochar application (C1 treatment) in the nine-day period after transplanting, when the maximum and minimum air temperatures around the rice plants were 42.9 and 26.6°C, respectively (**Figure 1C**).

In general, high temperature (or heat stress) can adversely affect the rice growth by disturbing photosynthetic activity and respiration rate (Fahad et al., 2019). However, unexpectedly and interestingly, the high temperatures seriously limited the growth of rice plants in the C0 treatment but seemed to not affect the growth of those in the C1 treatment in our pot experiment (**Figure 1A**). This observation indicates that the effect of heat stress on rice can be mitigated by applying biochar to regulate the root-zone environment.

It has been well documented that biochar application can improve soil properties and consequently promote root growth and nitrogen (N) utilization in rice (Huang et al., 2013, 2014, 2018; Xiang et al., 2017). In addition, the previous studies have shown that improving N availability can mitigate the negative effects of heat stress on several crops, such as rice, maize, and potato (Tawfik et al., 1996; Ordóñez et al., 2015; Liu et al., 2019). Heckathorn et al. (1996) found that N availability is closely related to patterns of accumulation of heat-shock proteins in plants, which are generally induced by heat stress and play an important role in the development of tolerance to heat stress (Parsell et al., 1993). Therefore, we hypothesized that the increased resistance to heat stress in rice plants in the C1 treatment compared to those in the C0 treatment is partly attributable to improved N utilization.

In this study, root-zone soil properties as well as some plant growth and physiological traits related to N utilization were compared between the C0 and C1 treatments. The objective of this study was to determine the eco-physiological processes

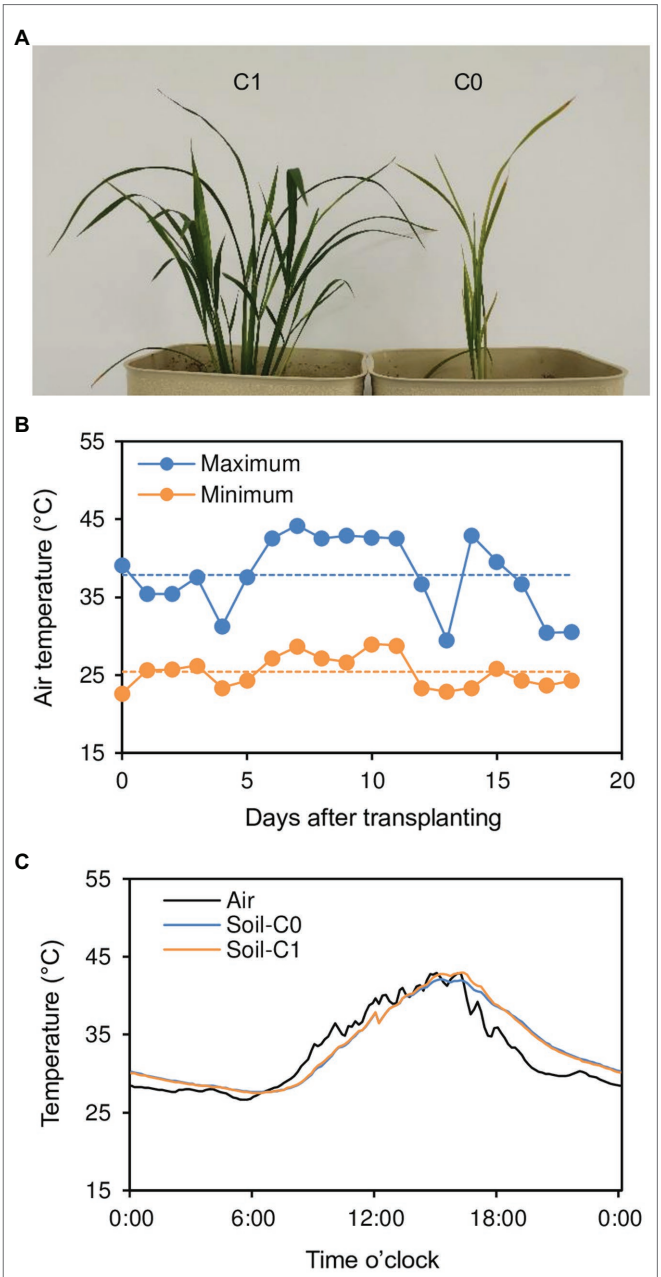


**FIGURE 1** | **(A)** Rice plants grown in pots with (C1) and without (C0) biochar application shown at 18 days after transplanting, **(B)** daily maximum and minimum air temperatures around rice plants during the experimental period, and **(C)** air and soil temperatures for the 24 h on day 9 after transplanting.

underlying resistance to heat stress in rice by altering the root-zone environment by adding biochar to the soil.

# MATERIALS AND METHODS

## Plants and Treatments

The pot experiment was conducted at the Crop and Environment Center, Hunan Agricultural University, Changsha, China, in 2020. "Longliangyouhuazhan," a high-yielding hybrid rice variety, was grown in pots without (C0) and with biochar application (C1). Each treatment consisted of six pots (replications). The pots were placed on concrete-covered ground exposed to the sun, causing high temperatures and heat stress for rice plants (see the Introduction section for details).

The soil used in the experiment was taken from the upper 20 cm layer of a rice field at the Experimental Farm of Hunan Agricultural University. The soil was a Fluvisol (FAO classification) with clay loam texture, pH = 5.99, organic matter = 22.7 g $kg^{-1}$, total $N$ = 1.32 g $kg^{-1}$, and available $N$ = 114 mg $kg^{-1}$. The soil was air-dried, sieved, mixed, and then used to fill plastic pots (25-cm height, 20-cm length, and 15-cm width) with a weight of 5 kg $pot^{-1}$ and a depth of ~20 cm.

The tested biochar was a rapeseed straw biochar with specific surface area = 3.02 $m^2$ $g^{-1}$, average bore size = 2.14 nm, pH = 10.8, total $C$ = 440 g $kg^{-1}$, and total $N$ = 10.7 g $kg^{-1}$. The application rate of the biochar was 200 g $pot^{-1}$ (or 40 g $kg^{-1}$ soil) for the C1 treatment. This biochar application rate was selected according to a preliminary screening, which indicated that rice plants grown under a biochar rate of 40 g $kg^{-1}$ soil performed better than those under a recommended rate (20 g $kg^{-1}$ soil) by Zhang et al. (2013). The biochar was applied 1 day before transplanting and was uniformly incorporated into the entire soil layer.

Pre-germinated seeds were sown in a seedling tray on 14 May. Twenty-day-old seedlings were transplanted into the pots with one hill per pot and one seedling per hill. Fertilizers used were urea for N, single superphosphate for phosphorus (P), and potassium chloride for potassium (K) at doses of 0.35 g N $pot^{-1}$, 0.10 g $P_2O_5$ $pot^{-1}$, and 0.10 g $K_2O$ $pot^{-1}$, respectively. The N fertilizer was split-applied: 0.25 g N $pot^{-1}$ 1 day before transplanting and 0.10 g N $pot^{-1}$ 7 days after transplanting. The P and K fertilizers were both applied 1 day before transplanting. A water depth of ~3 cm above the soil surface was maintained during the experimental period.

## Soil Sampling and Measurements

Soils were sampled from three pots (replications) selected at random 18 days after transplanting to determine bulk density, organic matter content, available N content, and the bacterial community. All the soil samples were taken from the upper 10 cm layer where the rice roots were mainly distributed on the sampling day.

The bulk soil density was determined using the core method (Blake and Hartge, 1986). The organic matter content was measured by the potassium dichromate method (Walkley, 1947), and the available N content was assayed by the alkali-hydrolysis and diffusion method (Cornfield, 1960).

The bacterial community was investigated by analysis of the 16S rRNA gene sequence, which was performed by Novagene (Beijing, China). Quality-filtered and non-chimeric sequences were clustered to generate operational taxonomic units (OTUs) at a similarity level of 97% using UPARSE, v7.0.1090 (Edgar, 2013). Taxonomic information for the OTUs was annotated against the GreenGene database (DeSantis et al., 2006) using the RDP classifier algorithm, v2.2 (Wang et al., 2007). Bacterial community diversity and richness indices, including observed species, and the Shannon, Simpson, Chao1, Good coverage, and abundance-based coverage estimators, and the phylogeny-based metrics (PD whole tree) were calculated using QIIME, v1.9.1 (Caporaso et al., 2010). The relative abundance of each phylum was calculated by dividing the number of OTUs affiliated with a phylum by the total number of OTUs.

## Plant Sampling and Measurements

On the day of soil sampling, roots and the uppermost fully expanded leaves were sampled from the three soil-sampled pots for proteomic analysis, and whole rice plants were sampled from the other three pots to measure root traits, including root length, diameter, surface area, and dry weight, and shoot traits, including tiller number per plant, leaf area per plant, specific leaf weight (SLW), leaf N content (LNC), shoot dry weight, and shoot N uptake.

The proteomic analysis was carried out by PTM BioLab Co., Ltd. (Hangzhou, China), using tandem mass tag (TMT) coupled to liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) in accordance with the following procedures:

### Protein Extraction

The sample was ground into fine powder in liquid $N_2$ and transferred to a 5 mL centrifuge tube. A four-fold volume of lysis buffer (including 10 mm dithiothreitol and 1% protease inhibitor cocktail) was added to each sample and then sonicated three times on ice using a high-intensity ultrasonic processor (Scientz, Ningbo, China). An equal volume of Tris-saturated phenol (pH 8.0) was added, and then, the mixture was vortexed for 5 min. After centrifugation (4°C, 10 min, 5,500 *g*), the upper phenol phase was transferred to a clean centrifuge tube. Proteins were precipitated by adding five volumes of 0.1 M ammonium acetate-saturated methanol and incubated at −20°C overnight. After centrifugation at 4°C for 10 min, the supernatant was discarded. The remaining precipitate was washed once with ice-cold methanol, followed by washing three times with ice-cold acetone. The protein was redissolved in 8 M urea, and the protein concentration was determined with the bicinchoninic acid kit (Thermo Fisher Scientific, Rockford, IL, United States) according to the manufacturer's instructions.

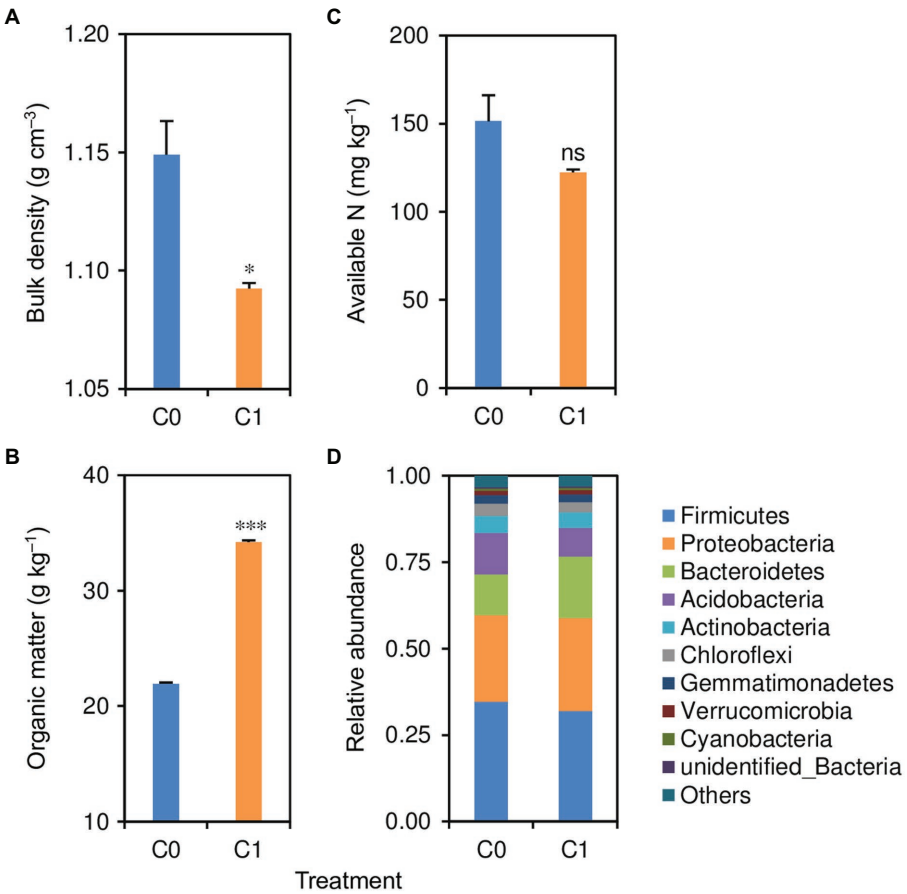


**FIGURE 2 | (A)** Soil bulk density, **(B)** organic matter content, **(C)** available N content, and **(D)** relative abundance of bacterial phyla in treatments with (C1) and without (C0) biochar application. * and *** indicate that the means of the rice plants in C1 treatment are significantly different from those in the C0 treatment at the 0.05 and 0.001 levels, respectively. "ns" indicates that the mean of the rice plants in C1 treatment is not significantly different from that in the C0 treatment at the 0.05 level.

**TABLE 1 |** Soil bacterial community diversity and richness for rice plants grown with biochar application (C1) and those grown without biochar application (C0).

| Index | C0 | C1 | Value of *p* |
|---|---|---|---|
| Observed species | 2,824 | 2,770 | 0.247 |
| Shannon | 9.41 | 9.31 | 0.248 |
| Simpson | 0.995 | 0.995 | 1.000 |
| Chao1 | 2,970 | 3,012 | 0.508 |
| Good coverage | 0.994 | 0.992 | 0.703 |
| Abundance-based coverage estimators (ACE) | 3,004 | 3,026 | 0.184 |
| Phylogeny-based metrics (PD whole tree) | 199 | 204 | 0.195 |

## Trypsin Digestion

The protein solution was reduced with 5 mm dithiothreitol at 56°C for 30 min and alkylated with 11 mm iodoacetamide at room temperature in darkness for 15 min. The protein sample was then diluted by adding 200 mm triethylammonium bicarbonate (TEAB) to insure the urea concentration <2 M. Finally, trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and at 1:100 trypsin-to-protein mass ratio for a second 4-h digestion.

## TMT Labeling

After trypsin digestion, the peptide was desalted by Strata X C18 SPE column (Phenomenex, Torrance, CA, United States) and dried by vacuum centrifuging. Peptides were reconstituted in 0.5 M TEAB and labeled by using TMT kit (Thermo Fisher Scientific, Rockford, IL, United States) according to the manufacturer's protocol. Briefly, one unit of TMT reagent was thawed and reconstituted in acetonitrile. The peptide mixtures were then incubated for 2 h at room temperature and pooled, desalted, and dried by vacuum centrifuging.

## High-Performance Liquid Chromatography Fractionation

The tryptic peptides were fractionated into fractions by high pH reverse-phase high-performance liquid chromatography (HPLC) using Agilent 300 Extend C18 column (5 μm particle size, 4.6 mm internal diameter, and 250 mm length). Briefly, peptides were first separated with a gradient of 8–32% acetonitrile (pH 9.0) over 60 min into 60 fractions. Then, the peptides were combined into nine fractions and dried by vacuum centrifuging.

### LC-MS/MS Analysis

The tryptic peptides were dissolved in solvent A (0.1% formic acid and 2% acetonitrile), directly loaded onto a home-made reversed-phase analytical column (15 cm length and 75 μm internal diameter). The gradient was comprised of an increase from 7 to 26% solvent B (0.1% formic acid and 90% acetonitrile) over 26 min, 26–38% in 8 min and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 500 nl/min on an EASY-nLC 1,000 ultra-performance liquid chromatography (UPLC) system (Thermo Fisher Scientific, Waltham, MA, United States). The peptides were subjected to a N solution index source followed by MS/MS in Q ExactiveTM Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled online to the UPLC. The electrospray voltage applied was 2.2 kV. The m/z scan range was 400–1,500 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 120,000. Peptides were then selected for MS/MS using a normalized collision energy setting at 28, and the fragments were detected in the Orbitrap at a resolution of 15,000. Automatic gain control was set at 5E4. Fixed first mass was set as 100 m/z.

### Database Search

The resulting MS/MS data were processed using Maxquant search engine (v.1.5.2.8). Tandem mass spectra were searched against UniProt *O. sativa*_indica database (37,383 sequences) concatenated with reverse decoy database. Trypsin/P was specified as cleavage enzyme allowing up to two missing cleavages. The mass tolerance for precursor ions was set at 10 ppm in the first search and at 5 ppm in the main search, and the mass tolerance for fragment ions was set at 0.02 Da. Carbamidomethyl-modified cysteine residues were specified as a fixed modification, and oxidation of methionine was specified as a variable modification. The quantitative method is set to TMT-6plex. Both the false discovery rate for the identification of protein and propensity score matching were adjusted to <1%.

The root length, diameter, and surface area were determined using a WinRHIZO root analyzer system (Regent Instruments Inc., Quebec, Canada). Leaf area was measured with a LI-3000C leaf area meter (Li-Cor Inc., Lincoln, United States). The N content in the shoot (leaf and stem) was assayed using a Skalar SAN Plus segmented flow analyzer (Skalar Inc., Breda, Netherlands). The root and shoot (leaf and stem) dry weights were determined after oven-drying at 70°C to a constant weight. The SLW was calculated by dividing the leaf dry weight by the leaf area. The shoot N uptake was calculated by multiplying the shoot dry weight by the shoot N content.

## Data Analysis

For statistical analysis of proteomics data, the log2-fold change of mean value of protein quantity in the C1 compared to the C0 treatment was calculated for each quantifiable protein and then subjected to two-tailed Fisher's exact test. The proteins with values of $p < 0.05$ and fold changes >1.3 or <1/1.3 were considered to be differentially modulated. The differentially modulated proteins were annotated based on the Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes pathway databases. For analysis of other data, differences between the C1 and C0 treatments were evaluated by Student's *t*-test, with significance levels of 0.05, 0.01, and 0.001.

# RESULTS

## Root-Zone Soil Properties

Biochar application significantly affected the soil bulk density and organic matter content but did not significantly affect soil available N content (**Figures 2A–C**). The C1 treatment had 5% lower soil bulk density but 56% higher soil organic matter content than did the C0 treatment.

Biochar application had no significant effects on soil bacterial diversity and richness (**Table 1**). However, the structure of the soil bacterial community was changed by the application of biochar (**Figure 2D**). Compared with the C0 treatment, the C1 treatment had 8–31% lower relative abundances of Firmicutes, Acidobacteria, Actinobacteria, Chloroflexi, and Gemmatimonadetes

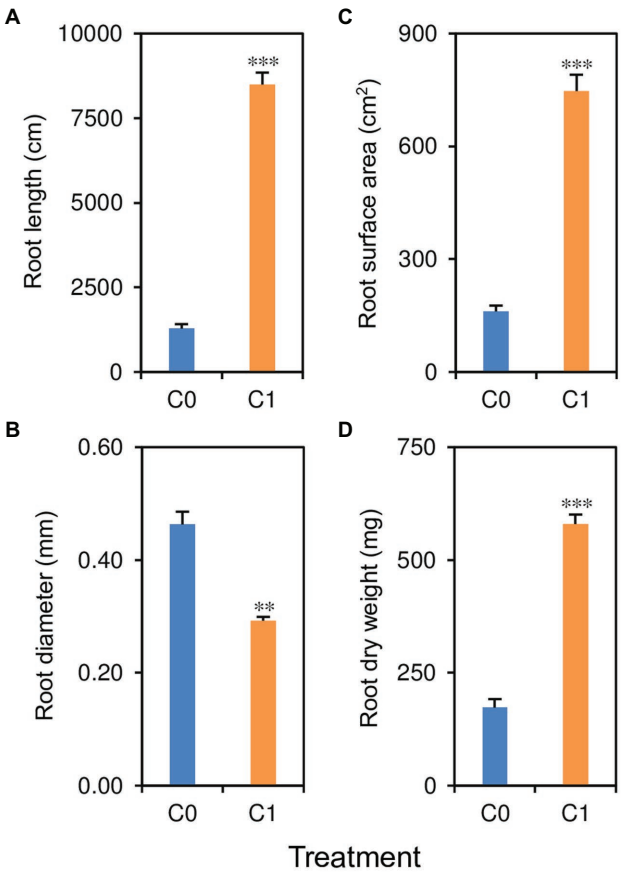


**FIGURE 3 | (A)** Root length, **(B)** root diameter, **(C)** root surface area, and **(D)** root dry weight in rice plants grown with (C1) and without (C0) biochar application. ** and *** indicate that the means of the rice plants in C1 treatment are significantly different from those in the C0 treatment at the 0.01 and 0.001 levels, respectively.

but 4–49% higher relative abundances of Proteobacteria, Bacteroidetes, Verrucomicrobia, and Cyanobacteria.

## Plant Growth Traits

Biochar application had significant effects on root length, diameter, surface area, and dry weight (**Figures 3A–D**). Rice plants in the C1 treatment had 235–561% greater root length, surface area, and dry weight but 37% lower root diameter compared to those in the C0 treatment.

Shoot traits, including shoot N uptake, tiller number, leaf area, SLW, LNC, and shoot dry weight, were significantly affected by biochar application (**Figures 4A–F**). Compared with rice plants in the C0 treatment, rice plants in the C1 treatment had 51–396% higher shoot N uptake, tiller number, leaf area, SLW, LNC, and shoot dry weight.

## Plant Physiological Traits

Total number of proteins identified in roots and leaves were 6,670 and 5,326, respectively, of which 4,975 and 4,013, respectively, were quantifiable (data not shown). Biochar application significantly modulated proteins in both roots and leaves. A total of 835 and 2084 differentially modulated proteins were detected in roots and leaves of C1 compared to C0 rice plants, respectively (data not shown). In particular, 10 and 22 differentially modulated heat-shock or related proteins were identified in roots and leaves, respectively (**Figure 5**). All 10 of the identified differentially modulated heat-shock or related proteins in roots and 20 of the 22 identified differentially modulated heat-shock or related proteins in leaves were down-modulated in rice plants in the C1 treatment compared to those in the C0 treatment with fold changes of 0.234–0.769.

In addition, we identified seven and three differentially modulated proteins in roots that are related to N metabolism and hormone activity, respectively (**Figure 6**). Five out of the seven differentially modulated proteins related to N metabolism were up-modulated with fold changes of 1.63–2.47, while the remaining two differentially modulated proteins related to N metabolism and all three differentially modulated proteins related to hormone activity were down-modulated with fold changes of 0.41–0.68 in rice plants in the C1 treatment compared to those in the C0 treatment. The five up-modulated proteins related to N metabolism were glutamine synthetase (OsI_08842 and OsI_10575), glutamine amidotransferase type-2 domain-containing protein (OsI_03285), and two ammonium transporters (OsI_08109

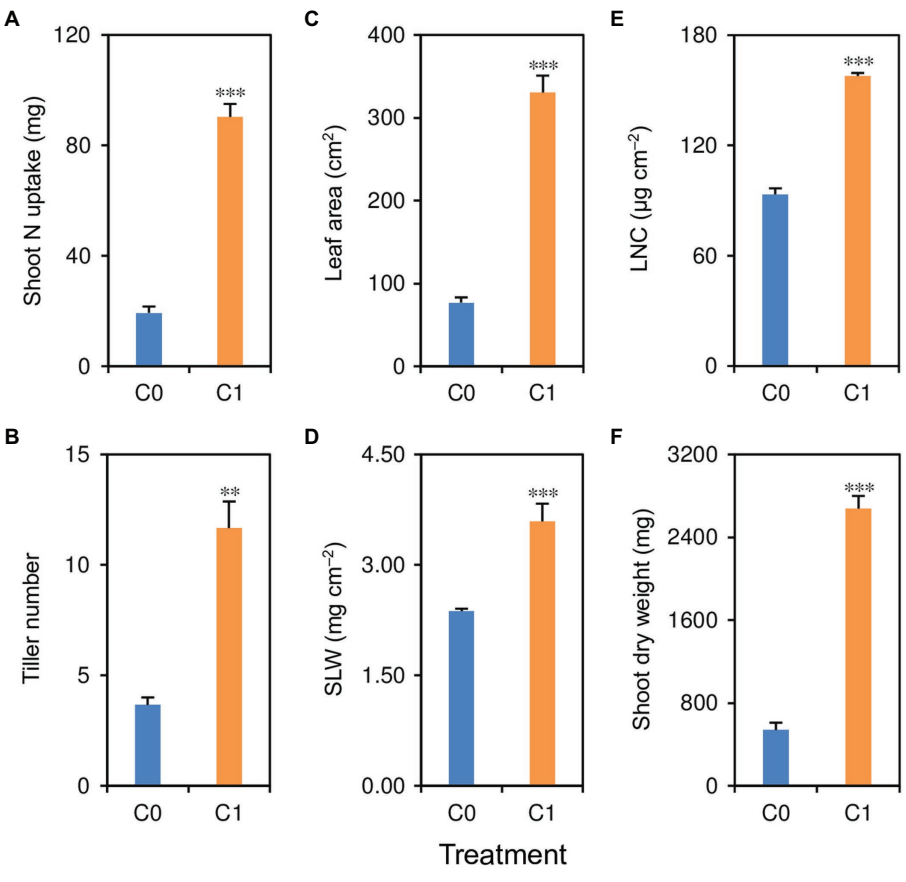


**FIGURE 4 | (A)** Shoot N uptake, **(B)** tiller number per plant, **(C)** leaf area per plant, **(D)** specific leaf weight, **(E)** leaf N content, and **(F)** shoot dry weight in rice plants grown with (C1) and without (C0) biochar application. ** and *** indicate that the means of the rice plants in C1 treatment are significantly different from those in the C0 treatment at the 0.01 and 0.001 levels, respectively.

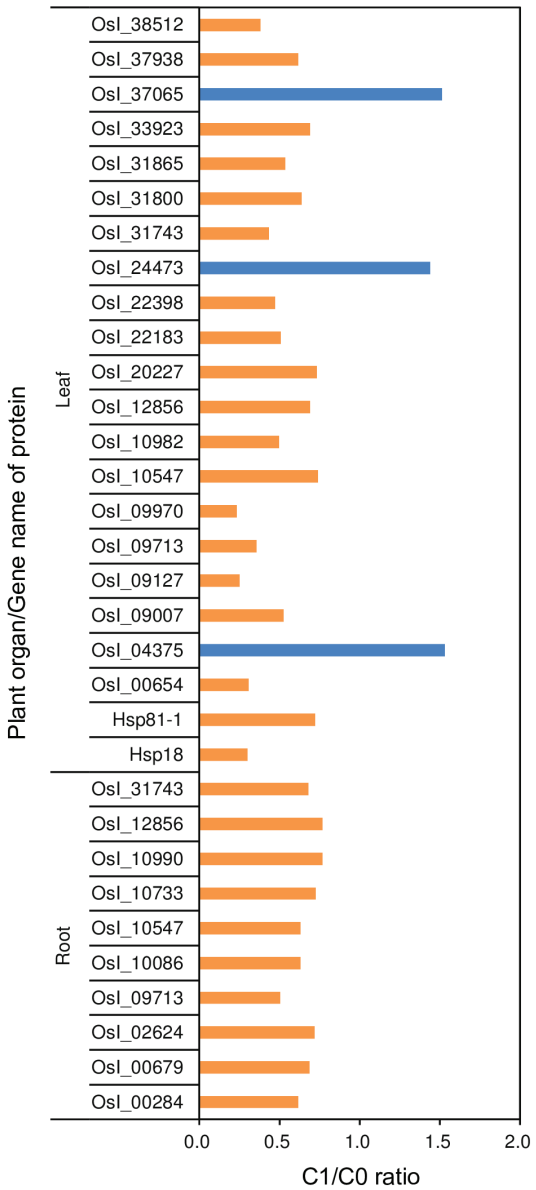


**FIGURE 5 |** Fold changes of differentially modulated heat-shock and related proteins in roots and leaves of the rice plants grown with biochar application (C1) compared to plants grown without biochar application (C0). Orange and blue bars represent the down- and up-modulated proteins in the C1 treatment compared to the C0 treatment, respectively, respectively.

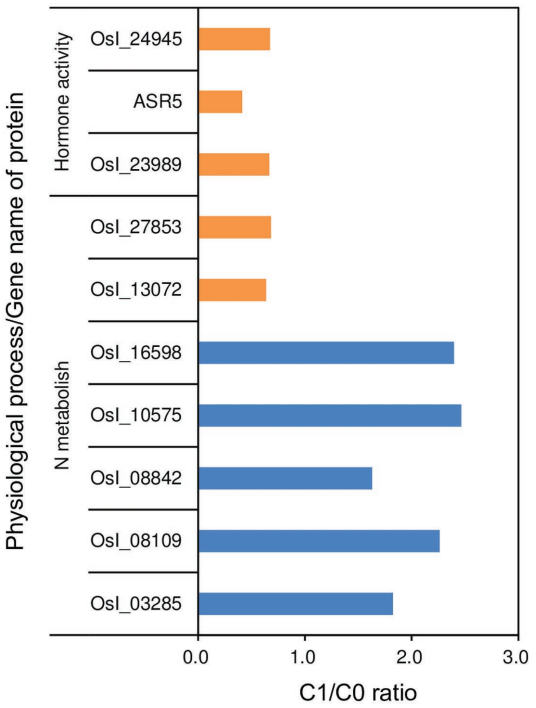


**FIGURE 6 |** Fold changes in differentially modulated proteins related to N metabolism and hormone activity in roots of rice plants grown with biochar application (C1) compared to plants grown without biochar application (C0). Orange and blue bars represent the down- and up-modulated proteins in the C1 treatment compared to the C0 treatment, respectively.

and OsI_16598), while the two down-modulated proteins related to N metabolism were both nitrate transporters (OsI_13072 and OsI_27853). The three down-modulated proteins related to hormone activity were abscisic stress-ripening protein 5 (ASR5), auxin efflux carrier component (OsI_23989), and ethylene-insensitive 2 (OsI_24945). We identified 49 differentially modulated proteins in leaves that are related to photosystems I and II, of which 44 differentially modulated proteins were up-modulated in rice plants in the C1 treatment compared to those in the C0 treatment with fold changes of 1.314–10.931 (**Figure 7**).

## DISCUSSION

Heat-shock proteins are generally up-modulated in response to high temperatures (Parsell et al., 1993). In this study, the down-modulation of several heat-shock or related proteins in C1 compared to C0 rice plants under high temperature conditions indicates that the effect of heat stress was mitigated in the C1 treatment. This finding is consistent with the performance of plant growth traits and, again, demonstrates that the effect of heat stress on rice can be mitigated by regulating the root-zone environment through biochar application.

In the present study, we found that biochar application improved the root-zone soil physical and chemical properties, including reduced bulk density and increased organic matter content. It is well known that reduced soil bulk density can reduce resistance to root penetration and increase root development (Lijima et al., 1991). In this study, we found that root length, surface area, and dry weight were consistently higher in rice plants in the C1 treatment with lower soil bulk density compared to those in the C0 treatment. The improvements in root length, surface area, and dry weight in the C1 treatment might also be partially attributed to the increased organic matter content, which plays an important role in maintaining soil quality because it has a positive effect on a wide range of soil properties, such as reducing soil compaction (Soane, 1990).

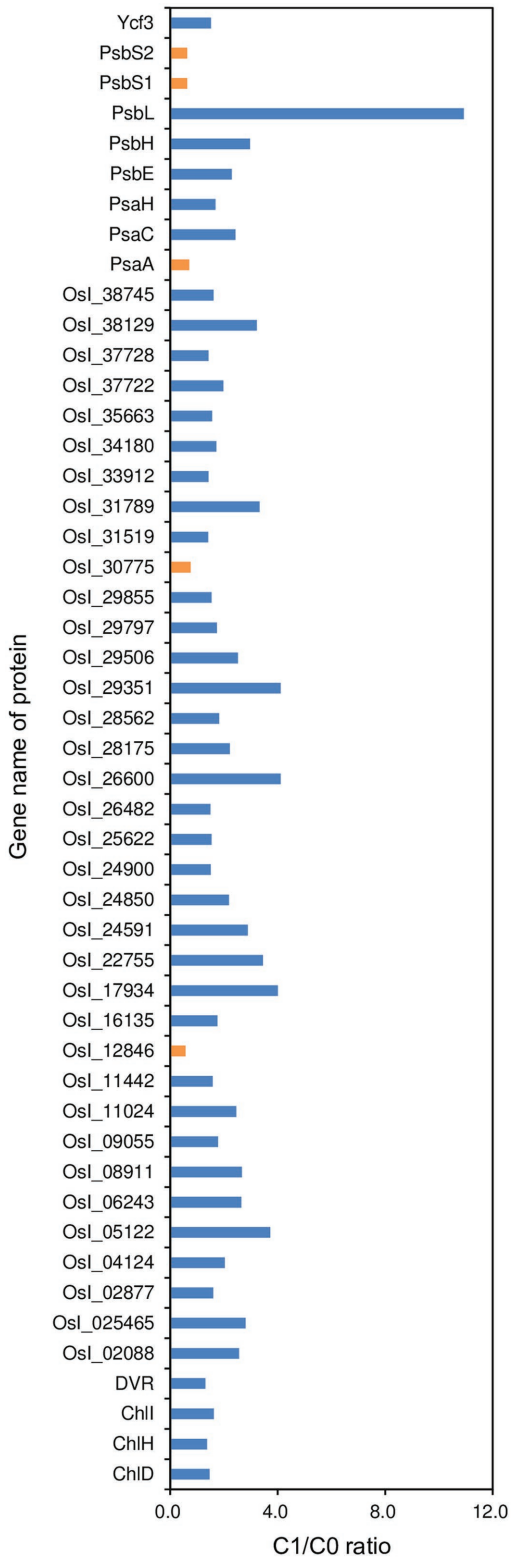


**FIGURE 7 |** Fold changes in differentially modulated proteins related to photosystems I and II in leaves of rice plants grown with biochar application (C1) compared to plants grown without biochar application (C0). Orange and blue bars represent the down- and up-modulated proteins in the C1 treatment compared to the C0 treatment, respectively.

This study also showed that biochar application altered the structure of the soil bacterial community, which is directly tied to soil nutrient recycling (Jacoby et al., 2017). In this regard, it has been reported that the abundances of Proteobacteria and Acidobacteria are related to the nutrient status of soils, and high Proteobacteria/Acidobacteria ratios are indicative of copiotrophic soils (Smit et al., 2001; Babujia et al., 2016; Huang et al., 2020). This could be partially explained or supported by that the Proteobacteria is responsible for several biogeochemical functions in the soil, such as symbiotic N fixation and nutrient cycling remineralization, while the Acidobacteria has a propensity to thrive in oligotrophic conditions, typically coupled with lower plant productivity (Lewis et al., 2012). In this study, biochar application resulted in an increase in the relative abundance of Proteobacteria but a decrease in the relative abundance of Acidobacteria. This means that a higher Proteobacteria/Acidobacteria ratio was induced by biochar application, suggesting that biochar application improved the soil nutrient status. This might also be partially responsible for the improvements in root length, surface area, and dry weight that resulted from biochar application.

In contrast to the effect of biochar application on increasing root length, surface area, and dry weight in rice plants, we found that root diameter was reduced in the biochar treatment (C1). This was because biochar application facilitated the growth of many more lateral roots with smaller diameter in the rice plants (**Figure 8**). This observation indicates that biochar application improved root architecture because the lateral roots are critical to allowing the plants to take up water and minerals effectively (von Wangenheim et al., 2020). Auxin is required for lateral root formation. In the present study, biochar application led to a down-modulation of an auxin efflux carrier component (OsI_23989) in roots, leading to the accumulation of auxin in lateral root initials and promotion of lateral root growth (Kazan, 2013). Moreover, it has been documented that increasing endogenous abscisic acid and ethylene can inhibit lateral root formation in plants (Negi, 2008; Guo et al., 2009). In this study, ASR5 induced by abscisic acid (Jia et al., 2016) and ethylene-insensitive 2 (OsI_24945) involved in ethylene biosynthesis (Singh et al., 2015) were down-modulated in roots by biochar application. This indicates that reductions in abscisic acid and ethylene biosynthesis could also be responsible for the increased number of lateral roots in the biochar treatment.

Nitrate acts as a crucial signal in the regulation of lateral root development, and nitrate transporters have negative effects on lateral root development under low nitrate conditions (Sun et al., 2017). In this study, (1) the experiment was conducted under flooding conditions, where the soil nitrate should be low because nitrate replaces oxygen as the terminal electron acceptor in microbial respiration leading to denitrification and/or nitrate ammonification under flooding (Laanbroek, 1990); and (2) the application of biochar resulted in down-modulation of two nitrate transporters (OsI_13072 and OsI_27853). These results show that the increased number of lateral roots in the biochar treatment could also be related to the repression of nitrate transporters.

In addition to improve the morphological and architectural traits, biochar application up-modulated glutamine synthetase

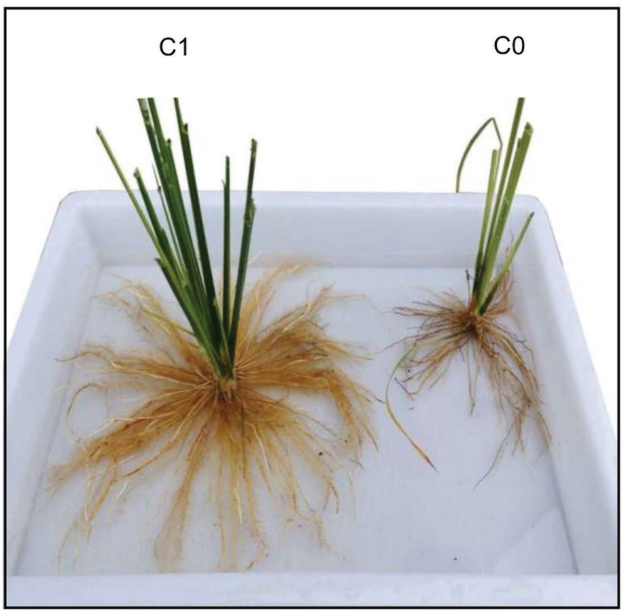


**FIGURE 8 |** Comparison on roots of rice plants grown with biochar application (C1) and without biochar application (C0).

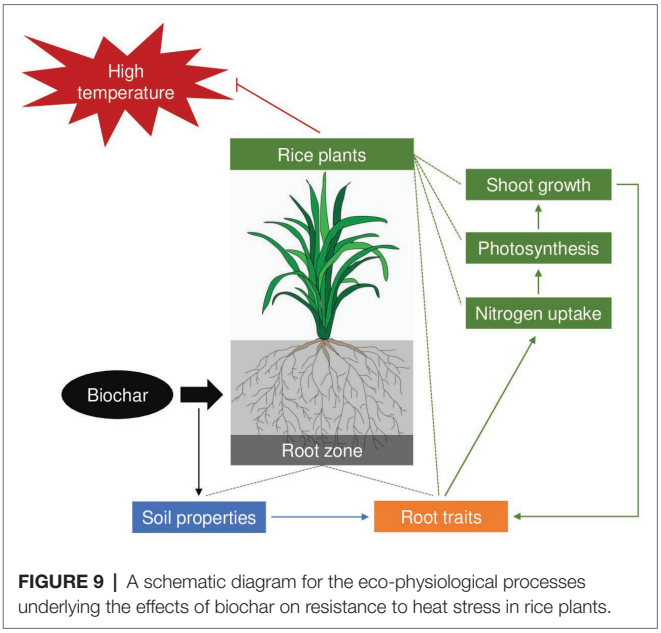


**FIGURE 9 |** A schematic diagram for the eco-physiological processes underlying the effects of biochar on resistance to heat stress in rice plants.

(OsI_08842 and OsI_10575), glutamine amidotransferase type-2 domain-containing protein (OsI_03285), and two ammonium transporters (OsI_08109 and OsI_16598) in the root. As a consequence, shoot N uptake in rice plants was increased by biochar application. It is well known that N plays an important role in improving photosynthesis and growth in rice (Yin et al., 2017). Consistently, in this study, leaf area, SLW, LNC, most differentially modulated proteins related to photosystems I and II, tiller number, and shoot dry weight in rice plants were increased or up-modulated in parallel with increased N uptake due to biochar application. These improvements in shoot traits could be in turn responsible for the improved root morphological, architectural, and physiological traits resulting from biochar application, because root establishment and maintenance require assimilates produced by the shoot (Yang et al., 2012). Furthermore, the synchronous improvements in root and shoot traits could accelerate the transport of water in the soil-plant-atmosphere system, might resulting in transpiration cooling, and helping plants to avoid heat stress damage (Jagadish et al., 2015). However, additional studies are required to confirm this potential mechanism.

Taken together, the results of this study suggest that the application of biochar can improve soil physiological, chemical, and biological properties and consequently improve root morphological, architectural, and physiological traits as well as shoot N uptake and utilization, which ultimately mitigate the effect of heat stress on rice plants (**Figure 9**). This finding not only increases our understanding of the fundamental eco-physiological processes underlying increased heat-stress tolerance in rice plants that results from biochar application, but also implies that improving the root-zone environment by optimizing management practices is an effective strategy to mitigate heat stress in rice production. The finding of this study also highlights the need for further investigations to compare the effects of biochar application on the growth and eco-physiological characteristics in rice plants between normal- and high-temperature conditions.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found at: https://figshare.com/articles/dataset/MS_identified_information_xlsx/14945382/1.

## AUTHOR CONTRIBUTIONS

MH conceived the experiment, analyzed the data, and wrote the manuscript. XY, JC, and FC performed the experiment. All authors have read and approved the final manuscript.

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# Identification of a New Giant Emrbryo Allele, and Integrated Transcriptomics and Metabolomics Analysis of Giant Embryo Development in Rice

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Rice embryos are rich in high-quality protein, lipid, vitamins and minerals, representing the most important nutritional part of brown rice. However, the molecular mechanism of rice embryo development is poorly understood. In this study, two rice cultivars with contrasting embryo size (the giant embryo cultivar Dapeimi and the normal embryo cultivar 187R) were used to explore excellent genes controlling embryo size, and the developed near-isogenic lines (NILs) (NIL-D, which has the giant embryo phenotype, and its matching line, NIL-X) were used to explore transcript and metabolic properties in the earlier maturation stage of giant embryo development under natural conditions. The map-based cloning results demonstrated that Dapeimi is a novel allelic mutant of the rice *GIANT EMBRYO* (*GE*) gene, and the functional mutation site is a single cytosine deletion in the exon1. A total of 285 differentially accumulated metabolites (DAMs) and 677 differentially expressed genes (DEGs) were identified between NIL-D and NIL-X. The analysis of DAMs indicated that plants lacking *GE* mainly promoted energy metabolism, amino acid metabolism, and lipid metabolism pathways in the rice embryo. Pearson correlation coefficient showed that 300 pairs of gene-metabolites were highly correlated. Among them, *OsZS_02G0528500* and *OsZS_12G0013700* were considered to be key genes regulating L-Aspartic acid and L-Tryptophan content during rice giant embryo development, which are promising to be good candidate genes to improve rice nutrition. By analyzing rice embryo development through a combination of strategies, this research contributes to a greater understanding of the molecular mechanism of rice embryo development, and provides a theoretical foundation for breeding high-nutrition varieties.

**Keywords: giant embryo, map-based cloning, metabolomics, transcriptomics, metabolic pathway**

## INTRODUCTION

Rice is one of the most important food crops in the world, with more than half the global population subsisting predominantly on rice grain (Seck et al., 2012). Rice grain contains abundant starch and cellulose, as well as a wealth of vitamins, trace elements, and bioactive substances. It not only provides the calories required for human productive labor, but also the nutrients necessary for human growth (Babu et al., 2009). With recent improvements in living standards, the demand for increased nutritional quality in rice grains has increased; thus, analyzing the breeding and molecular mechanisms of rice nutritional quality has become an important research area (Fontanelli et al., 2021). Rice grain consists chiefly of the endosperm, embryo, and seed coat, among which 70–80% of nutrients are enriched in the seed coat and particularly the embryo. The endosperm occupies approximately 90% of the rice grain volume, but is enriched with large amounts of starch (Zhou et al., 2002). Consequently, the composition, structure, and properties of the embryo directly determine the nutrition quality of rice grains.

Giant embryo rice, which refers to rice cultivars with larger embryos, whose volume is typically more than twice that of conventional rice, is an ideal material for studying rice embryo development. Moreover, compared with conventional rice, giant embryo rice exhibits greatly increased contents of amino acids, vitamin E, gamma-oryzanol, phenols, and minerals, especially gamma amino-butyric acid (GABA) (Zhang L. L. et al., 2005; Park et al., 2009; Seo et al., 2011; Jeng et al., 2012; Wang et al., 2013; Chen et al., 2020). GABA is a free amino acid that functions as an inhibitory neurotransmitter and typically accumulates in rice embryos. It has multiple physiological functions, such as normalizing blood pressure and aiding recovery from disorders of the autonomic nervous system (DeFeudis, 1983). Using different F2 populations, previous researchers mapped the giant embryo gene on chromosome 7 to a region within 19.2 cM (centiMorgan) and 5.8 cM, respectively (Koh et al., 1996; Qian et al., 1996). Moreover, Dong et al. (2003) suggested that embryo size is a quantitative trait, and genes controlling embryo length and embryo width are located on different chromosomes. Zhang G. H. et al. (2005) performed quantitative trait locus (QTL) analysis on multiple traits of a double haploid population and its parents, and showed that the size of the rice embryo was controlled by genes on chromosomes 3, 4, and 9. At present, *GE* is the first identified giant embryo gene, which encodes the cytochrome P450 designated as CYP78A13/CYP78B5 (Cahoon et al., 2003; Park et al., 2009; Nagasawa et al., 2013; Yang et al., 2013; Chen et al., 2015). CYP78B5 has three conserved domains: an N-terminal leucine-rich region, which is related to membrane localization; a P450 superfamily domain, which contains an oxygen binding site to perform monooxygenation function; and a heme-binding region, which combines with Fe ion to perform an enzyme catalytic function. Chen et al. (2015) showed that CYP78B5 functions in endoplasmic reticulum and peroxisome; loss of its function will cause a sharp decline in the auxin level, affect cell division and expansion, and eventually lead to giant embryo development. The *le* mutant showed mild enlargement in embryo size, which resulted from an increase in the size of scutellar parenchyma cell, and the *LE* gene encodes a C3H4-type RING finger protein (Lee et al., 2019).

Most previous studies on rice giant embryo mutants have focused on genetic analysis and functional research of single gene. Thus, the causal relationship between rice embryo metabolites and related genes has been rarely reported, and few studies have analyzed the relationship between the development regulation mechanism and biomolecular function of rice embryos. In recent years, with the continuous development and improvement of high-throughput sequencing technologies, the integration of multiple omics data has emerged and become a new direction for molecular mechanism research (Moreno-Risueno et al., 2010; Ma et al., 2016; Moschen et al., 2016; Shen et al., 2016; Rai et al., 2017; McLoughlin et al., 2018). Among these technologies, metabolomics and transcriptomics association analysis can achieve co-expression analysis of DAMs and DEGs. Furthermore, combining such analyses with metabolic pathway enrichment, functional annotation, and other biological function analysis can allow key metabolic pathways, key metabolites, and key genes to be identified (Ma et al., 2016; Moschen et al., 2016). Therefore, metabolomics and transcriptomics association analysis is a powerful tool for studying the molecular mechanism of metabolites.

In this study, we characterize the giant embryo rice cultivar Dapeimi. Map-based cloning is conducted to show that Dapeimi is a novel allelic mutant of *GE*. Its exon1 has a single cytosine deletion, which results in a truncated protein of CYP78B5. Furthermore, metabolomics and transcriptomics association analysis reveals that amino acid metabolism, energy metabolism, and lipid metabolism pathways are significantly enriched during embryo development in giant embryo rice. Further analysis revealed that 300 pairs of gene-metabolites are highly correlated. These results provide new insights into rice embryo development, which will help accelerate the breeding of high-nutrition rice.

**Abbreviations:** NIL, near-isogenic line; GE, GIANT EMBRYO; DAM, differentially accumulated metabolite; DEG, differentially expressed gene; cM, centiMorgan; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; QC, quality control; UHPLC, ultra high-performance liquid chromatography; RT, retention time; HMDB, Human Metabolome Database; m/z, mass-to-charge ratio; PCA, principal components analysis; OPLS-DA, orthogonal partial least-squares-discriminant analysis; VIP, variable importance in the projection; FPKM, fragments per kilobase of exon per million mapped reads; FDR, false discovery rate; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GEO, Gene Expression Omnibus; qRT-PCR, real-time quantitative PCR; $^{\Delta\Delta}$Ct, threshold cycle; EG, experiment group; CG, control group; RFO, raffinose family of oligosaccharide.

## MATERIALS AND METHODS

### Plant Materials and Growing Environments

Dapeimi is a local giant embryo indica rice from Jiangxi Province. In the autumn of 2017, the restoring-line 187R, a normal embryo indica rice, was used as the female parent and crossed with Dapeimi. Then, F1 seeds were harvested in the experimental field of the Life Science College of Fudan University, Shanghai (121°E,

31°N, altitude: 4 m, annual average temperature: 15.8°C, average annual sunshine: 1387 h, annual average evaporation: 1346.3 mm, and average annual rainfall: 1078.1 mm), China. In the same year, F1 seeds were planted in a paddy field in Sanya (109°E, 18°N, altitude: 7 m, annual average temperature: 25.4°C, average annual sunshine: 2287.3 h, annual average evaporation: 1950.7 mm, and average annual rainfall: 1826.5 mm), Hainan Province, China, and F2 seeds were collected in the spring of 2018. The obtained F2 segregating population and its family groups were planted in the same locations mentioned above. In the spring of 2020, after six generations of self-pollination, we developed the following NILs: NIL-D, which carries the Dapeimi allele, and NIL-X, which carries the homologous segment from 187R. The two NILs were planted in the experimental field of school in the summer of 2020.

## Field Experiment and Sampling Strategies

The soil type of experimental field in this study was clay, the tillage depth was 20 cm, and the total amount of nitrogen fertilized was 20 kg/667 $m^2$. From the critical leaf-age of productive tillers, low level water (3–5 cm) irrigation was kept until 1 week before harvest. The experimental field was subdivided into three plots, and each NIL separately planted three groups (40 plants per group; 5 rows × 8 columns) in one plot. The row spacing was 12 inches, and the plant spacing was 6 inches. The seedlings were transplanted into experimental field on the 19th of June, about 30 days after germination, and the rice plants grew under natural conditions.

The embryo samples were strictly collected in the middle area of each group on approximately the 15th day after flowering, and three copies (three groups) were collected in one plot for each NIL. Therefore, there were nine copies of samples for each NIL. Among these samples, two copies from each plot, six biological replicates totally, were used for metabolite profiling analysis, and the rest samples, three biological replicates totally, were used for transcriptome profiling analysis.

## Morphological and Agronomic Trait Analysis

Quantitative analysis of agronomic traits, including the 100-grain brown rice weight, endosperm weight, and embryo weight for the two parents, Dapeimi and 187R, were performed using 100 grains at maturity. The resulting values are the means ± SE of three biological repeats. Student's *t*-test was used to compare significant differences between samples. The related phenotype of transgenic lines was also detected according to the above method.

## Map-Based Cloning

Fresh rice leaves of plants at the grain filling stage were obtained from the two parents, the F2 population, and its family groups. Thirty normal embryo plants and 30 giant embryo plants were randomly selected from the F2 population; the same amount of leaves were mixed to form a normal embryo pool and a giant embryo pool. The genomic DNA of each pool was extracted using the Plant Genomic DNA kit (Tiangen, Beijing, China) and detected by single nucleotide polymorphism (SNP) chip array (RICE6K) to initially locate the target gene. HiScan scanner (Illumina Inc., San Diego, CA, United States) was used for chip scanning, and GenomeStudio software was used for raw data analysis. R platform was used for further analysis, such as genotype identification, comparison and map drawing (R Development Core Team, 2011). Simple sequence repeat (SSR) markers were identified from the Gramene database for grasses[1]. Molecular markers containing polymorphism between two parents were screened, and the genotypes of all markers in the target region were determined for recombinant plants in the F2 population and its family groups. Mapmaker/Exp v3.0 was used for linkage analysis and the Kosambi function was used to calculate the genetic distance (Lincoln et al., 1992). The candidate gene (*OsZS_07T0416900*) in the final positioning interval was amplified separately from the two parents, sequenced, and compared using GENtle software (v1.9.4, Magnus Manske, Cologne, NRW). All the primers used in the current study are listed in **Supplementary Table 1**.

## Rice Transformation

For *OsZS_07T0416900* over-expression, a 1,578 bp CDS fragment of *OsZS_07T0416900* was amplified from the total RNA of 187R and cloned into pMD19-T, and further inserted into the plant binary vector pCAMBIA1304 (GenBank accession number AF234300) to generate the expression vector pCaMV35S:$OsZS\_07T0416900^{187R}$. This construct was then introduced into the Dapeimi background via *Agrobacterium tumefaciens*-mediated transformation using standard protocols. The transgenic rice plants were further confirmed by PCR detection and direct sequencing.

## Metabolite Extraction and UHPLC-ESI-MS/MS Analysis

Prior to analysis, 100 mg of fresh grain embryos were accurately weighed, and the metabolites were extracted using a 400 μL methanol:water (4:1, v/v) solution with 0.02 mg $mL^{-1}$ L-2-chlorophenylalanine as internal standard. The mixture was settled at −10°C and treated by a high-throughput tissue crusher Wonbio-96c (Wanbo Biotechnology, Shanghai, China) at 50 Hz for 6 min, then followed by ultrasound at 40 kHz for 30 min at 5°C. The samples were placed at −20°C for 30 min to precipitate proteins. After centrifugation at 13000 *g* for 15 min at 4°C, the supernatant were carefully transferred to sample vials. The quality control (QC) sample was prepared by mixing equal volumes of all samples to monitor the stability of the analysis (Doppler et al., 2016).

Chromatographic separation of the metabolites was performed on a Thermo UHPLC (Ultra High-performance Liquid Chromatography) system in Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China). The mobile phases consisted of 0.1% formic acid in water:acetonitrile (95:5, v/v) (solvent A) and 0.1% formic acid in acetonitrile:isopropanol:water (47.5:47.5:5, v/v) (solvent B). The solvent gradient changed according to the following conditions: from 0 to 3.5 min, 0 to 24.5% B (0.4 mL $min^{-1}$);

[1]http://www.gramene.org/

from 3.5 to 5 min, 24.5 to 65% B (0.4 mL $min^{-1}$); from 5 to 5.5 min, 65 to 100% B (0.4 mL $min^{-1}$); from 5.5 to 7.4 min, 100 to 100% B (0.4 to 0.6 mL $min^{-1}$); from 7.4 to 7.6 min, 100 to 51.5% B (0.6 mL $min^{-1}$); from 7.6 to 7.8 min, 51.5 to 0% B (0.6 to 0.5 mL $min^{-1}$); from 7.8 to 9 min, 0 to 0% B (0.5 to 0.4 mL $min^{-1}$); from 9 to 10 min, 0 to 0% B (0.4 mL $min^{-1}$) for equilibrating the systems. The sample injection volume was 2 μL and the flow rate was set to 0.4 mL $min^{-1}$. The column temperature was maintained at 40°C. During the period of analysis, all these samples were stored at 4°C. The mass spectrometric data was collected using an AB Sciex TripleTOF 5600$^{TM}$ mass spectrometer system equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode with a capillary voltage 1.0 kV, sample cone, 40 V, collision energy 6 eV. The source temperature was set at 120°C, with a desolvation gas flow of 45 L $h^{-1}$. Centroid data was collected from 50 to 1000 m/z with a 30000 resolution.

The acquired MS data from UHPLC-ESI-MS/MS were imported into the Progenesis QI 2.3 (Non-linear Dynamics, Waters, MA, United States) for peak detection and alignment (Nanjegowda et al., 2018). The preprocessing results generated a data matrix that consisted of the retention time (RT), mass-to-charge ratio (m/z) values, and peak intensity. Mass spectra of these metabolic features were identified by using the accurate mass, MS/MS fragments spectra and isotope ratio difference with searching in reliable biochemical databases as Human Metabolome Database (HMDB)[2] and Metlin database[3]. Concretely, the mass tolerance between the measured m/z values and the exact mass of the components of interest was ± 10 ppm. For metabolites having MS/MS confirmation, only the ones with MS/MS fragments score above 30 were considered as confidently identified. Otherwise, metabolites had only tentative assignments.

## Data Analysis and Validation for Metabolites

A multivariate statistical analysis was performed using R package ropls version 1.6.2[4]. Principle component analysis (PCA) using an unsupervised method was applied to obtain an overview of the metabolic data, general clustering, trends, or outliers were visualized. Orthogonal partial least squares discriminate analysis (OPLS-DA) was used for statistical analysis to determine global metabolic changes between comparable groups. The model validity was evaluated from model parameters R2 and Q2, which provide information for the interpretability and predictability, respectively, of the model and avoid the risk of over-fitting. Variable importance in the projection (VIP) values were calculated in OPLS-DA model. *p*-values were estimated with paired Student's *t*-test on Single dimensional statistical analysis. Metabolites with VIP $\geq$ 1.0 and *p*-value $\leq$ 0.05 were defined as statistically significant. DAMs among two groups were summarized, and mapped into their biochemical pathways through metabolic enrichment and pathway analysis based on database search (KEGG[5]). scipy.stats (Python packages)[6] was exploited to identify statistically significantly enriched pathway using Fisher's exact test.

## RNA Isolation and Microarray Analysis

High-quality total RNA was isolated from fresh grain embryos using the PureLink® Plant RNA Reagent kit (Ambion, Austin, United States) according to the manufacturer's instructions. The RNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). The purity and integrity of total RNA was determined by 260/280 nm ratio and by NanoBioanalyzer RNA-6000 analysis (Agilent Technologies, Palo Alto, CA, United States). After that, 1μg of total RNA from each sample was used for RNA-Seq library construction following the specifications of the TruSeq® RNA Sample Preparation v2 Guide (Illumina), which was then sequenced using Illumina Hiseq 2500 in Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China).

The raw paired end reads were trimmed and quality-controlled by SeqPrep[7] and Sickle[8] with default parameters. The clean data were assembled using Cufflinks software, then mapped to the reference genome (ZS97RS3, RIGW3.0)[9] via TopHat with no more than two base mismatches allowed in the alignment (Trapnell et al., 2012). The basic information of the RNA-sequencing data is provided in **Supplementary Table 2**.

## Data Analysis and Validation for Transcripts

The expression level of each transcript was estimated by the fragments per kilobase of exon per million mapped reads (FPKM) with RSEM (Li and Dewey, 2011). The DEGs were determined using R package edgeR with a false discovery rate (FDR) $< 0.05$ and a logarithm two-fold change $|\log2FC| \geq 1$ (Anders and Huber, 2010; Robinson et al., 2010). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted based on the DEGs by Goatools[10] and KOBAS 2.1.1[11] (Xie et al., 2011). RNA-Seq data from this article can be found in Gene Expression Omnibus (GEO) under the accession number GSE173301.

## Real-Time Quantitative PCR (qRT-PCR) Based Validation of Genes

Total RNA was isolated as mentioned in the text using the PureLink® Plant RNA Reagent kit (Ambion, Austin, United States) and was reverse transcribed using the M-MLV Reverse Transcriptase kit (Promega, Madison, WI, United States) according to the manufacturer's instructions. qRT-PCR was performed using the SYBR I Premix ExTaq (Takara Bio, Kusatsu,

[2]http://www.hmdb.ca/

[3]https://metlin.scripps.edu/

[4]http://bioconductor.org/packages/release/bioc/html/ropls.html

[5]http://www.genome.jp/kegg/

[6]https://docs.scipy.org/doc

[7]https://github.com/jstjohn/SeqPrep

[8]https://github.com/najoshi/sickle

[9]http://rice.hzau.edu.cn/rice_rs3/

[10]https://github.com/tanghaibao/Goatools

[11]http://kobas.cbi.pku.edu.cn/download.php

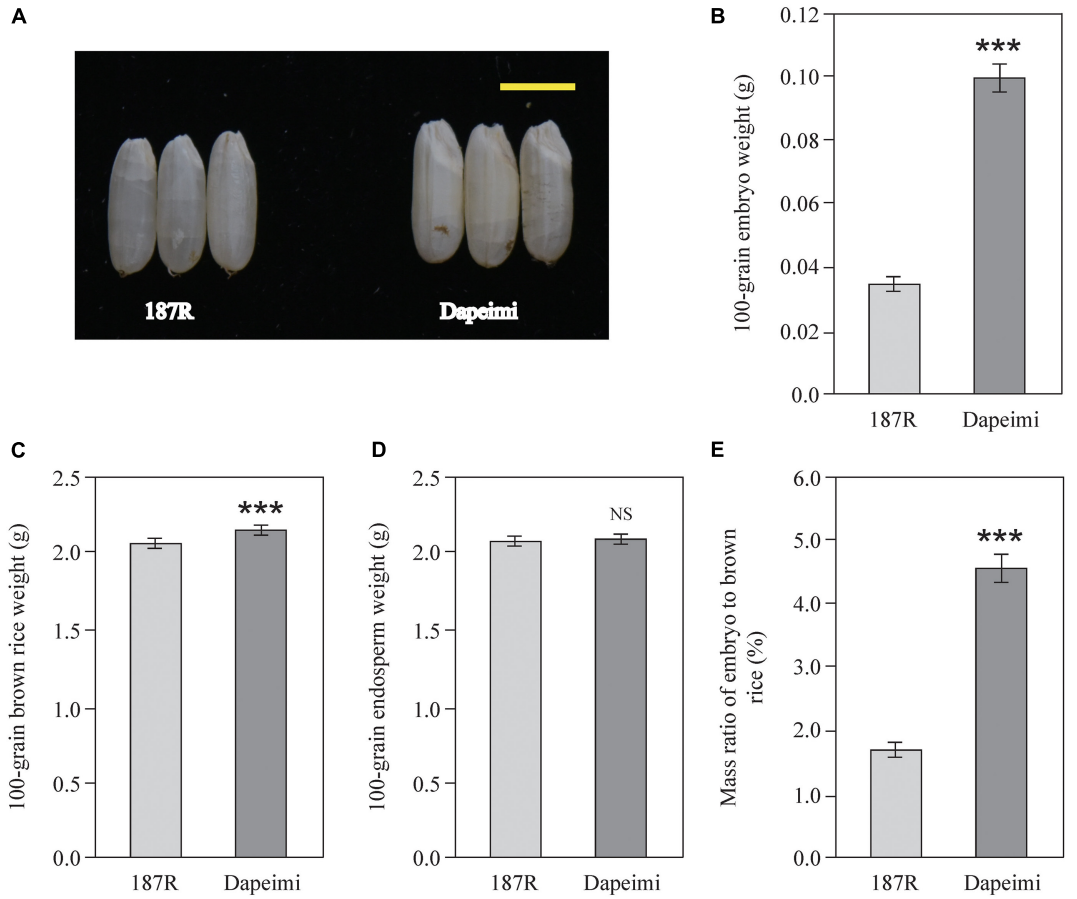


**FIGURE 1 |** Brown rice phenotypic characteristics of 187R and Dapeimi. **(A)** Brown rice of the parents used for gene mapping analysis. Scale bar, 0.5 cm. **(B–E)** Comparison of embryo weight **(B)**, brown rice weight **(C)**, endosperm weight **(D)**, and mass ratio of embryo to brown rice **(E)** between 187R and Dapeimi cultivars. Data are shown as means ± S.E.M. ($n = 3$). ***$P < 0.001$; NS, not significant at $P = 0.05$.

Shiga, Japan). The gene expression levels in three biological replicates were calculated using the $^{\Delta\ \Delta}$ Ct (threshold cycle) method (Livak and Schmittgen, 2001). Student's *t*-test was used to compare significant differences between samples. The primers for selected genes are listed in **Supplementary Table 1**.

# RESULTS

## Brown Rice Characteristics of Two Rice Cultivars

Dapeimi is a giant embryo rice cultivar grown in Jiangxi Province (**Figure 1A**). Its average 100-grain embryo weight was 0.099 g, and the mass ratio of embryo to brown rice was 4.57% (**Figures 1B,E**). 187R is a derivative line of the India wide-compatibility variety Dular, which has a normal embryo size (**Figure 1A**). Its average 100-grain embryo weight and mass ratio of embryo to brown rice were 0.036 g and 1.76%, respectively (**Figures 1B,E**). The two cultivars exhibited significant difference in the 100-grain brown rice weight, but the difference in the 100-grain endosperm weight was not obvious (**Figures 1C,D**). These data indicate that the size of embryo is the main difference between the two brown rice cultivars.

## Dapeimi Is a Novel Allelic Mutant of GE

According to the phenotypic investigation, there were 56 giant embryo plants, 60 normal embryo plants, and 92 varied embryo plants in the F2 population derived from Dapeimi × 187R. The segregation ratio agreed with the Mendelian ratio (1:2:1) for single locus segregation ($\chi^2 = 2.59 < \chi^2_{0.05,\ 2} = 5.99$), which suggests that the embryo phenotypic difference between Dapeimi and 187R was mainly controlled by a single gene. Base on the result of the SNP chip array, the candidate giant embryo gene was roughly mapped on chromosome 7 (**Supplementary Figure 1**). For fine mapping of this gene, a larger segregating population consisting of 1,000 plants derived from F2 heterozygous plants was constructed. Using 232 giant embryo plants, the locus was eventually narrowed down to a 127.8 kb interval defined by two SSR markers (*RM21931* and *RM21938*). Based on the published sequence annotation for indica rice Zhenshang97 (see text footnote 9), 18 predicted genes were identified in this interval, including the already identified

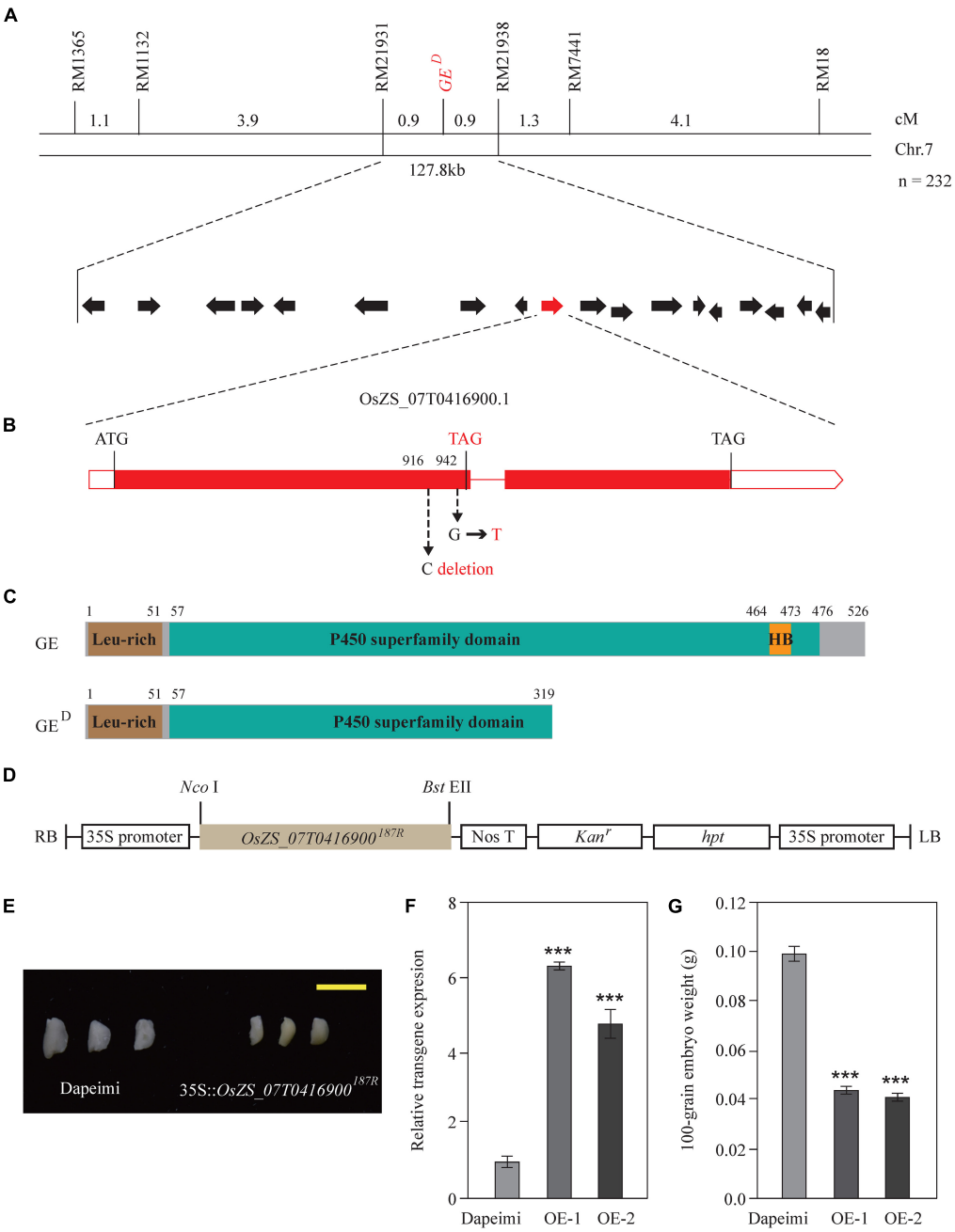


**FIGURE 2 |** Map-based cloning and confirmation of the *GE*$^{D}$ gene. **(A)** Gene location was performed with 232 giant embryo plants in the $F_3$ families. Genetic distance between adjacent markers is indicated above the linkage map. *GE*$^{D}$ locus was narrowed down to a 127.8-kb interval between the SSR marker *RM21931* and *RM21938* on chromosome 7. This region contains 18 predicated genes. **(B)** Gene structure and allelic variations of *OsZS_07T0416900* between 187R and Dapeimi. Filled boxes and red lines represent exons and introns, respectively. Start and stop codons are indicated above the gene, and allelic variations and a premature stop codon are shown in red letters. 187R was used as the reference for allelic comparison. **(C)** Comparison of protein structure between GE and GE$^{D}$. Start and stop positions of each domain are indicated above the protein. **(D)** The basic structure of the expression vector pCaMV35S:*OsZS_07T0416900*$^{187R}$. **(E–G)** Phenotypes of embryos **(E)**, transgene expression level of *OsZS_07T0416900* **(F)** and 100-grain embryo weight **(G)** from the control plants (Dapeimi) and the *OsZS_07T0416900* over-expression lines (OE, in Dapeimi background). Scale bar, 0.5 cm. Data are shown as means ± S.E.M. ($n = 3$). ***$P < 0.001$.

giant embryo gene *GE* (*OsZS_07T0416900*) (**Figure 2A**). The coding region of *OsZS_07T0416900* was comparatively aligned between Dapeimi and 187R, and a 1 bp base deletion mutation (C deletion) and a non-synonymous mutation (G→T) were detected in the exon1, separately occurring at nucleotide 916 and 942 (**Figure 2B**). *OsZS_07T0416900* was considered as the favorable candidate gene responsible for the embryo size, because the cytosine deletion mutation in Dapeimi results in a frame

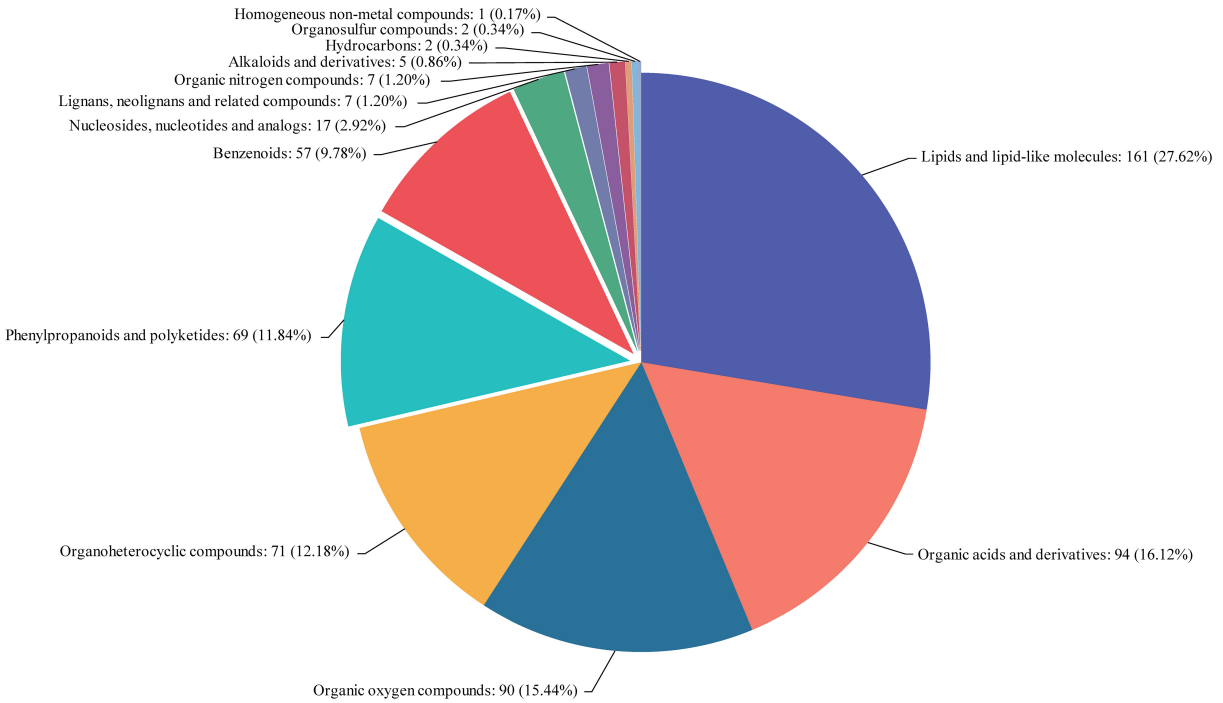


**FIGURE 3 |** Classification statistics of compounds. Names of the selected HMDB levels (superclass, class, or subclass) and percentage of metabolites are displayed in order from high to low depending on the number of metabolites. Different colors in each pie chart represent different HMDB classifications, whose area represents the relative proportion of metabolites in that classification.

shift, leading to premature translation termination of the encoded protein (**Figure 2C**).

To confirm the mapping results, the expression vector pCaMV35S:*OsZS_07T0416900*$^{187R}$ was used to generate *OsZS_07T0416900* over-expression transgenic rice plants (**Figure 2D**). Ten independent transgenic lines with the Dapeimi background were obtained, and two of them were used for measurements of embryo weight. Most of the 10 independent transgenic lines exhibited decreased embryo size in T1 seeds when compared with wild type (Dapeimi) seeds (**Figure 2E**), and the decrease in the embryo weight in T1 seeds was up to 60% (**Figures 2F,G**). These results reveal that disruption of the gene *OsZS_07T0416900* improves embryo size in Dapeimi. Sequence comparison indicated that the mutation type of *OsZS_07T0416900* in Dapeimi does not exist in other giant embryo varieties (Ping et al., 2015). Therefore, Dapeimi is a novel allelic mutant of *GE* with a truncated P450 superfamily domain compared to wild-type rice; we named this gene *GE*$^{D}$.

## Metabolite Profiling of Two NILs

The metabolites in rice embryos from NIL-D (EG) and NIL-X (CG) were investigated using UPLC-ESI-MS/MS. We identified 285 DAMs (148 increased and 137 decreased) from 13 classes. Lipids and lipid-like molecules, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds, phenylpropanoids and polyketides, benzenoids, nucleosides, nucleotides and analogs, lignans, neolignans and related compounds, organic nitrogen compounds, alkaloids and derivatives, hydrocarbons, organosulfur compounds, and homogeneous non-metal compounds accounted for 27.62, 16.12, 15.44, 12.18, 11.84, 9.78, 2.92, 1.2, 1.2, 0.86, 0.34, 0.17, and 18.5% of the DAMs, respectively (**Figure 3**). Detailed DAMs information was shown in **Supplementary Table 3**. In order to screen metabolites with similar expression patterns, we also conducted the correlation analysis of Top 50 by calculating the correlation and distance between various metabolites and speculating on the function of unknown metabolites (**Supplementary Figure 2** and **Supplementary Table 4**).

General differences on DAMs between EG and CG samples were revealed using PCA. Two principal components (PC1 and PC2) were extracted and found to represent 32.1 and 17% of the observed variation, respectively. In the PCA score plot, the EG and CG samples were clearly separated, and samples were collected in a compact and repeated way (**Figure 4A**). PLS-DA demonstrated that the two principal components explained 60.8% of the variation between EG and CG samples (**Figure 4B**). Moreover, OPLS-DA demonstrated that the two principal components explained 60.7% of the variation between EG and CG samples (**Figure 4C**). All data points in the figure formed tight clusters between the different comparison groups. To verify the model reproducibility and model fitting, it is imperative to avoid the classification obtained by the supervised learning method. We conducted 200 response-sorting tests of the OPLS-DA model, namely, the fixed X matrix and previously defined Y matrix variable classifications (e.g., 0 or 1) were randomly arranged n times (n = 200). The OPLS-DA model was established

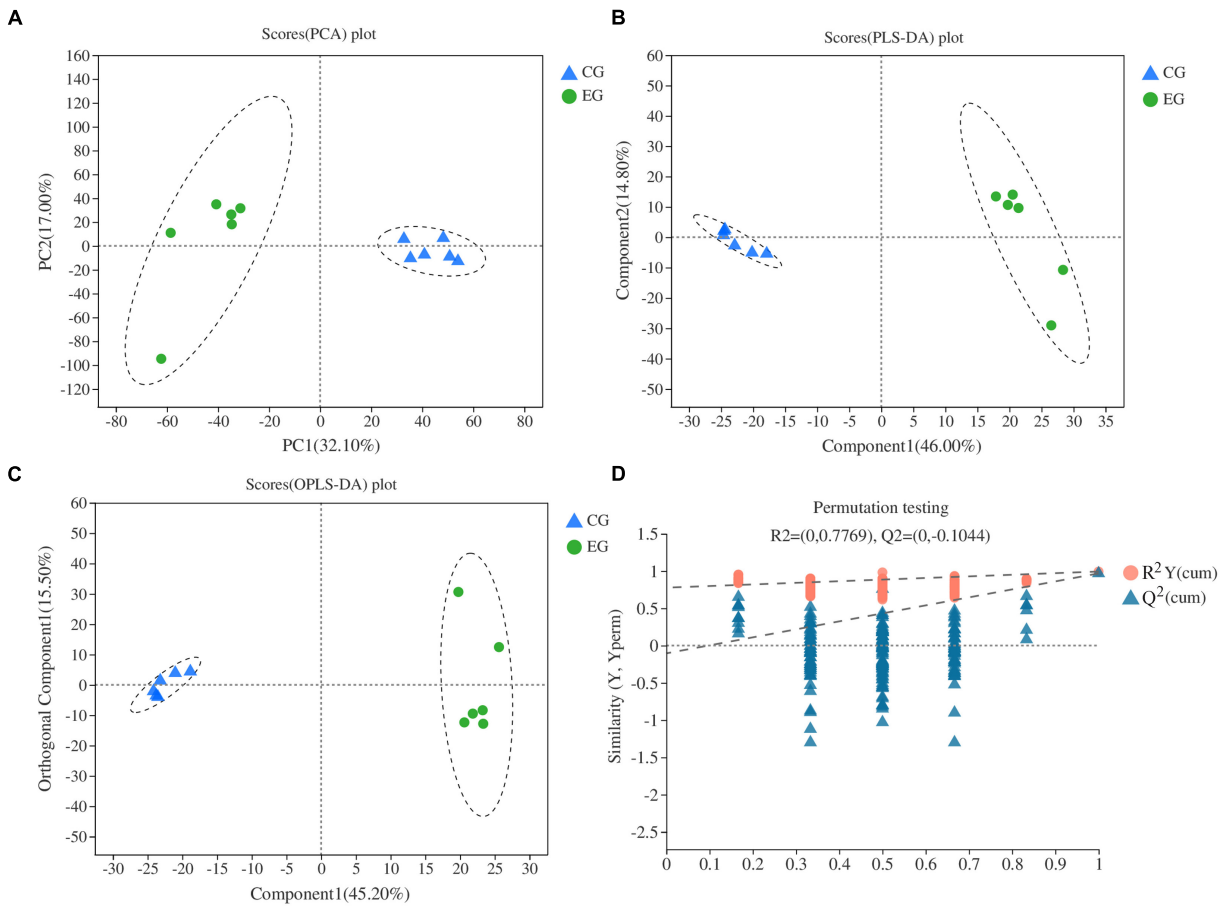


**FIGURE 4 |** Multivariate statistical score and response-sorting test diagram in two NILs. **(A)** PCA; **(B)** PLS-DA; **(C)** OPLS-DA; **(D)** response-sorting test of the OPLS-DA model. EG, experiment group (NIL-D); CG, control group (NIL-X).

to obtain the corresponding stochastic model of $R^2$ and $Q^2$ values. With the original model of $R^2Y$ and $Q^2Y$ linear regression, the regression line and the Y-axis intercept values ($R^2$ and $Q^2$) were used to measure whether the model was over fitted. The replacement test evaluation standard specifies that the intercept lies between the $Q^2$ regression line and *Y*-axis. An intercept of less than 0.05 indicates that the model is robust and reliable, and no overfitting occurs (**Figure 4D**).

## Transcriptome Profiling of Two NILs

To understand the genetic basis for the DAMs observed in EG and CG samples, transcriptome analysis of six samples was conducted in this study. A total of 50.26 Gb of clean data were used, with more than 7.86 Gb of clean data for each sample, and the percentage of Q30 bases was more than 93.03%. A total of 33,861 expressed genes were detected in the analysis, including 32,817 known genes and 1,044 new genes, as well as 64,166 expressed transcripts, including 49,510 known transcripts and 14,656 new transcripts. The genes and transcripts expressed in this study were analyzed by functional database annotation (NR, Swiss-prot, Pfam, COG, GO, and KEGG) (**Supplementary Table 2**). Three biological replicates were conducted and their repeatability was assayed by calculating the Pearson correlation coefficient (**Figure 5**). The results were found to be consistent and repeatable. The DEGs were identified between the EG and CG samples using DESeq. Of the EG and CG comparison groups, 677 DEGs were identified, including 313 up-regulated genes and 364 down-regulated genes (**Supplementary Table 5**). The FPKM value of the validated gene was closely correlated with its relative expression via RT-qPCR (**Supplementary Figure 3**).

## Metabolic Pathway Analysis of Two NILs

To further investigate the potential functions of the DEGs, we analyzed the metabolic processes. The DEGs in the amino acid metabolism, carbohydrate metabolism, energy metabolism, lipid metabolism, biosynthesis of other secondary metabolites, glycan biosynthesis and metabolism, metabolism of other amino acids, and metabolism of cofactors and vitamins were enriched in the metabolism pathway (**Figure 6**). Most of the DEGs in the metabolic processes were consistent with the GO enrichment analysis (**Supplementary Figure 4**). For the second metabolism, the enriched DEGs were correlated with the pathways of lysine degradation, fatty acid degradation, photosynthesis, starch and sucrose metabolism, fatty acid biosynthesis, citrate cycle (TCA

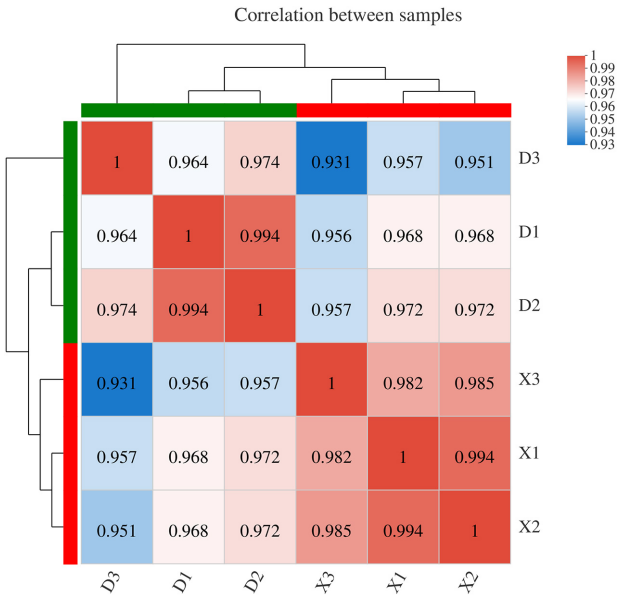


**FIGURE 5 |** Correlation analysis between different samples. Right and lower sides indicate sample names, X represents three replicates of the CG sample, and D represents three replicates of the EG sample. Left and upper sides are sample clustering, and squares of different colors represent the correlation between the two samples.

cycle), carbon fixation in photosynthetic organisms, flavonoid biosynthesis, amino sugar and nucleotide sugar metabolism, and glycolysis/gluconeogenesis.

## Genes and Metabolites Associated Network Analysis

The various DAMs and genes involved in the significantly enriched amino acid metabolism, energy metabolism, and lipid metabolism pathways of the two NILs are detailed in **Table 1** and **Supplementary Table 6**. To clarify the close correlation of genes and metabolites and screen out the key genes that regulate a certain metabolite, we perform a correlation network analysis of both DEGs and DAMs (**Figure 7** and **Supplementary Table 7**). By calculating the Pearson correlation coefficient of DEG-DAM, it was found that there were 300 pairs of gene-metabolites with the absolute value greater than 0.993, 150 pairs were positive and 150 pairs were negative (**Supplementary Table 7**). Therefore, these 300 pairs of gene-metabolites were highly correlated.

# DISCUSSION

## Experimental Design Provides Clues to Reveal the Underlying Molecular Basis of Nutrients Enrichment in Giant Embryo

Giant embryo rice is a special rice characterized by a particularly large embryo. Studies have shown that giant embryo rice is high nutrient functions (Zhao et al., 2019). It is therefore important to studying its molecular basis for breeding high-nutrition rice varieties. However, many previous studies focus on genetic basis of giant embryo rice or nutritional measurements performed on mature rice grains, which lead to little is known about the causal relationship between metabolites and related genes during rice embryo development (Koh et al., 1996; Qian et al., 1996; Dong et al., 2003; Zhang G. H. et al., 2005; Zhang L. L. et al., 2005; Park et al., 2009; Seo et al., 2011; Jeng et al., 2012; Wang et al., 2013; Chen et al., 2020).

Nagasawa et al. (2013) showed that the embryo cell volume in *GE* mutant was more than twice that of the wild-type, but the difference of cell number was not obvious, so the abundance of nutrients in giant embryo rice may be due to the increase of embryo cell volume (the larger the container, the more it could hold). Rice embryo development can be roughly divided into four successive stages: proembryo stage, differentiation stage, maturation stage and quiescence stage. In maturation stage (about 13–25 days), cells basically stop proliferating, embryo still grows slightly at earlier stage, and then is mainly the further accumulation of internal substances (protein, lipids, etc.) (Qin and Tang, 1982). Therefore, we inferred that maturation stage may be the key period for nutrients enrichment in giant embryo rice, and the genes expressed in this period are particularly important. Besides that, the genetic effects of genes on traits in different background could be significantly different, and eventually lead to the deviation of results (Zhao et al., 2017). To correctly evaluate the relationship between genes and metabolites in integrated transcriptomics and metabolomics analysis, the effect of genetic background should be eliminated. For this purpose, NILs are the appropriate materials, which had similar genetic background, but different in a specific trait or genetic basis (Zhang et al., 2009). Here, we characterized genetic basis of giant embryo rice Dapeimi, and analyzed metabolite content and transcript abundance of embryos in the earlier maturation stage of NILs (NIL-D and NIL-X). These offer good opportunities to study the underlying molecular basis of high nutrient functions in giant embryo.

## Energy Metabolism Pathway Involved in Rice Giant Embryo Development

In the process of rice embryo development, maturation stage is an important turning period. Synthesis of starch, as the development signal of this stage, took the lead. Its content increased rapidly from the early differentiation stage, slowed down in maturation stage, and gradually decreased in quiescence stage. The peak value is around the 21st day (Qin and Tang, 1982). Starch plays an important role during rice embryo development, which provides carbon frame and energy for the synthesis of protein, lipid, etc. It is a temporary material for energy storage in rice embryo, that is constantly accumulating and transforming (Qin and Tang, 1982).

According to the metabolomics analysis in this study, energy metabolism, carbohydrate metabolism, and galactose metabolism pathways were significantly enriched in the embryo of NIL-D (**Supplementary Table 6**). NADH and $NAD^+$ are a ubiquitous cellular redox couple, and NAD plays a central role in plant metabolism (Steinbeck et al., 2020). We found that NADH chemicals were significantly increased

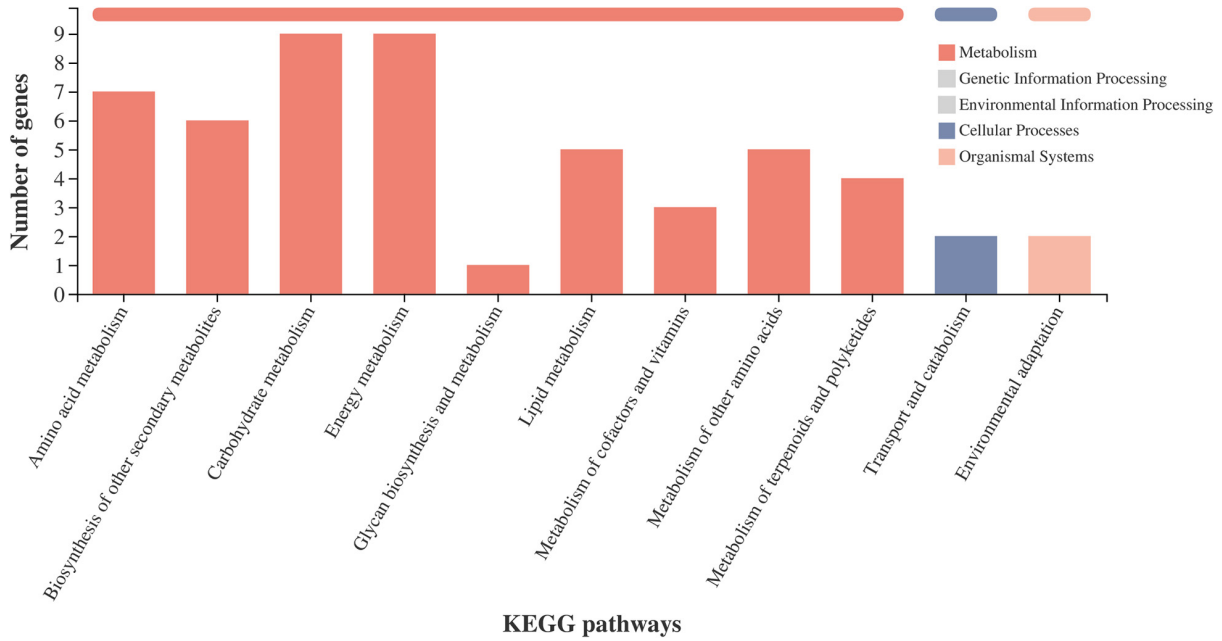


**FIGURE 6 |** KEGG pathway classification statistics. *X*-axis shows the name of the KEGG metabolic pathway; *Y*-axis shows the number of genes or transcripts annotated to the pathway. There are seven categories of KEGG metabolic pathways: metabolism, genetic information processing, environmental information processing, cellular processes, organismal systems, human diseases, and drug development.

in the oxidative phosphorylation pathway (**Figure 8** and **Supplementary Table 6**). Besides, carbon fixation in the photosynthetic organism pathway was also significantly enriched (**Figure 8** and **Supplementary Table 6**). Since NADH level is significantly positive correlated with utilization of carbon source (Wan et al., 2018). These imply that giant embryo has a more active anabolism of carbohydrate in earlier maturation stage. The embryo volume of giant embryo rice is 1–2 times greater than that of common rice, and the main components of embryos are protein, lipid, etc. (Azhakanandam et al., 2000; Lee et al., 2019). Therefore, giant embryo rice may need more carbon frame and energy for the synthesis of these substances, and then promoting the accumulation and transforming of energy-storage substances such as starch. Raffinose family of oligosaccharides (RFOs) are the most widespread D-galactose containing oligosaccharides in higher plants (Sengupta et al., 2015). We found that raffinose metabolites decreased significantly in the galactose metabolism pathway (**Supplementary Table 6**). In orthodox cereal seeds, RFOs are accumulated during latter grain development, and its accumulation is associated with loss of starch in the embryo (Black et al., 1996). It indicates that giant embryo had a lower transforming rate of starch to RFOs in earlier maturation stage. Besides, RFOs accumulation is considered to play an important role for the acquisition of desiccation tolerance during seed development (Sengupta et al., 2015). The ability of seeds to tolerate rapid drying, terminate metabolic activity and survive after rehydration is known as desiccation tolerance, and low desiccation tolerance means low seed vigor (Zhang et al., 2001). Our finding reveals that lower RFOs level maybe an important factor why the germination potential and emergence rate of giant embryo rice are significantly lower than that of corresponding rice.

## Amino Acid Metabolism Pathway Involved in Rice Giant Embryo Development

Relying on the early formation of protein synthesis mechanism and the increasing starch content to provide energy and carbon frame, the content of protein maintains the momentum of growth, even in maturation stage (Zhu et al., 1980). Amino acid is the basic unit of protein, and its metabolism is closely related to energy and carbohydrate metabolism, the carbon-nitrogen budget, and needed for protein synthesis and secondary metabolism (Yang et al., 2020).

According to the metabolomics analysis in this study, amino acid metabolic pathway was significantly enriched in the embryo of NIL-D (**Figure 6**). In plants, L-Aspartic acid is a precursor for the synthesis of several amino acids, including four essential ones: L-Methionine, L-Threonine, L-Isoleucine and L-Lysine. We found that L-Aspartic acid metabolites were increased significantly in the pathways of Cysteine and methionine metabolism, glycine, serine and threonine metabolism, and lysine biosynthesis etc. (**Figure 8** and **Supplementary Table 6**). Among the candidate genes, *OsZS_02G0528500* was significantly up-regulated (**Figure 8** and **Supplementary Table 6**). Correlation network analysis revealed that *OsZS_02G0528500* was positive correlated with L-Aspartic acid, and the value for Pearson correlation coefficient was 0.996 (**Figure 7** and **Supplementary Table 7**). Thus, it can be inferred that L-Aspartic acid metabolites

**TABLE 1 |** Candidate DEGs involved in KEGG pathways that are related to amino acid metabolism, energy metabolism, and lipid metabolism pathway.

| KEGG pathway | Pathway id | Metabolite | Gene id | FC | Annotation |
|---|---|---|---|---|---|
| Cysteine and methionine metabolism | map00270 | C00049 | *OsZS_09G0262200* | 0.049 | 1-aminocyclopropane-1-carboxylate oxidase 1, putative, expressed |
| | | | *OsZS_02G0118500* | 103.3 | Cysteine synthase, putative, expressed |
| | | | *OsZS_01G0494300* | 2.312 | Serine acetyltransferase protein, putative, expressed |
| | | | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| | | | *OsZS_09G0196800* | 18.51 | Expressed protein |
| Glycine, serine and threonine metabolism | map00260 | C00049 | *OsZS_09G0304800* | 0.263 | FAD dependent oxidoreductase domain containing protein, expressed |
| | | C00300 | | | |
| | | C00719 | | | |
| | | C00078 | *OsZS_09G0196800* | 18.51 | Expressed protein |
| Lysine degradation | map00310 | C00449 | *OsZS_09G0304800* | 0.263 | FAD dependent oxidoreductase domain containing protein, expressed |
| | | C00047 | | | |
| | | C04076 | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| Lysine biosynthesis | map00300 | C00047 | *OsZS_09G0196800* | 18.51 | Expressed protein |
| | | C00049 | | | |
| | | C00449 | | | |
| | | C04076 | | | |
| | | C00666 | | | |
| Phenylalanine, tyrosine and tryptophan biosynthesis | map00400 | C01179 | *OsZS_12G0349500* | 0.258 | Chorismate mutase, chloroplast precursor, putative, expressed |
| | | C00078 | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| Tryptophan metabolism | map00380 | C00632 | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| | | C00955 | | | |
| | | C02693 | | | |
| | | C00643 | | | |
| | | C00780 | | | |
| | | C00078 | | | |
| | | C02470 | | | |
| beta-Alanine metabolism | map00410 | C00049 | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| | | C00864 | *OsZS_04G0535900* | 0.402 | Amine oxidase, flavin-containing, domain containing protein, expressed |
| Valine, leucine and isoleucine degradation | map00280 | C00407 | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| Histidine metabolism | map00340 | C00049 | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| Phenylalanine metabolism | map00360 | C00180 | *OsZS_09G0291300* | 2.305 | caffeoyl-CoA O-methyltransferase, putative, expressed |
| | | C12621 | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| Arginine and proline metabolism | map00330 | C00300 | *OsZS_04G0007600* | 0.255 | Pyridoxal-dependent decarboxylase protein, putative, expressed |
| | | C00077 | *OsZS_04G0535900* | 0.402 | Amine oxidase, flavin-containing, domain containing protein, expressed |
| | | | *OsZS_03G0445500* | 3.445 | Expressed protein |
| | | | *OsZS_10G0381200* | 0.336 | Proline oxidase, mitochondrial precursor, putative, expressed |

*(Continued)*

**TABLE 1 |** Continued

| KEGG pathway | Pathway id | Metabolite | Gene id | FC | Annotation |
|---|---|---|---|---|---|
| | | | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| | | | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| Pyruvate metabolism | map00620 | C03451 | *OsZS_12G0142600* | 3.893 | Expressed protein |
| | | | *OsZS_02G0260900* | 0.028 | Retrotransposon protein, putative, Ty1-copia subclass |
| | | | *OsZS_01G0527400* | 0.288 | Phosphoenolpyruvate carboxylase, putative, expressed |
| | | | *OsZS_10G0129500* | 4.925 | Phosphoenolpyruvate carboxykinase, putative, expressed |
| | | | *OsZS_02G0528500* | 3.186 | Aldehyde dehydrogenase, putative, expressed |
| | | | *OsZS_03G0303200* | 2.104 | Pyruvate, phosphate dikinase, chloroplast precursor, putative, expressed |
| Arginine biosynthesis | map00220 | C00437 | *OsZS_04G0419400* | 0.483 | Glutamate dehydrogenase protein, putative, expressed |
| | | C00049 | | | |
| | | C03406 | | | |
| | | C00624 | | | |
| | | C00077 | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| Alanine, aspartate and glutamate metabolism | map00250 | C03406 | *OsZS_04G0419400* | 0.483 | Glutamate dehydrogenase protein, putative, expressed |
| | | C03794 | | | |
| | | C00049 | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| Tyrosine metabolism | map00350 | C01179 | *OsZS_07G0430000* | 0.319 | Dehydrogenase, putative, expressed |
| | | | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| | | | *OsZS_03G0598600* | 2.847 | Fumarylacetoacetase, putative, expressed |
| Cyanoamino acid metabolism | map00460 | C00407 | *OsZS_06G0198100* | 0.329 | Os6bglu24 – beta-glucosidase homolog, similar to G. max isohydroxyurate hydrolase, expressed |
| | | C00049 | | | |
| | | C15503 | | | |
| Pentose phosphate pathway | map00030 | C00620 | *OsZS_02G0412200* | 2.129 | Glucose-6-phosphate 1-dehydrogenase, cytoplasmic isoform, putative, expressed |
| Carbon fixation in photosynthetic organisms | map00710 | C00049 | *OsZS_12G0142600* | 3.893 | Expressed protein |
| | | | *OsZS_03G0303200* | 2.104 | Pyruvate, phosphate dikinase, chloroplast precursor, putative, expressed |
| | | | *OsZS_10G0129500* | 4.925 | Phosphoenolpyruvate carboxykinase, putative, expressed |
| | | | *OsZS_04G0149600* | 0.156 | Fructose-1,6-bisphosphatase, putative, expressed |
| | | | *OsZS_06G0303600* | 2.284 | Aminotransferase, classes I and II, domain containing protein, expressed |
| | | | *OsZS_01G0527400* | 0.288 | Phosphoenolpyruvate carboxylase, putative, expressed |
| Galactose metabolism | map00052 | C00492 | *OsZS_01G0149500* | 2.01 | UTP–glucose-1-phosphate uridylyltransferase, putative, expressed |

*(Continued)*

**TABLE 1 |** Continued

| KEGG pathway | Pathway id | Metabolite | Gene id | FC | Annotation |
|---|---|---|---|---|---|
| | | | *OsZS_02G0346800* | 0.104 | Glycosyl hydrolases, putative, expressed |
| | | | *OsZS_04G0308800* | 0.344 | Glycosyl hydrolases, putative, expressed |
| alpha-Linolenic acid metabolism | map00592 | C16343 | *OsZS_04G0329700* | 0.496 | Expressed protein |
| | | C16316 | | | |
| Linoleic acid metabolism | map00591 | C14827 | *OsZS_03G0520500* | 0.012 | Lipoxygenase, putative, expressed |
| Glycerophospholipid metabolism | map00564 | C00670 | *OsZS_01G0494000* | 3.093 | Phosphoethanolamine/phosphocholine phosphatase, putative, expressed |
| | | C04230 | | | |
| | | C04230 | *OsZS_05G0437300* | 0.146 | CPuORF26 – conserved peptide uORF-containing transcript, expressed |

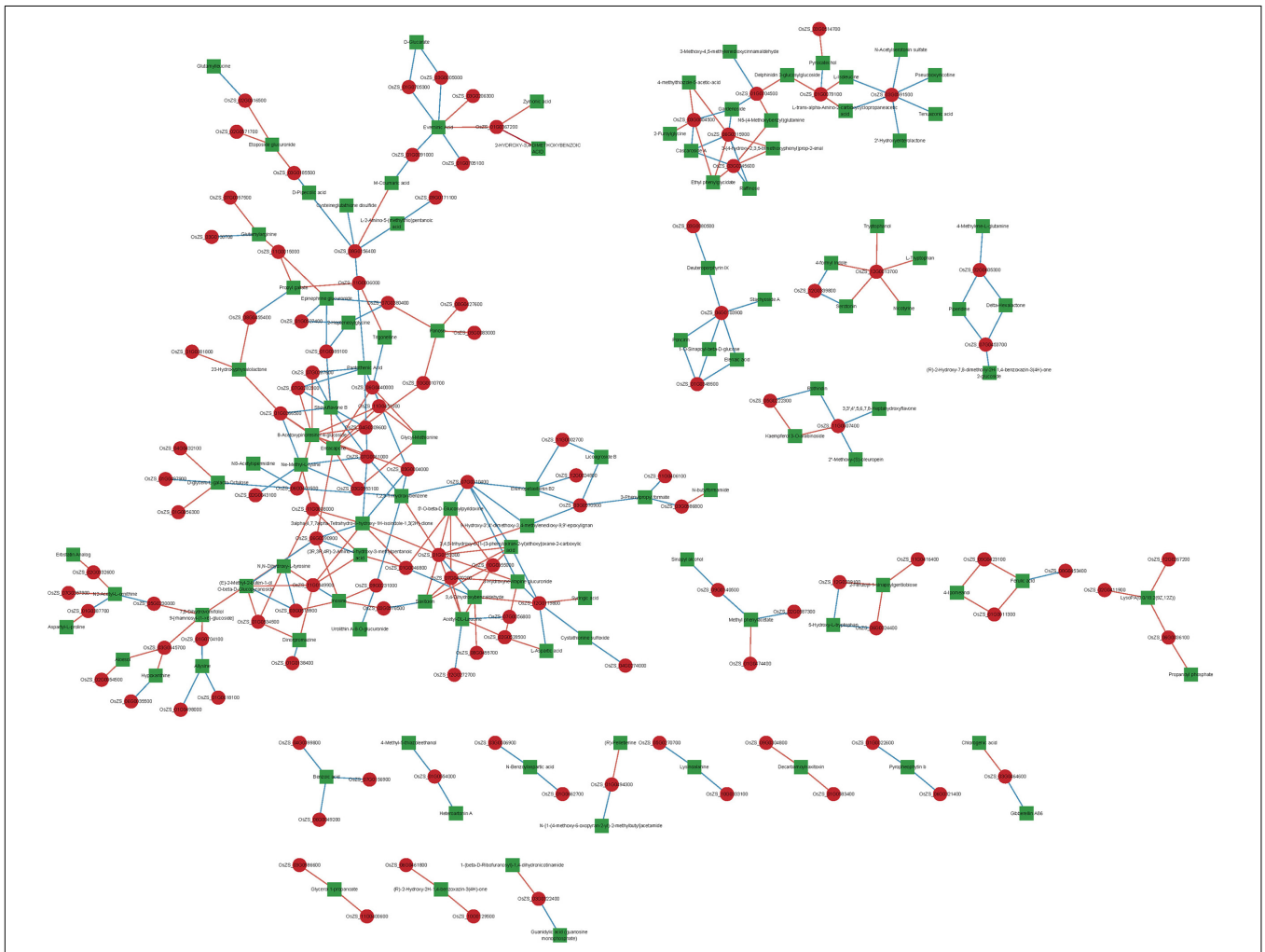

**FIGURE 7 |** Gene-metabolites associated network analysis. Each node in the figure represents a gene/metabolite, the red square dot represents the gene, the green square dot indicates the metabolites, the size of the node indicates the point connectivity; each connection indicates the interaction between the gene and the metabolites, the red line represents the positive correlation, the blue line indicates the negative correlation, the width of the connection indicates the size of the correlation coefficient, the larger the wider the correlation line.

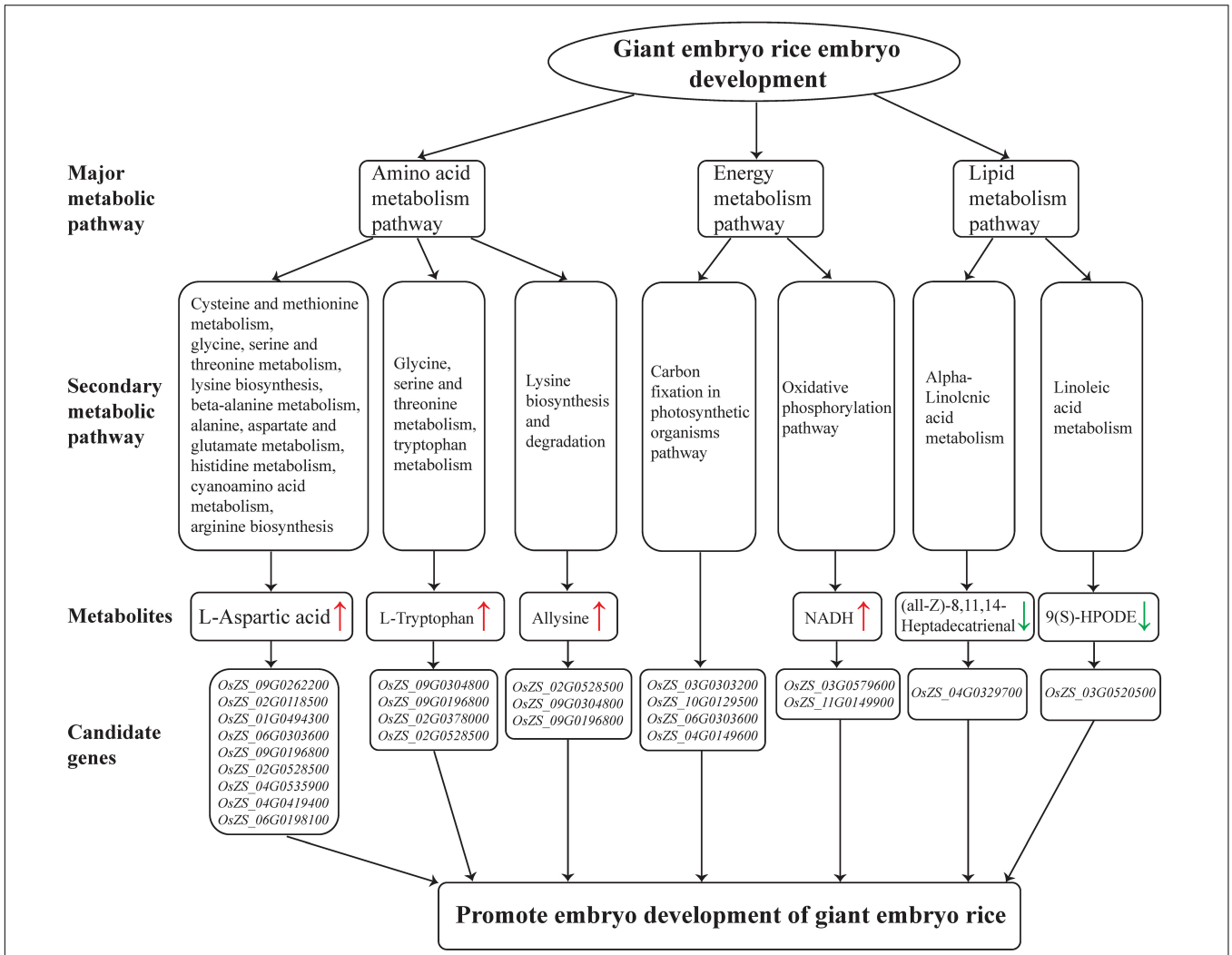


**FIGURE 8 |** Metabolic pathways involved in rice giant embryo development and candidate genes that regulate metabolites accumulation. Red and green arrows indicate increased metabolites and decreased metabolites, respectively.

may be important markers for the giant embryo development, and *OsZS_02G0528500* is a valuable potential candidate gene to regulate L-Aspartic acid content. L-Tryptophan is one of the essential amino acids for human body. It is similar to indole-3-acetic acid (IAA) in structure, and is an important precursor for the biosynthesis of IAA in plants (Zhao, 2018). The main pathway of IAA biosynthesis in higher plants is as follows: L-Tryptophan is firstly oxidized and deaminated to form indole acetone, and then decarboxylated to form indole acetaldehyde, which is finally oxidized to IAA under the catalysis of corresponding enzymes (Zhao, 2018). We found that L-Tryptophan metabolites were increased significantly in the pathways of glycine, serine, and threonine metabolism, and tryptophan metabolism (**Figure 8** and **Supplementary Table 6**). While IAA level was sharply decreased in the seeds of *GE* deficient mutant (Chen et al., 2015). The first step of IAA biosynthesis pathway is reversible. When indole acetone level is too high, the transaminase VAS1 would convert it back to L-Tryptophan (Gao et al., 2016). We speculate that the synthesis of IAA may be inhibited, which leading to the accumulation of upstream metabolites. It may also be due to the different positions and growth periods of materials. Further study is needed to investigate this. Among the candidate genes, *OsZS_12G0013700* was significantly up-regulated (**Supplementary Table 5**). Correlation network analysis revealed that *OsZS_12G0013700* was positive correlated with L-Tryptophan, and the value for Pearson correlation coefficient was 0.994 (**Figure 7** and **Supplementary Table 7**). Therefore, *OsZS_12G0013700* is a valuable potential candidate gene to regulate L-Tryptophan content. In addition, Allysine metabolites were increased significantly in the lysine biosynthesis and degradation pathway (**Figure 8** and **Supplementary Table 6**). Giant embryo rice is rich in protein and essential amino acids (Zhang L. L. et al., 2005). Our findings of higher L-Aspartic acid, L-Tryptophan, and Allysine level in NIL-D embryos in earlier maturation stage could explain the increased protein and essential amino acids level detected in mature giant embryo

rice, providing strong evidence for a causal relationship between amino acid metabolism and protein synthesis.

### Lipid Metabolism Pathway Involved in Rice Giant Embryo Development

Lipid is the third abundant nutrient component of brown rice, mainly distributed in embryo (Wu et al., 2020). Unlike starch, lipid is the ultimate form of energy storage in embryo, and is the main source of energy for rice seed germination. The change of lipid content in embryo is similar to that of starch, but the peak value is around the 15th day (Zhu et al., 1980).

According to the metabolomics analysis in this study, alpha-Linolenic acid metabolism, linoleic acid metabolism, and glycerophospholipid metabolism pathway were significantly enriched in the embryo of NIL-D (**Figure 8** and **Supplementary Table 6**). Specifically (all-Z)-8, 11, 14-Heptadecatrienal, and 13(S)-HOTrE metabolites were down-regulated in the alpha-Linolenic acid metabolism pathway; 9(S)-HPODE metabolite was down-regulated in the linoleic acid metabolism pathway; glycerophosphocholine, LysoPC (15:0), and LPC (16:0) metabolites were down-regulated in the glycerophospholipid metabolism pathway. As mentioned above, giant embryo rice is rich in lipids. Thus, we speculate that these decreased metabolites may be beneficial for the synthesis of lipid in giant embryo. Due to the low lipid content and difficult extraction, the mechanism of lipid regulation in rice embryo is still poorly understood, and more comprehensive and in-depth studies are needed.

## CONCLUSION

This study revealed that Dapeimi is a novel allelic mutant of *GE*, and the functional mutation site is a single cytosine deletion in the exon1. DAMs analysis of NILs indicated that a lack of *GE* promotes the energy metabolism, amino acid metabolism, and lipid metabolism pathways of rice embryos. In addition, the contents of NADH, L-Aspartic acid, and L-Tryptophan metabolites involved in the key metabolic pathways increased, whereas the raffinose content decreased. Further correlation network analysis of DAMs and DEGs screened out 300 pairs of gene-metabolites that highly correlated in the earlier maturation stage of rice embryo development. According to their functional annotations, we preliminary determined two potential candidate genes, *OsZS_12G0013700* and *OsZS_02G0528500*, for the content regulation of L-Aspartic acid and L-Tryptophan respectively. These findings can be used to provide genetic resources for the future cultivation of giant embryo rice with high nutritional value, and is significant to improve the nutritional value of endosperm and develop new rice varieties through synthetic biological methods. The functional identification of genes in metabolic pathways related to rice embryo development is in progress. Finally, we plan to construct some relevant vectors and express these genes in the rice endosperm in order to improve its nutritional value.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI GEO, accession no. GSE173301.

## AUTHOR CONTRIBUTIONS

ZH and QX performed most of the experiments. KW, LZ, and YY developed the rice mapping populations and NILs. LC and FN helped to map the gene. JZ and JH contributed to analysis of the data. ZH, QX, and SW designed the experiments, analyzed the data, and wrote the manuscript. All authors have discussed the results and contributed to the drafting of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.697889/full#supplementary-material

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# Targeting *Cis*-Regulatory Elements for Rice Grain Quality Improvement

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Rice is the most important source of food worldwide, providing energy, and nutrition for more than half of the population worldwide. Rice grain quality is a complex trait that is affected by several factors, such as the genotype and environment, and is a major target for rice breeders. *Cis*-regulatory elements (CREs) are the regions of non-coding DNA, which play a critical role in gene expression regulation. Compared with gene knockout, CRE modifications can fine-tune the expression levels of target genes. Genome editing has provided opportunities to modify the genomes of organisms in a precise and predictable way. Recently, the promoter modifications of coding genes using genome editing technologies in plant improvement have become popular. In this study, we reviewed the results of recent studies on the identification, characterization, and application of CREs involved in rice grain quality. We proposed CREs as preferred potential targets to create allelic diversity and to improve quality traits *via* genome editing strategies in rice. We also discussed potential challenges and experimental considerations for the improvement in grain quality in crop plants.

**Keywords: rice, *cis*-regulatory element, genome editing, grain quality, upstream open reading frame**

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## INTRODUCTION

Rice provides major nutrients and energy for more than half of the population worldwide. In the last 30 years, rice yield has been continuously improved through implementing a series of breeding programs (Xu et al., 2020). Meanwhile, the demand for high quality, multiresistance, and wide adaptability of rice variants is also increasing, especially for the high-quality rice under conditions of improved living standards (Rao et al., 2014). Generally, rice grain quality is a combination of milling, appearance, eating, cooking, nutritional, and hygiene traits (Zhou et al., 2020). Therefore, to meet the needs of consumers and producers, researchers have to understand the molecular mechanisms and genetic basis that determines rice quality, and breeders and seed companies have to develop rice varieties with excellent quality and high yield. However, the direct regulation of coding genes for target traits is difficult and is often accompanied by negative effects; therefore, it cannot achieve the expected effect. For instance, Pérez et al. (2019) used CRISPR/Cas9 to introduce mutations affecting the *Wx* gene [encoding granule-bound starch synthase I (GBSSI)] in the rice endosperm. The amylose content (AC) declined to as low as 5% in homozygous seeds, accompanied by abnormal cellular organization in the aleurone layer and amorphous starch grain structures. Plant genetic engineering efforts to improve grain quality in crop plants using well-characterized

promoter elements to modify the expression of regulatory genes and/or transcription factors (TFs) have proven to be advantageous (Yang et al., 2019). Therefore, in this perspective, we mainly discussed the identification and analysis of *cis*-regulatory elements (CREs) involved in rice grain quality and the modification of CREs *via* genome editing technologies for the improvement in rice grain quality.

## GENETICS AND GENOMICS OF RICE GRAIN QUALITY

In general, four main quality traits, namely, milling properties, appearance, nutritional value, and cooking quality, are widely used to assess rice grain quality (Zhou et al., 2020). With the rapid development of high-throughput technologies and functional genomics, many genes controlling important quality traits have been cloned in rice, and some molecular mechanisms have been characterized (Zhou et al., 2020). Milling quality is a complex grain trait, including the recovery of brown, milled, and head rice. Several major quantitative trait loci (QTLs) are associated with rice milling quality, such as *qBRR-10*, which influence brown rice recovery (Ren et al., 2016). Rice grain shape is an important index of appearance quality and yield, and several related genes and TFs have been studied (Huang et al., 2013). For example, *GS3*, *GW5*, *GLW7*, *GW8*, *GS2*, and *GS9* are the main genes or transcription regulators affecting variations in rice grain shape and size. In addition, genes such as *GL7/GW7*, *GL3.1*, *GS5*, *GW2*, and *GL3.3*, which were identified in natural variants, have profound effects on grain shape and functions, and their mechanisms have been studied (Huang et al., 2013). The composition and structure of starch play crucial roles in rice grain quality, especially for eating and cooking quality. Enzymes involved in the starch synthesis pathway (including GBSS, SS, SBE, ISA, and PUL) and associated TFs (i.e., *Dull*, *OsEBP89*, *OsRSR1*, and *OsbZIP58*) have been clearly determined in rice (Zhou et al., 2020). Many major functional genes have been identified, which affect nutrients such as seed proteins, essential amino acids, vitamins, minerals, and anthocyanins (Das et al., 2020). Similarly, grain quality-related genes and their metabolic pathways have been studied in other crops, and the results have been discussed in a recent review (Birla et al., 2017). The characterization of these quality-related genes has laid the foundation for the improvement in rice quality. Although many genes related to crop grain quality have been explored, and their genetic mechanisms have been analyzed, how these genes can be better used in breeding production and commercial utilization requires further study.

**Abbreviations:** ARFAT, auxin response factor; *AtHB1*, *Arabidopsis thaliana* HomeoBox 1; FZP, Frizzle panicle; GBSS, Granule-bound starch synthase; *GL3.*1, Grain length 3.1; *GL3.3*, Grain length 3.1; *GLW7*, Grain length and weight on chromosome 7; *GS2*, Grain size on chromosome 2; *GS3*, Grain size on chromosome 3; *GS5*, Grain size on chromosome 5; *GS9*, Grain shape on chromosome 9; *GSE5*, Grain size on chromosome 5; *GW2*, Grain width 2; *GW5*, QTL for grain width and weight on chromosome 5; *GW7*, Grain width 7; *GW8*, Grain width 8; ISA, Isoamylase; *LsGGP2*, GDP-L-galactose phosphorylase 2; *OsAAP6*, Amino acid permease 6; *OsbZIP18*, basic leucine zipper transcription factor 18; *OsbZIP58*, basic leucine zipper transcription factor 58; *OsEBP89*, *Oryza sativa* ethylene-responsive element binding protein, clone 89; *OsGluA2*, glutelin type-A2 precursor; *OsMADS1*, MADS-domain transcription factor; *OsPLDα1*, Phospholipase D alpha 1; *OsREM20*, *Oryza sativa* REPRODUCTIVE MERISTEM 20; *OsRSR1*, Rice starch regulator 1; *OsSPL16*, *squamosa* promoter binding protein-like 16; *PHO1*, PHOSPHATE1; PUL, pullulanase; *qBRR-10*, Brown rice rate on chromosome 10; *qSH1*, QTL of seed shattering in chromosome 1; *SAUR26*, SMALL AUXIN UP RNA gene 26; SBE, starch-branching enzyme; SEBF, silencing element binding factor; SS, soluble starch synthase; *TGW2*, 1000-grain weight 2.

## *CIS*-REGULATORY ELEMENTS AND THEIR MINING IN PLANTS

Fundamentally, in addition to genes, many elements present in the plant genome control the gene expression levels *via* interactions with DNA or regulatory proteins. Limited genetic diversity restricts the amount and effectiveness of the improvement in rice quality. Recent results showed that genetic changes in CREs of genes play important roles in shaping phenotypic diversity by altering gene expression (Swinnen et al., 2016).

### *CIS*-Regulatory Elements: Important Regulators of Gene Expression

The CREs are the noncoding DNA containing binding sites for TFs or other regulatory molecules that affect transcription, and they ultimately guide plant growth and development, cell differentiation, and responses to various stresses (Lu et al., 2018). Therefore, the whole genome identification and functional characterization of CREs involved in the DNA–protein interactions is a key aspect to understand plant transcription regulation (Lu et al., 2018). Generally, CREs in eukaryotes include promoters, enhancers, and other CREs, among which enhancers tend to be much more variable (Meng et al., 2021). Enhancers that drive transcription are independent of their distance and location from their cognate promoters, which allows a gene to be regulated by multiple remote enhancers with different spatiotemporal activities (Meng et al., 2021). The sequence structure of promoters of quality-related genes is relatively simple and conserved, and their functions have been clarified in rice. The identification and functional analysis of CREs are also in progress in rice (Swinnen et al., 2016). More comprehensive collections and analyses of CREs in rice are necessary, which will accelerate the fine-tuning of the improvement in rice quality *via* CRE editing.

### Mining CREs Based on "Omics"

The systematic identification of CREs in plant genomes is critically important to understand the transcriptional regulation and its exploitation for the improvement in quality. Some important results have been obtained using approaches involving sequence conservation within short distances from target genes (Huang et al., 2020a). Additionally, some databases, such as PlantCARE, which store all plant transcription sites, consensus sequences, and matrices described in the literature, can also be used to predict possible CREs for one or more genes (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Several limitations are as follows: sequence conservation can be restricted, CREs that are far from their target genes will not be

detected, and we have less information regarding their tissue specificity and functionality (Lu et al., 2018).

Generally, CREs are preferentially located in the accessible chromatin regions (España et al., 2017). Therefore, genomics and epigenomics are used to identify and analyze CREs. For example, the alignment of the upstream sequences of *OsPLDα1* orthologs across 34 rice accessions revealed sequence variations and identified CREs involved in differential transcription of orthologs, which resulted in the low expression of *OsPLDα1* and reduced free fatty acid content in the oil, facilitating good quality bran oil (Kaur et al., 2020). A genome-wide association study (GWAS) screen of 45 natural accessions and the *pif4* mutant in *Arabidopsis* identified CREs affecting *SAUR26* gene expression (Wang et al., 2021). Similar CRE mining strategies have been reported in rice (Ho and Geisler, 2019). The whole genome transcriptome profiling using microarrays was employed to discover CREs associated with drought and salinity stress tolerance in rice (Mishra et al., 2018).

In addition, CREs can be identified based on their elevated sensitivity to enzymes such as the bacterial transposase Tn5, DNase I, and micrococcal nuclease (MNase) (Zhang et al., 2016; Lu et al., 2017; Zhao et al., 2020). The coupling of chromatin accessibility assays with the high-throughput DNA sequencing, such as DNase-seq, MNase-seq, self-transcribing active regulatory region sequencing (STARR-seq), and assay for transposase-accessible chromatin using sequencing (ATAC-seq), represents an important technological development and has enabled the identification of CREs on a genome-wide scale (Lu et al., 2018). Numerous putative CREs were identified in plant species through the strategies of genetic, epigenomic, and functional molecular characterization (Lu et al., 2019). A major limitation to these assays is the lack of cell-type resolution and the paucity of information regarding which CREs function in specific tissues or cell types at the genome-wide scale, especially in endosperm tissue, which is closely related to rice quality.

Moreover, ribosome profiling (ribo-seq) provides a viable strategy to analyze active translation by determining ribosome occupancy in a transcriptome-wide manner, which can then be used to identify valid upstream open reading frames (uORFs), a type of CRE (Lulla et al., 2019). However, the large-scale identification of uORFs has not been reported in rice. Thus, ribo-seq is expected to be an effective tool to identify rice quality-related uORFs. Furthermore, combined with the multiomics data, such as RNA sequencing (RNA-seq), chromatin immunoprecipitation sequencing (ChIP-seq), and proteomics, CREs and their related TFs could be mined more accurately (Farmer et al., 2021).

## THE KEY ROLES OF *CIS*-REGULATORY ELEMENTS IN THE IMPROVEMENT IN RICE QUALITY

Unlike changes to protein-coding genes, which often result in easily interpretable loss-of-function alleles, the mutations of CREs offer the potential of fine-tuning gene expression without other adverse effects, leading to improved rice quality (Huang et al., 2020a; Zeng et al., 2020). Therefore, more attention should be paid to CREs and their influence on gene expression.

Employing both bioinformatic and experimental methods, CREs involved in gene regulation have been identified, and most of the genes affected by these CREs have been found to encode TFs that regulate plant development (España et al., 2017). Several universal CREs have been identified in the 5′ upstream region of the starch synthetase gene, lysine metabolism genes, and seed-storage protein genes in rice and/or other crops (**Table 1**) (Chen et al., 2012). Recent studies have shown that CREs play an important role in improving rice quality traits (**Table 1**).

The ARFAT and SEBF elements have been identified as CREs that might act as repressors in regulating *OsPLDα1* expression, which lead to decreased free fatty acid content in oil and improve the flavor and quality of rice bran oil (**Table 1**) (Kaur et al., 2020). Two common variations in the potential CREs of the *OsAAP6* 5′-untranslated region (5′-UTR) seem to be associated with grain protein content diversity and nutritional quality, mainly in *indica* cultivars (Peng et al., 2014). A single nucleotide polymorphism (SNP) located in the *OsGluA2* promoter region is associated with its transcript expression level and grain protein content diversity (Yang et al., 2019). For amino acids, natural variations in the *OsbZIP18* promoter contribute to branched-chain amino acid levels in rice (Sun et al., 2020). Two consensus nucleotide polymorphisms in the *Chalk5* promoter in rice varieties might partly account for the differences in *Chalk5* mRNA levels that contribute to natural variation in grain chalkiness (Li et al., 2014). *GRAIN WIDTH 7* (*GW7*) is an important gene that controls cell division in the spikelet hulls, and its expression is regulated by the repressive TF, i.e., *GRAIN WIDTH8* (*GW8*). A mutation in the CRE in the promoter of *GW7* led to enhanced *GW7* expression and ultimately to the improved yield and grain quality (Sakamoto and Matsuoka, 2008; Wang et al., 2015). Similarly, natural variation in the promoter of *GSE5* contributes to grain size diversity, and that in *TGW2* determines rice grain width and weight (Duan et al., 2017; Ruan et al., 2020). A novel variation in the *FRIZZLE PANICLE* (*FZP*) gene promoter improved rice grain number and yield (Wang et al., 2020).

## UPSTREAM OPEN READING FRAMES: IMPORTANT *CIS*-REGULATORY ELEMENTS IN THE 5′ LEADING SEQUENCE

Upstream open reading frames, as translational regulatory elements, are located in the 5′-UTR of eukaryotic mRNAs, and generally inhibit the translation initiation of downstream primary ORFs (pORFs) through ribosome stalling (Kurihara, 2020). In plants, uORFs have been predicted in ∼30% of the 5′-UTRs of genes, and some of these uORFs have been reported to regulate crucial growth and developmental processes (von Arnim et al., 2014). Increasing numbers of excellent crop genes have been identified and characterized (Zhou et al., 2020). Furthermore, to improve crop characteristics, many genes that regulate important traits are required to have a high translation rate, rather than their functional loss or reduction (Xu et al., 2017; Reis et al., 2020). Based on the knowledge that uORFs negatively

**TABLE 1 |** Improvement in rice quality achieved *via* the modification of *cis*-regulatory elements.

| Target trait | Gene | Target function | *Cis*-elements-dependent regulation | CRE | CRE core sequence | CRE location | Modification | Target gene expression pattern | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Gene expression | Tissue or organ | |
| Cooking and eating quality | *Wx* | Granule-bound starch synthase I | Apparent amylose content | unknown element, A-box, CAAT-box, Endosperm-box | TATAATAAT, GGCCAATCT | Upstream, Intron | Genome editing | Downregulation | Developing endosperm | Huang et al., 2020a; Zeng et al., 2020 |
| Nutritional quality | *OsbZIP18* | Basic leucine zipper transcription factor | Branched-chain amino acids levels | / | / | Upstream | Natural variation | Upregulation | Leaves | Sun et al., 2020 |
| | *OsBCAT1* | branched-chain aminotransferase1 | Branched-chain amino acids levels | ACE element | / | Upstream | Genome editing | Upregulation | Leaves | Sun et al., 2020 |
| | *OsBCAT2* | branched-chain aminotransferase2 | Branched-chain amino acids levels | C-box *cis*-element | GTCA | Upstream | Genome editing | Upregulation | Leaves | Sun et al., 2020 |
| | *OsAAP6* | Amino acid transporter | Grain protein content | copper-responsive element, Inr-element, sulfur-responsive element | / | Upstream | Natural variation | Diversity[#] | Endosperms | Peng et al., 2014 |
| | *OsGluA2* | Glutelin type-A2 precursor | Grain protein content | BIHD1OS | / | Upstream | Natural variation | Diversity | Endosperms | Yang et al., 2019 |
| | *REP-1* | Cysteine proteinase | Glutelin degradation | GA-responsive element | TAACAGA, TAACGTA, CAACTC | Upstream | Deletion and point-mutation | Upregulation | Seeds | Sutoh and Yamauchi, 2003 |
| | *Kala4* | bHLH transcription factor | Anthocyanin production | / | / | Upstream | Natural variation | Upregulation | Leaves | Oikawa et al., 2015 |
| | *OsPLDα1* | Lipolytic enzyme | Free fatty acid content and flavor | ARFAT element, SEBF element | TGTCTC, TTGTCTC | Upstream | Natural variation | Downregulation | Immature grains | Kaur et al., 2020 |
| | *Chalk5* | Vacuolar $H^+$-translocating pyrophosphatase | Grain chalkiness | RY/G-box, CACT tetranucleotide | CATGCA, CACT | Upstream | Natural variation | Downregulation | Endosperms | Li et al., 2014 |
| Appearance quality and yield | *GS5* | Serine carboxypeptidase | Grain size | ABA-responsive element | / | Upstream | Natural variation | Upregulation | Developing seeds | Li et al., 2011; Xu et al., 2015 |
| | *qSH1/RPL* | BEL1-type HomeoBox gene | Seed shattering | RY-repeat | / | Upstream | Natural variation | Upregulation | Abscission layer | Konishi et al., 2006 |

*(Continued)*

**TABLE 1** | Continued

| Target trait | Gene | Target function | *Cis*-elements-dependent regulation | CRE | CRE core sequence | CRE location | Modification | Target gene expression pattern | | References |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Gene expression | Tissue or organ | |
| | *Ghd7* | CCT domain protein | Grain number | / | / | Upstream | Natural variation | Diversity | Young stem and leaves | Lu et al., 2012, Xue et al., 2008 |
| | *GW8/SPL16* | SBP-domain transcription factor | Grain shape, quality, and size | / | / | Upstream | Natural variation | Downregulation | Developing panicles | Wang et al., 2012 |
| | *GW7* | TONNEAU1-recruiting motif protein | Grain shape and quality | GTAC motif | GTAC | Upstream | Natural variation | Upregulation | Young panicles | Wang et al., 2015 |
| | *FZP* | APETALA2/ETHYLENE response factor | Grain width and weight | CACTA transposon | CACTA | Upstream | Natural variation | Downregulation | Young panicles | Wang et al., 2020 |
| | *GW6* | Gibberellin-regulated GAST family protein | Grain size | CAAT-box | CACACAAATCT | Upstream | Natural variation | Upregulation | Young panicles | Shi et al., 2020 |
| | *OsACBP2* | Acyl-CoA-binding protein | Seed development and size | Skn-I-like motif | GTCAT | Upstream | Deletion mutation | Downregulation | Seeds | Guo et al., 2019 |
| | *Eui1* | GA-deactivating enzyme | Plant height, grain yield | RY motif-containing *cis*-silencing element | CATGCA | Intron | T-DNA insertion mutagenesis | Upregulation | Young panicles | Xie et al., 2018 |
| | *OsREM20* | B3 domain transcription factor | Grain number per panicle | CArG box-containing inverted repeat | CATTAATTAG | Upstream | Natural variation/Genome editing | Upregulation | 1-week-old seedlings | Wu et al., 2021 |
| | *GSE5* | Plasma membrane-associated protein | Grain size | / | / | Upstream | Natural variation | Downregulation | Spikelet hulls | Duan et al., 2017 |
| | *GLW7/SPL13* | plant-specific transcription factor OsSPL13 | Grain shape and yield | / | / | 5′-UTR | T-DNA insertion mutagenesis | Upregulation | Panicles and florets | Si et al., 2016 |
| | *TGW2* | Cell number regulator | Grain width and weight | / | / | Upstream | Natural variation | Upregulation | Glumes | Ruan et al., 2020 |
| | *OsMADS1* | CW domain-containing zinc finger protein | Grain width | CATTTC motif | CATTTC | Upstream | Genome editing | Downregulation | Young panicles | Huang et al., 2020b |

[#] *Represents diversity in the regulation region of the target gene.*

affect the translation of the pORF, the strategy of modulating uORFs to fine-tune the translation could be used to analyze gene function and improve crop traits. For example, uORF TBF1-mediated translation enabled engineered *Arabidopsis* and rice broad-spectrum disease resistance without any reduction in grain yield (Xu et al., 2017).

## GENOME EDITING TOWARD BETTER GRAIN QUALITY *VIA* TARGETING *CIS*-REGULATORY ELEMENTS

All the earlier studies suggested that CRE modification can regulate the expression of key genes for rice quality and effectively improve grain quality. However, the identification of natural mutations in the promoter regulatory regions of the gene is time-consuming and difficult, which slows the improvement in rice quality. Recently, genome editing technology has been developed and optimized and has been applied successfully to a large number of plants, which has accelerated the identification and application of regulatory elements in gene promoter regions in rice (Huang et al., 2020a; Zeng et al., 2020). Genome editing is a versatile, relevant, and preferred technique for functional genomics, as well as crop improvement, involving introducing DNA mutations in the form of deletions and/or insertions or base substitutions in target gene sequences (Fiaz et al., 2019; Tabassum et al., 2021).

The starch synthase gene *Wx* is very important for rice eating and cooking quality; therefore, it has been a popular target for study. Zeng et al. (2020) disrupted the Endosperm-box, A-box, and CAAT-box of the promoter sequence and intron region of the *Wx* gene using genome editing, which generated new *Wx* alleles producing various ACs by quantitative regulation of its expression. Novel *Wx* alleles, in which CREs in the *Wx* promoter near a predicted TATA-box were edited using the CRISPR/Cas9 system, produced fine-tuned amylose levels and improved the rice grain quality (Huang et al., 2020a). The deletion mutants of the CATTTC motif exhibited the lower expression of *OsMADS1* and produced narrower rice grains (Huang et al., 2020b). Wu et al. (2021) enhanced rice grain production by manipulating the CArG box-containing inverted repeat sequence of *OsREM20*. Although there are only a limited number of studies demonstrating successful editing of CREs for crop improvement, it is anticipated that genome editing techniques such as CRISPR/Cas9 will lead to further CRE editing to improve rice grain quality.

For uORFs, editing the uORF of *LsGGP2* increased oxidative stress tolerance and the ascorbate content by ∼150% in lettuce (Zhang et al., 2018). In *Arabidopsis*, a conserved peptide uORF (CPuORF33) was identified in the 5′-UTR of *AtHB1* mRNA, which ensures a relatively low level of *AtHB1* expression in aerial parts and avoids adverse phenotypes (Ribone et al., 2017). Similarly, there are several studies on the regulation of uORFs in morphogenesis, signaling pathways, and nutrient absorption stress response in *Arabidopsis* (Zhang et al., 2020). Reis et al. (2020) identified the *PHO1* uORF in the genomes of crops such as rice, maize, barley, and wheat, which improved plant growth under inorganic phosphorus (Pi)-deficient conditions. A tandem repeat sequence in the 5′-UTR of *GLW7* alters its expression by affecting transcription and translation, resulting in enhanced rice grain length and yield (Si et al., 2016). Hence, uORFs have great potential to improve rice grain quality by positively regulating target genes.

## CONCLUSION AND FUTURE PERSPECTIVES

The aim of the plant functional genomics is to explore the key genes in plant growth and development, to determine their regulatory mechanisms, and to fine-tune the gene expression effectively, with the aim of improving traits for research and/or commercial use. Crop quality is an important trait whose further improvement requires increased research resources. Recently, several crop quality-related genes and metabolic pathways have been identified and explored (Zhou et al., 2020). In addition, the high-throughput technologies have accelerated the identification and analysis of these functional quality-related genes (Hernandez-Garcia and Finer, 2014). However, it is difficult to achieve the desired improvement in quality through the direct manipulation of these elite genes. The identification and modification of the CREs of these genes would provide an appropriate approach to modulate their expression (Huang et al., 2020c). The popularity of genome editing further confirms that CREs are good potential targets to create new alleles for the improvement in rice quality in transgene-free derivatives (**Figure 1**).

Recently developed omics approaches, such as DNase-seq, ATAC-seq, and ribo-seq, have identified certain CREs and uORFs. Thus, a more comprehensive analysis of CREs in promoter regions and uORFs in 5′ leading sequences will increase opportunities for quality-related genome editing. Additionally, these techniques are complex and have limitations, especially in the study of CREs and uORFs related to endosperm traits in rice. Hence, the development of an effective and simple method or system for the CRE identification in rice seeds is essential to improve rice quality. Moreover, CREs are very short (usually only a few nucleotides), and constructing a uORF mutant requires the start codon to be modified; therefore, more precise genome editing techniques should be considered to avoid limited protospacer adjacent motif (PAM) sites, off-target mutations, and low homology-directed repair (HDR) efficiency.

In addition to the technological issues, there are significant gaps in our knowledge of gene regulation in most species. At present, studies on CREs and uORFs have mainly focused on their identification and functional analysis (Hernandez-Garcia and Finer, 2014). Given the importance of CREs for gene expression in crops, in-depth studies, such as CRE regulatory mechanisms and related metabolic connections, require further research. Thus, the analyses of the CREs related to crop quality and their transcriptional and translational regulatory modifications are essential. It is hoped that the combination of CREs and genome editing technologies will enable the simultaneous manipulation of multiple traits in rice (**Figure 1**).

Moreover, it is not easy to forensically detect genome editing events at the molecular level, especially as no foreign DNA exists

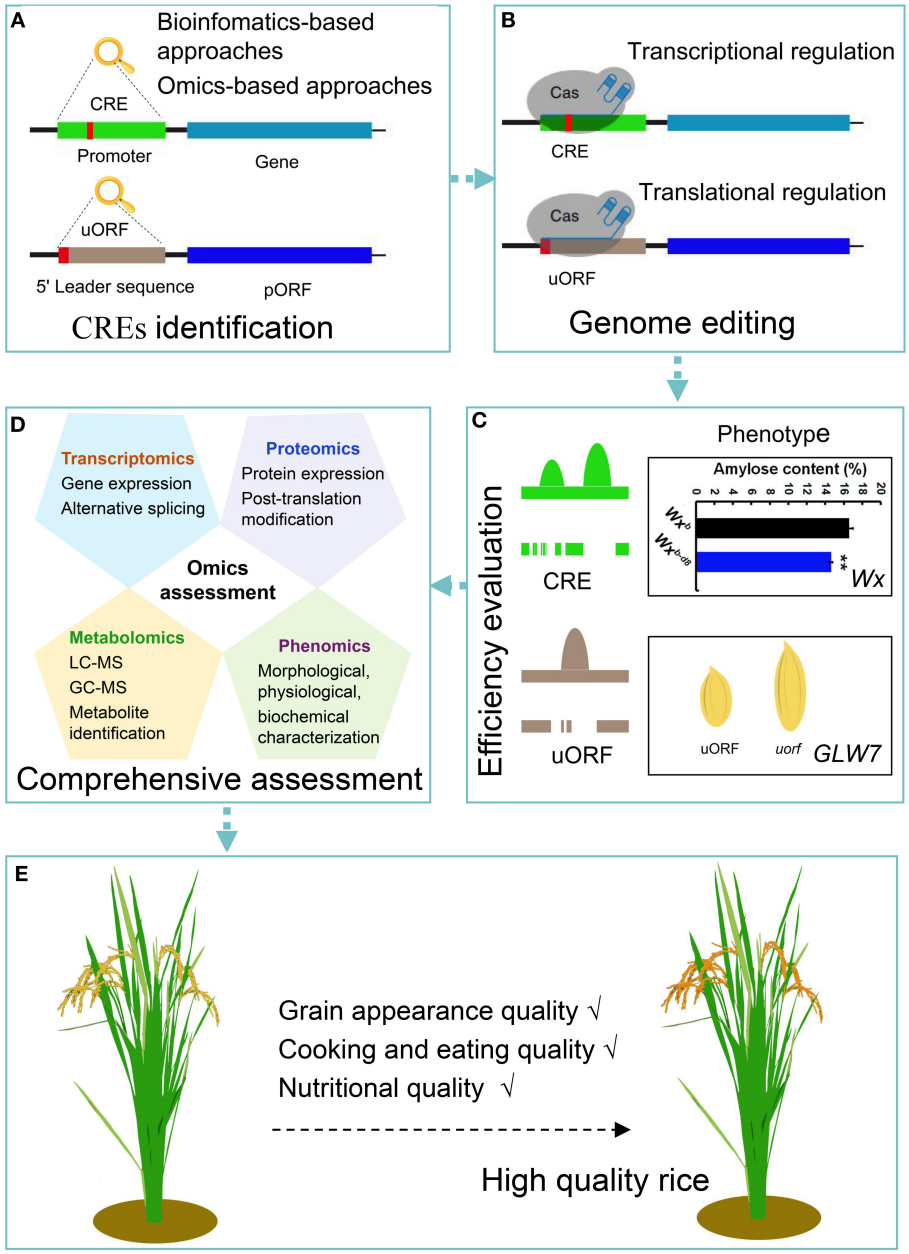


**FIGURE 1 |** Schematic representation of the workflow depicting the application of genome editing approaches to obtain high grain quality rice. **(A)** CREs are identified by multiomics strategies and bioinformatic approaches. *Cis*-regulatory sequences are linear nucleotide fragments of non-coding DNA. Their localization and orientation in relation to genes and activity vary (Verter and Botha, 2010). uORFs, as translational regulatory elements, are located in the 5′ leader sequence of eukaryotic mRNAs and generally inhibit the translation initiation of downstream pORFs (Kurihara, 2020). **(B)** Selection of the desired target DNA sequences of CREs and uORFs, and recognition of PAM sequences for genome editing, which regulates gene expression at the transcription and translation levels, respectively. **(C)** Efficiency evaluation using molecular identification and phenotypic analysis. For example, a genome editing strategy was used to edit the core promoter region of the *Wx* gene, and the obtained $Wx^{b-d8}$ mutant had a nine nucleotide deletion (Huang et al., 2020a). In the $Wx^{b-d8}$ mutant, the predicted core promoter region was disrupted, which decreased *Wx* expression compared with the wild-type $Wx^{b}$ and reduced the amylose content (%) in the mature rice seeds (Huang et al., 2020a). The same method is applicable to edit the predicted uORF of *GLW7* (Si et al., 2016). The deletion of predicted uORF in the *GLW7* 5′ leader sequence causing increased expression levels of the GLW7 protein and enhanced rice grain length. Colored peaks represent different TF-binding events within CREs or uORFs, and peak height indicates the chromatin accessibility from genomic data sets (i.e., DHase-seq and ATAC-seq). Lines with spaces beneath the TF-binding peak indicate destroyed CRE or uORF sequences. **(D)** Comprehensive assessment of edited rice using multiomics strategies. **(E)** Obtaining the desired high-quality rice. CRE, *Cis*-regulatory element; uORF, upstream open reading frame; pORF, primary open reading frame; PAM, protospacer adjacent motif; *Wx*, the *Waxy* gene encoding granule-bound starch synthase I, which controls amylose synthesis in rice endosperm; $Wx^{b}$, Nipponbare carrying the $Wx^{b}$ allele; $Wx^{b-d8}$, the *Wxb-d8* mutant with nine nucleotide deletion of the core promoter region in Nipponbare ($Wx^{b}$). GLW7, which is encoding the plant-specific TF OsSPL13, positively regulates cell size in the grain hull, resulting in enhanced rice grain length and yield (Si et al., 2016); TF, transcription factor.

in the line in which the regulatory element(s) are subtly edited. Given this limitation, the downstream "omics" technologies that can reveal the effects of the edits, such as proteomics and metabolomics, should be considered to fully assess the changes of proteins and/or their compositions in novel foodstuffs from the edited crops. The integration of the in-depth understanding of gene regulatory mechanisms and related networks, and genome editing to identify and modify CREs at the single nucleotide level in plant genomes, might represent a promising strategy for future crop improvement.

## AUTHOR CONTRIBUTIONS

QY, YD, and TZ organized and wrote the manuscript. JZ collected and summarized some points. QL and DZ provided critical evaluation and edited the text. All the authors have read and approved the manuscript.

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# Combining Genome and Gene Co-expression Network Analyses for the Identification of Genes Potentially Regulating Salt Tolerance in Rice

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Salinity stress tolerance is a complex polygenic trait involving multi-molecular pathways. This study aims to demonstrate an effective transcriptomic approach for identifying genes regulating salt tolerance in rice. The chromosome segment substitution lines (CSSLs) of "Khao Dawk Mali 105 (KDML105)" rice containing various regions of DH212 between markers RM1003 and RM3362 displayed differential salt tolerance at the booting stage. CSSL16 and its nearly isogenic parent, KDML105, were used for transcriptome analysis. Differentially expressed genes in the leaves of seedlings, flag leaves, and second leaves of CSSL16 and KDML105 under normal and salt stress conditions were subjected to analyses based on gene co-expression network (GCN), on two-state co-expression with clustering coefficient (CC), and on weighted gene co-expression network (WGCN). GCN identified 57 genes, while 30 and 59 genes were identified using CC and WGCN, respectively. With the three methods, some of the identified genes overlapped, bringing the maximum number of predicted salt tolerance genes to 92. Among the 92 genes, nine genes, *OsNodulin*, *OsBTBZ1*, *OsPSB28*, *OsERD*, *OsSub34*, *peroxidase precursor* genes, and three expressed protein genes, displayed SNPs between CSSL16 and KDML105. The nine genes were differentially expressed in CSSL16 and KDML105 under normal and salt stress conditions. *OsBTBZ1* and *OsERD* were identified by the three methods. These results suggest that the transcriptomic approach described here effectively identified the genes regulating salt tolerance in rice and support the identification of appropriate QTL for salt tolerance improvement.

**Keywords: transcriptome analysis, gene co-expression network, salt-tolerant genes, rice, clustering co-efficient**

## INTRODUCTION

Salinity is a major environmental stressor that affects rice production worldwide. Salt stress decreases crop yield and limits agricultural productivity (Munns, 2002), particularly in non-irrigated farmlands by triggering two primary effects on plants, osmotic stress, and ion toxicity (Boyer, 1982). In most rice cultivars, the seedling and early booting stages are the most sensitive to salt stress (Lafitte et al., 2007). High concentrations of sodium ions are toxic to most plants (Dionisio-Sese and Tobita, 2000). A combination of ion toxicity and osmotic stress inhibits growth and affects plant development or cause cell death (Hasegawa et al., 2000; Zhu, 2001, 2002). Moreover, these factors affect enzyme activities, which lead to a reduction in photosynthetic rate, metabolism, growth, and development; additionally, pollen germination may also be affected, lowering fertility. These effects contribute to the lower yield of crops exposed to salt stress (Abdullah et al., 2001).

Salt tolerance is a polygenic trait, and although several genes regulating salt tolerance have been identified, there are still some genes regulating salt tolerance in different rice varieties that are yet to be identified. Thai jasmine rice or "Khoa Dawk Mali 105" ("KDML105") rice is one of the most popular Thai rice cultivars among consumers. The high quality KDML105 grains are produced in rain-fed farms in the northeastern part of Thailand, and the farmlands are characterized by high soil salinity (2–16 $dS.m^{-1}$). Kanjoo et al. (2012) developed a drought tolerant line by generating chromosome substitution lines (CSSLs) in the KDML105 rice genetic background. The introgressions in these CSSLs contain drought-tolerant quantitative trait loci (QTL) on chromosome 1 and were engineered *via* marker-assisted breeding by crossing KDML105 to a drought-tolerant donor, DH212. Chutimanukul et al. (2018a,b). Chutimanukul et al. (2019) reported that CSSL16, a CSSL from this population, exhibited salt tolerance when compared to other CSSLs and KDML105 at the vegetative and seedling stage.

RNA-seq has been widely used to investigate transcriptomes under biotic and abiotic stress conditions in several plants (Song et al., 2014; Garg et al., 2015). High-throughput information can be analyzed to understand plant responses at the transcriptional level using various methods. The gene co-expression network (GCN) is a simplified method used in investigating the biological functions of genes under different conditions using the node degree or hub centrality. GCN analysis was applied to identify the gene modules that regulate drought tolerance (Sircar and Parekh, 2015), salt tolerance (Chutimanukul et al., 2018b), and osmotic stress tolerance (Nounjan et al., 2018). However, this type of network is an undirect graph, which contains nodes corresponding to genes and edges representing neighborhood relations (Stuart et al., 2003; Lee et al., 2004). Recently, the analysis of complex data is being carried out using high-performance computing systems. Consequently, the clustering coefficient method was developed to identify genes in plants or animals exposed to different environment (Zhang and Horvath, 2005).

In the analysis of network topological features, the node degree is one of the most generally used analytical techniques to identify the connection between the number of hub genes and neighboring nodes in the network. The consideration of the important genes can refer to the high number of neighboring nodes. The local density of the connection, referred to as the clustering coefficient (CC), is the measurement of the local density that quantifies the network's tendency of the connections (Watts and Strogatz, 1998; Ravasz et al., 2002). Furthermore, CC was developed from a simple binary network to a weighted network to fulfill the prediction constant degree of any real-world network (Humphries and Gurney, 2008). There have been reports of CC in GCN datasets from yeast and cancer microarrays (Zhang and Horvath, 2005). Moreover, the data analysis of degree on weighted gene co-expression network (WGCN) can be used to construct the signed gene co-expression network to define transcriptional modules (Horvath, 2011). This technique can identify the hub genes in plants or animals subjected to different conditions and the genes responsible for human diseases (Horvath, 2011; Mukund and Subramaniam, 2015; Riquelme Medina and Lubovac-Pilav, 2016).

To perform the expression network analysis for the identification of genes regulating salt tolerance in rice, we used the expression datasets from a single pair of rice lines with similar genetic backgrounds, but different levels of salt tolerance. Therefore, we selected the CSSL population because the lines share a similar genetic background but possess different levels of salt tolerance. To create an expression network, transcriptome datasets of the selected lines at seedling and booting stages were used to identify the major (hub) genes responsible for salt tolerance, as these two stages are the most susceptible to salt stress in rice.

In this study, we compared various CSSLs with different size segments of the putative abiotic stress tolerance genomic region to validate the salt tolerance of CSSL16 at the booting stage. The transcriptome data from leaves at the seedling stage, second leaf, and flag leaf at the booting stage of CSSL16 were analyzed using GCN, CC, and WGCN to predict the major genes responsible for salt tolerance. The expression of some predicted genes was investigated in both salt-tolerant and-susceptible lines.

## MATERIALS AND METHODS

### Plant Materials

Rice (*Oryza sativa* L.) seeds of CSSL lines (CSSL10, CSSL14, and CSSL16) with "KDML105" rice genetic background, and their parents (DH212 and KDML105) were obtained from the Rice Gene Discovery Unit (RGDU), National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. CSSL16 contained the full segment of the putative salt tolerance region between RM1003–RM3362 (Chutimanukul et al., 2018b),

**Abbreviations:** CC, Clustering coefficients; *Ci*, internal $CO_2$ concentration; CSSL, chromosome segment substitution line; *E*, transpiration rate; $F_v/F_m$, maximum PSII efficiency; GCN, Gene co-expression network; GO, Gene ontology; $g_s$, stomatal conductance; KDML105, Khao Dawk Mali 105; PAR, Photosynthetically active radiation; Pi, Photosynthetic performance index; *Pn*, Net photosynthetic rate; PSI, Photosystem I; PSII, Photosystem II; RNA-seq, RNA sequencing; ROS, Reactive oxygen species; SES, Standard Evaluation System; WGCN, Weighted gene co-expression network.

while CSSL10 contained the segment between RM1003–RM6827, and CSSL14 contained the segment between RM3468–RM3362 (**Figure 1**). The three CSSL lines, CSSL10, CSSL14, and CSSL16, were compared with KDML105 and DH212 for salt stress responses. Then the best CSSL candidate for salt tolerance was selected for transcriptomic analysis.

## Determination of the Photosynthetic Rate and Yield Components of the Lines at Booting Stage

### Plant Growth Condition

CSSL10, CSSL14, CSSL16, and their parental lines, "KDML105" and DH212 were grown in plastic pots containing soil. We supplied the necessary nutrients by applying Bangsai nutrient solution (1:100) to the soils. At the booting stage, 75 mM NaCl was added to the nutrient solution of the treatment groups, but not to the control group. The addition of NaCl increased the soil EC to 8 $dS.m^{-1}$, thus inducing salt stress. The experiment was performed in randomized complete block design with four replicates. Three plants per replicate were used for collecting the data. Analysis of variance was performed and means were compared with Duncan's multiple range test.

### Measurement of Physiological Parameters

After 6 days of salt-stress at the booting stage, standard physiological responses, such as net photosynthetic rate ($P_n$), stomatal conductance ($g_s$), internal $CO_2$ concentration ($Ci$), transpiration rate ($E$), $F_v/F_m$, and performance index (Pi), were evaluated. In parallel, every 3 days from day 0 to 9 during salt-stress treatment, we classified rice responses using the standard evaluation system (SES) of rice (IRRI, 1996). After 9 days of salt stress, the saline solution was washed out to reduce soil salinity to 2 $dS.m^{-1}$. Plants were then grown until seed harvest and yield components were determined.

At day 6 of salt stress, we measured gas exchange parameters in the middle portion of the flag leaves using a portable photosynthesis system (LI-6400 XT; LI-COR, Lincoln, NE). We used three plants per group as a replicate. The leaves were examined under the following conditions: 500 mmol $m^{-2}$ $s^{-1}$ air flow per unit leaf area, 1,200 mol $m^{-2}$ $s^{-1}$ photosynthetically active radiation (PAR) at leaf surface, leaf temperature ranged from 31.0 to 35.0°C, and a $CO_2$ concentration of 380 mol $mol^{-1}$.

$F_v/F_m$ and Pi were measured according to the recommended procedures of FMS 2 (Hansatech, King's Lynn, UK). Leaves were dark-adapted for 40 min using dark-adapted leaf clips before measurement.

### Experimental Design and Statistical Analysis

The study was laid out in a completely randomized design (CRD), with four replicates per treatment group (samples from three plants in a group constituted a replicate). Data of physiological parameters were subjected to analysis of variance (ANOVA) and significant means were compared using Duncan's multiple range tests (DMRT) by using SPSS version 21 (IBM Corp, Armonk, USA). Values were considered statistically significant at $p < 0.05$.

## Identification of the Putative Salt Tolerant Genes *via* Transcriptome Analysis

### RNA Extraction and Sequencing

To identify the genes regulating salt tolerance in rice, we focused on transcriptome analysis of CSSL16, which had the highest salt tolerance in the seedling and booting stages. Three replicates were used for each condition (CSSL16 grown under normal condition and under salt stress (75 mM NaCl treatment), respectively). Leaf tissues were collected at the seedling and booting stages. We harvested leaf samples from 21 days old seedlings after 0 and 2 days of salt stress, while flag leaves and second leaves were harvested at the booting stage on days 0 and 3. Leaves from the seedlings, flag leaves, and second leaves of untreated plants were used as the control. Three biological replications were conducted for this experiment. Total RNA was extracted from the leave samples using plant RNA purification reagent (Invitrogen, USA), and contaminated genomic DNA was removed with DNaseI (Invitrogen). cDNA libraries were constructed using the KAPA Stranded RNA-Seq Library Preparation Kit from Illumina® (Kapa Biosystem, USA). All short reads with a size of ∼300 bp were selected and connected with adaptors. Thereafter, all fragments were enriched by PCR for 12 cycles. The cDNA libraries were sequenced using Illumina Next-Generation sequencing (Illumina, USA).

For transcriptome analysis, all short-sequence reads were classified into the right category and QC was performed using a pipeline created by Missirian et al. (2011). The transcriptome sequences were uploaded to the NCBI database with BioProject ID, PRJNA507040. The sequence reads were aligned and mapped to the rice genome database (Ouyang et al., 2007) using Bowtie 2 (Langmead and Salzberg, 2012). The DESeq program (version 1.24.0) was used to identify differentially expressed genes (Anders and Huber, 2010). Genes with $p$-value $< 0.01$ were identified as differentially expressed genes.

## Identification of Marker Genes by GCN and CC Analysis

The read count of the RNA-Seq was analyzed and normalized using the DESeq package in software R (Anders and Huber, 2010). We constructed the gene co-expression network of the rice lines under normal and salt stress conditions at the growth stages following the method of Suratanee et al. (2018), and these constructs were combined as whole-state networks. The expression levels of whole-state networks were mixed. The edges in the network were recognized by calculating and selecting gene pairs with highly correlated ($r \geq 0.9$) levels of expression. Node degree is the number of edges connected to a node in a network, and clustering coefficient is a measure of the proportion of true connections and the number of all possible connections among neighbors of a gene node. The nodes represent the investigated genes, and the edges represent the significant co-expression level of any of the gene pairs. GCN identifies genes by using the degree or hub centrality. The clustering coefficient (CC) is a common measure of the true proportion of the link between the gene nodes and neighbors. The original clustering coefficient (small-world

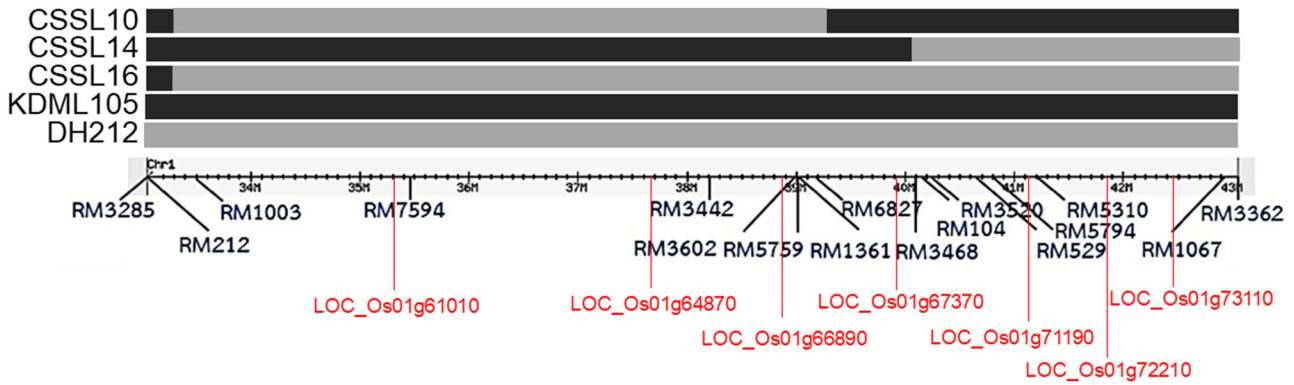


**FIGURE 1 |** The chromosomal segment substitution line of CSSL10, CSSL14, and CSSL16 with regions between RM1003 and RM3362 markers on chromosome 1. Some genes with putative functions, *Nodulin* (*LOC_Os01g61010*), *BTBZ1* (*LOC_Os01g66890*), *PSB28* (*LOC_Os01g71190*), and *ERD* (*LOC_Os01g72210*), are included.

network) by Watts and Strogatz (1998) is as follows:

$$C(i) = \frac{\sum_j \sum_{q \neq j} (a_{(ij)} a_{(iq)} a_{(jq)})}{k_i(k_i - 1)} \quad (1)$$

$C(i)$ varies from 0 to 1. $a_{ij}$ is a binary value from the connection between node $i$ and node $j$. The degree of node $i$ is $k_i$. If all neighbors of $i$ are themselves connected to another, CC equals 1, and if the neighbors of $i$ do not connect to each other, CC equals 0. Based on a real-world network, their nodes are mostly connected with some level of strength connections or weights. Moreover, the clustering coefficient for a weighted graph was constructed from the total weights of the neighbors (Onnela et al., 2005).

## Identification of Marker Genes by Weighted Co-expression Network (WGCN)

For WGCN, the connection of the network has its own values as a binary network of 0 or 1. Therefore, a weighted degree is the sum of all edges connecting the given node and neighbors. According to Onnela et al. (2005), a weighted graph of the clustering coefficient is obtained by taking the geometric mean of the total weights of its neighbors. Moreover, these connection weights can be positive or negative. While 0 represents no connection with neighbors, 1 represents the highest connection with all neighbors. The formula for using the real weights in the network is as follows:

$$C_{\text{realweight}}(i) = \frac{\sum_j \sum_{q \neq j} \left| w_{(ij)} w_{(iq)} w_{(jq)} \right|^{\frac{1}{3}}}{k_i(k_i - 1)} \quad (2)$$

The weight of the edge connecting nodes $i$ and $j$ is $w_{ij}$. The connection weights can be categorized as positive or negative. The value of $C_{realweight}(i)$ is distributed in the range [0, 1], where 0 means that there were no neighbors to connect to each other, and 1 means that there were high connections with neighbors. This formula was used to calculate the clustering coefficient for the real weights in the network, while the original formula was performed using a cut-off for the weight estimation into a binary class.

To clarify the analysis of GCN, CC, and WGCN, **Figure 2** shows an example of a gene co-expression network in the form of a binary network (**Figure 2A**) and in the form of a weighted network (**Figure 2B**). Gene identification by GCN analysis involves calculating the degree for each gene in the binary network in **Figure 2A**. Then, the highly connected nodes are recognized as marker genes. Therefore, $G_1$ with degree of 4, $G_2$ with degree of 6, and $G_3$ with degree of 4 have more connections than the other genes and are identified as important markers. On the other hand, gene identification of CC explores the possibility of connections among the neighbors of a certain node. There are no connections among the neighbors of $G_1$ and among the neighbors of $G_3$, while there is one connection among the neighbors of $G_2$. Therefore, the CC values of $G_1$ and $G_3$ are zero while the CC value of $G_2$ is 1/15 since 15 is the total number of all possible connections among the six neighbors.

Gene identification by WGCN involves the direct calculation of a weighted degree, that is the sum of all edge weights for a certain node in **Figure 2B**. With the use of weighted network, there are more edges with known strength as more information needs to be considered. Thus, the weighted degree of $G_1$ is 4.6, the weighted degree of $G_2$ is 5, and the weighted degree of $G_3$ is 4.3. Comparing with the degree values above, the weighted network indicates that $G_1$ is more important than $G_3$ while they have the same level of importance in the binary network.

## Validation of the Salt Tolerant Candidate Genes by Gene Expression Analysis

To validate regulation of salt-tolerance candidate genes by qRT-PCR, CSSL16, which had the highest salt tolerance at the seedling and booting stages, was compared with KDML105. The seeds of CSSL16 and KDML105 were soaked in water to induce germination. After 7 days, the seedlings were transplanted to nutrient solution (Udomchalothorn et al., 2014) with three replicates (three seedlings per replicate). Subsequently, after 7 days, the seedlings were transferred to nutrient solution without NaCl (control) and nutrient solution containing 75 mM NaCl (treatment group).

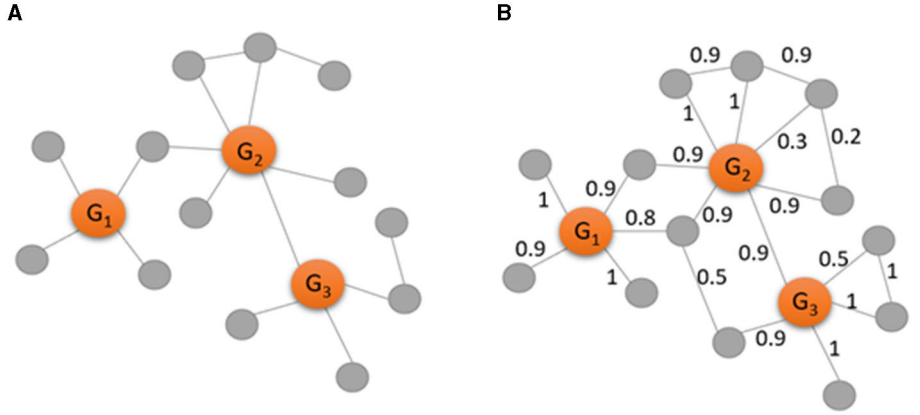


**FIGURE 2 |** Examples of a binary gene co-expression network **(A)** and a weighted gene co-expression network **(B)** consisting of three observed genes (in orange) and 13 genes (in gray). The edges in the network **(A)** are recognized by calculating and selecting gene pairs with highly correlated ($r \geq 0.9$), while the edges in the network **(B)** are weighted by the absolute values of the correlation.

Seedlings were harvested after salt stress treatment for 0, 3, 6, 12, 24, and 48 h for the early response and for the late response, seedlings were harvested on days 0, 3, and 6 of treatment.

## Gene Expression Analysis

Total RNA was extracted from the shoots of seedlings from the control and treatment groups using GENEzol GZR100 (Geneaid Biotech, Taiwan). The RNA was treated with DNase I (Thermo Scientific, USA) and converted into cDNA. cDNA synthesis was performed using an Accupower RT premix (Bioneer Inc., Alameda, USA). The synthesized cDNA was used as template for the PCR. qRT-PCR was conducted using Luna Universal qPCR master mix M3003L (New England Biolabs Inc., USA).

Quantitative RT-PCR reactions were conducted on three technical replicates for each sample. No template (NTC) was used as a negative control, and *EF-1α* primers (Chutimanukul et al., 2018b) were used as an internal control to standardize the equal template in the reaction. Gene sequences were obtained from the rice genome database (Ouyang et al., 2007) and then submitted to Primer3 to generate specific primers for the nine selected genes (**Table 1**). Relative gene expression was determined by qRT-PCR. The PCR conditions were as follows: an initial denaturation step at 95°C for 60 s, followed by 35 cycles of denaturation at 95°C for 15 s, annealing steps with the temperature shown in **Table 1** for 30 s, and continued with an extension step at 75°C for 30 s. The melt curve and plate read were set at 60–94°C with increasing temperature at the rate of 5°C per 5 s. Average cycle threshold (Cq) values of all genes were normalized to the level of *EF-1α* reference genes in the same sample and then used to measure relative gene expression by following the ΔΔCt method as described by Pfaffl (2001). The gene expression analysis was interpreted based on the relative expression levels, and SPSS software was used for the analysis of variance ($p < 0.05$).

## Analysis of Arabidopsis Mutant Lines for Salt Stress Responses

The selected mutant seeds were ordered from Arabidopsis Biological Resource Center (ABRC). The homozygous mutant lines were screened according to SALK T-DNA primer design. The homozygous mutant lines used in this experiment were *bt3*, *psb28*, *AT5G45310*, *sbt3.3*, *sbt3.4*, and *per3* mutants. Col-0 wild type (WT) was used as a control. The evaluation of salt stress response was performed with complete randomized design with three replicates. Each replicate contained 20 seedlings. Mutant lines and WT seeds were sterilized and germinated for 7 days after stratification at 4°C for 48 h. Then, 7 day-old seedlings were transferred to the freshly prepared MS medium with or without 100 mM NaCl addition. After 7-day incubation under light intensity of 35 $mmol.m^{-2}.s^{-1}$, 16/8 light/dark cycle at 22 °C, dry weight was measured with 15 plants per treatment. Photosynthetic pigment contents were determined from 5 plants per treatment according to Wellburn (1994). The absorbance at $A_{470}$, $A_{646.8}$, and $A_{663.2}$ were measured to determine Chlorophyll *a*, chlorophyll *b* and carotenoid contents by using the following equations:

$$Chlorophyll\ a\ (Chl\ a)\ content = 12.25A_{663.2} - 2.79A_{646.8} \quad (3)$$

$$Chlorophyll\ b\ (Chl\ b)\ content = 21.5A_{646.8} - 5.1A_{663.2} \quad (4)$$

$$Totalcarotenoids = (100A_{470} - 1.82Chla - 85.02chlb)/198 \quad (5)$$

## Putative Promoter Analysis

The putative promoter region (2 kb upstream from coding region) of *OsBTBZ1* gene of KDML105 and CSSL16 was retrieved from PRJNA659381. Sequence alignment was performed by using Needle tool *via* EMBOSS. Cis-elements were searched against PLACE database (Higo et al., 1999).

**TABLE 1 |** Quantitative RT-PCR rice primers.

| Name/annotation | | Sequence 5'à3' | Product size | Position | Annealing temperature |
|---|---|---|---|---|---|
| *LOC_Os01g61010 (Nodulin)* | FW | CCGCGAAAAGTGGCTACTCCA | 101 bp | 1,179–1,282 | 60.0°C |
| | RV | AAAGAAGTCCCGCTGGTTGAG | | | |
| *LOC_Os01g64870* | FW | CGAGCAGTTTGCCAGGTTGAAT | 183 bp | 974–1,156 | 61.5°C |
| | RV | AGCCTTTGGAATGCAAGCTCCT | | | |
| *LOC_Os01g66890*(*BTBZ1*) | FW | TTCCTGCCTGCAAGGGCATC | 172 bp | 1,108–1,280 | 61.5°C |
| | RV | TCCTTGAAATGCCTACAGAGGGG | | | |
| *LOC_Os01g67370* | FW | GGCGGATTTACCGAACATATTTGA | 173 bp | 260–432 | 60.5°C |
| | RV | TGTCAGCCAGGAAGGTTGGA | | | |
| *LOC_Os01g72210* (*ERD*) | FW | GGTTCTAACAAGCTTTGGGTGC | 141 bp | 562–703 | 61.5°C |
| | RV | TTGGTCAGGCCGTTTCCTGT | | | |
| *LOC_Os01g71190 (PSB28)* | FW | GATGCCCCGCAGGTTCGTC | 170 bp | 218–387 | 60.0°C |
| | RV | GGTGCCCTGGATGAACTGGA | | | |
| *LOC_Os01g73110* | *FW* | CCGATGGTGATGGTTGGCTG | 180 bp | 160–339 | 61.0°C |
| | RV | CCGATCCAGCTTGCGCTCT | | | |
| *LOC_Os04g03050* (*Sub34*) | FW | TGTGGTTATCACCTTGGGCG | 124 bp | 1,164–1,287 | 61.0°C |
| | RV | ATTGTCGGCATTGCAGTCGT | | | |
| *LOC_Os06g46799* (*Peroxidase*) | FW | CCTCTCCTCCTTCCAGAGCAA | 97 bp | 629–725 | 61.0°C |
| | RV | GCTGAACGAGTTGCAGTGCG | | | |
| *EF1α* | FW | ATGGTTGTGGAGACCTTC | 127 bp | 1,326–1,435 | 60.0°C |
| | RV | TCACCTTGGCACCGGTTG | | | |

# RESULTS

## CSSL16 Sustained Photosynthetic Responses Under Salt Stress at Booting Stage

The physiological study showed that the net photosynthesis rate (*Pn*) of the flag leaves of the rice lines under normal grown condition was not significantly different (**Figure 3A**). However, the *Pn* of the second leaves of the lines were significantly different, with the second leaves of "KDML105" recording the highest *Pn* values, while the second leaves of CSSL14 grown under normal conditions had the lowest *Pn* values.

Salt stress caused a decrease in the *Pn* of the flag leaf and second leaf of the lines (**Figure 3B**). The flag leaves of CSSL10, CSSL16, and DH212 had significantly higher *Pn* than those of "KDML105" and CSSL14, while the second leaves of CSSL10 had similar *Pn* values to those of "KDML105" and DH212. A similar response was also found in stomatal conductance (**Figures 3C,D**). The *Ci* levels of rice grown under normal conditions were not significantly different; contrarily, the *Ci* levels of both flag leaves and second leaves of rice lines grown under salt stress were significantly different with the second leaves of "CSSL16" recording the highest *Ci* level (**Figures 3E,F**). The transpiration rate of these plants was consistent with their $g_s$ (**Figures 3G,H**).

Salt stress did not affect the PSII efficiency ($F_v/F_m$) of the flag leaves (**Figures 4A,B**). Additionally, the Pi's of the flag leaves were not significantly different under normal growth condition; contrarily, salt stress significantly affected the Pi's of the flag leaves, with CSSL14 recording the highest Pi, while "KDML105" recorded the lowest. The second leaves of CSSL16 recorded the highest Pi both under normal growth condition and under salt stress, while the second leaves of "KDML105" had the lowest Pi both under normal growth condition and under salt stress. Overall, the Pi's of the second leaves of the rice lines were significantly different both under normal growth conditions and under salt stress (**Figures 4C,D**). During the first 6 days and after 9 days under salt stress conditions (**Figure 5**), CSSL16 and DH212 had significantly lower SES than the other lines.

## CSSL16 Had Higher Yield Components Than That Did "KDML105" and Other CSSLs

After exposing the rice seedlings to salt stress at 8 $dS.m^{-1}$ for 9 days, soil salinity was reduced to 2 $dS.m^{-1}$ and the plants were grown under this condition until grain harvest. The yield components of the different lines were determined after harvest (**Table 2**). Results showed that rice lines with KDML105 genetic background recorded higher tiller numbers per plant than the corresponding, introgression-free line DH212. Salt stress decreased tiller numbers per plant, panicle numbers per plant, panicle length, total seed number, and number of filled grains per plant. Moreover, shoot fresh weight, dry weight, and height were affected by salt stress (**Table 2**). CSSL16 had the highest tiller numbers per plant, panicle number per plant, total seed number per panicle, filled grain, and seed number per plant, compared to the other lines. Based on gas exchange parameters, PSII efficiency and yield component, CSSL16 was the most tolerant line under high salt stress at the booting stage. This suggested that the presence of the whole QTL region was required to achieve the

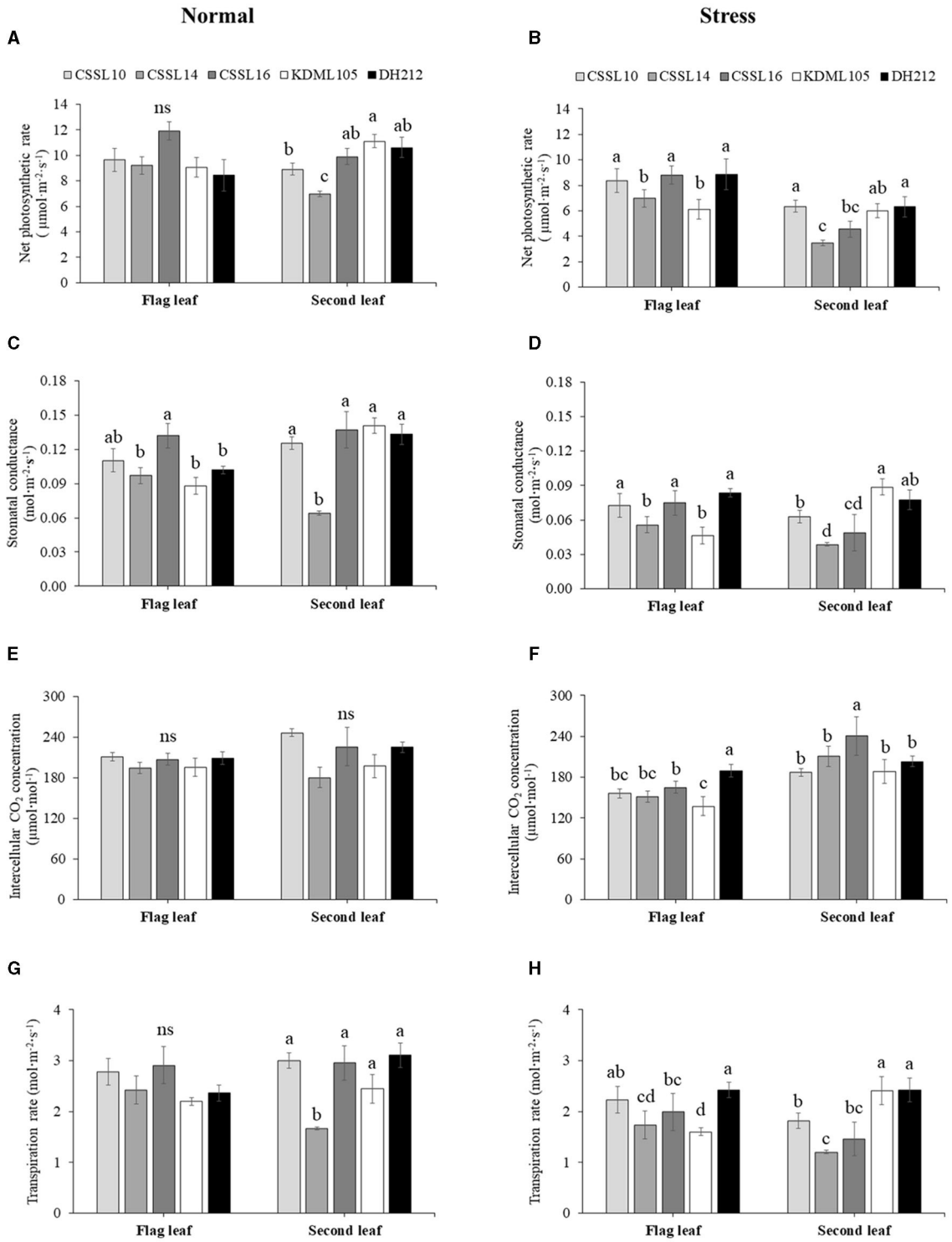


**FIGURE 3 |** Gas exchange parameters, net photosynthesis rate [Pn, **(A,B)**], stomatal conductance [$g_s$, **(C,D)**], internal $CO_2$ concentration [*Ci*, **(E,F)**], and transpiration rate **(E,G,H)** of flag leaves and second leaves of CSSL10, CSSL14, CSSL16, "KDML105," and DH212 under normal and salt stress conditions. Values are represented as mean ± SE ($n = 4$). Different letters above bars indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.

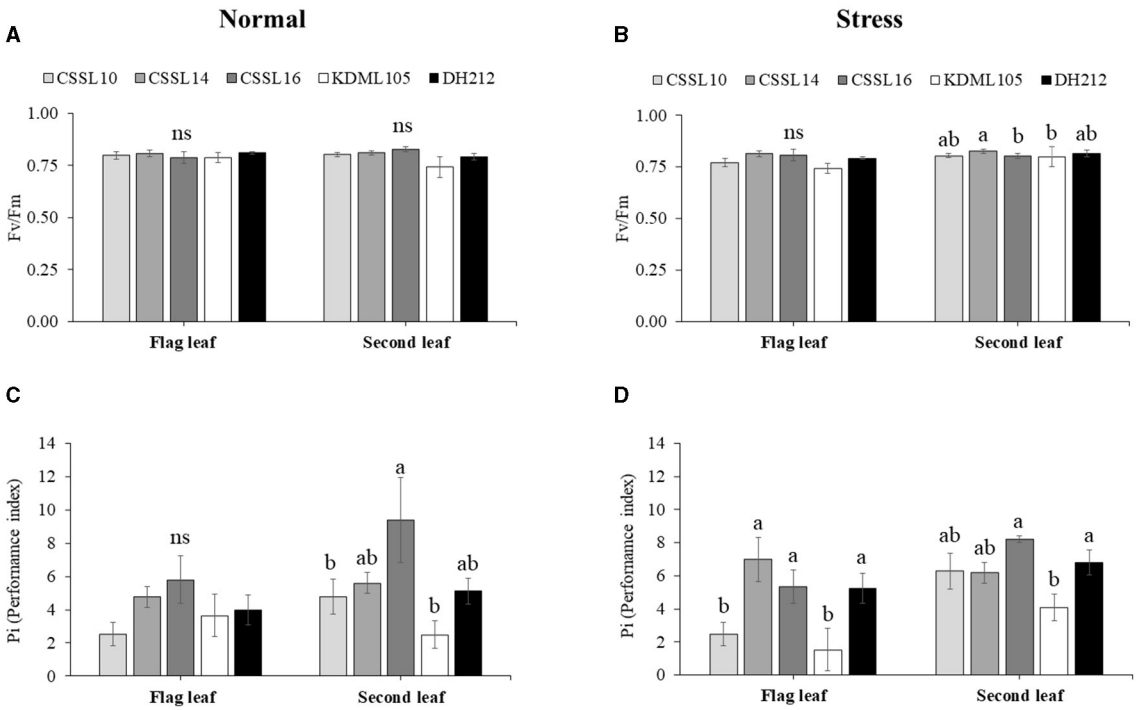


**FIGURE 4 |** Maximum PSII efficiency ($F_v/F_m$) **(A,B)** and Performance index (Pi) **(C,D)** of flag leaves and second leaves in CSSL10, CSSL14, CSSL16, "KDML105." and DH212 under normal and salt stress conditions. Values are represented as mean ± SE ($n = 4$). Different letters above bars indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.

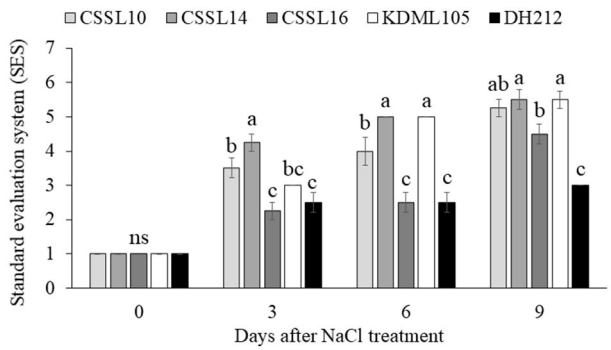


**FIGURE 5 |** Standard evaluation system (SES) determined from the appearance of plants under salt stress condition for 0, 3, 6, and 9 days. Values are presented as mean ± SE ($n = 4$). Different letters above bars indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.

best tolerance, implicating the action of two or more genes Therefore, CSSL16 was chosen for transcriptome analysis.

## Transcriptomics Profile of CSSL16 Rice at Seedling and Booting Stages

To identify genes regulating salt tolerance in rice, we analyzed the transcriptome of three seedling leaves, and from the flag and second leaves of CSSL16 plants exposed to normal growth condition and salt stress, respectively. Gene expression was examined by RNA sequencing of the leaves of seedlings at 0 and 2 days of treatment. At the booting stage, RNA sequencing was performed from flag leaf and second leaf samples at 0 and 3 days of treatments. We identified 511 differentially expressed genes in the leaves of the seedling, while 520 and 584 differentially expressed genes were identified in the second leaf and flag leaf, respectively (**Supplementary Files 1**, **2**). More than 50% of the differentially expressed genes were downregulated by salt stress at the seedling stage and in the flag leaves at the booting stage. Contrarily, <50% of the differentially expressed genes were downregulated by salt stress in the second leaf.

We used the ClueGo tool to screen for gene ontology (GO) terms that were significantly enriched by the DEGs. The results showed that genes enriched in biological processes, such as response to inorganic substances, oxygen-containing compounds, alcohol, heat, and temperature stimulus were downregulated in the leaves of the seedlings, while the genes involved in cell wall biogenesis, cellular glucan metabolism, and glucan metabolism were upregulated (**Supplementary Figure 1**). We compared the transcriptomes of the second leaf before and after 3 days of salt stress. The GO enrichment analysis of the second leaf indicated a significant upregulation of genes regulating temperature and heat responses, and the sizes of cellular components and anatomical structures (**Supplementary Figure 2**), while genes enriched in cellular chemical homeostasis and chemical homeostasis were

**TABLE 2 |** Yield components of CSSL10, CSSL14, CSSL16, "KDML105," and DH212 grown under normal or salt stress conditions (8 $dS.m^{-1}$) at booting stage for 9 days.

| Yield components† | Condition | Rice lines | | | | | *F*-test |
|---|---|---|---|---|---|---|---|
| | | CSSL10 | CSSL14 | CSSL16 | KDML105 | DH212 | |
| Tiller number per plant | Normal | $14.25 \pm 0.85^{a}$ | $15 \pm 1.77^{a}$ | $16.75 \pm 0.63^{a}$ | $13.25 \pm 0.85^{a}$ | $8.5 \pm 1.19^{b}$ | * |
| | Salt stress | $10.25 \pm 0.85^{bc}$ | $12 \pm 0.71^{ab}$ | $14 \pm 1.08^{a}$ | $12.25 \pm 0.47^{ab}$ | $7.75 \pm 1.03^{c}$ | * |
| Panicle number per plant | Normal | $9.25 \pm 0.85^{b}$ | $9.75 \pm 0.85^{b}$ | $14.5 \pm 0.29^{a}$ | $10.25 \pm 0.95^{b}$ | $8.5 \pm 1.55^{b}$ | * |
| | Salt stress | $7.75 \pm 1.11$ | $9.25 \pm 0.95$ | $11 \pm 1.47$ | $9.75 \pm 1.11$ | $7.5 \pm 1.19$ | ns |
| Panicle length (cm) | Normal | $27.78 \pm 0.54^{a}$ | $26.14 \pm 0.33^{b}$ | $24.98 \pm 0.29^{c}$ | $25.96 \pm 0.17^{bc}$ | $27.31 \pm 0.21^{a}$ | * |
| | Salt stress | $24.31 \pm 0.17^{b}$ | $24.12 \pm 0.36^{b}$ | $23.49 \pm 0.26^{b}$ | $21.46 \pm 0.53^{c}$ | $25.92 \pm 0.26^{a}$ | * |
| Total seed per panicle | Normal | $130.25 \pm 3.94^{bc}$ | $116.75 \pm 4.31^{c}$ | $152.25 \pm 3.68^{a}$ | $124.25 \pm 5.78^{c}$ | $143.75 \pm 4.40^{ab}$ | * |
| | Salt stress | $121.50 \pm 4.13^{bc}$ | $114.75 \pm 1.75^{c}$ | $142.75 \pm 4.05^{a}$ | $113.5 \pm 3.10^{c}$ | $134.75 \pm 2.63^{ab}$ | * |
| Filled grains per plant | Normal | $91.75 \pm 0.48^{c}$ | $94.5 \pm 2.10^{c}$ | $130.25 \pm 2.25^{a}$ | $109.75 \pm 5.20^{b}$ | $114.25 \pm 4.85^{b}$ | * |
| | Salt stress | $74.25 \pm 2.50^{d}$ | $75 \pm 1.68^{d}$ | $117 \pm 3.03^{a}$ | $90.25 \pm 2.29^{c}$ | $98.25 \pm 2.87^{b}$ | * |
| 100 Seeds weight (g) | Normal | $1.89 \pm 0.07^{b}$ | $1.90 \pm 0.10^{b}$ | $1.92 \pm 0.04^{b}$ | $2.20 \pm 0.10^{a}$ | $2.08 \pm 0.02^{ab}$ | * |
| | Salt stress | $1.14 \pm 0.15^{b}$ | $1.67 \pm 0.08^{a}$ | $1.65 \pm 0.07^{a}$ | $1.85 \pm 0.07^{a}$ | $1.55 \pm 0.16^{a}$ | * |
| Plant height (cm) | Normal | $178.25 \pm 2.62^{a}$ | $171.75 \pm 5.21^{ab}$ | $156 \pm 8.95^{b}$ | $121.25 \pm 3.35^{c}$ | $164.75 \pm 4.19^{ab}$ | * |
| | Salt stress | $152.5 \pm 2.75^{a}$ | $160 \pm 5.05^{a}$ | $139 \pm 2.68^{b}$ | $109.5 \pm 0.65^{c}$ | $152.75 \pm 2.56^{a}$ | * |
| Shoot fresh weight (g) | Normal | $179.25 \pm 8.01^{a}$ | $147.5 \pm 10.13^{ab}$ | $157.7 \pm 10.40^{ab}$ | $116.22 \pm 11.54^{b}$ | $132.19 \pm 22.07^{b}$ | * |
| | Salt stress | $138.5 \pm 7.053$ | $118.25 \pm 11.44$ | $125.75 \pm 12.30$ | $86.5 \pm 13.37$ | $117.25 \pm 16.12$ | ns |
| Shoot dry weight (g) | Normal | $28.74 \pm 2.19$ | $28.67 \pm 2.10$ | $32.76 \pm 0.95$ | $24.56 \pm 1.27$ | $29.07 \pm 2.27$ | ns |
| | Salt stress | $24.49 \pm 1.41^{bc}$ | $27.39 \pm 1.02^{ab}$ | $29.01 \pm 0.53^{a}$ | $22.13 \pm 0.88^{c}$ | $27.94 \pm 1.71^{ab}$ | * |

† *Values are represented as mean ± SE (n = 4). Different letters indicate significant difference between lines at $p < 0.05$. "ns" indicates no significant difference.* * *Significant difference at $p < 0.05$.*

downregulated. When the plants were exposed to salt stress, the upregulated genes were enriched in response to heat and temperature stimulus (**Supplementary Figure 3**).

## Combining the Gene Co-expression Network Analysis With SNP Information Can Identify Salt Tolerant Genes

The co-expression networks under salinity and normal conditions were constructed by calculating the correlation of the expression levels of DEGs in the plants (leaves of the seedlings, flag leaves, and second leaves). Genes that were highly correlated ($r > 0.9$) under normal condition were used to construct the normal-state network. Similarly, genes that were highly correlated under salinity stress were used to construct the salinity-state network. We found 579 DEGs in the normal-state network and 573 DEGs in the salinity-state network. The results showed that the network created from expression data under normal conditions had higher number of nodes, edges, connection per node, and average degree than those of the network created from the expression data under the salt stress condition. The genes involved in salt tolerance were selected from genes with high connections per node under salt stress conditions and low connections per node under normal conditions. Fifty-seven candidate genes (**Supplementary File 2**) were selected. Most of the selected genes were on chromosome 1. Four of them, *LOC_Os01g64870*, *LOC_Os01g66890*, *LOC_Os01g67370*, and *LOC_Os01g72210* were located in the salt/drought tolerant QTL reported by Kanjoo et al. (2012). *LOC_Os01g72210* and *LOC_Os01g67370* encoded unknown expressed proteins, while *LOC_Os01g66890* was annotated as *BTBZ1* and *LOC_Os01g72210*, was annotated as a protein part of the early response to dehydration (*ERD*) protein. Both *BTBZ1* and *ERD* displayed SNPs between CSSL16 and "KDML105" in the promoter, 5'UTR, exons, introns, and 3'UTR.

We analyzed the distributions of the clustering coefficients for the binary network by comparing a dense local cluster between salt stress and normal conditions. The clustering coefficient analysis identified 30 genes involved in salt tolerance (**Supplementary File 3**). Four genes were located in the salt/drought tolerant QTL (Kanjoo et al., 2012), *LOC_Os01g61010*, *LOC_Os01g66890* (*BTBZ1*), *LOC_Os01g72210* (*ERD*), and *LOC_Os01g73110*. The CC analysis identified *BTBZ1* and *ERD*, which were also identified by GCN analysis. *LOC_Os01g61010* was annotated as encoding a *Nodulin,* while *LOC_Os01g73110* encoded an unknown expressed protein.

Furthermore, we identified 59 genes using weighted co-expression network analysis (**Supplementary File 2**). *LOC_Os01g64870*, *LOC_Os01g66890* (*BTBZ1*), *LOC_Os01g71190*, *LOC_Os01g72210* (*ERD*), and *LOC_Os04g03050* were located in the salt/drought QTL (Koyama et al., 2001; Kanjoo et al., 2011, 2012). Moreover, three out of the five genes (*LOC_Os01g64870, BTBZ1*, and *ERD*) were identified by both the co-expression network and clustering coefficient analyses. The other three genes included *LOC_Os01g71190* (*PSB28*), which was annotated to encode the protein involved in photosystem II reaction center, while

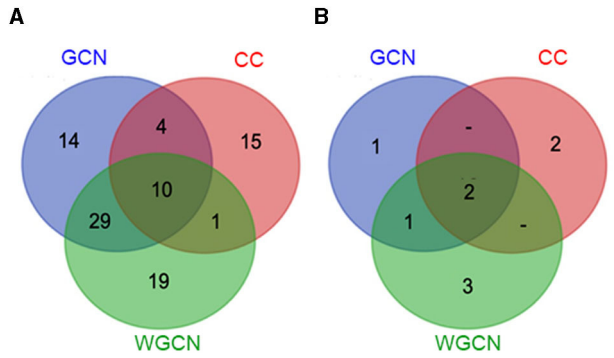


**FIGURE 6 |** Venn diagram **(A)** showing the number of salt-responsive genes from co-expression network analysis (blue circle), Clustering coefficient analysis (red circle), weighted co-expression network analysis (green circle), and Venn diagram **(B)** showing number of salt-responsive genes containing the SNPs in each method analysis.

**TABLE 3 |** Salt-tolerant genes consistently predicted by GCN, CC, and WGCN.

| Locus | Annotation |
|---|---|
| LOC_Os01g66890 (*BTBZ1*) | BTBZ1—Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains, expressed |
| LOC_Os01g72210 (*ERD*) | Early-Responsive to Dehydration protein-related, putative, expressed |
| LOC_Os02g08100 | AMP-binding domain containing protein, expressed |
| LOC_Os02g45950 | cytochrome $b_6f$ complex subunit, putative, expressed<br>expressed protein |
| LOC_Os03g55720 | Cytochrome $b_6f$ complex subunit, putative, expressed |
| LOC_Os06g28630 | Expressed protein |
| LOC_Os07g02540 | HLS, putative, expressed |
| LOC_Os09g26880 | Aldehyde dehydrogenase, putative, expressed |
| LOC_Os09g39910 | ABC transporter, ATP-binding protein, putative, expressed |
| LOC_Os11g42500 | Dirigent, putative, expressed |

*LOC_Os04g03050* and *LOC_Os06g46799* encoded subtilisin (*OsSub34*) and peroxidase precursor, respectively.

**Figure 6A** displays a Venn diagram of the genes identified using the three network analyses. The blue, red, and green circles included genes identified by GCN, CC, and WGCN, respectively (**Figure 6A**). In total, we identified 92 genes using the three methods. Among the genes, 10 were identified by each of the three methods (**Table 3**). The co-expression network of 92 genes identified by GCN, CC, and WGCN is shown in **Figure 7**. The 10 genes, identified by these three techniques (GCN, CC, and WGCN), are displayed as red circles.

Using SNPs found in CSSL16 and "KDML105," the number of genes identified by GCN, CC, and WGCN were 4, 4, and 6, respectively (**Figure 6B**). Together with the three methods of transcriptome analysis and SNP information of the salt tolerant and susceptible lines, we identified nine genes, which were responsible for salt tolerance in rice (**Figure 6B** and **Table 4**). Two out of these genes, which are *LOC_Os01g66890* (*BTBZ1*) and *LOC_Os01g72210* (*ERD*), contain SNPs between CSSL16 and "KDML105" rice. In addition, these two genes are connected to each other in the network (**Figure 7**). We hypothesize that the nine genes were responsible for the salt tolerance of CSSL16 compared with KDML105 rice.

## Significantly Different Expression Levels of the Candidate Genes in CSSL16 After Salt-Stress Treatment

To examine the salt-tolerance candidate gene expression, we used qRT-PCR to study the expression response to salt stress of the nine genes in **Table 4**. After growing rice seedlings for 14 days, 75 mM NaCl was added to the nutrient solution. We compared their expression in CSSL16, the salt-tolerant genotype, and in its salt-susceptible parent, "KDML105." The comparison was performed in two sets of experiments to investigate the early (0, 3, 6, 12, 24, and 48 h after stress) and late (0, 3, and 6 days after stress) responses. After 6 days of salt stress, morphology of the plants is displayed in **Figures 8A,B**. For early stress responses, *OsNodulin* expression did not vary much during this period of salt stress (**Figure 9A**), while *LOC_Os01g64870* expression in the salt-treated CSSL16 after 12 h of salt treatment was increased to more than 7-fold higher than treated KDML105 (**Figure 9B**). The expression levels of *OsBTBZ1* (**Figure 9C**), *LOC_01g67370* (**Figure 9D**), and *OsPeroxidase* (**Figure 9I**) in the salt-treated CSSL16 were also significantly higher than those of the salt-treated KDML105 after 12 h of the treatment, while the expression levels of *OsERD* (**Figure 9E**), *LOC_01g73110* (**Figure 9G**), and *OsSub34* (**Figure 9H**) in CSSL16 was dramatically higher than KDML105 after 6 h of salt stress. It is worth mentioning that the expression of *OsBTBZ1*, *OsERD*, *OsSub34*, and *LOC_01g73110* was induced more than 15-fold by salt stress in the early response. The expression level of *OsPSB28* (**Figure 9F**) was higher in CSSL16 after 6 and 48 h of stress, but the level of expression was fluctuating and did not show much difference during this early response.

For the late response, the expression of *Nodulin* (**Figure 10A**), *LOC_Os01g64870* (**Figure 10B**), *BTBZ1* (**Figure 10C**), *LOC_Os0167370* (**Figure 10D**), and *PSB28* (**Figure 10F**), increased significantly in CSSL16, but decreased in KDML105 at 3 days of exposure to salt stress. However, the expression of *ERD* (**Figure 10E**) and *LOC_Os01g73110* (**Figure 10G**) increased in both CSSL16 and KDML105 at 3 days of salt stress. After 6 days of salt stress, the expression of *Nodulin*, *LOC_Os01g64870*, and *BTBZ1* was still higher in CSSL16 compared with that of KDML105, but the expression of *LOC_Os01g73110* decreased, while the expression of *ERD* increased. After 6 days of salt stress, the expression of *ERD* increased by more than 4.5- and 4-fold in CSSL16 and KDML105, respectively. The expression of *OsSub34* was reduced by salt stress in both lines; however, this decrease was more pronounced in CSSL16 than that in "KDML105" (**Figure 10H**). Peroxidase increased after 6 days of salt stress

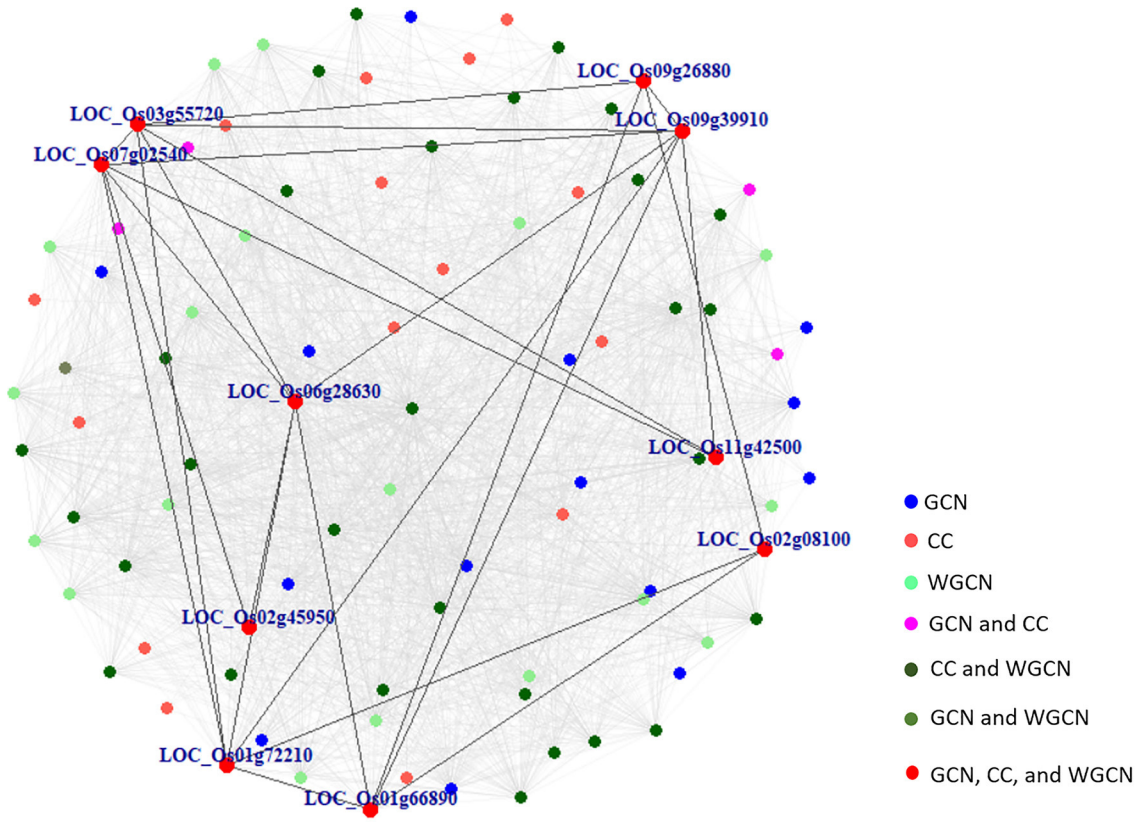


**FIGURE 7 |** The co-expression network of 92 marker genes identified by GCN, CC, and WGCN. Ten genes in red were detected by all methods and the connections among them were shown in darked lines. All gray lines represent the connections among these 92 marker genes.

in both lines (**Figure 10I**). The results suggest that the nine candidate genes may be involved in salt tolerance in rice.

## The Predicted Genes Have the Potentials to Function in Salt Tolerance

In order to investigate the potential of these predicted genes for functioning in salt tolerance, Arabidopsis mutant lines containing T-DNA insertion in the genes orthologous to the predicted rice genes, were analyzed for their salt responsive phenotypes. Due to the dramatically higher induction at early response of *OsBTBZ1*, *OsSub34*, and *LOC_01g73110*, the Arabidopsis mutants of their ortholgous genes (**Table 4**), namely *bt3*, *sbt3.3*, *sbt3.4*, and *at5g45310* mutants, were analyzed. Although *OsERD4* displayed high level during early induction, the *erd4* mutant was not included in this analysis because no homozygous insertion lines could be obtained. Finally, the *psb28* and *per3* mutants were included in this experiment and Col-0 wild type (WT) was used as a control.

Under normal growth condition, *sbt3.4*, *psb28*, and *per3* mutants showed significantly higher dry weights than WT, while the *bt3* mutant had significantly lower dry weight. The photosynthetic pigment contents were also different among these lines. The *at5g45310* mutant displayed a similar phenotype to the WT, and so did *sbt3.3*, except that *sbt3.3* had higher chl *a* content than the WT (**Table 5**).

Salt stress caused dry weight reduction in the WT, but it had decreased effects on the *sbt3.3*, *sbt3.4*, *at5g45310, per3*, and *psb28* mutants. A negative effect of salt stress on dry weight was detected in the *bt3* mutant, with more than 60% reduction in dry weight. Salt stress conditions caused the reduction of photosynthetic pigments content in all lines, especially the *bt3* mutant, whose photosynthetic pigments content was decreased more than 65%. Interestingly, the carotenoid content in *sbt3.3* and *per3* mutants was dramatically decreased by salt stress (more than 80% reduction), but both mutants displayed better Chl *b* maintenance than the WT (**Table 5**). These changes in salt stress responses in these mutant lines, when compared to WT, suggest a role for these genes in salt stress adaptation in Arabidopsis and reinforce the hypothesis of functions of these gene families in other plant species, including rice.

Because the *bt3* mutant displayed the highest growth inhibition and photosynthesis pigment reduction and the *OsBTBZ1* gene was highly-induced under salt stress, we focused on its promoter. We compared putative regulatory sequences 2 kb base pairs upstream from the coding region of *OsBTBZ1* in the KDML105 and CSSL16 accessions analyzing it for putative regulatory cis-elements (**Supplementary Figure 4**).

**TABLE 4 |** Putative salt tolerance genes predicted by GCN, CC, and WGCN containing SNPs between CSSL16 and "KDML105" rice.

| Locus | Annotation | Types of network analysis | Orthologous gene in Arabidopsis |
|---|---|---|---|
| *LOC_Os01g61010* (*Nodulin*) | Nodulin, putative, expressed | CC | - |
| *LOC_Os01g64870* | expressed protein | GCN, WGCN | *AT1G71240* |
| *LOC_Os01g66890* (*BTBZ1*) | BTBZ1—Bric-a-Brac, Tramtrack, and Broad Complex BTB domain with TAZ zinc finger and Calmodulin-binding domains, expressed | GCN, CC, WGCN | *AT1G05690* (*BT3*) |
| *LOC_Os01g67370* | Expressed protein | GCN | *AT3G59300* |
| *LOC_Os01g71190* | Photosystem II reaction center PSB28 protein, chloroplast precursor, putative, expressed | WGCN | *AT4G28660* (*PSB28*) |
| *LOC_Os01g72210* (*ERD*) | Early-responsive to dehydration protein-related, putative, expressed | GCN, CC, WGCN | *AT3G54510* (*ERD4*) |
| *LOC_Os01g73110* | Expressed protein | CC | *AT5G45310* |
| *LOC_Os04g03050* | OsSub34—Putative Subtilisin homolog, expressed | WGCN | *AT1G32940* (*SBT3.5*)<br>*AT1G32950* (*SBT3.4*)<br>*AT1G32960* (*SBT3.3*)<br>*AT4G10510*<br>*AT4G10540* (*SBT3.8*)<br>*AT4G10550* |
| *LOC_Os06g46799* | Peroxidase precursor, putative, expressed | WGCN | *AT1G05260* (*PER3*) |

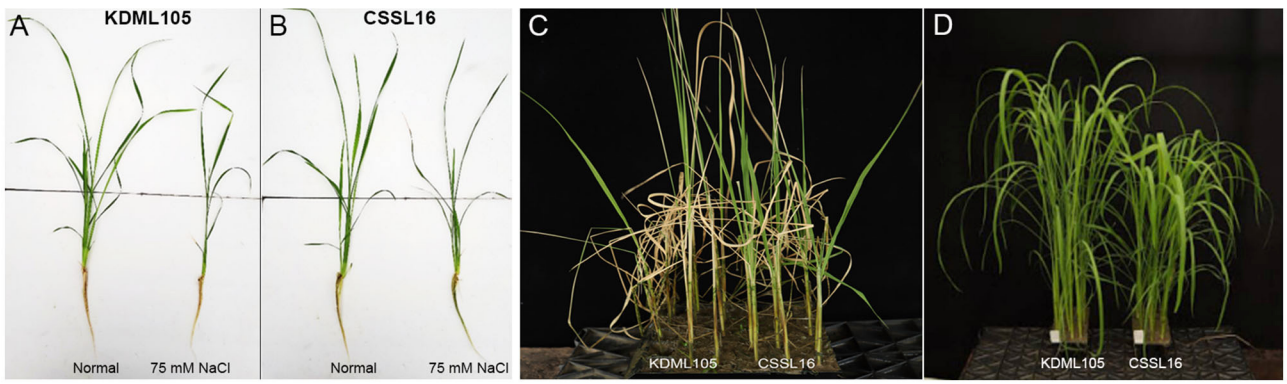


**FIGURE 8 |** Fourteen day-old KDML105 and CSSL16 seedlings after growing in nutrient solution in nomral condition or supplementaed with 75 mM NaCl for 6 days **(A,B)** and the seedlings that were soil-grown and treated with 75 mM for 12 days **(C)** or grown in normal condition **(D)**.

Three ABA responsive elements (ABREs) are located within 250 base pairs upstream of the gene. Moreover, four MYC binding sites, which represent water-stress responsive elements, are located within this region, and two out of four overlapped with the ABREs. Beyond this region, −251 to −2,000 bp, 12 more MYC binding sites are found. The MYB transcription factor was also reported for water stress and salt stress regulation (review; Ponce et al., 2021). Five MYB binding sites are located in the putative regulatory sequence of *OsBTBZ1* gene.

Two elements that are found only in the putative regulatory region of CSSL16's *OsBTBZ1* gene, but not in KDML105's are an endosperm-specific element (AAAG) and GAGA-binding site. The insertion and base substitution in KDML105 eliminate the two elements found in CSSL16. This polymorphism may contribute to the difference in *OsBTBZ1* gene expression level in these two rice lines.

## DISCUSSION

In the present study, the results of the gas exchange parameters and yield components indicated that CSSL16 was more resistant to salt stress than KDML105 at the booting stage, as it recorded higher *Pn* and yield components than KDML105 (**Table 2**). This was consistent with the reports of Chutimanukul et al. (2018a,b), who examined salt tolerance in rice at the seedling and vegetative stages. Salt-tolerant rice varieties can maintain their photosynthetic ability after a short period of salt stress (Moradi and Ismail, 2007); however, shoot biomass may decrease (Bhowmik et al., 2009; Krishnamurthy et al., 2009). In the present study, we documented higher stomatal conductance in CSSL16 than in "KDML105," which may have contributed to the higher net photosynthetic rate observed in CSSL16 (**Figures 1B,D**). Robinson (1988) reported that stomatal

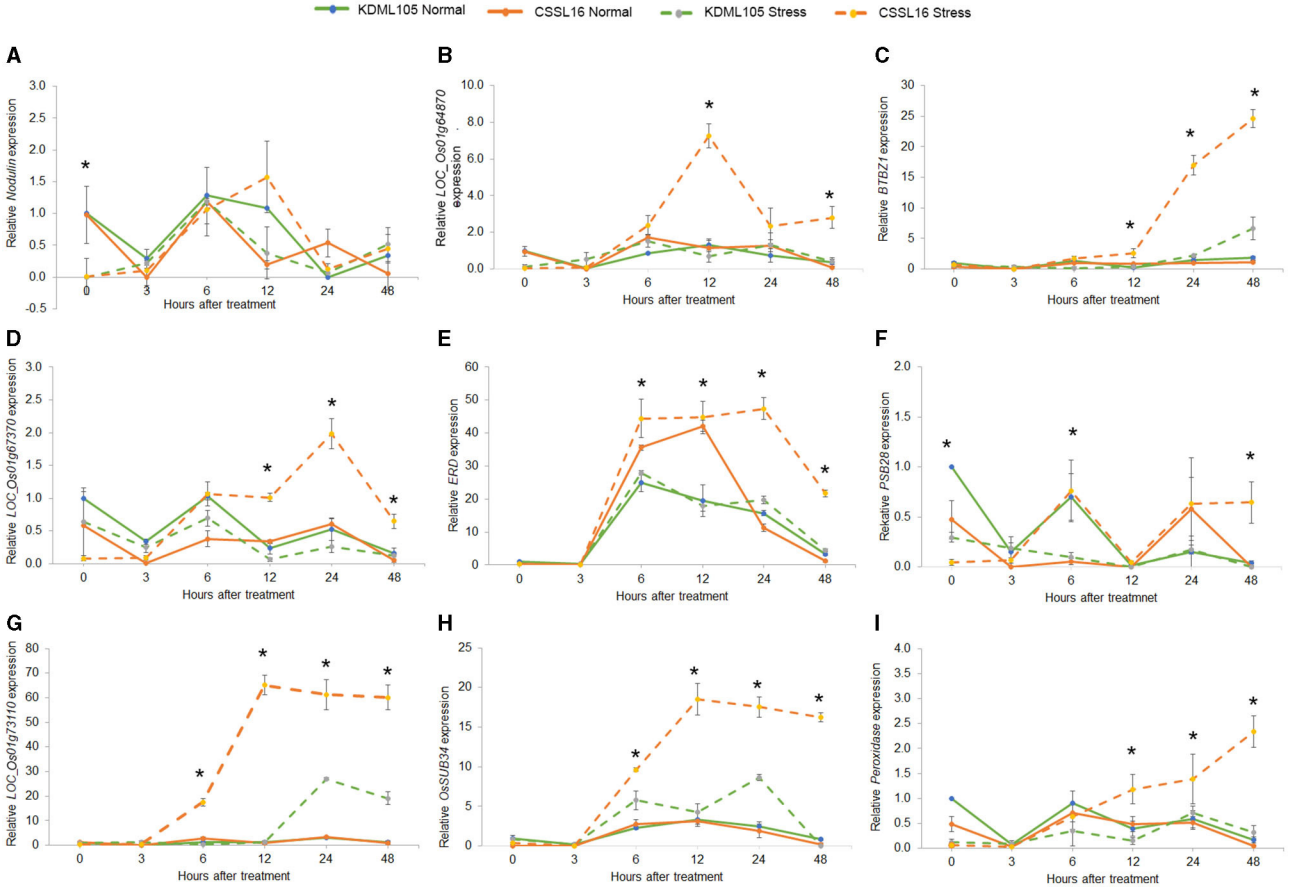


**FIGURE 9 |** Gene expression analysis of nine candidate genes, *Nodulin* **(A)**, *Os01g64870* **(B)**, *BTBZ1* **(C)**, *Os01g67370* **(D)**, *ERD* **(E)**, *PSBS28* **(F)**, *Os01g73110* **(G)**, *Sub34* **(H)**, and *Peroxidase* **(I)** in CSSL16 and KDML105 under normal and salt stress conditions after 0, 3, 6, 9, 12, 24, and 48 h of salt stress. *indicates the significant difference among mean of the gene expression at $p < 0.05$.

conductance and transpiration rate adaptation were the most important mechanisms for salt tolerance. Although the *Pn* of the second leaves of CSSL16 was lower than the *Pn* of the second leaves of KDML105, the tiller number per plant and filled grain number of CSSL16 were higher than those of KDML105 after salt stress. These results suggest that photosynthetic activity in the flag leaves contributed more to grain filling than that of the second leaves. However, salt stress during the booting stage did affect the overall yield of the rice lines (**Table 2**).

Studies in various plant species have shown that salt stress results in a decrease in $F_v/F_m$ (Huang et al., 2014; Martins et al., 2020; Sun et al., 2021). A reduction of $F_v/F_m$ can be used as an indicator of photo-inhibition in stressed plants (Hichem et al., 2009). In the present study, the $F_v/F_m$ values of the flag leaves were unaffected by salt stress at the booting stage. Lisa et al. (2011) reported an increase in the expression of photosynthesis-related genes in salt tolerant rice cultivars. In the present study, photosynthesis was sustained in the CSSL16 at the vegetative stage under salt stress and this may be due to the higher expression of the *PsbS1* gene encoding the chlorophyll binding protein in photosystem II (Chutimanukul et al., 2018b). Contrarily, the "KDML105" rice had the lowest *Pn*, suggesting that it was the most susceptible compared with the other lines. Pi refers to the quantum efficiency of primary photochemistry, the concentration of reaction centers, and excitation energy conversion in electron transport (Melis, 1999; Strasser et al., 2000). At the booting stage, CSSL14 and CSSL16 had higher Pi values under salt stress (**Figure 3F**), indicating that they were able to maintain the quantum efficiency of primary photochemistry.

A comparison of the three methods of transcriptomic analysis showed that WGCN identified the highest number of salt tolerance candidate genes, while CC identified the lowest number of candidate genes. Among the 92 genes identified by the three methods, nine genes contained SNPs in CSSL16 and KDML105. The expression level of the nine genes was different in CSSL16 and KDML105, consistent with the notion that they may be involved in regulating salt tolerance. Moreover, seven of the genes were located in the salt tolerance QTL reported by Kanjoo et al. (2012) (**Figure 1**).

The expression analysis of these nine genes within 48 h (**Figure 8**) showed much higher induction in *OsBTBZ1*, *OsERD*, *LOC_Os01g73110*, and *OsSUB34* genes, when compared to the

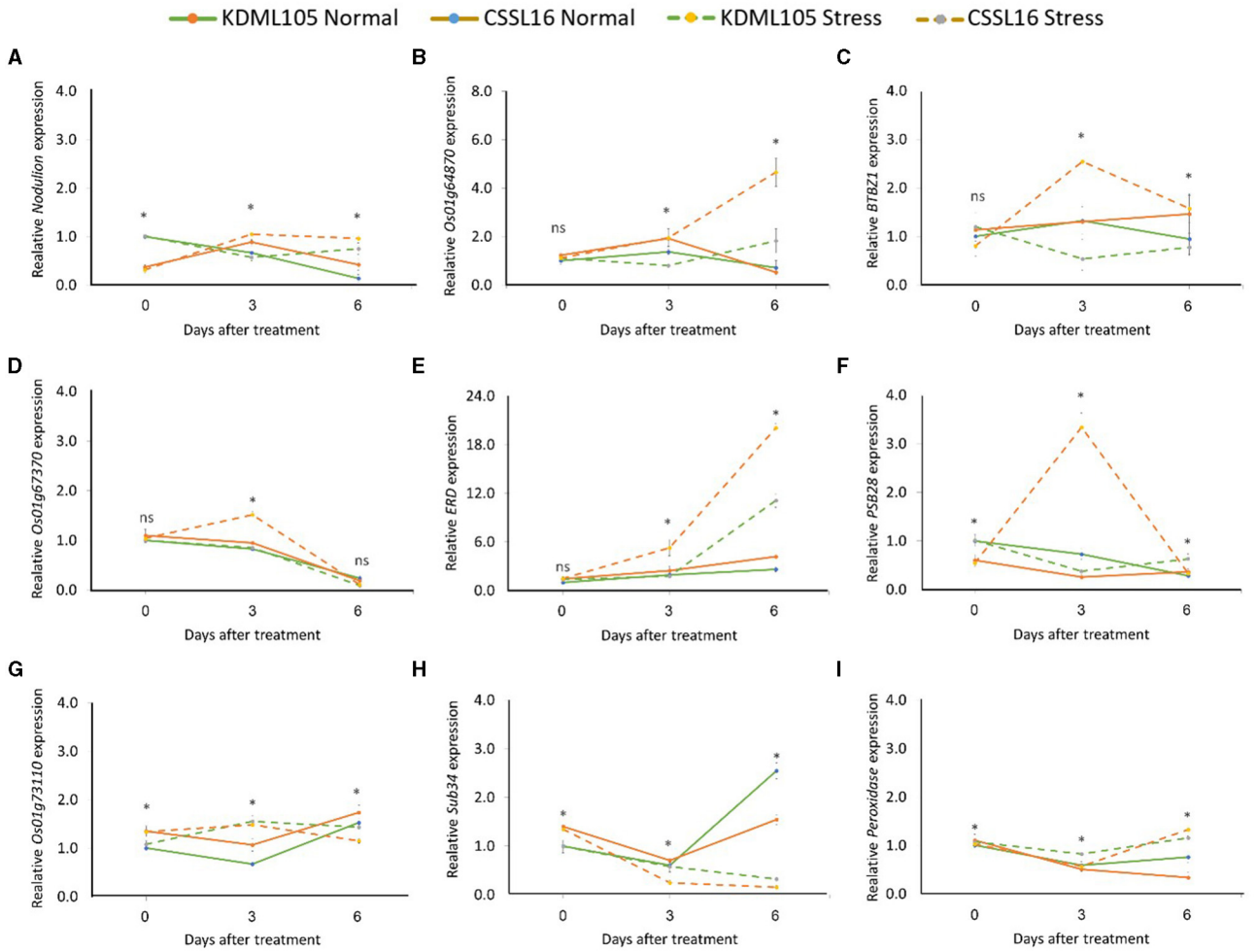


**FIGURE 10 |** Gene expression analysis of nine candidate genes, *Nodulin* **(A)**, *Os01g64870* **(B)**, *BTBZ1* **(C)**, *Os01g67370* **(D)**, *ERD* **(E)**, *PSBS28* **(F)**, *Os01g73110* **(G)**, *Sub34* **(H)**, and *Peroxidase* **(I)** in CSSL16 and KDML105 under normal and salt stress conditions at day 0, 3, and 6 of salt stress. *indicates the significant difference among mean of the gene expression at $p < 0.05$.

expression at later stages (**Figure 9**), suggesting that these four genes may function in the early response to salt stress. Therefore, we have tried to investigate the roles of these genes in salt stress tolerance by using the Arabidopsis mutant with T-DNA insertion in these orthologous genes. Unfortunately, we cannot obtain homozygous of Arabidopsis mutant with T-DNA insertion in *ERD4* at this moment. We also investigate the Arabidopsis mutant with T-DNA insertion in *PSB28* and *Per3* gene. The decrease in photosynthetic pigments and changes in dry weight response in the mutant lines support the role of the genes in salt tolerance.

Some of the nine genes were reported to be involved in stress responses. *LOC_Os01g61010 (Nodulin)* encodes a member of a family of highly conserved proteins involved in regulating membrane transporters. Wallace et al. (2006) found that *Nodulin* contributed to water permeability under osmotic stress in soybean. Moreover, *Nodulin* stimulated phosphorylation to regulate the process of cellular transport during osmotic adaptation in soybean exposed to salt or drought stress (Guenther et al., 2003). *LOC_Os01g73110* has not been characterized. However, Sircar and Parekh (2015), who investigated the function of *LOC_Os01g73110* using the AraNet and RGAP database identified its homolog in Arabidopsis as *AT5G45310,* whose product is involved in the biosynthesis of abscisic acid (ABA). *LOC_Os01g67370* Arabidopsis ortholog, *AT3G59300*, encodes a pentatricopeptide-repeat (PPR) superfamily protein. Some PPR proteins in Arabidopsis have been associated with abiotic stress responses, including oxidative stress and ABA responses (Liu et al., 2016). *PSB28* was found to be associated with photosystem II reaction center and water splitting in light-dependent reactions. Suorsa and Aro (2007) reported the molecular function of *PSB28*. The *PSB28* rice mutant identified from the T-DNA insertion population exhibited a pale green plant (Jung et al., 2008). The expression of *PSB28* was reduced under water stress and heat stress in tomato seedlings (Zhang et al., 2018) and *Populus tomentosa* (Ren

**TABLE 5 |** Dry weight per plant, chlorophyll *a*, chlorophyll *b* and carotenoid contents of 14 day-old Col-0 wild type, *bt3*, *sbt3.3*, *sbt3.4*, *at5g45310*, *psb28*, and *per3* mutants grown in MS medium or MS medium supplemented with 100 mM NaCl for 7 days.

| | Line | Dry weight* (mg/pl) | Chlorophyll *a** (μg·mg$^{-1}$ FW) | Chlorophyll *b** (μg·mg$^{-1}$ FW) | Carotenoid* (μg·mg$^{-1}$ FW) |
|---|---|---|---|---|---|
| Normal | Col-0 | 0.471 ± 0.062$^{de}$ | 0.482 ± 0.039$^{b}$ | 0.179 ± 0.028$^{ab}$ | 0.149 ± 0.001$^{a}$ |
| | *bt3* | 0.379 ± 0.023$^{f}$ | 0.378 ± 0.022$^{d}$ | 0.203 ± 0.003$^{a}$ | 0.138 ± 0.006$^{bc}$ |
| | *sbt3.3* | 0.496 ± 0.020$^{d}$ | 0.525 ± 0.028$^{a}$ | 0.189 ± 0.036$^{ab}$ | 0.177 ± 0.003$^{a}$ |
| | *sbt3.4* | 0.667 ± 0.089$^{b}$ | 0.418 ± 0.011$^{c}$ | 0.166 ± 0.004$^{b}$ | 0.133 ± 0.007$^{bc}$ |
| | *at5g45310* | 0.467 ± 0.039$^{de}$ | 0.480 ± 0.006$^{b}$ | 0.164 ± 0.005$^{b}$ | 0.161 ± 0.001$^{ab}$ |
| | *psb28* | 0.604 ± 0.072$^{c}$ | 0.512 ± 0.016$^{ab}$ | 0.180 ± 0.002$^{ab}$ | 0.117 ± 0.005$^{cd}$ |
| | *per3* | 0.704 ± 0.098$^{ab}$ | 0.354 ± 0.033$^{d}$ | 0.105 ± 0.005$^{c}$ | 0.102 ± 0.016$^{d}$ |
| Salt stress | Col-0 | 0.416 ± 0.023$^{ef}$ | 0.222 ± 0.009$^{ef}$ | 0.068 ± 0.007$^{d}$ | 0.070 ± 0.008$^{e}$ |
| | *bt3* | 0.147 ± 0.016$^{g}$ | 0.077 ± 0.013$^{h}$ | 0.031 ± 0.02$^{c}$ | 0.044 ± 0.021$^{ef}$ |
| | *sbt3.3* | 0.518 ± 0.015$^{d}$ | 0.213 ± 0.036$^{ef}$ | 0.116 ± 0.05$^{c}$ | 0.030 ± 0.046$^{gh}$ |
| | *sbt3.4* | 0.758 ± 0.02$^{a}$ | 0.231 ± 0.010$^{ef}$ | 0.107 ± 0.008$^{c}$ | 0.038 ± 0.005$^{gh}$ |
| | *at5g45310* | 0.469 ± 0.015$^{de}$ | 0.199 ± 0.033$^{f}$ | 0.088 ± 0.004$^{cd}$ | 0.058 ± 0.015$^{ef}$ |
| | *psb28* | 0.667 ± 0.095$^{b}$ | 0.243 ± 0.012$^{e}$ | 0.099 ± 0.011$^{cd}$ | 0.045 ± 0.008$^{ef}$ |
| | *per3* | 0.713 ± 0.04$^{ab}$ | 0.162 ± 0.017$^{g}$ | 0.120 ± 0.007$^{c}$ | 0.015 ± 0.004$^{h}$ |

*The values are shown in mean ± SD with three replicates. Each replicate contains 20 plants. The different letters above value represent the significantly different among means compared in the same column at $p < 0.05$.

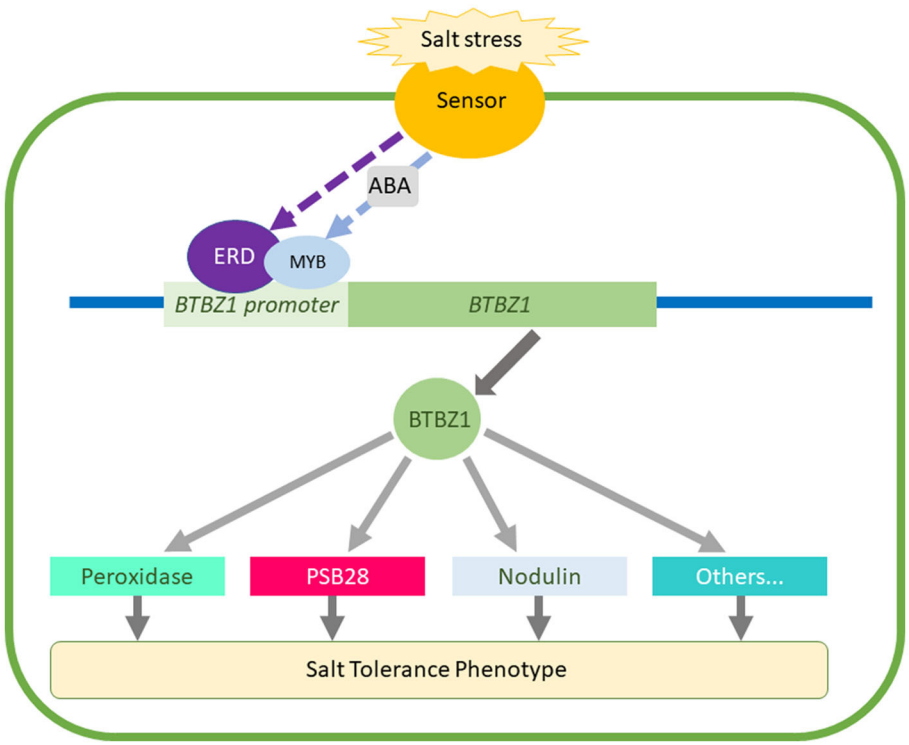


**FIGURE 11 |** Hypothetical model for the function of the predicted genes obtained from the combining of genome and gene co-expression network analysis.

et al., 2019), respectively. Moreover, Kosmala et al. (2009) found that expression of the *PSB28* gene responded to cold stress in *Festuca pratensis*. These results indicated that PSII and PSI were suppressed under stress conditions. Consequently, the accumulation of *PSB28* might enhanced the electron transport rate and photochemical efficiency.

*OsSub34* encodes a subtilisin protein associated with serine peptidase. Subtilisin contributes to plant responses under biotic and abiotic stress, organ abscission, senescence, and programmed cell death (Schaller et al., 2018). In rice, *LOC_Os06g46799* encodes a peroxidase precursor that is highly responsive to various abiotic stress stimuli and plays an important role in

the regulation of reactive oxygen species (ROS) by converting $H_2O_2$ to water (Hiraga et al., 2001). Hiraga et al. (2001) identified a group of genes that encodes redox regulation-related proteins, including ascorbate peroxidase, peroxidase precursor, glutathione synthetase, and glutathione S-transferase, in rice exposed to drought stress. Moreover, Chutimanukul et al. (2019) reported that CSSL16 had higher peroxidase activity than that did KDML105 under salt stress at the seedling stage, which supports the role of *LOC_Os06g46799* in the present study.

*BTBZ1* and *ERD* are proposed to be the genes with the highest correlation with salt tolerance in the rice lines, as both were predicted by three methods of gene co-expression network analysis. Additionally, *BTBZ1* and *ERD* contained SNPs in CSSL16 and KDML105 and both genes were located in the salt/drought QTL previously identified by Kanjoo et al. (2011). Consistent with a joint requirement for both genes for optimal stress tolerance, CSSL10 and CSSL14 carry, respectively, either the *BTBZ1* or the *ERD* allele of DH212 and neither displays the full tolerance phenotype of CSSL14. *BTBZ1* belongs to the Bric-A-Brac/Tramtrack/Broad Complex (BTB) protein superfamily (subfamily C1) and contains a TAZ zinc finger and calmodulin-binding domain. The homologous gene in Arabidopsis, *AtBT1*, encodes a nuclear CaM-binding protein. The expression of *AtBTs* can be triggered by stress stimuli (Du and Poovaiah, 2004). BTB-ZF proteins are known as the POK, POZ, and Krüppel zinc finger proteins (Deweindt et al., 1995). Moreover, Stogios et al. (2005) reported that the *BTB* domain is a protein-protein interaction motif that is involved in cellular functions, including transcriptional regulation, cytoskeleton dynamics, ion channels, and targeting proteins for ubiquitination. Moreover, *BTB-ZF* genes constitute a supergene family encoding proteins that are thought to be transcription factors. Additionally, the analysis of protein-protein interactions from the Predicted Rice Interactome network (PRIN) indicated that the *BTBZ1* protein interacted with a cullin protein (*LOC_Os02g51180*), which may be involved in the degradation of the target protein through the ubiquitin/proteasome pathway (Figueroa et al., 2005). Several reports have described the important role of BTB proteins in developmental programs, defense, and abiotic stress responses (Weber and Hellmann, 2009; Prasad et al., 2010). Nutrient, stress, and hormone responses were regulated by *AtBT2* in Arabidopsis (Mandadi et al., 2009). However, an ortholog of the *BTBZ* gene in Arabidopsis (AT1G05690) was involved in plant development (Robert et al., 2009).

*ERD* was associated with early response to dehydration, which could be rapidly induced during drought stress and other abiotic stresses. *ERD* is a member of a large gene family, whose protein products are associated with triphosphate (ATP)-dependent proteases, heat shock proteins (HSPs), membrane proteins, proline, sugar senescence-related genes, chloroplasts, biosynthesis, protein transporters, dehydrogenase, and ubiquitin extension proteins (Kiyosue et al., 1994; Taji et al., 1999; Simpson et al., 2003). Borah et al. (2017) reported that "Dhagaddeshi rice," a drought-tolerant cultivar, had higher expression levels of *ERD1* and responded faster than the susceptible cultivar (IR20) to drought stress. Moreover, Liu et al. (2009) found that the *ERD4* gene played a key role in the adaptation of maize to the early stages of stress and enhanced the plant's tolerance to abiotic stress conditions. In transgenic tobacco, the overexpression of *ERD15* increased the efficiency of PSII ($F_v/F_m$) through the protection of cellular membranes (Ziaf et al., 2011). Additionally, transgenic Arabidopsis plants overexpressing the *BjERD4* gene from *Brassica juncea* displayed increased tolerance to salt stress and drought, while the *Bjedr4* knockdown lines were susceptible to salt and drought stress (Rai et al., 2016). Therefore, *ERD* may contribute to salt tolerance in rice.

The *bt3* Arabidopsis mutant showed the highest reduction in growth and photosynthesis pigment content, while in rice, more than 20-fold induction of *OsBTBZ1* gene was detected after 48 h of salt stress treatment. This is consistent with the *cis*-regulatory elements found in the putative *OsBTBZ1* promoter (**Supplementary Figure 4**), which include 3 ABREs, 5 MYB binding sites, and 16 MYC binding sites. Many MYB proteins regulate salt tolerance through regulation of the ABA signaling pathway (review: Wang et al., 2021). Both MYB and MYC proteins function as the transcriptional activators in ABA signaling in Arabidopsis (Abe et al., 2003). Together with this literature information our finding support an *OsBTBZ1* contribution to salt tolerance phenotype of CSSL16. The upstream region of *OsBTBZ1* consists of multiple *ERD* binding sites. We identified *OsERD* as one of the key genes because it was highly induced prior to *OsBTBZ1* (45-fold induction) in CSSL16, while it was up-regulated only 25-fold in KDML105. Therefore, the interaction between *OsBTBZ1* and *OsERD* and their involvement in salt tolerance in rice should be further characterized.

Due to insertion and base substitution in the putative promoter region of *OsBTBZ1* in KDML105, GAGA binding site was detected only in CSSL16. In Arabidopsis, bHLH34 binds to GAGA element and is involved in ABA and salinity response (Min et al., 2017). Moreover, rice Trithorax factor ULTRAPETALA 1 (OsULT1) was found to bind the promoter region of the *OsDREB1b* gene during transcriptional activation. The binding of OsULT1 to GAGAG elements decreases tri-methylation of lysine 27 on histone H3 (H3K27me3), which antagonizes the transcriptional repression effect of H3K27me3, favoring transcriptional activation of the gene (Roy et al., 2019). A similar phenomenon may occur in the regulation of *OsBTBZ1* leading to the higher expression in CSSL16 than KDML105.

Considering all our findings, we hypothesize that the predicted key regulatory genes in the network reported here coordinate a response that makes the rice plants more tolerant to salt stress. The earlier and higher expression of *LOC_Os01g64870*, *OsBTBZ1*, *LOC_Os01g67370*, *OsERD*, *LOC_Os01g73110*, *OsSUB34*, and *OsPeroxidase* in CSSL16 leads to higher salt tolerance when compared to KDML105. Further investigations should be performed to validate this hypothesis in the future. Based on the ERD binding site in *OsBTBZ1* putative promoter, we hypothesize that the ERD protein regulates *OsBTBZ1* gene expression and regulates other genes such as PSB28, and Peroxidase. The proposed model for this hypothesis is shown in **Figure 11**.

According to the comparison of the predicted alleles from chromosome 1 of DH212 in CSSLs, 3 candidate alleles

from DH212, *Nodulin* (*LOC_Os01g61010*), *LOC_Os01g64870*, and *BTBZ1* (*LOC_Os01g66890*) are located in CSSL10, while CSSL14 contains another 3 candidates from DH212, which are *PSB28* (*LOC_Os01g71190*), *ERD* (*LOC_Os01g72210*), and *LOC_Os01g73110*. The salt tolerance phenotype of CSSL16 was significantly higher than CSSL10 and CSSL14 in all stages, seedling (Chutimanukul et al., 2018a, 2019), vegetative (Chutimanukul et al., 2018b) and booting stages. Therefore, we explicitly propose that the whole QTL in this region is necessary for salt tolerance in rice.

## CONCLUSION

In the present study, we demonstrate an effective transcriptomic approach for identifying genes regulating salt tolerance in rice using two rice lines with close genetic relationships, but different salt tolerance ability. Combining GCN, CC, and WGCN analyses with available SNP information, we identified nine genes involved in salt tolerance in rice. Under salt stress, the expression levels of the nine genes differed in the two rice lines. Moreover, most of the genes were involved in abiotic stress responses. Therefore, we can conclude that the combination of the three methodologies for transcriptome analysis, GCN, CC, and WGCN with SNP information is an effective approach for the identification of genes involved in abiotic stress tolerance and it can support the identification of appropriate QTL for salt tolerance improvement.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

SC, LC, TT, and KP: study conception and design. PC, PM, MS, and TBS: data collection. PC, TBS, PM, KP, SC, and TB: analysis and interpretation of results. PC, TB, KP, LC, and SC: draft manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.704549/full#supplementary-material

**Supplementary File 1 |** Differentially Expressed Genes in seedling leaves, flag leaves, second leaves.

**Supplementary Figure 1 |** The gene set enrichment analysis of differentially expressed genes in seedlings when compared between the transcriptome of seedlings grown under normal (0 day) condition and after 75 mM NaCl treatment for 2 days in term of biological process by using ClueGO tool.

**Supplementary Figure 2 |** The gene set enrichment analysis for biological process of differentially expressed genes when compared between the transcriptome of second leaves from rice plants grown under normal (0 day) condition and after 75 mM NaCl treatment for 3 days at booting stage with ClueGO tool.

**Supplementary Figure 3 |** The gene set enrichment analysis for biological process of differentially expressed genes when compared between the transcriptome of flag leaves from rice plants grown under normal (0 day) condition and after 75 mM NaCl treatment for 3 days at booting stage with ClueGO tool.

**Supplementary Figure 4 |** The putative regulatory element analysis of *OsBTBZ1* gene from CSSL16 and KDML105.

**Supplementary File 2 |** The number of differentially expressed genes in leaves at seedling stage, second leaf, and flag leaf at booting stage under salt stress condition.

**Supplementary File 3 |** List of significant candidate genes identified by gene co-expression network, clustering co-efficient and weighted gene co-expression network.

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# Improvement of Phosphorus Use Efficiency in Rice by Adopting Image-Based Phenotyping and Tolerant Indices

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Phosphorus is one of the second most important nutrients for plant growth and development, and its importance has been realised from its role in various chains of reactions leading to better crop dynamics accompanied by optimum yield. However, the injudicious use of phosphorus (P) and non-renewability across the globe severely limit the agricultural production of crops, such as rice. The development of P-efficient cultivar can be achieved by screening genotypes either by destructive or non-destructive approaches. Exploring image-based phenotyping (shoot and root) and tolerant indices in conjunction under low P conditions was the first report, the epicentre of this study. Eighteen genotypes were selected for hydroponic study from the soil-based screening of 68 genotypes to identify the traits through non-destructive (geometric traits by imaging) and destructive (morphology and physiology) techniques. Geometric traits such as minimum enclosing circle, convex hull, and calliper length show promising responses, in addition to morphological and physiological traits. In 28-day-old seedlings, leaves positioned from third to fifth played a crucial role in P mobilisation to different plant parts and maintained plant architecture under P deficient conditions. Besides, a reduction in leaf angle adjustment due to a decline in leaf biomass was observed. Concomitantly, these geometric traits facilitate the evaluation of low P-tolerant rice cultivars at an earlier stage, accompanying several stress indices. Out of which, Mean Productivity Index, Mean Relative Performance, and Relative Efficiency index utilising image-based traits displayed better responses in identifying tolerant genotypes under low P conditions. This study signifies the importance of image-based phenotyping techniques to identify potential donors and improve P use efficiency in modern rice breeding programs.

**Keywords: rice, phosphorus use efficiency, image J, geometric traits, breeding**

## INTRODUCTION

Phosphorus, a macronutrient placed in phosphorus (P) block, maintains overall plant morphometry, regulates metabolic processes, and is a fundamental element of several essential biomolecules (nucleic acid, ATP, NADPH, and phospholipids) involved in reproduction, pointing to its indispensability in crop growth and development. However, its less solubility and very low

concentration in the soil rhizosphere of about 0.05–0.3 μg P $ml^{-1}$ (Bolan, 1991) severely limit crop yield. The phosphorus use efficiency (PUE) of rice is only about 25% (Dobermann and Frairhurst, 2000), and it absorbs 1.07 M tonnes of $P_2O_5$ at the rate of 24.3 kg $P_2O_5$ $ha^{-1}$ alone, providing enormous scope for its development. Meanwhile, the report suggests that India demands 19% of the global production of phosphate fertiliser (Tirado and Allsopp, 2012), bagging its limelight. The chelation of P with Fe and Al in acidic; Ca in alkaline soil and immobilised in fine textured clay-loam soil intensifies P deficiency. Moreover, statistics shows that 20 million hectares of world rice cultivation area are Pi-deficient (Neue et al., 1990), and that 61.02% of Indian soil is low in P (Muralidharudu et al., 2011). To alleviate this issue, the idea of developing P use-efficient rice cultivars has been introduced as an efficient strategy, especially in India, to reduce the cost of production, import demand, and effect of eutrophication (Mahender et al., 2017).

In rice, screening strategies for the identification of genotypes with low P tolerance or improved P acquisition ability the plant need to be harvested or destroyed at certain stages and evaluated based on several parameters, such as relative tillering ability (RTA), shoot and root biomass production, and root architectural response, such as root hair, axial root angle, elongation of lateral roots with high specific root length value under low P conditions (Zhu and Lynch, 2004; Vandamme et al., 2013). Among these traits, those of root is the critical aspect for improving crop performance *via* improving P acquisition (Richardson et al., 2011; Rose et al., 2013a,b). However, observing root traits is a labour-intensive, time-consuming process that only includes selective traits. It provides inaccurate data as it depends on means of subsets of plants at each harvest rather than follows the growth trajectories of individuals (Berger et al., 2013). To overcome this difficulty, the advent of proximal sensing technology allows for critical non-destructive support in measuring performance and predicting crop yield under controlled and field environments (Araus and Cairns, 2014; Araus and Kefauver, 2018). A non-invasive technique, such as high-throughput image-based phenotyping, e.g., visible imaging or RGB (red-green-blue) imaging, has a potential application, as it screens the varieties at earlier stages, covering crucial parameters such as vegetative mass associated with important traits such as grain yield in cereals (Ghamkhar et al., 2019; Anandan et al., 2020). It allows the measurement of dynamic changes in the plant form over time and quantifies based on multiple parameters such as tillering, leaf area index, leaf angle, and convex hull (Anandan et al., 2020).

Consequently, the imaging technique is gaining momentum among researchers to screen genotypes for several biotic and abiotic factors such as salinity, nitrogen, water deficiency, nodal root angle, and early seedling vigour in barley, rice, and sorghum (Araus et al., 2012; Crowell et al., 2014; Atkinson et al., 2015; Turner et al., 2018; Narisetti et al., 2019; Wu et al., 2019; Anandan et al., 2020). However, the phenotypic screening of rice genotypes under low phosphorus conditions is not established. In this study, we followed a systematic phenotypic strategy of destructive and non-destructive image-based phenotyping methodologies applied to examine the complex of genotype × phenotype × environment interactions in a low phosphorus regime at an early stage, which, to the knowledge of the authors, is the very first case study. Initially, to understand the plant response to low P using manual morphometric traits, 65 popular improved rice varieties were screened, and selected rice genotypes were evaluated to identify the traits through destructive (morphology and physiology) and non-destructive (geometric characteristics by imaging) techniques. The screening methodologies in both experiments were helpful in identifying the tolerance genotypes, and used the derivative index traits for low P tolerance, which is demonstrated here. This approach is most suitable for low P tolerance and improving the PUE in rice breeding programs. Based on this rationale, this experiment was set to (i) differentiate rice genotypes by exploring all possible physiological and geometric (non-destructive) traits by multivariate analysis, (ii) identify geometric characteristics by imaging and with associated morphological traits to use them as a surrogate in the absence of an imaging system, and (iii) demonstrate different adaptive mechanisms involved in low P tolerance.

# MATERIALS AND METHODS

## Plant Materials

Sixty-five popular rice genotypes developed for Odisha province and three checks (Dular, Kasalath, and IC459373) (**Supplementary Table 1**) were collected from Regional Research and Technology Transfer Station (RRTTS), Coastal zone, Bhubaneswar; Orissa University of Agriculture and Technology (OUAT); and ICAR-National Rice Research Institute (NRRI), Cuttack, Odisha.

## Experiment-1: Soil-Based Screening

This experiment was conducted using a cemented tank (1 m × 10.29 m × 2.3 m) at NRRI, Cuttack (20°27'09" N, 85°55'57" E, 26 msl), Odisha, containing low P soil (<3 kg/ha; 0–15 cm layer, pH 4.9) collected from Central Farm, OUAT, Bhubaneswar, Odisha. Before sowing, the seeds of all the genotypes were heat-treated to break seed dormancy in a hot-air oven at 50°C for 45 h. The experiment was conducted in January 2019 for 45 days (day/night temperature of 23.5/16°C, RH ∼70%, bright sunlight) without the addition of any fertiliser. Seeds of the 68 genotypes were directly sown 15 cm apart in a 1-m long row with a spacing of 20 cm between rows in a randomised block design with three replications. The uniform plant population of two seedlings per hill was maintained across genotypes by thinning on the 14th day after sowing, and irrigated on alternate days until the end of the experiment. For post-uprooting of plants, on the 45th day, morphometry traits such as shoot length (cm), maximum root length (cm), number of tillers per plant, root dry weight (g), shoot dry weight (g), percent dry matter partitioning for root, and root: shoot ratio (based on length) were taken into consideration for the identification of genotypes and for the subsequent hydroponic experiment.

## Experiment-2: Hydroponic Study

From experiment 1, 18 genotypes (**Supplementary Table 1**) were selected based on the level of tolerance from all quarters

of principal component analysis (PCA) to evaluate further under hydroponics and identify the traits through destructive (morphology and physiology) and non-destructive (geometric traits by imaging) techniques in a low phosphorus regime. Uniform size seeds of respective genotypes were handpicked carefully to avoid admixture and heat-treated in the hot air oven at 50°C for 45 h to break the seed dormancy. Subsequently, surface sterilisation with 75% ethanol for 1 min and 2.5% sodium hypochlorite for 20 min (NaClO) was performed. To remove the traces of the sterilising agent, the seeds were washed four to five times in sterile distilled water for several minutes. Two seeds of each genotype were placed on Styrofoam fixed with mesh. The Styrofoam containing seeds was placed in a plastic tray containing 10 L of tap water for 3 days in the dark to promote germination. Later, the Styrofoam with well-germinated and healthy seedlings was transferred to a tray containing full strength of Yoshida solution (10 L/tub) (Yoshida et al., 1976) as control. Parallel to the control, another set of genotypes was placed in Yoshida solution containing 0.5 ppm (deficient P) of $NaH_2PO_4.H_2O$. The experiment was performed with three replications for 35 days. The pH of the nutrient solution was adjusted between 4.5 and 4.55 on an alternate day, and tap water was added to compensate for the loss in the solution. Once every 7 days, a new solution was used with respective concentrations by maintaining the same pH.

## Sample Collection and Data Observation by Destructive and Non-destructive Technique

On the penultimate day of the experiment, SPAD (SPAD-502, Konica Minolta, Tokyo, Japan) value was observed on the fourth leaf from the bottom to measure chlorophyll concentration. The plants were separated from Styrofoam carefully to record the plant images 36 days after sowing (DAS). Photographs of three plants per genotype of three biological replicates were taken, and 10 plants were used to measure the morphometric data to estimate growth parameters on the same day. The images were captured using a 12-megapixel Nikon camera (Nikon, Tokyo, Japan) at 1.5 m with high precaution. The plants were imaged under a high-intensity artificial light source inside a closed chamber. To calculate the whole plant area (WPA), a ruler was placed near the plants with proper labelling. The images were recorded from three different angles; front and backside views (at 90°) and top view, which fall in the visible range or the so-called RGB spectrum (400–700 nm). Uniformity was maintained in all aspects, such as light intensity and distance from plants, throughout the process.

The recorded images were analysed for geometric traits using the open-source Image J software. Briefly, excess areas on all four sides of the selected images were cropped, and only potted plants were composed of one object. A straight line with known distance was drawn over the ruler for further image analysis. The plant images were then separated from the background with the colour threshold system. To measure individual or whole plant area, the greenness of the plant was determined by converting RGB images into Hue Saturation and Brightness (HSB) system. Using a known scale, the individual or whole plant area was interchanged from pixels to $mm^2$. The summed area of all three images (top and two side views) was used to estimate the whole plant area (WPA) and expressed in square millimetres.

Additionally, other geometric traits such as convex hull (the smallest possible mathematically solved perimeter that envelopes the imaged plant), calliper length (the longest dimension of the canopy when viewed from above), minimum enclosing circle (MEC) (the minimum circle that can enclose the plant), and eccentricity (the degree of radial symmetry) were measured from images of the top view (Neilson et al., 2015; Anandan et al., 2020), while leaf inclination at all levels (adaxial side) was analysed using Image J from the image taken from the front view (**Figure 1**). To measure the leaf inclination, the stem was considered as the vertical axis. The leaf blade inclined from the lamina joint junction was noted, and the angle was drawn between the two points (leaf blade and lamina joint) to determine the angle (**Figure 1**).

The same plants and additional seven plants per replication of each genotype were used to observe morphological traits such as shoot length (cm), maximum root length (cm), number of roots, number of leaves $plant^{-1}$, number of $tillers^{-1}$, shoot dry weight (g), stem dry weight (g), root dry weight (g), and individual leaf weight (g) at respective positions. The first formed leaf from base was counted as the first leaf, and successive leaves were numbered accordingly. Besides, root traits such as total root length (cm), projected root area ($cm^2$), root surface area ($cm^2$), average root diameter (mm), root volume ($cm^3$), and the number of root tips, were determined from three plants per biological replicate using WinRHIZO$^{TM}$ (Régent Instruments Inc., 2013, Quebec City, Canada).

The dry weight of shoot, root, stem, and leaves was taken after drying the detached part in a hot air oven at 60°C for 5 to 6 days until the samples were dried completely. Thereafter, the dried samples were finely ground, and around 300 mg shoot and 90 mg root samples were used for total P quantification following the ternary acid method. To quantify P from the shoot and root, samples were taken in a digestion tube and kept in the digestion unit for 1:45 to 2:15 h at 150 to 170°C for digestion in the ternary acid mixture (conc. $HNO_3$ + conc. $H_2SO_4$ + conc. $HClO_4$: 5:1:2). The digest P concentration was determined using Systronics (Gujarat, India) UV Spectrophotometer at 420 nm, and total shoot and root P contents were determined on an mg/g dry weight basis. Phosphorus utilisation efficiency of the shoot (PUE_S) and phosphorus utilisation efficiency of root (PUE_R) were calculated using the following formula.

$$\text{Phosphorus utilization efficiency (PUE)} = \frac{(\text{P content in control} - \text{P content in deficient conditions})}{\text{Phosphorus applied}}$$

## Statistical Analysis

Principal component analysis was performed with 68 rice genotypes for seven traits to identify the genotypes based on their performance under low P conditions. Initially, the mean data of all the traits were calculated. The PCA analysis was executed using the PCA function from the Facto Mine R package (Lê et al., 2008) in R version (3.6.3) (R Core Team, 2015). Among the 68

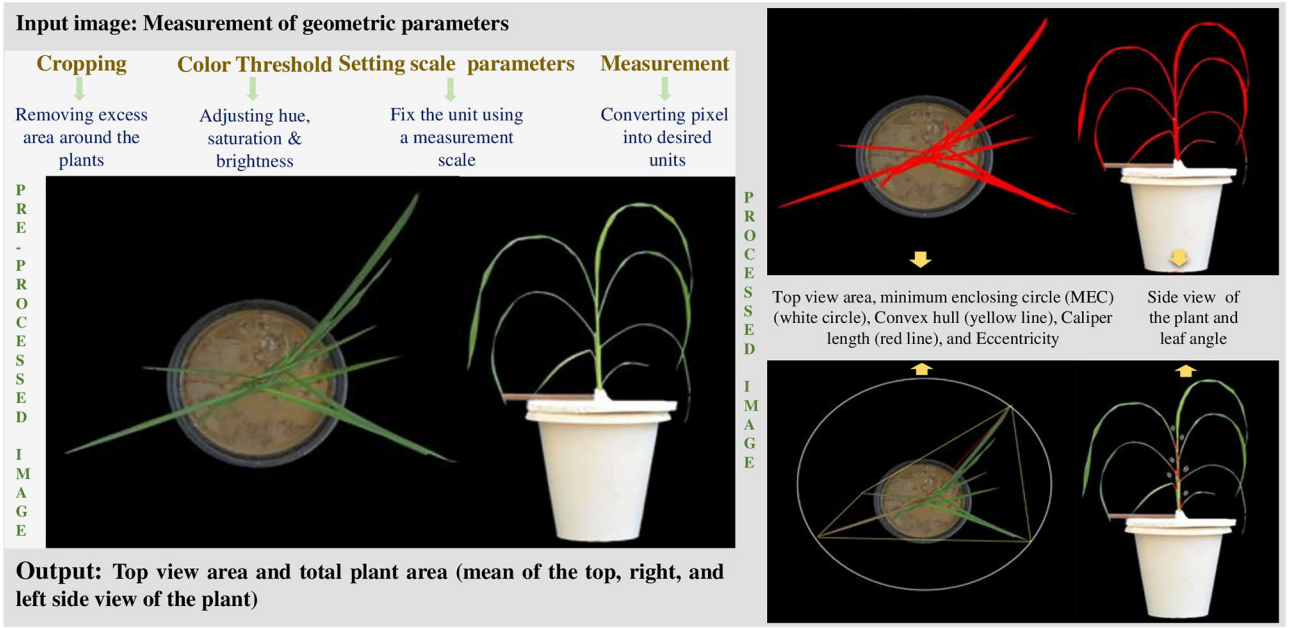


**FIGURE 1 |** Image processing and derivation of geometric parameters with representative plant image.

genotypes, 18 representative genotypes were selected based on their response under low P conditions and were further analysed under hydroponic conditions.

Descriptive statistics, ANOVA, variability and heritability analyses were performed for both control and P deficiency conditions for the selected 18 genotypes evaluated under hydroponic conditions to assess the effect of P-induced changes under deficient conditions using PBTools v1.4 (PBTools, 2014). The model used for ANOVA was

$$Yijk = \mu + \alpha i + \beta j + \gamma ij + \varepsilon ijk$$

where $\mu$ is the overall mean, $\alpha i$ is the effect of the $i$th genotype, $\beta j$ is the effect of the $j$th P concentration, $\gamma ij$ is the effect due to any interaction between the ith genotype, jith P is the concentration, and $\varepsilon ijk$ is the error. The genotypes and P levels were considered as fixed, while replicates and interactions were considered as random.

Broad sense heritability ($H^2$), for each trait in different concentrations of P, was estimated as

$$H^2 = \sigma^2 g/\sigma^2 p \times 100 \; and \; \sigma^2 p = \sigma^2 g + \sigma^2 e/r$$

where $\sigma^2$g = genotypic variances, $\sigma^2$p = phenotypic variances, $\sigma^2$e is the error variance, and r is the number of replications.

The variability in traits related to phosphorus deficiency tolerance was studied by principal component analysis on a matrix of 38 morphometric and geometric traits of the 18 genotypes. To examine the effect of low P on the different genotypes, the Pearson correlation among all the morphological and geometric traits was analysed using the corrplot functions from the corrplot package (Wei et al., 2017) in R. The clustering approach was executed separately for the genotypes and traits studied under P deficiency conditions. The output of the clustering technique would be useful to identify genotypes having similar phenotypic expression and traits that are similar under P deficiency conditions. To carry out the heatmap analysis, genotypes of both treatments are arranged in rows and traits in columns. The cluster heat map function (x, dendrogram = "both," scale = "row," method = "ward.$D^2$") of the R package made4 (Culhane et al., 2005) was used to identify the possible tolerance mechanisms for P deficiency using the 18 genotypes (both treatments) and 38 traits. In keeping with the rest of the functions, dendrogram = "none" was used to avoid phylogeny, and to understand the differential trait expression between treatments." Row Z-score scaling method was used to normalise the data of each row (trait). $Z = (x - \mu)/\sigma$, where x is the trait value, $\mu$ is the population means of the trait, and $\sigma$ is the population standard deviation. The colour spectrum (legend) illustrates the Z-score across the range of −2 to 2. A Z-score of '0' indicates that the trait value is identical to the mean, and values greater than the mean are categorised into +2 and vice versa.

## Contribution of Individual Plant Parts Towards Its Overall Development

It explains the nature of the contribution of the individual plant part, especially leaf in orchestrating plant growth and development, calculated using the following formulas:

- Contribution of the individual leaf for its own development = (individual leaf weight/total leaf weight) *100.
- Contribution of the individual leaf towards total development = (individual leaf dry weight/total dry weight) *100.

- Percentage partitioning of dry matter = (individual plant part/total dry weight) *100.
- Contribution of the leaf towards shoot development = (individual leaf dry weight/shoot dry weight) *100.
- Contribution of the leaf towards root development = (individual leaf dry weight/root dry weight) *100.

## Tolerance Indices

Several researchers have proposed screening indices to determine the level of tolerance among genotypes evaluated in controlled and stress environments (**Table 1**). These stress indices may be used as an indicator to identify tolerant genotypes that perform well under P deficiency conditions. In this study, the secondary trait that generates maximum variability from the above-mentioned statistical analysis was substituted in the indices to distinguish genotypes that perform well under deficient conditions.

# RESULTS

## Experiment 1

### Multivariate Analysis to Assess Low P-Tolerant Rice Genotypes in P-Deficient Soil

To generate a core set of low P tolerant genotypes for experiment 2, the growth and performance of 68 genotypes (**Supplementary Table 1**) were studied in P-deficient soil by PCA considering eight morpho-physiological parameters. Principal component 1 (PC1) and PC2 individually accounted for 53.6 and 20.9% of total variability among traits and individuals, respectively, representing a cumulative variance of 74.5%. PCA revealed that total biomass showed the highest degree of positive correlation to PC1, followed by root and shoot biomass. Similarly, root: shoot ratio (based on length) contributes maximally to PC2 followed by dry matter partitioning for root. Genotypes plotted on the right side of PCA of origin were found to exhibit better tolerance, as all the traits clustered together, showing maximum variation to these two components (**Supplementary Figure 1**). Conversely, genotypes belonging to –PC1 and +PC2 are sensitive, as no other traits possess variability. Based on the output of the PCA, 18 genotypes were selected from all quarters based on the superiority or inferiority of the traits such as root length, shoot dry weight, root dry weight, and shoot length. Traits such as root length (mean = 11.63 cm) shoot dry weight (mean = 0.157 g), root dry weight (mean = 0.066 g), and shoot length (mean = 22.46 cm) that exhibited maximum contribution in the PCA analysis were selected to identify genotypes for hydroponic experiment. The mean of the respective traits was considered as a threshold in the selection of the genotypes.

## Experiment 2

### Degree of Variation in Physio-Morphological Traits Under P Deficient and Sufficient Conditions

Morphological, physiological, and geometric traits of the 18 rice genotypes were studied under P sufficient (control) and deficient conditions. Analysis of variance reflected significant differences among the genotypes, P concentrations (C), and their interaction (G × C) for most of the studied parameters (**Table 2**). Among the morphological and physiological traits, shoot length, leaf number, maximum root length, number of roots, SPAD, dry biomass of third to seventh leaf angle, stem, shoot and root, shoot and root P content, and their utilisation efficiency significantly ($P < 0.01$) differed between genotypes under deficient, control, and interaction conditions. The geometric trait, convex hull, root volume, and root tips significantly differed at both genotype and P concentration, while, calliper length, minimum enclosing circle (MEC), and eccentricity had a significant difference only at the genotype level. However, tiller number, whole plant area (WPA), top view area (TVA), and third to seventh leaf angles were strongly influenced by P concentration.

The results revealed that traits other than leaf angle, genotype, and P concentration significantly influence most of the traits in comparison with G × C interaction. All these outcomes suggest that the genetic basis of the trait under study can be exploitable for a further breeding program. The G × C interaction provides deeper insight into the adaptability of genotypes in a deficient environment. Among the traits studied, genotype explained most variation (28–86%), while traits, namely, tiller number, leaf number, sixth, and seventh leaf weight, WPA, TVA, shoot and root P, fourth to sixth leaf angle, and PUE of shoot and root were explained by P concentration.

Descriptive statistics for 38 traits of the 18 genotypes under control and low P conditions are shown in

**TABLE 1 |** Tolerance and susceptibility indices formula used in this study.

| Index | Formula | Selection pattern | References |
|---|---|---|---|
| Mean Productivity Index (MPI) | MPI = $(Y_i)$ ns + $(Y_i)$ s/2 | Maximum value | Hossain et al., 1999 |
| Mean Relative Performance (MRP) | [$(Y_i)$ s/(Ys)] + [$(Y_i)$ ns/(Yns)] | Maximum value | – |
| Relative Efficiency Index (REI) | [$(Y_i)$ s/(Ys)] * [(Yi) ns/(Yns)] | Maximum value | – |
| Stress Tolerance Level (TOL) | TOL = $(Y_i)$ ns - $(Y_i)$ s | Minimum value | Rosielle and Hamblin, 1981 |
| Stress Tolerance Index (STI) | [$(Y_i)$ ns*$(Y_i)$ s]/$(Yns)^2$ | Maximum value | Fernandez, 1992 |
| Stress Susceptibility Index (SSI) | SSI = [1– $(Y_i)$ s/$(Y_i)$ ns]/SI. | Minimum value | Fischer and Maurer, 1978 |
| Drought Tolerant Efficiency (DTE) | (Specific trait under stress/Specific trait under non-stress)* 100 | Maximum value | Fischer and Wood, 1981 |

*$(Y_i)$ ns, specific trait of ith genotype under non-stress conditions; $(Y_i)$ s, specific trait of ith genotype under stress conditions; SI (stress intensity), 1– (Ys/Yns); Ys, mean of the specific trait of all the genotypes evaluated under stress conditions; Yns, mean of the specific trait of all the genotypes evaluated under non-stress conditions.*

**TABLE 2 |** Analysis of variance for various traits under deficiency (0.5 ppm) and control concentration of phosphorus and genotype interaction effect.

| Variate | Genotype (G) MSS (0.5 ppm) | Genotype (G) MSS (control) | Genotype (G) MSS of interaction | G SS% | Conc. MSS | Con. SS% | G x C MSS | G × C SS% |
|---|---|---|---|---|---|---|---|---|
| Shoot length | <0.01 | <0.01 | <0.01 | 85.95 | <0.01 | 5.41 | <0.05 | 4.48 |
| Tiller number | ns | ns | ns | 15.54 | <0.01 | 25.11 | ns | 16.26 |
| Leaf number | <0.01 | <0.01 | <0.01 | 36.11 | <0.01 | 41.77 | <0.01 | 12.18 |
| Root number | <0.01 | <0.01 | <0.01 | 65.34 | <0.01 | 15.44 | <0.01 | 11.76 |
| Root length | <0.01 | <0.01 | <0.01 | 59.35 | <0.01 | 19.76 | <0.05 | 9.92 |
| SPAD | <0.01 | <0.01 | <0.01 | 53.61 | <0.05 | 2.34 | <0.05 | 23.85 |
| 1st leaf weight | ns | ns | ns | 31.06 | ns | 3.64 | ns | 29.93 |
| 2nd leaf weight | ns | <0.01 | <0.01 | 61.65 | <0.05 | 4.60 | ns | 9.45 |
| 3rd leaf weight | <0.01 | <0.01 | <0.01 | 62.07 | ns | 0.04 | <0.01 | 20.94 |
| 4th leaf weight | <0.01 | <0.01 | <0.01 | 79.73 | ns | 0.25 | <0.05 | 10.30 |
| 5th leaf weight | <0.01 | <0.01 | <0.01 | 78.44 | <0.01 | 2.63 | ns | 7.32 |
| 6th leaf weight | <0.01 | <0.01 | <0.01 | 27.91 | <0.01 | 38.76 | <0.01 | 20.78 |
| Stem dry weight | <0.01 | <0.01 | <0.01 | 80.67 | <0.01 | 3.82 | <0.01 | 10.26 |
| Shoot weight | <0.01 | <0.01 | <0.01 | 59.13 | <0.01 | 20.24 | <0.01 | 11.86 |
| Root dry weight | <0.01 | <0.01 | <0.01 | 70.55 | <0.01 | 15.31 | <0.01 | 8.33 |
| Whole plant area | <0.05 | ns | ns | 19.26 | <0.01 | 32.32 | ns | 16.48 |
| Top view area | ns | <0.05 | <0.05 | 26.33 | <0.01 | 27.17 | <0.05 | 21.88 |
| Shoot P | <0.01 | <0.01 | <0.01 | 2.17 | <0.01 | 96.08 | <0.01 | 1.47 |
| Root P | <0.05 | <0.01 | <0.01 | 4.37 | <0.01 | 90.37 | <0.01 | 3.65 |
| Convex hull | <0.01 | <0.05 | <0.01 | 57.05 | <0.01 | 11.69 | ns | 10.19 |
| Calliper length | <0.01 | ns | <0.01 | 55.31 | ns | 0.14 | ns | 16.21 |
| Eccentricity | <0.01 | <0.05 | <0.01 | 70.22 | ns | 0.05 | ns | 6.94 |
| Mini enclosing circle | <0.01 | <0.05 | <0.01 | 61.98 | ns | 1.40 | <0.05 | 16.54 |
| 1st Leaf angle | ns | <0.01 | ns | 28.46 | ns | 1.29 | ns | 27.74 |
| 2nd Leaf angle | ns | <0.01 | <0.01 | 40.18 | ns | 0.24 | <0.05 | 29.10 |
| 3rd Leaf angle | ns | ns | <0.01 | 37.59 | <0.01 | 16.95 | ns | 16.83 |
| 4th Leaf angle | ns | ns | <0.01 | 28.73 | <0.01 | 36.90 | ns | 10.24 |
| 5th Leaf angle | ns | ns | ns | 16.23 | <0.01 | 33.49 | ns | 21.76 |
| 6th Leaf angle | ns | ns | ns | 17.60 | <0.01 | 28.27 | ns | 18.26 |
| T root Length | ns | <0.01 | <0.01 | 45.46 | <0.01 | 10.93 | <0.01 | 22.82 |
| Proj. root Area | ns | <0.01 | <0.01 | 55.72 | ns | 0.53 | <0.05 | 22.72 |
| Root Surf. Area | ns | <0.01 | <0.01 | 55.72 | ns | 0.53 | <0.05 | 22.72 |
| Root avg diameter | <0.01 | ns | <0.01 | 38.90 | <0.01 | 20.91 | ns | 16.70 |
| Root volume | <0.05 | <0.01 | <0.01 | 57.29 | <0.05 | 2.52 | ns | 18.12 |
| Tips | <0.05 | <0.01 | <0.01 | 35.11 | <0.01 | 15.78 | ns | 26.44 |
| PUE_S | <0.01 | <0.01 | <0.01 | 2.52 | <0.01 | 95.16 | <0.01 | 1.71 |
| PUE_R | <0.01 | <0.01 | <0.01 | 5.06 | <0.01 | 88.24 | <0.01 | 4.28 |

*P-values shown as "ns" (non-significance) >0.05. PUE_S, phosphorus utilisation efficiency of shoot; PUE_R, phosphorus utilisation efficiency of root.*

**Supplementary Table 2**. Under deficient conditions, stem dry weight exhibited the highest (92%) degree of heritability followed by root dry weight, shoot length, fourth leaf weight, shoot P content, shoot PUE, and root length exhibited more than 80% (**Supplementary Table 2**). The image-based geometric traits such as MEC (77%) and convex hull (73%) showed better heritability responses than the other geometric traits. More than 60% of the traits were normally distributed with absolute values of skewness of <1. The coefficient of variation (CV) was high for most of the studied parameters, 3 to 64% under low P conditions, and 6 to 78% under control conditions (**Supplementary Tables 2, 3**).

The correlation matrix (**Figure 2**) displayed traits with significance under P deficient conditions. Among the geometric traits, the MEC, convex hull, calliper length, and eccentricity showed a significant strong positive correlation (>0.90, $P < 0.01$) between them. Similarly, they had a strong positive association with TVA, WPA, third, fourth, and fifth leaf weight, shoot length, stem dry weight, shoot dry weight, root volume, and root length, which is in support of the PCA-derived results (**Figure 3**). For the physiological traits, root P content showed a significant positive association with root PUE (0.9731, $P < 0.01$). However, shoot PUE and shoot P content displayed a greater negative association with the convex hull (−0.6829, $P < 0.05$; −0.6828, $P < 0.05$) and eccentricity (−0.6419, $P < 0.05$; −0.6418, $P < 0.05$). Similarly, shoot length is significantly negatively correlated with shoot PUE (−0.7887, $P < 0.05$) and shoot P content (−0.7886, $P < 0.05$). Coherently, root surface

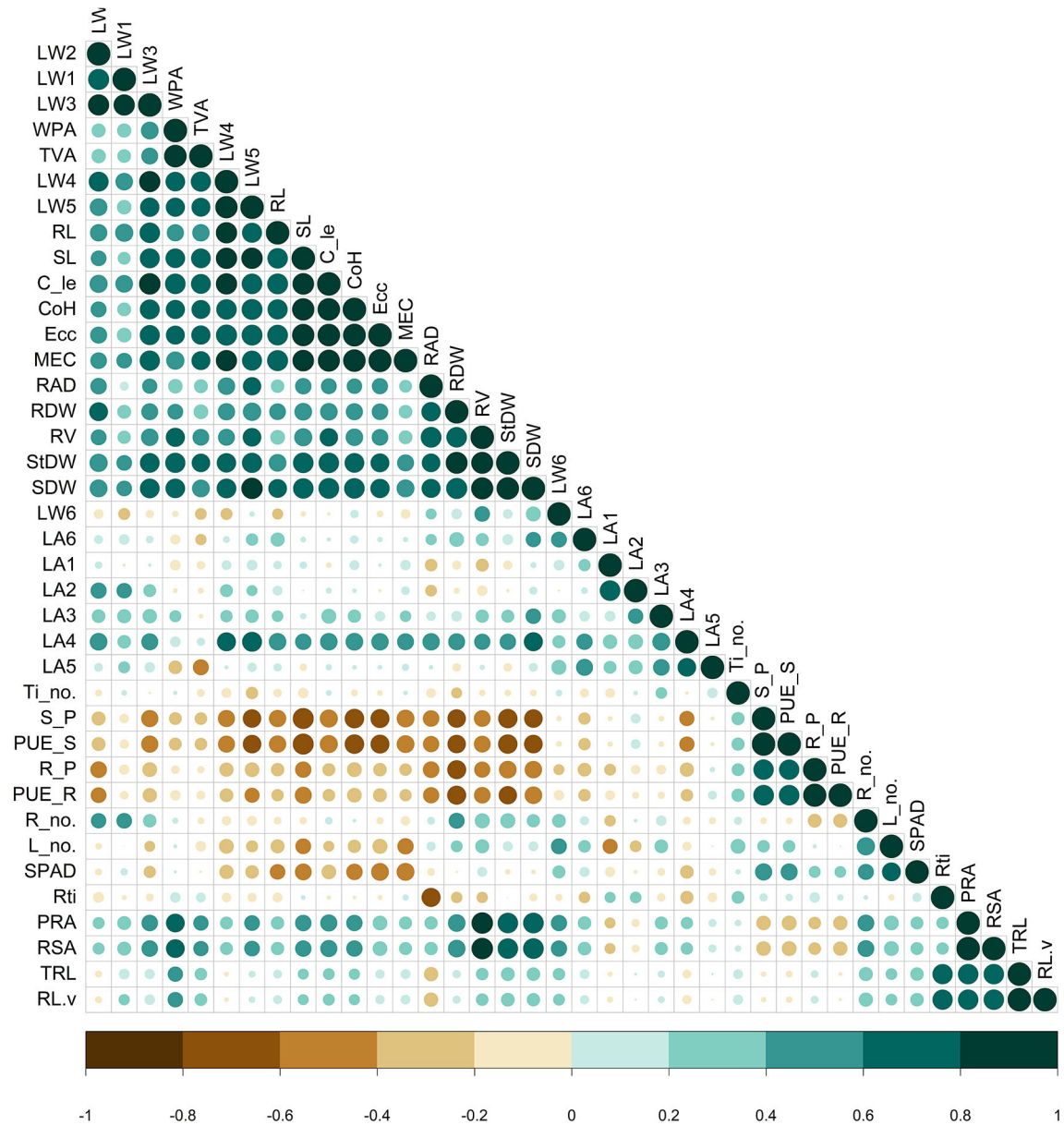


**FIGURE 2 |** Pearson correlation matrix of the measured traits in the phosphorus (P) stress environment. Colour (green-positive correlation; brown-negative correlation) intensity and the size of circle are proportional to the correlation coefficient. RAD, average root diameter; RDW, root dry weight; RV, root volume; StDW, stem dry weight; SDW, shoot dry weight; LW, leaf weight; WPA, whole plant area; TVA, top view area; RL, maximum root length; SL, shoot length; C_le, calliper length; CoH, convex hull; Ecc, eccentricity; MEC, minimum enclosing circle; LA, leaf angle; Ti_no, tiller number; S-P- shoot P content; PUE_S, PUE of the shoot; R_P, root P content; PUE_R, PUE of the root, Rti, root tip; TRL, total root length; PRA, projected root area; RSA, root surface area; R_no.- number of roots, L_no., leaf number.

area and root volume positively correlated with shoot weight (0.7662) and fifth leaf weight (0.626, $P < 0.05$). Interestingly, the average root diameter exhibited a strong positive association with root volume, shoot, stem, and root biomass, and negatively correlated with the number of root tips (−0.6532, $P < 0.05$). Physiological traits such as individual leaf weight also manifested positive responses among each other. The dry weight of all leaves at each level had positively correlated with the preceding two leaves.

## Genotype and Trait Grouping Reveals the Significance of Traits Under Deficient Conditions

Hierarchical clustering classified the 18 genotypes into two major clusters, *viz.*, cluster 1 (12 genotypes) and cluster 2 (6 genotypes), differing in their level of expression of morpho-physiological and geometric parameters under deficient P conditions (**Supplementary Figure 2**). Each sub-cluster serially from first to three comprises four (sub-cluster 1), five (sub-cluster 2), and three (sub-cluster 3) genotypes, respectively. Among the

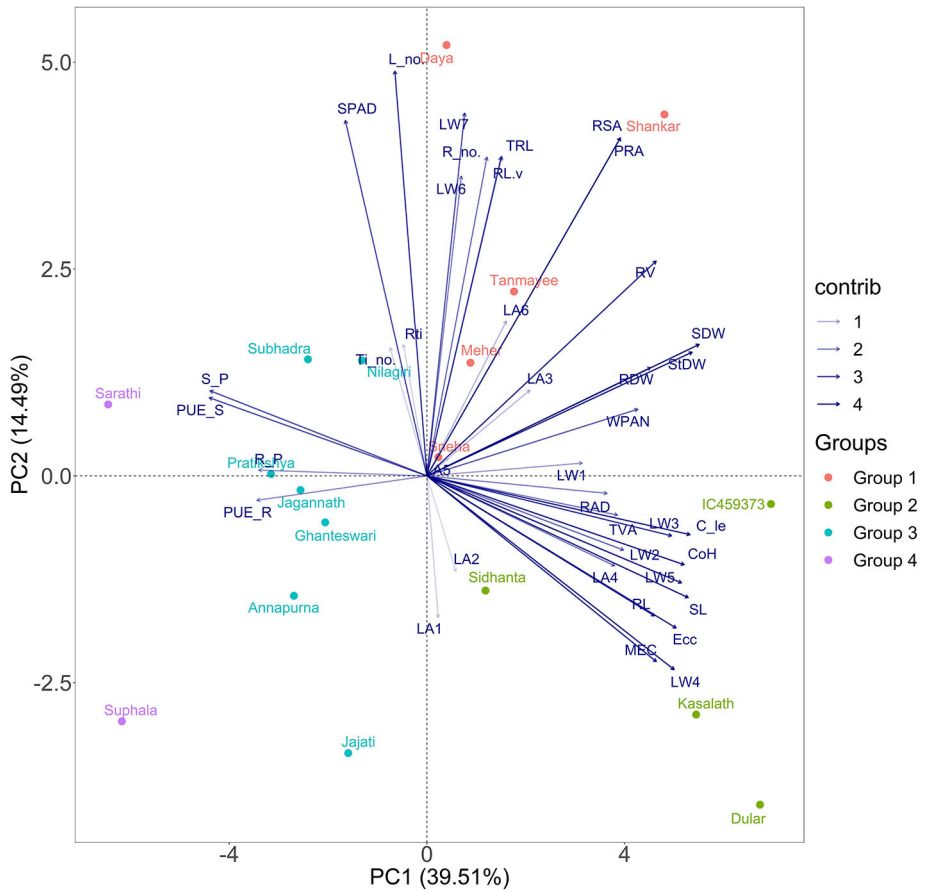


**FIGURE 3 |** Principal component analysis (PCA) biplot of the 18 genotypes based on variance in 38 morpho-physiological and geometric traits measured in P stress environment, explained by two axes. Together, the two PC axes explained 54% of the total variance. The transparency of the vector indicates the contribution to the variance in the dataset, ranging from 1 (lightest) to 4% (darkest). The direction and length of the vector represent trait contribution to the first two components of the PCA. Genotypes are grouped into four based on their expression pattern of morpho-physiological and geometric traits measured under deficient P conditions. Group 1 (orange circle) shares high values of root surface area, Group 2 (green circle) shares high values of leaf area and weight, and Group 3 (cyan colour) and Group 4 (purple colour) share high values of the shoot and root P content with reduced growth.

three sub-clusters, sub-cluster 3 exhibited maximum values for all geometric, morphological, and physiological traits, namely, leaf inclination except root number, SPAD, total root length, root tips, project, and surface area of roots. Interestingly, sub-cluster 1 displayed a bit lower values in all the above-mentioned traits of sub-cluster 3 with maximum total root length, root tips, project, and surface area of roots. On the other hand, the contribution of all the traits declined in sub-cluster 2 when compared with sub-clusters 3 and 1. Contrastingly, cluster 2 displayed lower contributions from almost all the traits except for root (0.64) and shoot P (0.66) contents.

Clustering of genotypes reflected the significance of the geometric traits as well as the morpho-physiological traits, which are again reflected from trait clusters based on Ward's D distance (**Supplementary Figure 3**). It grouped all the traits into six clusters. MEC and convex hull formed individual clusters, terming them as the most valuable non-destructive predictors of low P tolerance under deficient conditions. Similarly, clusters 3 and 4 encompassed 30 and 4 traits, respectively. Additionally, WPA and TVA form cluster 5 separately showed their significance under P deficient conditions. Traits in each cluster exhibit more closeness compared with those of the other clusters, which are again throw-back by the tree form of trait cluster representation.

## PCA Revealed the Tendency of Genotypes and Contributions of Traits Under P-Deficient Conditions

Principal component analysis was performed to determine the principal components of morpho-physiological and geometric parameters of the 18 rice genotypes, which best explains the response to low P conditions for screening tolerant rice varieties under hydroponic study. The first two principal component vectors contribute 39.51 (PC1) and 14.49% (PC2) to the total variation, which collectively describes a cumulative variance of 54% (**Figure 3**). From the selected traits, shoot dry weight followed by stem dry weight exhibited a very strong positive correlation to dimension 1. Among the geometric traits, which are new to this study, convex hull and calliper length showed

promising results compared with the other geometric traits. In contrast, root and shoot P contents, and root and shoot PUEs illustrate a negative correlation on PC1. Similarly, morphological traits such as leaf numbers have a strong correlation followed by SPAD for dimension 2. However, root traits such as root number, surface and projected root area, total root length, and root volume contributed more towards dimension 2. Dimension 3 explained 9.93% of the total variation. The number of root tips preceded by TRL possessed strong interrelations among the traits.

As PC1 and PC2 contributed more than half of the total variation and held significant importance in separating the genotypes into several categories, the 18 rice genotypes were classified into four distinct groups, similar to hierarchical clustering. The genotypes present at the third quadrant exhibited a maximum level of expression of geometric traits having the highest degree of tolerance in the low P regime, which are IC459373, Dular, Kasalath, and Sidhanta. Similarly, genotypes such as Shankar, Meher, Tanmayee, Sneha, and Daya at the second quadrant, depending upon their position in the ordinal plane and direction, exhibited maximum root growth with the moderate geometric trait. Genotypes Jajati, Annapurna, Ghanteswari, Jagannath, Pratikshya, Nilagiri, and Subhadra were grouped as moderately sensitive based on their response under low P conditions. On the left plane of the plot, Suphala and Sarathi fell at the end of –PC1 and +PC2, forming the most sensitive ones.

### Variation in Low P Tolerance Exists Across Landraces and Improved Rice Genotype Mechanism

The performance of the 18 rice genotypes was further scrutinised with a clustered heat map approach under both control and low P conditions. Albeit this approach is complex, it is more informative. It not only provides information on essential traits but also visualises the responses of genotypes in different colour shades. Hierarchical clustering separates the genotypes into two major clusters, which are further divided into several sub-clusters. In major cluster 1, Dular, Kasalath, IC459373, and Sidhanta performed better under deficient conditions, grouping them as tolerant ones, and were clustered with genotypes (Sidhanta, Shankar, Tanmayee, Jajati, and Meher) that performed well under control conditions. The majority of the traits, such as leaf weight, root dry weight, stem dry weight, and average root diameter at all levels, and geometric traits such as MEC and Convex hull positively attributed to this cluster and followed the same grouping pattern as was evident from the trait cluster. Major cluster 2 is represented by two sub-clusters as deficient and control groups. In sub-cluster 1, genotypes (Sneha, Subhadra, Nilagiri, Suphala, Pratikshya, Sarathi, and Jagannath) poor in their performance under low P conditions were grouped. Sub-cluster 2 is a group of a mixture of several genotypes, and included those that were moderately tolerant to low P. Among them, Shankar and Tanmayee illustrated two contrasting scenarios where the former was in a better position than the latter under deficient conditions, making Shankar more tolerant than Tanmayee. On the other hand, genotypes Jagannath and Suphala underperformed under both deficient and control conditions, indicating their sensitivity and making them more susceptible than the other genotypes. Therefore, this approach provides a better look at variation among genotypes under different conditions.

### Contribution of Individual Plant Parts Towards Its Overall Development

The results of this study shed light on the physiological aspect of plant development. Leaves, the major photosynthetic organ of plants, allocate assimilates to different plant parts and contribute towards its overall development. Fifth leaf (31.44%) followed by the fourth leaf (22.96%) and sixth leaf (16.01%) from the base contribute photosynthates maximally for its development across all the genotypes under P deficient conditions. Similarly, the fifth leaf (9.02%) followed by the fourth leaf (7.06%) exhibited a greater assimilated contribution towards overall plant development. Moreover, under low P conditions, the greater dry matter that was diverted to stem (15.94%) followed by root (10.48%) indicated that maintenance of above-ground parts in a deficient environment is more important than that of below-ground parts for balancing yield. Additionally, the fifth leaf (20.14, 89.22%) preceded by the fourth leaf (15.78, 69.47%) contributed maximally towards both shoot and root development, irrespective of all genotypes displaying their importance.

### Variation in Root Diameter and Number of Root Tips Across Various Rice Genotypes

Both root diameter and number of root tips are key components of the underground plant part, mainly associated with nutrient sensing and its uptake. The results depicted the percentage variation in average root diameter and number of root tips across various rice genotypes under limited P conditions. Most of the genotypes exhibited variation in average root diameter that ranged from 5.78 to 55.77% (**Supplementary Table 4**). This suggests that there is an increase in root diameter under low P conditions over the control environment. However, some cultivars such as Annapurna (−3.63%) and Ghanteswari (−3.26%) displayed a reverse trend. Nilagiri showed the highest positive variation in average root diameter (55.77%) followed by Dular (55.18%), Kasalath (39.07%), and IC459373 (35.74%). However, most of the cultivars showed negative variation in the number of root tips under stress conditions compared with the control that ranged between −2.25 and −65.81%. Several genotypes reported positive changes, such as Sarathi (38.72%), Ghanteswari (13.49%), Daya (3.72%), and IC459373 (32.08%). However, genotypes such as Dular (−65.81%) suggested the highest negative variation followed by Shankar (−63.04%), Pratikshya (−50.29%), Meher (−43.9%), Nilagiri (−40.01%), Jagannath (−28.93%), Tanmayee (−20.22%), and Suphala (−6.94%).

### Comparison of Specific Traits Based on Stress Indices

Several stress indices had been developed to be used as an indicator for identifying tolerant genotypes evaluated in abiotic stress environments. Based on the contribution of the specific

trait, the stress indices were grouped into two categories; tolerant indices (MPI, MRP, REI, STI, and DTE) and susceptible indices (TOL and SSI). The correlation between the indices and various traits considered, MPI, MRP, and REI, had a strong correlation that ranged between 0.646 and 0.988 (**Table 3**). Among the various traits, shoot length, displayed a greater degree of positive correlation with MPI ($r = 0.982$, $P < 0.01$), MRP ($r = 0.984$, $P < 0.01$), and REI ($r = 0.988$, $P < 0.01$) followed by stem dry weight and 5th leaf weight. Out of various geometric traits, MEC revealed a better positive relationship with MPI ($r = 0.953$, $P < 0.01$), MRP ($r = 0.96$, $P < 0.01$), and REI ($r = 0.962$, $P < 0.01$) followed by the convex hull and calliper length. Besides, other traits such as shoot dry weight manifested positively with the above-described tolerance indices (MPI, MRP, and REI) followed by average root diameter, fourth leaf weight, root dry weight, third leaf weight, fourth leaf angle, total root length, and number of root tips.

## DISCUSSION

Nutrients and their uptake and/or utilisation efficiency are the most evergreen debatable topics haunting the scientific community. Since P, an element with immense importance, not only nurtures all biological species but also forms the basis of life, including the plant community. Exasperated with the conventional method, non-invasive RGB imaging has been included to identify the tolerant plant in early stages encompassing crucial geometric traits, such as leaf angle, MEC, and leaf area, associated with grain yield. To unfold the traits related to low P, conventional and geometric traits measured by imaging technique were applied for the 18 genotypes identified from initial soil-based screening selected from 65 genotypes by applying multivariate analysis, and mean of root length, shoot length, and biomass of shoot and root were considered as a threshold (**Supplementary Figure 1**). In this study, we have established the possibility of using non-destructive imaging techniques to differentiate genotypes at an early stage of crop growth in a P-deficient environment, identification of the most informative traits indicating the tolerant source, and their potential underlying mechanisms for low P tolerance.

The analysis of variance suggests leaf angle as an adaptive trait, as it is greatly influenced by P concentration and unravels geometric traits such as calliper length, MEC, and eccentricity significantly differed at the genotype level. Among the variance, genotype (G) has a maximum contribution followed by concentration (C) and G × C interaction. However, traits, namely, number of tillers, WPA, TVA, and leaf angle of position from third to seventh were immensely influenced by P availability, hinting at their sensitivity towards it. The trait stem dry weight exhibits the highest heritability at a low P concentration, which is the most important quantitative parameter considered in the evaluation of low P-tolerant crop plants. However, physiological traits stem and root dry weight, fourth leaf weight, shoot P content, and its utilisation efficiency exhibited higher heritability in both P regimes, possibly because of constitutive gene expression in a particular tissue. Additionally, most of the traits displayed a greater degree of variation, as it is evident from their CV and controlled genetically, clearly showing from GCV values (**Supplementary Tables 2**, **3**). Therefore, the higher CV specifies exploitation of a higher degree of genetic variability among the studied parameters and the possibility of a greater potential in selecting these parameters in developing low P-tolerant genotypes.

To assess the performance of the genotypes under phosphorus-deficient conditions, several traits were reported, such as shoot and root biomass, tiller number, total root length, volume, and phosphorus content of shoot on the destructive basis (Rose et al., 2016; Anandan et al., 2021). In this study, we found that the traits such as shoot and root biomass, stem weight, root length, root volume, and P content of shoot were highly correlated and significant with low P tolerance. Therefore, we have focussed the attention on these representative traits, and the geometric traits measured using an imaging technique have become the epicentre of this study. The geometric trait calliper length positively correlated with third, fourth, and fifth leaf weights (**Figure 2**). Similarly, the convex hull displayed a highly significant correlation with calliper length and with the weight of third, fourth, and fifth leaves. The stronger association between them suggests greater biomass accumulation, full expansion of leaves, and wider convex hull under P-deficient conditions clearly supports that these traits would play an essential role in differentiating lines. Fully expanded active leaves absorb a high amount of solar radiation, have a high $CO_2$ assimilation rate, and translocate large amounts of assimilates to other parts of the plant. Furthermore, MEC also positively correlated with third, fourth, and fifth leaf weights, calliper length, convex hull, eccentricity, shoot length, and root length, indicating dependency of those traits influencing yield. The top view area and WPA were positively correlated with the leaf weight of third to fifth levels and fourth leaf angle along with the above-described geometric traits (calliper length, convex hull, eccentricity, and MEC) and morphological trait shoot length. This indicates that leaves positioned at 3rd to 5th perform better nutrient mobilisation among different parts of plants by maximising leaf area when viewed from the top. Furthermore, the fully expanded leaves with a good number of tillers maintain higher ground coverage by foliage, thus reducing the weed population by facilitating rapid vegetative growth under low P conditions. The trait WPA was positively associated with calliper length, convex hull, eccentricity, fourth and fifth leaf weights, combining root architectural traits such as root volume, projected area, and surface area. This suggests that increased root area and volume increases WPA because of greater excavation of nutrients either by diffusion and/or by ion exchange by contact with nutrients through enhancement in root traits (Reddy et al., 2020). This analysis revealed that genometric traits (MEC, convex hull, calliper length, eccentricity, etc.) and morphological traits (shoot length, shoot and root dry weight, total root length, root surface area, root volume, root diameter, projected root area, etc.) have been further utilised as predictors for low P tolerance under P-deficient conditions. From a physiological point of view, the fifth leaf followed by the fourth and sixth leaves

**TABLE 3 |** Relationship (*r*) between tolerance indices and some selected traits measured under P deficient conditions.

| Trait | TOL | STI | SSI | DTE% | MPI | MRP | REI |
|---|---|---|---|---|---|---|---|
| Shoot length | 0.161 | 0.056 | −0.056 | 0.056 | 0.982 | 0.984 | 0.988 |
| Stem dry weight | 0.389 | 0.024 | −0.024 | 0.024 | 0.971 | 0.978 | 0.97 |
| 5th leaf weight | −0.074 | 0.397 | −0.397 | 0.397 | 0.973 | 0.973 | 0.983 |
| Mini. enclosing circle | 0.243 | 0.27 | −0.27 | 0.27 | 0.953 | 0.96 | 0.962 |
| Convex hull | −0.05 | 0.61 | −0.61 | 0.61 | 0.932 | 0.953 | 0.918 |
| Avg. root diameter | −0.911 | 0.838 | 0.838 | 0.838 | 0.963 | 0.952 | 0.952 |
| Shoot dry weight | 0.424 | −0.191 | 0.191 | −0.191 | 0.923 | 0.938 | 0.945 |
| 4th leaf weight | −0.2 | 0.175 | −0.175 | 0.175 | 0.936 | 0.938 | 0.936 |
| Calliper length | 0.183 | 0.021 | −0.021 | 0.021 | 0.934 | 0.935 | 0.938 |
| Root dry weight | −0.511 | 0.037 | 0.037 | 0.037 | 0.957 | 0.918 | 0.919 |
| SPAD | −0.787 | 0.792 | −0.792 | 0.792 | 0.911 | 0.915 | 0.913 |
| 4th Leaf angle | 0.042 | 0.345 | −0.345 | 0.345 | 0.803 | 0.907 | 0.94 |
| 3rd leaf weight | −0.491 | 0.549 | 0.549 | 0.549 | 0.862 | 0.861 | 0.851 |
| Leaf number | −0.177 | 0.311 | −0.311 | 0.311 | 0.825 | 0.846 | 0.839 |
| 3rd Leaf angle | −0.263 | 0.41 | −0.41 | 0.41 | 0.764 | 0.839 | 0.881 |
| Total root length | −0.203 | 0.334 | −0.334 | 0.334 | 0.72 | 0.759 | 0.741 |
| Root tips | −0.475 | 0.57 | −0.57 | 0.57 | 0.646 | 0.734 | 0.745 |
| Top view area | −0.074 | 0.294 | −0.294 | 0.294 | 0.417 | 0.622 | 0.608 |
| 5th Leaf angle | −0.568 | 0.76 | −0.76 | 0.76 | 0.197 | 0.619 | 0.657 |
| Whole plant area | 0.021 | 0.11 | −0.11 | 0.11 | 0.381 | 0.599 | 0.585 |

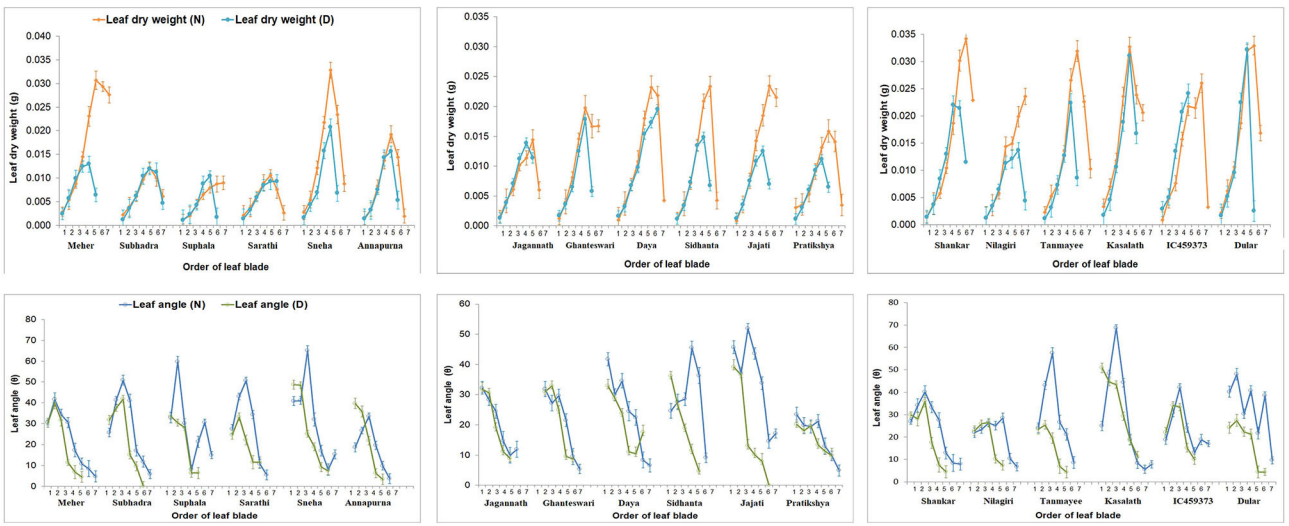


**FIGURE 4 |** Effect of P on leaf blade weight and leaf angle of the 18 genotypes at all levels and relative comparison between the treatments. Individual points with error bars refer to leaf position from bottom to top. Leaf blade weight and leaf angle were reduced at all levels of leaves. Tolerant genotypes exhibited minimum relative differences for leaf blade and angle.

supported their growth maximally, indicating them to be the most active photosynthetic organs, but, due to the senescence of the first leaf and very young growth of the seventh leaf, did not contribute much to its development. Similarly, the fifth and fourth leaves also maximally contributed to overall plant shoot and root development, indicating these to be the most active photosynthetic reservoir. Greater dry matter diversion towards shoot rather than root implies the importance of shoot architecture in determining final crop yield. While considering the root-associated traits, maximum root length and root volume were found to have a prime role under P deficient conditions. They exhibited the strongest association ($r = > 0.7$, $P < 0.05$) with most of the traits (**Figure 2**). Both of them (root length and volume) had no negative association with the other traits

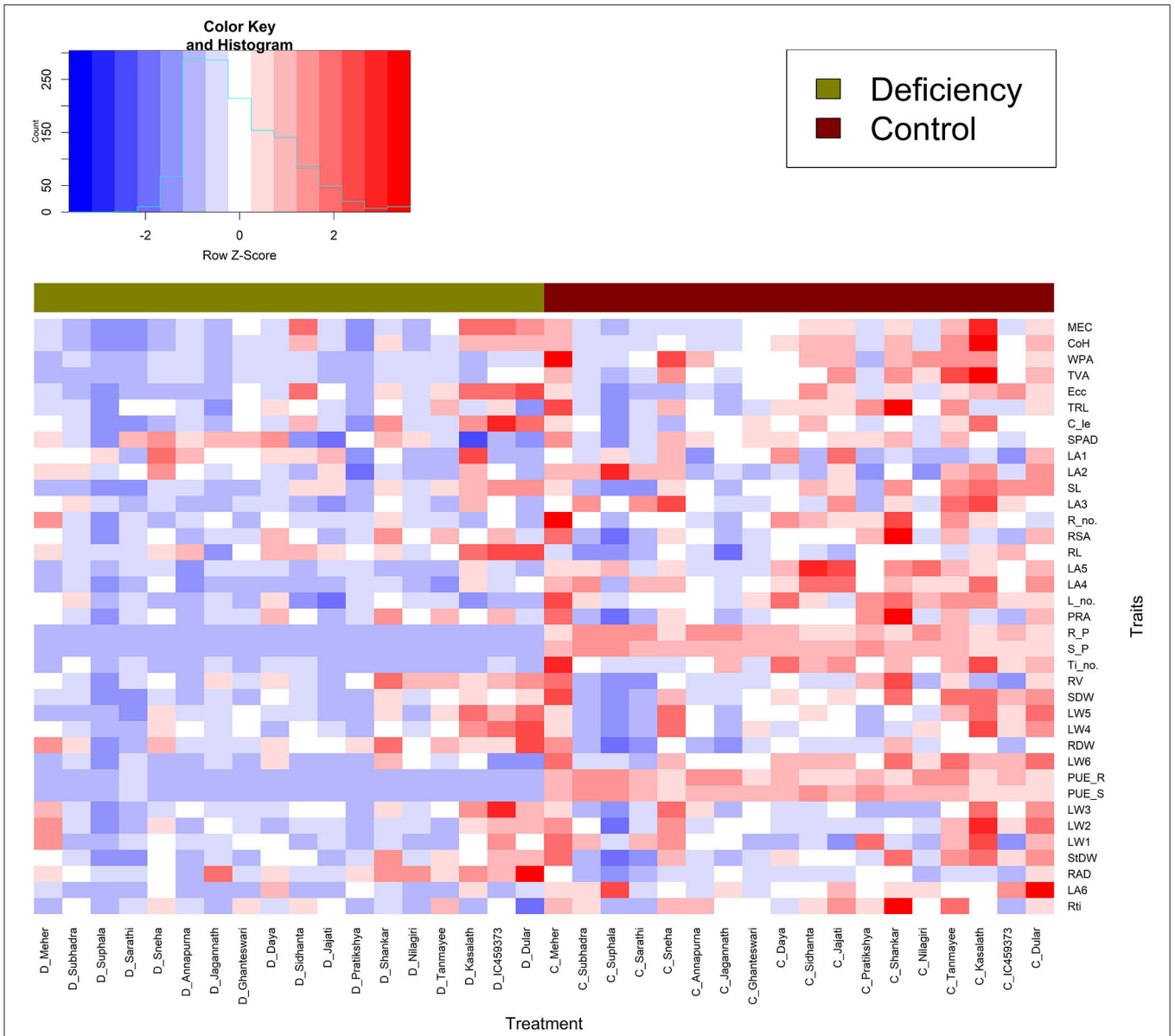


**FIGURE 5 |** Heat map of two treatments (control and deficit) of the 18 genotypes and 38 morpho-physiological and geometric traits. Each column represents a genotype, and each row represents a trait. The horizontal bar with two colours (olive and maroon) represents the treatment difference.

but had a moderate association ($r = 0.4$) between them, having some traits in common with a positive association. Therefore, by improving root length and volume together, there is a possibility to explore greater soil volume to enhance P uptake from a distant region of soil under P-deficient conditions. On the contrary, the increase in average root diameter had a negative effect on the number of root tips, P content, and PUE of shoot and root. The average root diameter was found to increase with a decrease in the availability of P (Anandan et al., 2021). Compared with control, root diameter was increased by 18.91%, and root tips decreased by 25.46% at 0.5 ppm. The decrease in the number of root tips might be due to an increase in root diameter with aerenchyma, root length (maximum and total root length), and restricted branching. This could be a strategy to reduce the energy requirement for root growth and maintenance (Steffens and Rasmussen, 2016) under P-deficient conditions.

In this experiment, the testing of different genotypes revealed that root dry weight showed a negative correlation with root and shoot phosphorus contents, and utilisation efficiency, hinting that the observed P was not available in sufficient quantity under phosphorus-deficient conditions. Plants with large roots have the opportunity to explore more nutrients, but that does not necessarily mean higher P uptake, which is clear from the negative correlation between root dry weight and root or shoot P content. Further, the negative association between root length and shoot P or shoot utilisation efficiency supports poor uptake

or poor translocation of P from root to shoot. Similarly, a negative association was observed for shoot length, and shoot and stem biomass (**Figure 2**). Among the various geometric and physiological traits, the negative association of shoot P content and its utilisation efficiency with leaf weight (third to fifth), convex hull, MEC, calliper length, and eccentricity explained why those traits need more P than any of the other traits to maintain the plant in required architecture. Presumably, those traits may compensate the P deficit through remobilization from fully and partly senescenced leaves that might be acting as organic P pools.

The hierarchical clustering that separated MEC, and convex hull into two separate clusters from the rest of the other traits suggests their importance in differentiating the genotypes under deficient conditions compared with other traditionally (morphological and physiological) measured traits. The two geometric traits (MEC and convex hull) that form separate clusters depend on the development and inclination of fully functional leaves. They are directly related to the area, biomass accumulation, and inclination of leaves under both control and deficient P conditions. Widely, all cultivars maintained higher leaf biomass under control than deficient conditions ($0.085 \pm 0.034$). Interestingly, susceptible genotypes Subhadra, Sarathi, Jagannath, and Suphala had minimum differences in leaf biomass at all levels under limited P conditions with high tissue P (>0.7 mg/g) and PUE (>0.0036) (**Figures 3**, **4**). This could be the dilution effect. The smaller leaves appeared to have higher P concentration compared with genotypes having larger leaves. Besides, these susceptible genotypes exhibited the least TVA (<1,600 $mm^2$) under P-deficient conditions. High tissue P, and the PUE of smaller and narrow leaves would be a strategy of plants to improve their survival under P-deficient conditions. Further research on these genotypes would help gain insight into understanding the minimum differences in leaf biomass between control and deficient conditions.

The close relationship between leaf dry weight and angle summarises that more biomass accumulation in leaves would lead to a higher rate of leaf inclination and vice versa. This can be well-observed from the positive association between them (**Figure 4**). However, the negative correlation between first leaf weight and angle might be because the first formed leaf (primary leaf) is small and acts as a protective covering for the succeeding leaf (Dunand and Saichuk, 2014), and that it is associated with initial root growth (Anandan et al., 2020). On the other hand, Anandan et al. (2021) reported reduced leaf area and increased root growth in 2-week-old rice seedlings under P-deficient conditions. Apart from that, a reduction in leaf inclination might be due to the adaptiveness of rice plants under P-deficient conditions. P-deficient plants reduce photosynthesis to conserve Pi. Plants raised under low P conditions inhibit the export of triose-P from chloroplast stroma to the cytosol by the Pi translocators (Natr, 1992), leading to the conversion of photosynthate into starch in the chloroplast. The Pi that liberated on the conversion of triose-P into starch and consumed in large amounts during photosynthesis for the synthesis of ATP was stopped to conserve the use of Pi (Dietz and Foyer, 1986). This highlights that the geometric traits MEC and convex hull, based on leaf inclination, would involve manifesting their importance as non-destructive parameters to differentiate the genotypes in a low P environment. Additionally, other traits such as total root length, root surface area, root volume, projected root area, shoot P content, and utilisation efficiency establish their vitality in the selection of low P-tolerant rice genotypes. The heat map in **Figure 5** gives an enhanced picture of traits expression between the two groups (control and deficit). Traits such as an increase in pigmented/bluish-green leaves, reduction in leaf inclination (third to fifth leaves), shoot dry weight, root tips, MEC, convex hull, and increase in average root diameter and root dry weight were observed under P-deficient conditions (Anandan et al., 2021). Subsequently, hierarchical clustering (**Supplementary Figure 4**) sub-clustered the tolerant genotypes into three clades on the basis of geometric traits and root parameters. The contribution of greater values of geometric traits made tolerant genotypes (Dular, Kasalath, and IC459373) into a separate clade. Compared with the other genotypes, the relative expression of traits in Kasalath was least affected by deficient P, and this can be observed from **Supplementary Figure 4**, where it formed a separate clade. Besides, The Kasalath clade joined with the neighbouring clade have better-performing genotypes under deficient and control conditions, while the genotypes represented by greater total root length, root surface area, projected root area, and root tips under P-deficient conditions separated from the susceptible clade but grouped with the known tolerant clade. This hypothesis was confirmed by PCA further (**Figure 3**). The genotype with high biomass (shoot or root) exhibited a major trait that distinguishes it from the other genotypes. Genotypes with the greater geometric value used to have more shoot biomass with larger root systems were categorised as low P-tolerant, while genotypes with poor biomass and high tissue P were regarded as a poor performer under low P conditions. This signifies the importance of biomass in differentiating genotypes in a low P environment. The varying tolerant traits (geometric or root biomass) between groups of the genotypes may suggest the possibility of adopting different tolerant mechanisms to maintain their growth at the seedling stage under low P conditions. Irrespective of genotypes and treatments, P content was more in root than the shoot. Besides, the concentration of P was found to be lower in shoots and roots of all highly tolerant genotypes (Dular, Kasalath, and IC459373) compared with moderately tolerant or susceptible genotypes. The increased shoot biomass with reduced root numbers (**Figure 3**; **Supplementary Figure 4**), total root length, projected root area and surface root area, and root tips were observed intolerant than moderately tolerant genotypes. Reducing few numbers of lateral/crown roots and root tips might have diverted metabolites/photosynthates and P for increasing shoot biomass under P-deficient conditions. This was very well-supported by the negative correlation between root diameter and the number of root tips. An increase in root circumference (diameter) with maximum root length might have increased dry root biomass. Therefore, dry biomass should be used as a scale to classify genotypes, and this has been widely acknowledged (Dissanayaka et al., 2018). The increase in maximum root length and root thickness

is directly proportional to the increase in root volume. The tolerant genotypes were observed to have higher volumes under P-deficient conditions.

Measuring economically relevant trait grain yield is not possible in all experiments, specifically at the seedling stage or handling large segregating generations where destructive sampling to study biomass is not viable. In a low P environment, shoot biomass, leaf number, and area are the most affected traits (Neilson et al., 2015), as most photosynthates are translated into root biomass in susceptible and moderately tolerant genotypes. Therefore, an imaging technique that measures MEC and convex hull based on leaf number, area, and inclination is observed to be an important trait contributing to plant biomass. The methodology (imaging technique) used here could provide insights into the mechanism by which low P affects leaf growth. The heat map generated combining traits, and genotypes of both the treatments were quite informative. They grouped the genotypes behaving in a similar manner across treatments. This supports the outcome of classification of traits *via* Ward's D distance where MEC, convex hull, WPA, and TVA form separate clusters indicated their significance among the traits under P-deficient conditions. Hence, these geometric traits may be considered as selection criteria for identifying low P-tolerant rice genotypes. The above highlighted geometric and root traits were explored to identify tolerant genotypes using stress-tolerant indices such as MPI, MRP, REI, TOL, STI, SSI, and DTE. Among them, MPI, MRP, and REI were found to be useful indices having r of >0.91 for the following traits: MEC, convex hull, calliper length, SPAD, shoot length, stem dry weight, fourth and fifth leaf weights, average root diameter, and shoot and root dry weight. The tolerance indices of MPI, MRP, and REI, and 11 mentioned morphological and geometric traits (**Supplementary Table 5**) helped to clearly differentiate low P-tolerant (Kasalath, IC459373, Dular, Sidhanta, Shankar, Meher, and Tanmayee) and susceptible genotypes (Suphala, Sarathi, Pratikshya, and Subhadra). The output of the above-mentioned indices were similar to the output of the PCA analysis (**Figure 3**). We switched from the destructive to the non-destructive way of evaluating genotypes by adapting geometric traits (MEC, convex hull, and calliper length), as surrogate traits would yield promising results in identifying low P-tolerant genotypes at the seedling stage. The methodology and traits identified with non-destructive imaging techniques in this experiment would be highly valuable for plant physiologists and breeders involved in identifying and developing rice genotypes for low P environments.

## CONCLUSION

This study explains the importance and utilisation of image-based phenotyping to select geometric traits, and identifies tolerant rice genotypes under low P conditions. The geometric traits such as MEC and convex hull highlighted their supremacy over the others in differentiating various rice genotypes, which is quite evident from various statistical analyses. In addition, shoot length, stem and root biomass, total root length, root volume, and root surface area could be further utilised as surrogate traits in the absence of an imaging technique at the seedling stage of the crop. Leaves positioned from third to fifth played a crucial role in P mobilisation to different plant parts; thus, maintaining plant architecture under P-deficient conditions to stabilise final yield. In addition, leaf angle, a critical yield determining factor, is reduced in the low P environment. Thus, the decline in leaf biomass and changes in the photosynthetic process create the scope for further determination of the underlying mechanism behind it. Concurrently, these geometric traits and other morphological and physiological traits were further utilised to identify the tolerant genotypes based on several stress indices. MPI, MRP, and REI displayed better responses for several traits establishing their usefulness under low P conditions. Overall, this study not only emphasises the importance of image-based phenotyping, which screens genotypes at the seedling stage, but also highlights the role of geometric traits in determining the final yield. Still, further research is needed to study the variation at the transcriptome and proteome levels, and the genes responsible for modification in underground traits accompanying alteration in photosynthetic processes and exudation of organic acids responsible for P mobilisation under P-deprived conditions.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

AA, RP, and BB designed the experiments. BB and NP executed the experiments and performed data collection. AA performed data analysis and visualisation. BB and AA interpreted the data. BB, AA, AM, RP, BP, and JA wrote and critically revised the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.717107/full#supplementary-material

**Supplementary Figure 1 |** PCA biplot of 68 genotypes based on the variance in eight morpho-physiological traits measured in P stress environment, explained by two axes [PC1 and PC2, selected genotypes (orange) and non-selected genotypes (green)].

**Supplementary Figure 2 |** Hierarchical clustering of the genotypes based on the level of expression of morpho-physiological traits in low P environment.

**Supplementary Figure 3 |** Hierarchical clustering of the 38 morpho-physiological and geometric traits based on Ward's method.

**Supplementary Figure 4 |** Hierarchical clustering and heat map of the 18 genotypes and 38 morpho-physiological and geometric traits. Each column represents a genotype, and each row represents a trait. The horizontal bar represents the treatment difference. Deficient (D) represented by pink colour, and Control (C) is represented by grey colour. The genotypes are grouped into two major clusters, and those having better shoot growth under deficient conditions and control are separated from the other genotypes.

**Supplementary Table 1 |** List of genotypes used in this study.

**Supplementary Table 2 |** Variability parameters of traits measured in rice genotypes raised under 0.5ppm of phosphorus.

**Supplementary Table 3 |** Variability parameters of traits measured in rice genotypes raised in hydrophonics with full phosphorous.

**Supplementary Table 4 |** Percent variation for significant traits of rice genotypes under both P deficient and control condition.

**Supplementary Table 5 |** Stress tolerance indices and significant traits measured under P deficient and control condition.

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# An Integrated Approach for the Efficient Extraction and Solubilization of Rice Microsomal Membrane Proteins for High-Throughput Proteomics

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The preparation of microsomal membrane proteins (MPs) is critically important to microsomal proteomics. To date most research studies have utilized an ultracentrifugation-based approach for the isolation and solubilization of plant MPs. However, these approaches are labor-intensive, time-consuming, and unaffordable in certain cases. Furthermore, the use of sodium dodecyl sulfate (SDS) and its removal prior to a mass spectrometry (MS) analysis through multiple washing steps result in the loss of proteins. To address these limitations, this study introduced a simple micro-centrifugation-based MP extraction (MME) method from rice leaves, with the efficacy of this approach being compared with a commercially available plasma membrane extraction kit (PME). Moreover, this study assessed the subsequent solubilization of isolated MPs in an MS-compatible surfactant, namely, 4-hexylphenylazosulfonate (Azo) and SDS using a label-free proteomic approach. The results validated the effectiveness of the MME method, specifically in the enrichment of plasma membrane proteins as compared with the PME method. Furthermore, the findings showed that Azo demonstrated several advantages over SDS in solubilizing the MPs, which was reflected through a label-free quantitative proteome analysis. Altogether, this study provided a relatively simple and rapid workflow for the efficient extraction of MPs with an Azo-integrated MME approach for bottom-up proteomics.

**Keywords: rice, label-free quantitative proteomics, microsomal membrane extraction, ultracentrifugation, AZO, sds, MaxQuant, Perseus**

## INTRODUCTION

Microsomes are cell membrane-derived vesicles that are formed during the lysis of plant tissues (LaMontagne et al., 2016). They are known to consist of the plasma membrane (PM), endoplasmic reticulum (ER), Golgi apparatus, intracellular vesicles, and tonoplast (Yang and Murphy, 2013; LaMontagne et al., 2016). The successful enrichment and proteomic analyses of microsomes have gained considerable interest in recent years, as researchers aim to understand the functions of numerous membrane proteins that augment our comprehension of the diverse biological pathways operating in different subcellular compartments (Wang et al., 2015; Alqurashi et al., 2017; Meisrimler et al., 2017; Pontiggia et al., 2019). For instance, a recent study investigated various microsomal proteins that were responsive to abscisic acid (ABA) and hydrogen peroxide to understand their mode of action using a shotgun proteomic approach (Alqurashi et al., 2017). However, due to various technical challenges the isolation of microsomal membrane proteins (MPs) remains difficult. This challenge is encountered because these proteins are of low abundance and highly hydrophobic, which is why they are devoid of contaminating molecules such as organic acids, polyphenols, lipids, carbohydrates, and other secondary metabolites that are predominantly present in the plant cells (Cox and Emili, 2006; Vilhena et al., 2015; Mehraj et al., 2018). To date, the large-scale isolation of MPs for proteome analysis has primarily relied on ultracentrifugation-based methods due to multiple advantages such as the higher purity of products, suitability in a large volume of samples, and accurate separation of subcellular compartments based on own sedimentation rates (Yang D. et al., 2020). However, these ultracentrifugation-based methods require expensive instrumentation, skilled technicians, and a large amount of starting material, which limits their large-scale utilization (Wang et al., 2015; Alqurashi et al., 2017; Meisrimler et al., 2017; Yang D. et al., 2020). Thus, commercial kits that facilitate the isolation of MPs and PM proteins have been introduced to address the discussed limitations. However, these kits are often expensive and offer limited extraction, hindering the large-scale preparation of the targeted proteins (Kaundal et al., 2017; Kim et al., 2019; Yang Z. et al., 2020; Kato et al., 2021). Therefore, the development of relatively simpler methods for the isolation of MPs is necessary and is also a prerequisite for microsomal proteomic studies.

There were significant efforts in the past studies that were made to develop approaches for the efficient isolation and identification of MPs without ultracentrifugation (Tu and Kawai, 1998; Cox and Emili, 2006; LaMontagne et al., 2016; Moloney et al., 2016). For instance, the study by Nagahashi et al. (1978) used low-speed centrifugation to separate a crude mitochondrial fraction from the primary roots of barley and showed that about 88% of the mitochondria were sedimented with a centrifugal force of 13,000 *g* (Nagahashi et al., 1978). In a similar study by Giannini et al. (1988), a microsomal membrane fraction was successfully isolated from the roots of red beets (*Beta vulgaris* L. cv. Detroit Dark Red) by centrifugation at 13,000 *g* for 3–4 min, while a further increase in the duration of centrifugation gave a substantial recovery of the tonoplast and PM adenosine triphosphatase (ATPase) (Giannini et al., 1988). However, there has been no research on the implementation of these methods in the isolation of total MPs from plant tissues for large-scale proteomics.

The solubility of extracted MPs is another major concern that hinders their large-scale analysis. Being highly hydrophobic in nature, MPs are currently solubilized either in anionic detergent sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), or Triton X-100 (Kalipatnapu and Chattopadhyay, 2005). Among these agents, SDS has been a widely used surfactant for the solubilization of MPs in proteomic studies. However, SDS is not compatible with mass spectrometry (MS) as it interferes with chromatographic separation and suppresses peptide ionization during MS analysis (Alfonso-Garrido et al., 2015). Therefore, SDS must be removed prior to MS analysis through rigorous washing, which eventually results in the inevitable loss and degradation of the MPs (Alfonso-Garrido et al., 2015). Fortunately, various MS-compatible surfactants have been developed for the solubilization of proteins. Among these surfactants, 4-hexylphenylazosulfonate (Azo) has shown considerable potential in solubilizing various types of hydrophilic and hydrophobic proteins. Interestingly, Azo can be subjected to rapid degradation through ultraviolet irradiation, and, most importantly, it is compatible with MS analysis (Brown et al., 2019, 2020). The potential of Azo to be used in protein solubilization has already been shown in animal tissues for top-down and bottom-up proteomics studies. Nonetheless, its efficiency in the plant samples still needs to be tested.

In this study, we introduced a microcentrifuge-based method for the enrichment of MPs (MME) using rice as a model system. Furthermore, the extraction efficiency of this method was compared with a commercially available plasma membrane protein extraction (PME) kit using a label-free quantitative proteomic approach. Using the same approach, this study further compared the protein solubilization efficacies of Azo and SDS isolated by the MME method for a bottom-up proteomic analysis.

## MATERIALS AND METHODS

### Plant Materials and Growth Condition

Rice (*Oryza sativa* L. cv. Dongjin) seeds were sterilized in a 0.05% spotak solution (Bayer Crop Science, Seoul, South Korea) overnight at 28°C and then washed with distilled water five times as described in a previous study (Gupta et al., 2019). The sterilized seeds were then germinated on wet tissue paper at 28°C in the dark and transferred to a Yoshida solution in a growth chamber maintained at 70% humidity at 25°C with a light and dark cycle of 16 and 8 h, respectively (Yoshida et al., 1976). For the proteomic analysis, 4-week-old rice leaves were harvested and immediately stored at −70°C for further analysis.

### Synthesis of the Azo Surfactant

The synthesis of Azo surfactant was carried out as reported in previous studies (Brown et al., 2019, 2020). Briefly, the synthesis

involved 0.857 ml of 4 mM of 4-n-hexylaniline ($n$ = 4, C6) that was stirred with a mixture of 4.8 ml 10% hydrochloric acid (HCl) and 8 ml of deionized dihydrogen monoxide ($H_2O$) at 10°C for 10 min. This was followed by the dropwise addition of 4 ml of 1 mM of ice-cold sodium nitrite ($NaNO_2$) into the mixture. After 5 min of stirring at 10°C, 12 ml of 12 mM of sodium carbonate ($Na_2CO_3$) was added dropwise into the solution under constant stirring for an additional 3 min. Afterward, the solution was filtered into a prepared ice-cold flask containing 8 ml of 8 mM of sodium sulfite ($Na_2SO_3$) and incubated at 4°C overnight to allow for the complete precipitation of the surfactant. Finally, the yellow compound was purified through recrystallization, and the structure of Azo was confirmed using electrospray ionization (ESI)-MS and $^1$H-nuclear magnetic resonance (NMR) analyses as described in a previous study (Brown et al., 2019).

## Microsomal Membrane Protein Extraction From Rice Leaves and Western Blot Analysis

The extraction efficiencies of the two different microsomal protein extraction methods, namely, MME and PME (ab65400, Abcam, Cambridge, United Kingdom), were compared. For the MME method, 200 mg of rice leaf powder were homogenized in an High density sucrose (HDS) buffer, which consisted of 37.5 mM of hydroxyethyl piperazine ethane sulfonic acid (HEPES), pH 8, 37.5% (w/w 1.215 M) sucrose, 7.5% (v/v) glycerol, 15 mM of ethylene-diamine-tetraacetic acid (EDTA), 15 mM of ethylene-glycol-tetraacetic acid (EGTA), 1 mM of dithiothreitol (DTT), and a 100 × Halt$^{TM}$ protease inhibitor cocktail (Thermo Fisher Scientific, MA, United States). Afterward, the supernatant was collected after centrifugation at 600 *g* for 3 min at 4°C (Abas and Luschnig, 2010). The obtained pellet was re-extracted, first with half, and then a third of the initially used HDS buffer at the same centrifugation conditions as the first extraction. After centrifugation, both the supernatants were combined and centrifuged again at 600 *g* for 3 min at 4°C to remove any remaining debris. Subsequently, the 1 ml of supernatant was taken out and was diluted with 2.167 ml of double-distilled water ($ddH_2O$) to adjust the sucrose to a final concentration of 12–13%. After centrifugation at 21,000 *g* for 90 min at 4°C, the supernatant, referred to as the soluble protein fraction, was precipitated with 12.5% (w/v) trichloroacetic acid (TCA)/acetone containing 0.07% (v/v) β-mercaptoethanol overnight at –20°C and centrifuged at 14,000 *g*. Meanwhile, the pellet, referred to as the microsomal membrane protein fraction, was resuspended in a wash buffer (20 mM HEPES, pH 8.0, 5 mM EDTA, and 5 mM EGTA) and re-centrifuged at 21,000 *g* for 45 min at 4°C. Finally, all of the resulting pellets were additionally washed with 80% acetone and stored –20°C for further analysis. The extraction of MPs using the PME kit was carried out following the instructions of the manufacturer.

A Western blot analysis was performed as described in a previous study (Min et al., 2015). The antibodies, such as the anti-luminal binding protein (BiP), anti-PM intrinsic protein 2-1 (PIP 2-1), and anti-tonoplast intrinsic protein 1-1 (TIP 1-1), were used in the Western blot analysis to confirm the presence of the resident proteins of the Golgi apparatus, PM, and vacuolar membrane, respectively.

## Protein Solubilization and In-Solution Digestion Using the Filter-Aided Sample Preparation Method

The isolated MPs were subjected to in-solution trypsin digestion employing the filter-aided sample preparation (FASP) approach (Wiśniewski et al., 2009; Min et al., 2020a,b). In brief, the acetone-precipitated MPs (300 μg) were dissolved in 30 μl of a denaturation buffer [4% SDS and 100 mM of DTT in 0.1 M of tetraethylammonium tetrahydroborate (TEAB), pH 8.5], sonicated for 3 min, and heated at 99°C for 30 min followed by the loading of the proteins onto a 30K spin filter (Amicon Ultra-0.5 ml, Merck Millipore, Darmstadt, Germany). The protein extract was diluted with a Urea-TEAB (UA) buffer (8 M urea in 0.1 M TEAB, pH 8.5) to a final volume of 300 μl. The protein extract was washed three times with 300 μl of a UA buffer by centrifugation at 14,000 *g* to remove the SDS. Cysteine alkylation was accomplished through the addition of 200 μl of an alkylation buffer [50 mM of iodoacetamide (IAA), 8 M of urea in 0.1 M of TEAB, pH 8.5] for 1 hat room temperature in the dark. This was followed by switching the UA buffer with a TEAB buffer (50 mM of TEAB, pH 8.5) in a spin filter unit. The protein was digested with trypsin [enzyme-to-substrate ratio (w/w) of 1:100] dissolved in 50 mM of the TEAB buffer containing 5% acetonitrile (ACN) at 37°C overnight. The digested peptides were collected by centrifugation, and the filter device was rinsed with 50 mM of TEAB and 50 mM of sodium chloride (NaCl). The procedure for protein solubilization and digestion using the Azo surfactant was similar to the procedures discussed in the previous sections, which used Azo (1% as final concentration) as a replacement for SDS. Prior to reduction and alkylation, the Azo was degraded by exposing the samples to UV-vis irradiation using a 100-W high-pressure mercury lamp (Nikon housing with Nikon HB-10101AF power supply; Nikon, Tokyo, Japan) as described in a previous study (Brown et al., 2019). After the UV irradiation of the samples, the alkylation and incubation with a trypsin solution were applied following the procedures discussed in the previous sections. Finally, the peptide concentrations were measured using the Pierce Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, MA, United States) following the instructions of the manufacturer. Further peptide desalting (Gupta et al., 2020; Min et al., 2020b) and pre-fractionation using a basic pH reverse-phase (BPRP) column were also carried out as described in previous studies (Kim et al., 2018; Min et al., 2020b). Detailed procedures for peptide desalting, BPRP peptide fractionation, Q-Exactive Orbitrap liquid chromatography–tandem MS (LC-MS/MS) analysis (Pajarillo et al., 2015; Meng et al., 2019), and data analyses with functional annotations using MaxQuant (Savaryn et al., 2016; Tyanova et al., 2016a), Perseus (Tyanova et al., 2016b), MetaboAnalyst (Chong et al., 2018), AgriGO v2.0 (Tian et al., 2017), CELLO2GO (Yu et al., 2014), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2017; Kanehisa and Sato, 2020) are described in the **Supplementary Material**. The generated proteomics data

were deposited to the ProteomeXchange Consortium *via* the PRIDE partner repository with the dataset identifier PXD025132 (Perez-Riverol et al., 2019).

# RESULTS

## Label-Free Proteomic Analysis of Proteins Isolated by the MME and PME Methods

To compare the protein profiles of the isolated MPs, a label-free quantitative proteomic approach was utilized, which led to the identification of 18,240 peptides and 15,737 unique peptides that matched with 4,045 protein groups, resulting in an average sequence coverage of 13% (**Figure 1A**). Specifically, the three replicates of the same sample in PME showed less than 12.5% of the coefficient of variation (CV) values, while less than 4.2% of the CV values were observed in the case of the MME method (**Figure 1A**). Further removal of potential contaminants and proteins with >30% missing values (70% valid values in the three replicates in at least one sample) narrowed down the identification list to 2,384 proteins (**Figure 1B**).

Among these 2,384 proteins, 302 (12.7%) and 192 (8.1%) proteins were uniquely identified in the MME and PME samples, respectively, while 1,890 (79.3%) proteins were commonly identified in both approaches (**Figure 1C** and **Supplementary Table 1**). The subcellular localization prediction of commonly identified proteins revealed that 19.8 (375 proteins) and 38.9% (735 proteins) of the proteins were localized in the cytoplasm and organelle, respectively. On the other hand, 41.3% (780 proteins) of the proteins were localized to the cellular membranes as determined with Uniport and the CELLO2GO web-based database (**Figure 1D**; Yu et al., 2014). The results also showed that, among the 192 proteins specific to the PME method, 30 (15.6%) and 69 (35.9%) proteins were found to be localized in the cytoplasm and organelles, respectively. Furthermore, 93 (48.4%) proteins were determined to be MPs (**Figure 1E**). Regarding the 302 proteins specific to the MME method, 46 (15.2%) and 148 (49%) proteins were predicted to be localized to the cytoplasm and organelles, respectively. Additionally, 108 (35.8%) proteins were determined to be MPs (**Figure 1F**). Proteins associated with the PM, chloroplast membrane, ER membrane, mitochondrial membrane, Golgi membrane, vesicle membrane, vacuolar membrane, and endosome membrane were identified in both methods, while lysosome membrane proteins were only identified in the MME method. The PM, chloroplast membrane, ER membrane, and mitochondrial membrane proteins were the major MPs accounting for 34 (38.2%), 25 (28.1%), 8 (9%), and 8 (9%) of the total specific MPs in the PME method, respectively (**Figure 1E**). Meanwhile, the PM, mitochondrial membrane, ER membrane, and vacuolar membrane proteins were the main MPs, accounting for 82 (66.1%), 12 (9.7%), 9 (7.3%), and 8 (6.5%) of the total specific MPs in the MME method, respectively (**Figure 1F**). The Western blot analysis of the soluble and microsomal proteins demonstrated a clear and better enrichment of a plasma membrane marker protein, plasma membrane-localized PIP2-1, in the MME as compared with the PME sample (**Supplementary Figure 1**).

For examining the correlation and variations between the two sample sets and the reproducibility of different replicates of the same sample, multi-scatter plot and principal component (PCA) analyses were performed (**Supplementary Figures 2A,B**). The multi-scatter plots showed Pearson correlation coefficient values among the same sample ranging from 0.962 to 0.970 and from 0.972 to 0.973 in the three replicates of PME and MME, respectively. This suggests a strong correlation among the replicates of each sample set (**Supplementary Figure 2A**). In addition, the PCA plot showed a clear separation between the two samples in principal component 1 that accounted for 93.5% of the total variation (**Supplementary Figure 2B**). The application of a multiple ANOVA test controlled by a Benjamini-Hochberg false discovery rate (FDR) threshold of 0.05 with a fold change of more than 1.5 on the 2,384 high-confidence proteins revealed the identification of 1,222 significantly modulated proteins between the two sample sets. Among these, 614 and 608 proteins showed increased abundance when extracted with the MME and PME methods, respectively (**Supplementary Figure 2C**).

## Functional Classification of the Identified Proteins

A volcano plot and sequential hydrophobic cluster analysis (HCA) segregated all the significantly modulated proteins into two major clusters. The results revealed that cluster_1, containing 614 proteins, showed an increased abundance in the MME sample and cluster_2, having 608 proteins, had an increased abundance in the PME sample (**Figure 2A** and **Supplementary Table 2**). Functional classification of the identified proteins by Gene Ontology (GO) enrichment analysis (Tian et al., 2017) revealed that the intracellular and membrane categories were found to be the most enriched terms in both the clusters in the cellular component category (**Supplementary Table 3**). In the intracellular category, two subgroups consisting of organelle (with 67 and 86 proteins identified in clusters 1 and 2, respectively) and cytoplasm (comprising 74 and 78 proteins found in clusters 1 and 2, respectively) were enriched in the analysis (**Supplementary Table 3**). In the membrane category, five different subgroups, namely, intrinsic to the membrane, photosynthetic membrane, organelle membrane, proton-transporting ATPase, and membrane (miscellaneous), were determined in both clusters. However, the associated proteins were majorly enriched in the MME method as compared with the PME method (**Supplementary Table 3**). Moreover, the membrane coat proteins were specifically identified in the MME sample, while photosynthetic membrane proteins were only identified in the PME sample (**Supplementary Table 3**).

## Kyoto Encyclopedia of Genes and Genomes and Subcellular Localization Analysis of the Identified Proteins

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis using the KEGG brite database

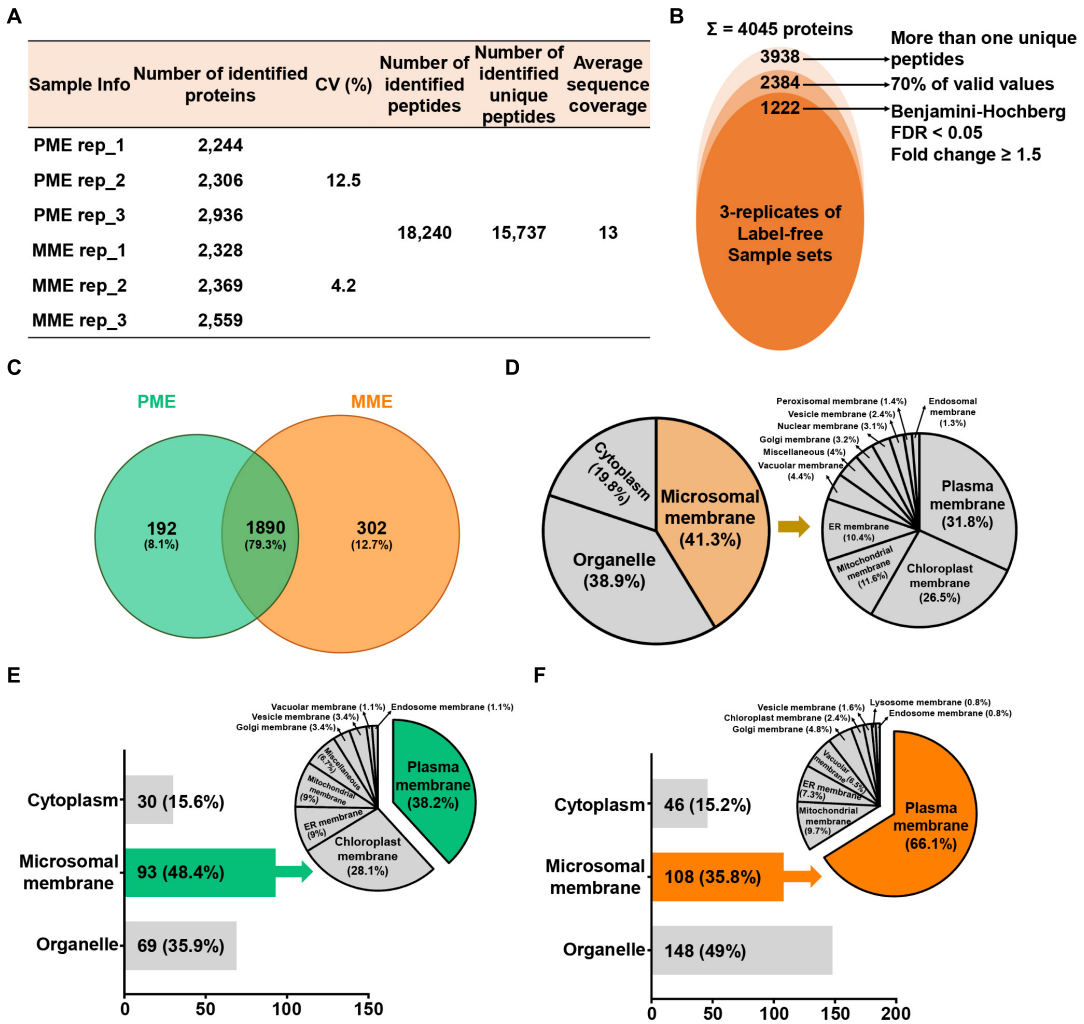



**FIGURE 1 |** Label-free quantitative proteomic analysis of microsomal membrane proteins (MPs) extracted from rice leaves using the MME and PME methods. **(A)** Table revealing the information of identified proteins in two different sample sets with replicates. **(B)** Circular diagram showing the distribution of the total identified and significantly modulated proteins followed by narrow down approaches. **(C)** Comparison and determination of subcellular localization using 2,384 identified proteins after the removal of potential contaminants. Venn diagram showing the distribution of specifically and commonly identified proteins extracted by the PME and MME methods. Subcellular localization analysis was carried out using commonly **(D)** and uniquely identified proteins in the PME **(E)** and MME **(F)** methods. MME, microsomal membrane protein extraction; PME, plasma membrane extraction kit.

(Kanehisa et al., 2017; Kanehisa and Sato, 2020) resulted in the identification of various biological objects including enzymes and other proteins related to the exosome, membrane trafficking, mitochondrial biogenesis, ribosome, and transport, among many others (**Supplementary Table 4**). In addition, the proteins related to cellular transport reactions including the exosome, membrane trafficking, transport, protein kinase, ion channel, and secretion system showed enriched abundance profiles in the MME sample as compared with the PME sample (**Figure 2B** and **Supplementary Table 5**). A total of 62, 59, 27, 11, 5, and 3 proteins showing increased abundances in the MME sample were found to be associated with the exosome, membrane trafficking, transport, protein kinase, ion channel, and secretion system, respectively. These proteins included syntaxin, the SNARE protein, ABC transporter, mitochondrial phosphate carrier protein, sugar transport protein, potassium transporter, mitochondrial outer membrane protein porin, and signal recognition particle receptor subunit alpha, among others. In contrast, only 57 proteins related to cellular transport reactions showed increased abundance in the PME sample (**Figure 2B** and **Supplementary Table 5**).

The prediction of the subcellular localization of significantly modulated proteins showed the enhanced abundance of membrane-resident proteins in both the MME and PME samples (**Figures 2C,D**; Yu et al., 2014). The results showed that, among the 1,222 significantly modulated proteins between the MME and PME methods, 42.8% were localized to various organelles in the samples of the former and 47.7% in those of the latter. However,

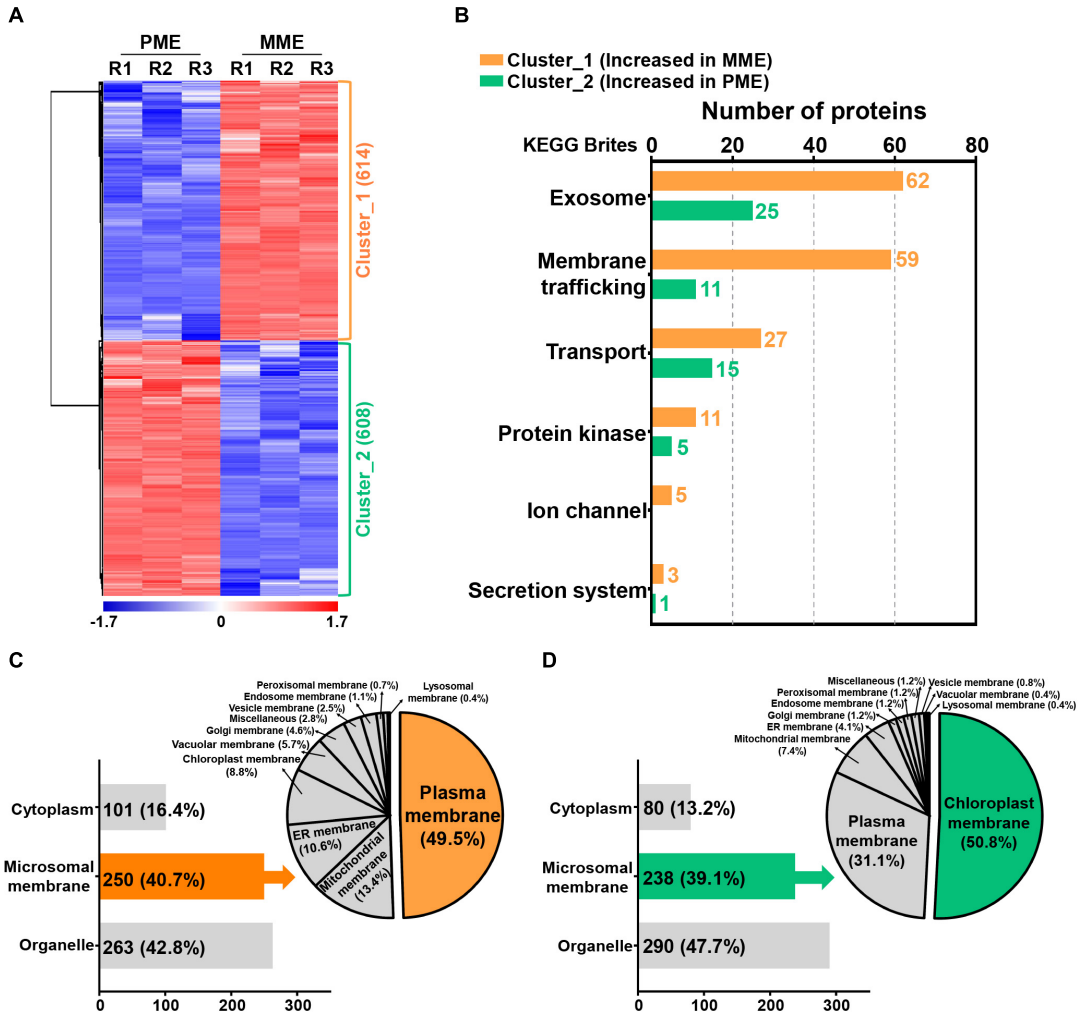



**FIGURE 2 |** Quantification and functional characterization of the significantly modulated microsomal membrane proteins of rice leaves. **(A)** Hydrophobic cluster analysis (HCA) of 1,222 significantly modulated proteins grouped into two major clusters based on their abundance patterns. **(B)** Functional hierarchical classification of proteins with increased abundance in the MME and PME samples, respectively, using the List of identified Kyoto Encyclopedia of Genes and Genomes (KEGG) brite database. Subcellular localization analysis of significantly modulated proteins is represented as clusters, wherein cluster_1 corresponds to the increased abundance in MME **(C)** and cluster_2 corresponds to the increased abundance in PME **(D)**.

the MPs were enriched by a similar fraction (39–41%) in both the methods (**Figures 2C,D**). Microsomal membrane proteins showing an increased abundance in the MME sample were also predicted to be localized in the PM (49.5%), mitochondrial membrane (13.4%), ER membrane (10.6%), chloroplast membrane (8.8%), vacuolar membrane (5.7%), Golgi membrane (4.6%), vesicle membrane (2.5%), endosome membrane (1.1%), peroxisomal membrane (0.7%), lysosomal membrane (0.4%), and other membranes (miscellaneous) (2.8%) (**Figure 2C**). Meanwhile, the chloroplast membrane-, PM-, mitochondrial membrane-, and ER membrane-associated proteins were the major MPs accounting for 50.8, 31.1, 7.4, and 4.1% of the total specific MPs in the PME method, respectively (**Figure 2D**). Overall, these results showed that the MME method yielded a highly enriched microsomal protein fraction of rice leaves with minimal chloroplast contamination as compared with the PME method. Moreover, a further comparison using the same approach with the above analysis for the evaluation of the solubilization efficacy of the two different surfactants, Azo and SDS, was carried out. This was carried out to increase the dynamic resolution of the microsomal membrane proteome in the rice leaves.

## Label-Free Quantitative Proteomic Analysis of Azo- and SDS-Solvated Proteins

The synthesis and validation of Azo surfactant were carried out following a previous report before conducting the comparative proteomic analysis (Brown et al., 2019). After the affirmation of the mass, the accurate structure (**Supplementary Figure 3**) and rapid degradation of Azo under UV irradiance

(**Supplementary Figure 4**) and MPs from rice leaves were isolated using the MME method. Subsequently, the obtained MPs were solubilized in either Azo or SDS for comparison. Quantitative analysis showed the solubilization of 96 ± 7 μg of proteins in Azo and 74 ± 9 μg of proteins in the SDS sample per 100 μg of membrane proteins used. This highlighted the better protein solubilization efficiency of Azo compared with SDS. However, subsequent Western blot analyses of the Azo- and SDS-dissolved proteins demonstrated organellar enrichment profiles in the MP fractions utilizing antibodies against membrane-localized BiP, PIP2-1, and TIP1-1 proteins (**Figure 3**). This suggested the comparable technical efficiencies of SDS and Azo in the solubilization of MPs.

A quantitative proteomic assessment of Azo- and SDS-solubilized MPs (labeled as MME_Azo and MME_SDS, respectively) showed the identification of 42,289 peptides and 35,560 unique peptides matched with 5,880 protein groups (**Figures 4A,B**). The three replicates of each sample set in MME_SDS and MME_Azo samples revealed CV values less than 4.3 and 1.3%, respectively. This suggested that the MME_Azo sample yielded a much reproducible result as compared with MME_SDS. Furthermore, the sequential downstream processing led to the identification of a total of 3,972 proteins with 70% valid values within three replicates (**Figure 4B**). Pearson's correlation coefficient values of the triplicates of the same datasets showed high degrees of correlation and ranged from 0.973 to 0.978 and from 0.972 to 0.976 in the MME_SDS and MME_Azo samples, respectively (**Figure 4C**). In addition, the PCA plot analysis showed a clear separation between the two samples in principal component 1 that accounted for 92.2% of the total variation (**Figure 4D**). A multiple ANOVA test controlled by a Benjamini-Hochberg FDR threshold of 0.05 with a fold change of more than 1.25 was applied, which showed a total of 828 significant differentially modulated proteins (**Figure 4B** and **Supplementary Table 6**).

## Functional Annotation and Subcellular Localization Analysis of the Identified Proteins

The volcano plot and HCA of the significantly modulated proteins from the MPs fraction solubilized in two different surfactants were performed to determine protein abundance patterns (**Figures 5A,B**). The statistically significant proteins were sorted into two major clusters, of which 589 proteins (cluster_1) and 289 proteins (cluster_2) exhibited patterns of increased abundance in MME_Azo and MME_SDS, respectively (**Figures 5A,B** and **Supplementary Table 6**). The functional annotation of proteins exhibiting significant differences indicated that intracellular (99 and 67 proteins identified in cluster 1 and 2, respectively) and macromolecular complex (46 and 57 proteins identified in clusters 1 and 2, respectively) were the major enriched GO terms of the cellular component category in both the MME_Azo and SDS samples (**Supplementary Table 7**). Furthermore, the increased abundance of the 61 proteins related to membrane (GO:0016020) function, including the ABC transporter, transmembrane protein, importin, vesicle transport SNARE protein, mitochondrial carrier protein, and potassium transporter, among others, was uniquely observed in the MME_Azo samples (**Supplementary Table 7**).

The KEGG analysis further confirmed the functional association of the proteins showing an increased abundance in the MME_Azo samples to the exosome, membrane trafficking, transporter, protein kinase, and ion channel (**Figure 5C** and **Supplementary Tables 8**, **9**). Subcellular localization showed that, out of 589 proteins showing an increased abundance in MME_Azo (cluster_1), 88 (14.9%) and 317 (53.8%) proteins were predicted to be localized in the cytoplasm and organelles, respectively. On the other hand, 184 (31.2%) proteins were specifically localized to different cellular membranes (**Figure 5D**). Moreover, 289 proteins showing an increased abundance in the MME_SDS sample (cluster_2) were found to be located at the cytoplasm (51 proteins, 21.3%), organelles (144 proteins, 60.3%), and cellular membranes (44 proteins, 18.4%) (**Figure 5E**). In particular, the subcellular localization prediction of highly abundant MME_Azo proteins showed their association with the PM (76 proteins, 37.6%), chloroplast membrane (27.2%), and mitochondrial membrane (9.2%). The endoplasmic reticulum, nucleus, vesicle, and Golgi membrane, among others, accounted for 26% of the proteins in the MME_Azo sample (cluster_1) (**Figure 5D**). In contrast, the highly abundant protein cluster of MME_SDS was distinctly represented by the PM (16 proteins, 35.6%), chloroplast (37.8%), mitochondrial membrane (8.9%), and ER membranes (4.4%), while the remaining organelles were represented by 13.3% of the total proteins in MME_SDS sample (cluster_2) (**Figure 5E**).

# DISCUSSION

Membranes perform specific functions depending on their associated proteins and carry out the task of demarcating the boundaries between cells and cellular organelles (Luschnig and Vert, 2014; LaMontagne et al., 2016). In particular, microsomal membrane proteins play key roles in essential biological processes such as plant development and tolerance to environmental stressors by activating diverse signaling pathways (Komatsu et al., 2007; Takagi et al., 2011; Gomez-Navarro and Miller, 2016). Some of these signaling events and other functions of MPs are well-characterized, but the majority of these remain understudied because of the technical difficulties in the isolation of these highly hydrophobic proteins. Therefore, this study developed a relatively simpler protocol for the isolation and subsequent solubilization of MPs using rice as a model system. The method is referred to as the MME method in this study. The efficacy of the developed protocol was assessed by two consecutive label-free quantitative proteomic analyses. The foremost analysis dealt with a comparative assessment of the efficacies of the MME and PME methods in the isolation of MPs and the second strategy included a comparison of the solubilization efficiencies using two different surfactants (SDS and Azo surfactants).

The isolation of MPs using ultracentrifugation was first reported by the study of Blackburn and Kasper in 1976, when they used a centrifugation speed of 394,000 *g* for

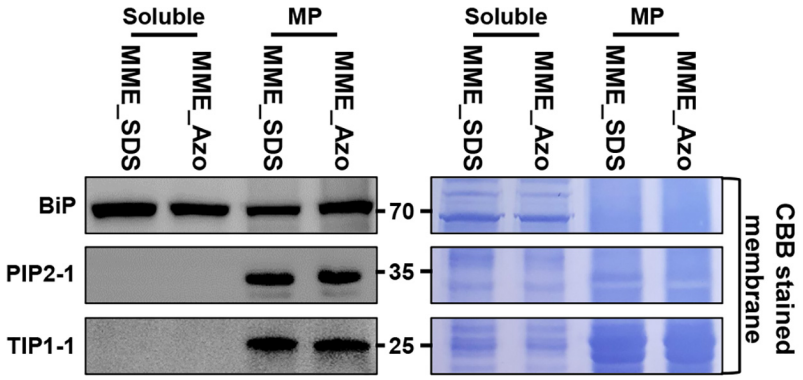
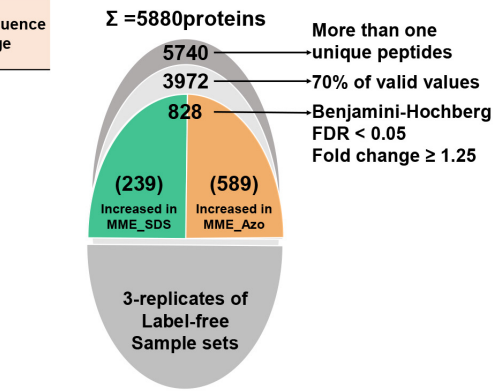
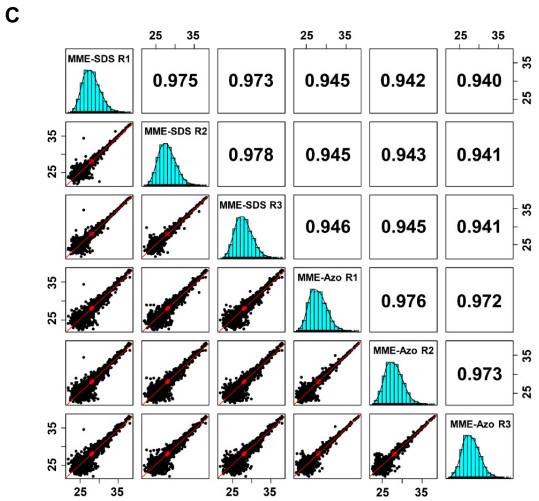



**FIGURE 3 |** Extraction of microsomal membrane proteins using the MME method and sequentially solubilized in the sodium dodecyl sulfate (SDS) and 4-hexylphenylazosulfonate (Azo) surfactants for label-free quantitative analysis. Further validation of the solubility of microsomal membrane proteins in the SDS and Azo surfactants and Western blot analyses using BiP, PIP2-1, and TIP1-1 antibodies were carried out.

**A**

| Sample Info | Number of identified proteins | CV (%) | Number of identified peptides | Number of identified unique peptides | Average sequence coverage |
|---|---|---|---|---|---|
| MME_SDS Rep_1 | 4,292 | | | | |
| MME_SDS Rep_2 | 4,323 | 4.3 | | | |
| MME_SDS Rep_3 | 4,715 | | | | |
| | | | 42,289 | 35,560 | 23.2 |
| MME_Azo Rep_1 | 4,252 | | | | |
| MME_Azo Rep_2 | 4,243 | 1.3 | | | |
| MME_Azo Rep_3 | 4,362 | | | | |



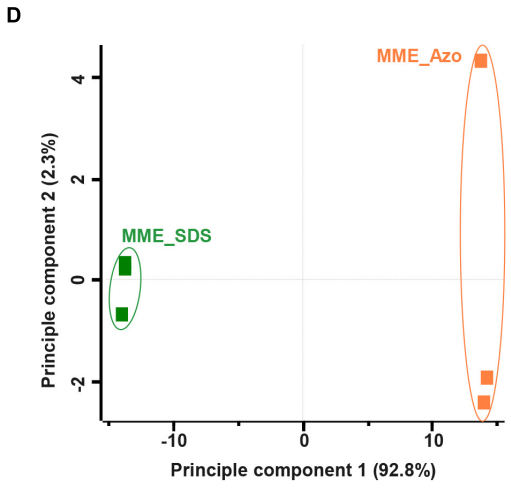




**FIGURE 4 |** Label-free quantitative proteomic analysis of microsomal membrane proteins solubilized in the SDS and Azo surfactants, respectively. **(A)** Table showing the information of identified proteins in two different sample sets with replicates. **(B)** The narrow-down approach for the identification of significantly modulated proteins. **(C)** Multi-scatter plots represent the Pearson's correlation coefficient values of each sample solubilized in SDS and Azo. **(D)** Principal component analysis of significantly modulated proteins. MME_SDS, microsomal membrane extraction and dissolved in SDS buffer; MME_Azo, microsomal membrane extraction and dissolved in Azo buffer.

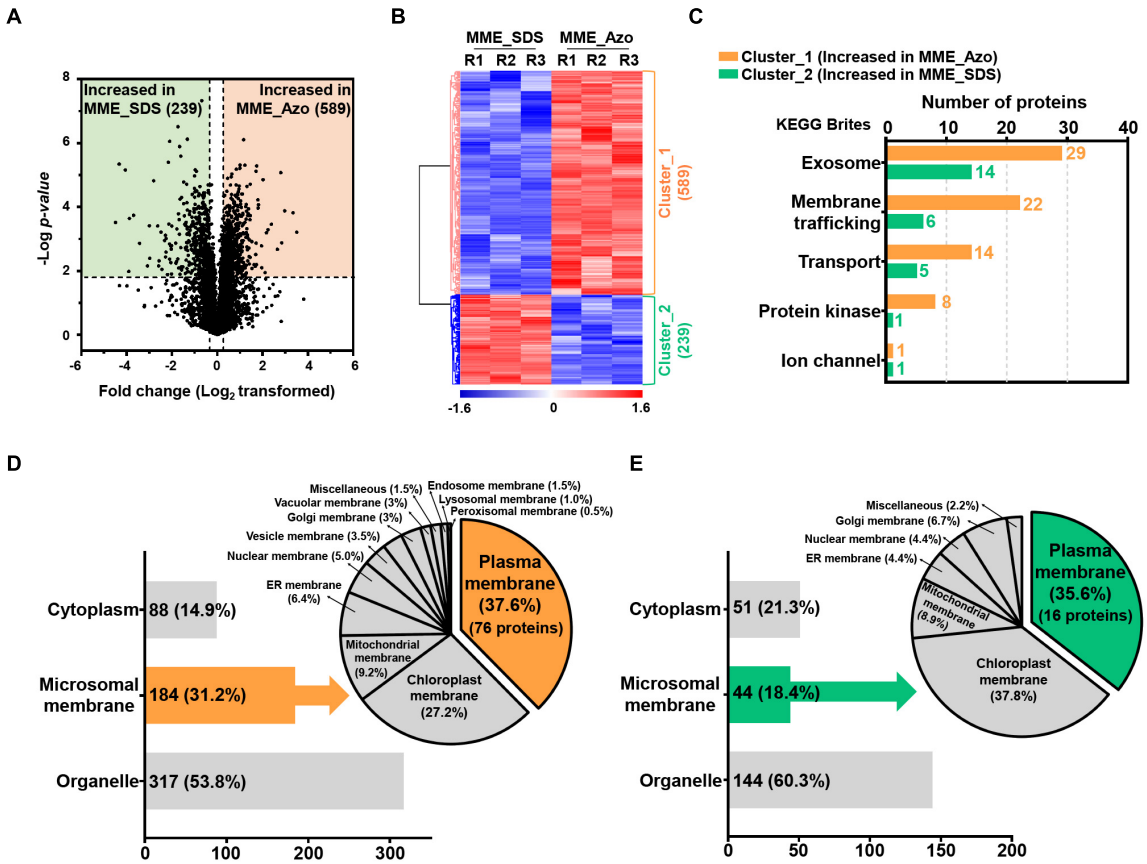



**FIGURE 5 |** Determination of the abundance profiles and functional classifications of the significantly modulated proteins solubilized in SDS and Azo. Volcano plot **(A)** and HCA **(B)** highlighting the relative fold change differences of the identified proteins from the microsomal membrane fractions solubilized by SDS and Azo. **(C)** The functional classifications of significantly modulated proteins by KEGG pathway analysis using the KEGG brite database. **(D)** Subcellular localization analysis of the proteins with increased abundance in MME_Azo (cluster_1) **(D)** and MME_SDS (cluster_2) **(E)**, respectively. Representatively, three major localizations including the cytoplasm, microsomal membrane, and organelles were predicted by UniProt and the CELLO2GO web-based database.

2 min to isolate insoluble membrane fraction from the rat hepatocytes (Blackburn and Kasper, 1976). In the case of plants, ultracentrifugation was first utilized to purify the PM proteins from barley roots, through which the enrichment of PM proteins was achieved using density gradient centrifugation (Nagahashi et al., 1978). As of today, these ultracentrifugation-based methods are still the most efficient and successful methods for the isolation of MPs from plants (LaMontagne et al., 2016; Meisrimler et al., 2017; Junková et al., 2018). In a recent report, the enrichment of MPs was attempted for the identification of the potential interaction partner of flotillins in *Arabidopsis thaliana* which led to the identification of ATPase 1, early-responsible to dehydration stress protein 4, and syntaxin-71, to be flotillins interacting proteins (Junková et al., 2018). In addition, a study by Meisrimler et al. (2017) employed differential centrifugation for 10 min at 10,000 *g* and 30 min at 50,000 *g* to remove cell debris and soluble proteins, respectively, and subsequently isolate the root-microsomal proteins from four pea cultivars exhibiting variable root lengths during germination (Meisrimler et al., 2017). In particular, the authors identified 33 (55%) MPs associated with the plastid, Golgi membrane, mitochondria, nucleus, tonoplast, and vesicle, of which 20% had a transmembrane region and 27 proteins (45%) were predicted to be localized in the cytoplasm (Meisrimler et al., 2017). Similarly, a few other research groups have also performed the identification and characterization of MPs in plant systems. However, limited efforts have been invested toward the development of a rapid, simple, and reproducible method for plant samples.

The results of this study suggested that the MME method led to the successful enrichment of several classical MPs as compared with the PME method. Moreover, the majority of the MPs such as sucrose transport protein SUT1, ABC transporter, sugar transporter, receptor-like serine/threonine-protein kinase, calcium transporting ATPase, elicitor responsive protein 1 (ERG1), aquaporin PIP2, sodium/calcium exchanger NCL1, and voltage-gated potassium channel protein, among others, represented higher abundance profiles in the MME methods as compared with the PME method. This difference between the extraction efficiencies of the two methods could be because of the use of different extraction and wash buffers (**Figure 6A**;

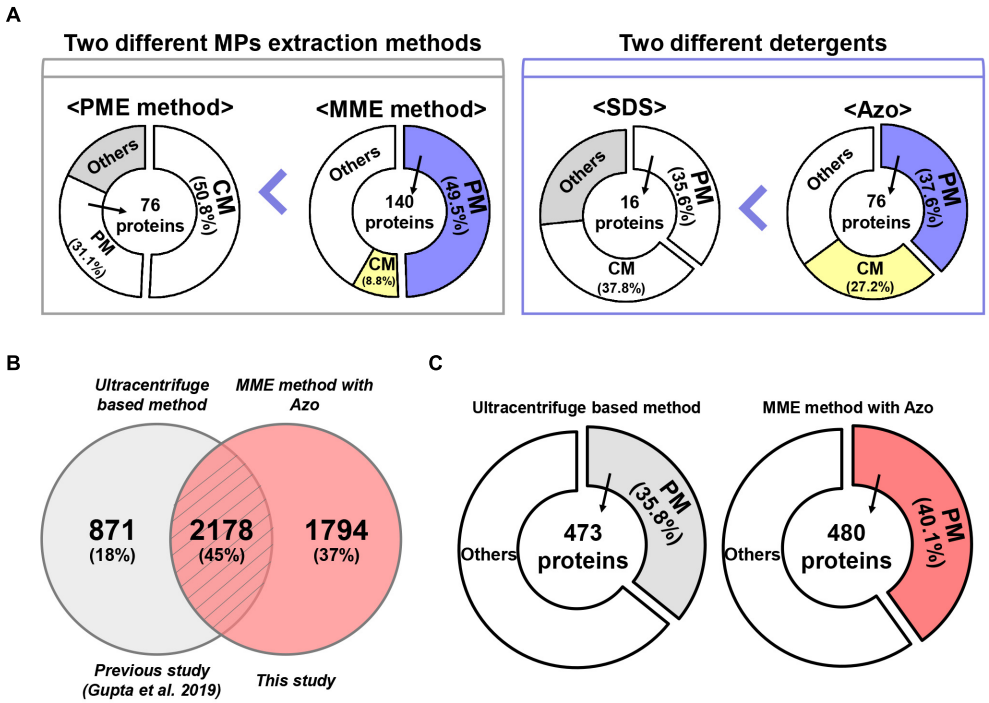



**FIGURE 6 |** A comparative analysis of MME vs. PME methods and SDS vs. Azo utilizing variable methods for microsomal proteomics. **(A)** The MME method revealed the successful enrichment of MPs, a particularly higher fraction of PM proteins in bottom-up proteomic analysis. Moreover, MPs, especially PM proteins, were solubilized more effectively in Azo and demonstrated differential proteomic identifications when compared with the SDS-based method. **(B)** Venn diagram and **(C)** subcellular localization showing a further comparison of the number of identified proteins in the current study and previous study.

Shen et al., 2015; Meisrimler et al., 2017). Furthermore, in this study, we recorded the presence of chloroplast and cytoplasmic proteins in both MME and the PME methods, with the former having a lesser abundance. However, the detection of membrane proteins in most microsomes indicated that chloroplast and cytoplasmic contaminations have minor or no impacts on the identification of MPs. This suggested that the complete removal of chloroplast and cytoplasmic contaminations are somewhat unnecessary. The label-free quantitative proteomic analysis and the functional annotation also provided convincing evidence that the MME method was more effective than the PME method in isolating MPs, leading to an improvement in PM protein recovery.

Plasma membrane proteins contribute a large part of the MPs and play crucial roles in sensing signals, catalysis, transport, adhesion, and construction, which ensure the survival and development of the cells. However, the enrichment of PM proteins is also one of the most significant hurdles for plant proteomics because of their low abundance and high hydrophobicity (Santoni et al., 2000; Kalipatnapu and Chattopadhyay, 2005; Speers and Wu, 2007; Helbig et al., 2010). Conventional methods using ultracentrifugation (centrifugation speed ranging from 40,000 to 156,000 *g*) coupled with sucrose density gradient centrifugation allowed the achievement of relatively pure PM fractions from roots of various plants such as barley, corn, and oats. However, these approaches are known to be complicated and are not adapted universally for green tissues due to unavoidable contaminations by fragmented chloroplasts (Nagahashi et al., 1978; Yoshida et al., 1983; Yang and Murphy, 2013). A previous study focusing on the isolation of transport competent vesicles from plant tissues reported that mitochondrial membrane proteins can be enriched using centrifugation at 13,000 *g* for 3–4 min. In contrast, if the centrifugation was prolonged to 20 min, most of the PM ATPase activity was suspended (Giannini et al., 1988). This procedure nonetheless requires a higher amount of starting material, usually 20–25 g. In the case of this study, only 200 mg or less of starting material was adequate for the isolation of a sufficient amount of MPs for proteome analysis. Moreover, this study also demonstrated that centrifugation time plays a crucial role in the enrichment of microsomal/PM proteins. A higher number of PM proteins were also identified here by the MME method in this study compared with the PME method. This could be due to the prolonged centrifugation (1.5 h) used in the MME method to sediment the PM proteins in our experiment (Giannini et al., 1988).

Protein solubility, especially in the case of MPs, is another major challenge in proteomic studies (Speers and Wu, 2007). Particularly, the anionic detergent SDS has been the most commonly used detergent for the processing of protein samples because it can denature, solubilize, stabilize, and establish proteins separation through SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Bhuyan, 2010).

Although techniques such as exchange liquid chromatography, metal-organic frameworks MIL-101, gel filtration, dialysis, SDS precipitation, affinity spin column, and FASP method have been developed for the effective removal of SDS from protein extracts, SDS at a concentration higher than 0.01% results in a decrease in the signal-to-noise ratio and resolution of the ions of interest during the MS analysis (Wiśniewski et al., 2009; Botelho et al., 2010; Sun et al., 2012; Ilavenil et al., 2016; Žilionis, 2018). To address these problems, we used a photocleavable anionic surfactant, namely, Azo, as a replacement for SDS in the MME method (Brown et al., 2019, 2020). Our results showed that the solubilization of MPs in Azo was advantageous over SDS as the number, amount, and abundance of MPs were higher in the MME_Azo sample as compared with the MME_SDS sample. Interestingly, 184 proteins in the MME_Azo sample were found to be MPs, with 76 proteins, including ABC transporter, aquaporin, sugar transporter protein MST4, transmembrane receptor family protein, potassium transporter, and voltage-gated potassium channel protein, among others, were specifically localized to the PM (**Figure 6A**). In the case of SDS solubilized proteins, only 16 PM proteins were identified out of 44 MPs (**Figure 6A**). A comparison of the identified MPs was made with a previous study that characterized various MPs associated with plant-pathogen interaction from rice leaves using an ultracentrifugation-based method (Gupta et al., 2019). Results showed 45% of the commonly identified MPs in both studies of which 18% (871 proteins; Gupta et al., 2019) and 37% (1,794 proteins; this study) of the MPs were specifically identified in each study, respectively (**Figure 6B**). Besides, a subcellular localization analysis of the total identified proteins in each study revealed that 480 PM proteins (40.1%) were identified specifically in the current study (**Figure 6C**), while only 473 (35.8%) of the proteins were found to be located at PM in the previous study (**Figure 6C**; Gupta et al., 2019). Further subcellular localization analysis of the identified proteins between the two studies provided convincing evidence that the MME method may comparatively be useful for isolating various MPs, especially for the enrichment of the PM proteins. Moreover, the results from the present study have suggested that Azo can effectively solubilize the MPs from rice leaves for bottom-up proteomics with a similar or higher performance that is comparable with that of SDS. Further, the advantageous effects of Azo in solubilizing various membrane-localized proteins have been validated with respect to SDS, which is in concordance with a similar methodology followed in the study by Brown et al. (2019) in animals. Since Azo is compatible with MS analysis and can easily be degraded under UV radiation, it can be safely implemented as an SDS replacement for bottom-up proteomics (Brown et al., 2020).

In conclusion, this study reported a simple, reproducible, and cost-effective method for the isolation of MPs for bottom-up proteomics. So far, Azo has only been used in the proteomics field for the top-down proteomics of animal proteins. Therefore, this is the first study in which the efficacy of Azo was shown in the solubilization of plant proteins for a bottom-up proteomic analysis (Brown et al., 2019). The Azo-integrated MME approach has its advantages, including being simple, time-saving, and easy to scale up in laboratory conditions. Furthermore, it is also highly effective in isolating and solubilizing the MPs with performance comparable with that of the ultracentrifugation-based methods.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: PRIDE (ID: PXD025132).

## AUTHOR CONTRIBUTIONS

RG, CWM, and STK conceptualized the experiments. TVN, GHL, and JWJ prepared samples for the proteomic analysis. DA and KHP synthesized the Azo surfactant with ESI-MS and $^{1}$H-NMR analyses. CWM, JY, and Y-JK performed the functional analysis of the proteomic data. TVN, CWM, RR, RG, and K-HJ wrote and made the English corrections. All authors read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.723369/full#supplementary-material

**Supplementary Figure 1 |** Validation of the extraction efficiencies of microsomal membrane proteins using the PME and MME methods, respectively, and the Western blot analysis using the plasma membrane-localized PIP2-1 antibody was carried out.

**Supplementary Figure 2 |** Statistical validation of the label-free quantitative proteomic analysis. **(A)** Pearson correlation coefficient values of the triplicates of each sample by a multi-scatter analysis. **(B)** Principal component analysis of significantly modulated proteins. **(C)** Volcano plot highlighting the relative fold change between the samples.

**Supplementary Figure 3 |** Structural validation of the synthesized Azo surfactant. The mass **(A)** and structure **(B,C)** of the Azo surfactant were measured by electrospray ionization–tandem MS (ESI-MS/MS) and $^{1}$H-nuclear magnetic resonance ($^{1}$H-NMR) analyses.

**Supplementary Figure 4 |** Photo-degradation kinetics of the Azo surfactant upon UV irradiation with normal **(A)** and heating **(B)** conditions after dissolving in a denaturation buffer.

**Supplementary Table 1 |** Identification of 2,384 proteins by label-free quantitative analysis using microsomal proteins extracted by two different methods in rice leaves.

**Supplementary Table 2 |** Identification of differentially modulated proteins by comparison between two different microsomal protein extraction methods.

**Supplementary Table 3 |** Gene ontology (GO) enrichment analysis of differentially modulated proteins by comparison between two different microsomal protein extraction methods.

**Supplementary Table 4 |** List of identified KEGG brite using differentially modulated proteins by comparison between two different microsomal protein extraction methods.

**Supplementary Table 5 |** KEGG pathway analysis of differentially modulated proteins by comparison between two different microsomal protein extraction methods using the KEGG brite database.

**Supplementary Table 6 |** Identification of differentially modulated proteins by comparison between the samples dissolved in different surfactants.

**Supplementary Table 7 |** GO enrichment analysis of differentially modulated proteins by comparison between the samples dissolved in two different surfactants.

**Supplementary Table 8 |** List of identified KEGG brite using differentially modulated proteins by comparison between the samples dissolved in two different surfactants.

**Supplementary Table 9 |** KEGG pathway analysis of differentially proteins by comparison between the samples dissolved in two different surfactants using the KEGG brite database.

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# Loss of Function of *OsFBX267* and *OsGA20ox2* in Rice Promotes Early Maturing and Semi-Dwarfism in γ-Irradiated IWP and Genome-Edited Pusa Basmati-1

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Targeted mutagenesis is now becoming the most favored methodology to improve traits in popular rice cultivars selectively. Understanding the genetic basis of already available mutants could be the first step in designing such experiment. Improved White Ponni (IWP), a popularly grown South Indian rice variety, was subjected to γ irradiation to develop WP-22-2, an $M_6$ line superior in semi-dwarfism, early flowering, and high yield, and it has grain qualities similar to those of IWP. The exogenous application of gibberellic acid ($GA_3$) on WP-22-2 resulted in the elongation of shorter internodes to a level similar to IWP. The expression profiling of six genes regulating plant height showed their differential expression pattern at different time points post $GA_3$ treatment. Furthermore, the sequencing of WP-22-2 and IWP genomes revealed several single nucleotide polymorphisms (SNPs) and large-scale deletions in WP-22-2. The conversion of functional codons to stop codons was observed in *OsGA20ox2* and *OsFBX267*, which have been reported to have roles in regulating semi-dwarfism and early flowering, respectively. The loss of function of *OsGA20ox2* and *OsFBX267* in WP-22-2 resulted in reduced plant height as well as early flowering, and the same has been confirmed by editing *OsGA20ox2* in the rice variety Pusa Basmati1 (PB1) using the CRISPR-Cas9 approach. The targeted editing of *OsGA20ox2* in PB1 conferred shorter plant height to the edited lines compared with the wild type. Altogether, the study provides evidence on mutating *OsGA20ox2* and *OsFBX267* genes to develop early maturing and semi-dwarf varieties that can be released to farmers after functional characterization and field trials.

**Keywords: dwarfing, early flowering, F-box protein, genome editing, genome sequencing, *Sd1***

## INTRODUCTION

Improved White Ponni is a medium-duration rice variety widely cultivated in Southern India. The high commercial consumer preference of this variety is due to superior grain quality traits, such as fine grain structure and high head rice recovery. This variety also has high yield potential and resistance to diseases such as rice tungro bacilliform virus infection, leaf yellowing, blast, and bacterial leaf blight (Rajendran, 2014). As Improved White Ponni (IWP) is tall (>140 cm in height) and has a medium duration in maturity, the variety is prone to lodging, which causes heavy yield loss to farmers (Subramanian et al., 1986). Thus, improving the architecture of the plant by reducing plant height and days-to-maturity could significantly enhance lodging resistance and prevent yield loss. Among the different approaches deployed for trait improvement, mutation breeding has been a preferred and successful method. Gamma-ray-induced mutations were shown to be effective in altering the improvement of agronomic growth and stress tolerance traits of several crops, which include rice. Such mutant lines were released as new varieties for commercial cultivation or had been used as donors of improved traits in breeding programs (Ahloowalia et al., 2004). Although the phenotypic characteristics of the gamma-induced mutants are extensively studied, the nucleotide-level changes hold the key to understanding the alterations in the genes that confer those specific phenotypes.

Gamma-ray irradiation causes genome-wide mutations, wherein identifying genes that underwent base changes is imperative to understand the molecular roles of those genes. Although conventional marker systems, such as simple-sequence repeats (SSRs), could identify major mutation sites, the resolution of those markers is not powerful enough to identify minor variations and single-base changes in the genes. This bottleneck was substantially ameliorated by the intervention of next-generation sequencing (NGS), as it allowed for the identification of genome-wide variations at base pair-level in the mutants (Barabaschi et al., 2016; Heuermann et al., 2019). Being the first crop genome to be sequenced, rice has gold-standard sequence data and enormous sequence information available in public domains (Eckardt, 2000; IRGSP, 2005; Song et al., 2018). This is a valuable resource for deploying comparative genomics approaches to compare any mutant genotype with the wild type to identify the mutations underlying the genetic determinants of improved traits in the mutants. Further characterization of those mutant genes derives their precise roles in regulating the desired phenotype, further aiding in breeding programs.

Dwarf plant type and short duration are two important attributes that breeding programs have been targeting to achieve in rice. IR8, a semi-dwarf cultivar released by the International Rice Research Institute in the Philippines has become the most popular cultivar in research to study these traits. The incomplete recessive gene, *d47*, caused semi-dwarfism in Dee-Geo-Woo-Gen, the parent line of IR8. Later, the recessive gene in the Calrose 76 variety was found to be *sd1,* which was allelic to *d47*. Similar allelic relationships were found in cv. Taichung Native 1 derived from Dee-Geo-Woo-Gen and cv. Shiranui derived from Jukkoku. The *d49* identified in the mutant cultivar Reimei was also allelic to *sd1*. It was proven that *sd1* is the most common locus that controls semi-dwarfism despite having different parentage (Tomita and Ishii, 2018). Despite this information, several other genes in the gibberellin pathway or that epistatically control the pathway have been known to control plant height in rice (Aach et al., 1997; Hedden and Phillips, 2000; Helliwell et al., 2001; Hong et al., 2005; Ishikawa et al., 2005; Ueguchi-Tanaka et al., 2005; Yamaguchi, 2008; Hedden and Sponsel, 2015). In the case of flowering, several studies have reported the genetic control of flowering time in rice. The FLOWERING LOCUS T/Heading date3a (*Hd3a*) protein identified in rice and *Arabidopsis* constitutes the flowering signal called florigen (Turck et al., 2008). In rice, *Hd3a* has functional variations because of its facultative short-day nature, and it is activated by several unique genes that are non-orthologous to *Arabidopsis*, such as *Ehd1*, *Ehd2*, *Ehd3*, *Ehd4*, *Ghd7*, and *MADS51*. These genes mediate the florigen pathway and control flowering time (Doi et al., 2004). Further, regulatory genes such as *OsLFL1* (Ryu et al., 2009), *OsMADS14* (Kyozuka et al., 2000), and *OsFKF1* (Han et al., 2015) are known to control flowering time in rice. However, because of the complex mechanisms regulating this trait, comprehensive genome-wide studies are essential to gain mechanistic insights into flowering and early maturity.

The previous study showed that the WP-22-2 mutant is sensitive to gibberellic acid (Andrew-Peter-Leon et al., 2021). Responsiveness to exogenous gibberellin (as measured by the reversal of internode elongation in dwarf mutants) indicates a defective gibberellin pathway in rice (Ashikari et al., 2002), and the famous mutant *sd1*allele is also sensitive to gibberellin. This study aimed to validate these observations further.

In this study, stable mutant lines of IWP with dwarf plant type and short duration were developed with γ-irradiation. The sequencing of mutant and wild-type lines detected mutations in the *OsFBX267* and *OsGA20ox2* genes subjected to further characterization. The results showed that the two genes regulate dwarf plant type and earliness in flowering, and further editing of *OsGA20ox2* using CRISPR-Cas9 in Pusa Basmati 1 validated the experimental findings of this study. Altogether, this study has identified two players that regulate dwarf plant type and earliness in flowering in rice, which could be further exploited in crop improvement programmes.

## MATERIALS AND METHODS

### Genetic Material

This study used two rice cultivars popularly cultivated in South (Improved White Ponni) and North India (Pusa Basmati 1) as experimental materials. IWP is a medium-duration (115 days to flowering) cultivar with fine slender grains (Subramanian et al., 1986). The seed materials were collected from the germplasm collections of the Tamil Nadu Agricultural University. The plants were raised at the Agricultural College and Research Institute, Killikulam, and Agricultural Research Station, Thirupathisaram, from 2011 to 2016.

## Mutagenesis, Selection, and Evaluation of Mutant Lines

Five hundred well-filled seeds of IWP were treated with different doses of gamma rays (100, 200, 300, 400, and 500 Gy) using Gamma Chamber (Model GC 1200, BRIT, India) installed at the Tamil Nadu Agricultural University, Coimbatore, India. Immediately after the treatment, the seeds were allowed to germinate in the fields of Agricultural College and Research Institute, Killikulam, India, following standard agricultural practices. Non-mutagenized IWP wild-type plants were also raised as control. The M1 plants were harvested individually at maturity, and 184 plants were advanced to $M_2$ in 2012. In $M_2$, 152 early flowering and dwarf mutants were identified and advanced to $M_3$ generation for further evaluation and validation. The $M_3$ population was evaluated for a reduction in days to flowering (early flowering), dwarfism, high yield, and fine grain quality traits compared with the IWP. In the $M_5$th generation, 70 mutants were chosen and screened for high yield and quality traits similar to IWP. Twenty such mutants were forwarded to the $M_6$ generation. In $M_6$ generation, the mutants were evaluated for morphological characteristics, such as plant height (PH, in cm), days to 50% flowering (DFF), number of productive tillers (NOPT), panicle length (PL, in cm), number of grains per panicle (GPP), single plant yield (SPY, in g), and 1,000-grain weight (TGW, in g). Furthermore, grain quality traits, namely, milling percent, head rice recovery (HRR) percent, grain length before cooking (LBC, in mm), grain breadth before cooking (BBC, in mm), grain length and breadth ratio (LB), grain length after cooking (LAC, in mm), grain breadth after cooking (BBC, in mm), linear elongation ratio (LER), breadthwise elongation ratio (BER), and alkali spreading value (ASV) were also evaluated. The chlorophyll content of the genotypes were measured (in SPAD units) using a portable chlorophyll meter (Soil Plant Analytical Development, SPAD, Model 502). The average leaf area (in $cm^2$) was measured by following the standard method (Yoshida, 1981).

As all the selected mutants were fixed and found to be stable in $M_6$ generation, a mutant designated as WP-22-2 was chosen for multiplication for further analyses. WP-22-2 was found to be superior in semi-dwarfism, early flowering, high yield, and similar grain qualities of IWP (fine-slender grains and cooking quality).

## Exogenous Application of Gibberellic Acid and Expression Profiling by qRT-PCR

Earlier, we reported the high sensitivity of WP-22-2 to 50 μM gibberellic acid (Andrew-Peter-Leon et al., 2021). This was tested by raising WP-22-2 along with IWP (wild-type) in a completely randomized design and spraying the 10-day-old seedlings with 50 μM of $GA_3$. Untreated plants served as the control, and three biological replicates for five technical replicates were maintained. Five days post sowing, the seedling height in cm, first internode length (in cm), and second leaf length (in cm) were measured and analyzed by Student's *t*-test.

For expression profiling, the leaves were harvested from treated and control plants 0, 6, 12, and 24 h post spraying of $GA_3$, and total RNA was extracted following standard procedures (Chomczynski and Mackey, 1995). Following the instructions of the manufacturer, the first-strand cDNA synthesis was performed using the Protoscript M-MuLVRT kit (NEB, Ipswich, MA, United States). Gene-specific primers for six regulatory genes involved in the gibberellin pathway of rice (*OsSLR1*, LOC_Os03g49990; *OsGA20Ox2*, LOC_Os01g66100; *OsKOL4*, LOC_Os06g37300; *OsKO2*, LOC_Os06g37364; *OsMAX2*, LOC_Os06g06050; *OsBRD2*, LOC_Os10g24780) were developed using GenScript Real-time PCR Primer Design tool (https://www.genscript.com/ssl-bin/app/primer) with default parameters. The qRT-PCR analysis was performed using SYBR Green detection chemistry on 7900HT Sequence Detection System (Applied Biosystems, Waltham, MA, United States). The reaction mixture in a final volume of 20 μl containing 2 μl of 5X diluted cDNA, 250 nM of each primer, and 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, United States) was subjected to initial denaturation for 2 min at 50°C and 10 min at 95°C, and 40 cycles of 15 s at 95°C and 1 min at 60°C. Three technical replicates for three independent biological replicates were maintained during the experiment.

## Whole-Genome Sequencing and Processing of Sequence Data

Total DNA was isolated from IWP and WP-22-2 following the standard CTAB method, and the quality and quantity of the samples were ascertained by resolving on 1.2% agarose gel and with a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), respectively. The DNA samples were then subjected to sequencing at 30X coverage on the Illumina HiSeq2500 platform (AgriGenome Labs Pvt Ltd, Hyderabad, India). Based on previous reports, more than 100 potential sites that control plant height and days to maturity in rice were chosen; their promoter and gene sequences were compared against the IWP and WP-22-2 and analyzed for indels and SNPs. SNPs that qualify in SNP calling were tested for their significance and filtered to obtain high-quality SNPs. Mutations identified in three genes, namely, *B3DNA* (LOC_Os03g42290), *OsFBX267* (LOC_Os08g09466), and *OsGA20ox2* (LOC_Os01g66100), were found to have frameshifts or premature terminations that affected the protein synthesis. These were further confirmed by Sanger sequencing. A multiple sequence alignment of Sanger sequencing data and corresponding reference genes was performed using MUSCLE (Madeira et al., 2019) under default parameters. The data was further analyzed on BioEdit (Hall, 1999). The genes and sequence variations were illustrated using Illustrator for Biological Sequences (Liu et al., 2015).

## Annotation and Analysis of Candidate Genes and Proteins

The gene sequences were BLAST-searched against rice genome (release 7) available in the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml) to identify the CDS (coding DNA sequence) of each gene. Furthermore, the alignment of candidate genes sequenced from IWP and WP-22-2 was performed in the

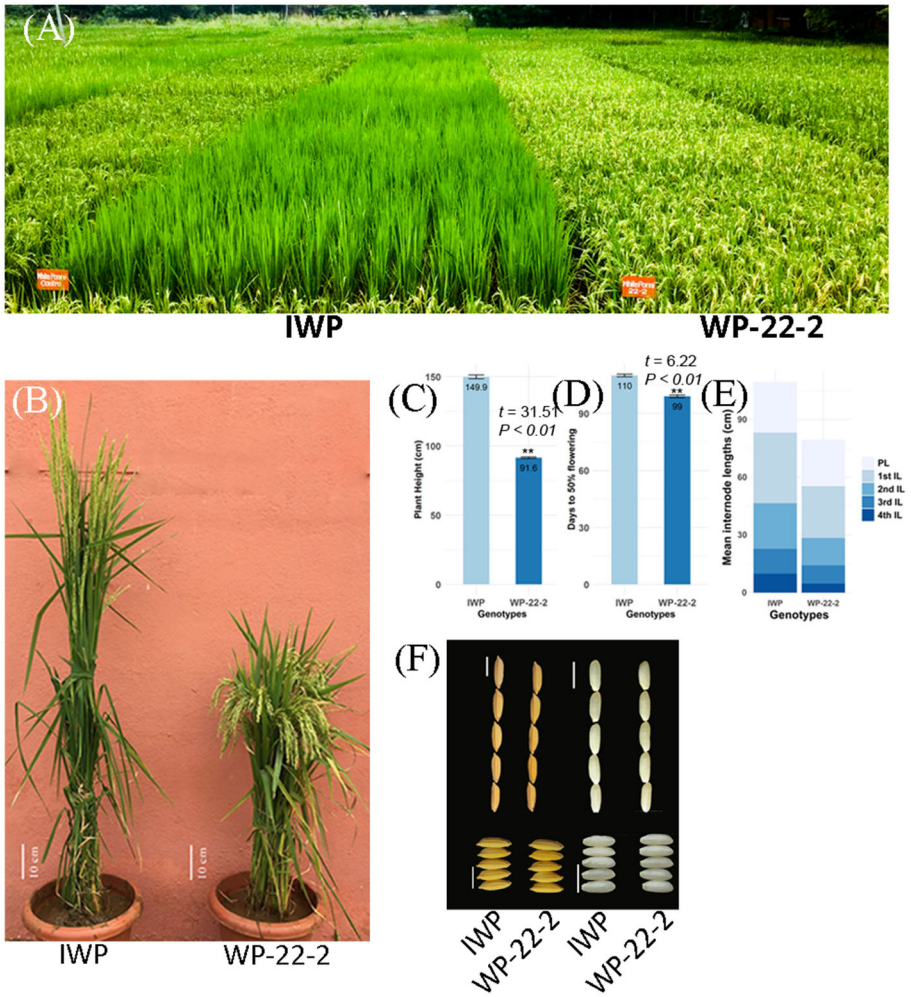


**FIGURE 1 |** Comparison between the rice variety Improved White Ponni (IWP) and superior mutant WP-22-2. **(A)** Field view of IWP and WP-22-2. IWP is clearly tall and still in vegetative stage, whereas WP-22-2 is dwarf and has already reached maturity. **(B)** Plants of IWP and WP-22-2 (scale bar: 10 cm). **(C,D)** Barplot showing difference in days to 50% flowering and plant height between c- IWP and m-WP-22-2. **(E)** Comparison of internode and panicle lengths of IWP and WP-22-2. **(F)** Length and breadth of rice grains of IWP and WP-22-2 (scale bars: 5 mm).

LALIGN server (https://embnet.vital-it.ch/software/LALIGN_form.html). The DNA sequences were then translated to amino acid sequences using EMBOSS Transeq (https://www.ebi.ac.uk/Tools/st/emboss_transeq/) and EMBOSS Sixpack (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/) (Madeira et al., 2019). The predicted protein sequences were aligned using EMBOSS Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). The SNPs identified were classified as synonymous (coding for the same amino acid) and non-synonymous (coding for a different amino acid) based on amino acid changes. The substituted SNPs were analyzed for their functional effects using Sorting Intolerant From Tolerant (SIFT: https://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) (Ng and Henikoff, 2003) and sorted as "tolerated" (score:.05 to 1) or "deleterious" (score: 0–0.05) according to their scores. The SIFT prediction was compared with PROVEAN (http://provean.jcvi.org/seq_submit.php) for similar functional effects (Choi et al., 2012; Choi and Chan, 2015) with a threshold value of −2.5, below which a particular mutation was considered deleterious. Furthermore, protein 3D-structure was predicted using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/) (Kelley et al., 2015).

## Targeted Editing of *OsGA20ox2* in Pusa Basmati

*OsGA20ox2* (LOC_Os01g66100), popularly known as "Semi-dwarf 1", has three exons in its coding region. Mutations (SNPs and indels) in this gene cause dwarfism in rice cultivars (Sasaki et al., 2002). The first exon of this gene was targeted for gene silencing in the rice cultivar Pusa Basmati-1 (PB-1). The CRISPR-Cas9 vector, pRGEB32, from Yinong Yang (Xie et al., 2015) was acquired through Addgene (Watertown, MA, United States) (Addgene plasmid #63142; http://n2t.net/addgene:63142; RRID: Addgene_63142). A unique 20-nucleotide guide sequence for

the targeted region was designed using the CRISPR-PLANT tool (http://www.genome.arizona.edu/crispr) and following the standard procedures (Ren et al., 2014; Doench, 2017). The target regions were selected to have suitable restriction enzyme sites at the Cas9 endonuclease cutting site (3-bp upstream 5′-NGG) to detect genome editing in the plants by PCR/RE assay (**Figure 6**). The constructs were prepared following the standard protocol (Xie et al., 2014), and then transformed into an *Agrobacterium tumefaciens* AGL-1 strain using the triparental mating method (Ditta et al., 1980). Well-matured and healthy seeds of PB-1 were cultured to produce transformable calli on a Murashige and Skoog medium (Murashige and Skoog, 1962), with minor modifications. An *Agrobacterium*-mediated genetic transformation was performed following an established protocol (Kumar et al., 2005). The transformants were screened for the presence of hygromycin phosphotransferase gene (*hptII*) by PCR, and the mutants were characterized using gene-specific PCR primers (**Supplementary Material 13**). The genome-edited lines as well as the control plants (three technical replicates for three independent biological replicates) were further phenotyped for PH and other parameters.

## Statistical Analysis

### Biometrical Data

The biometrical data were obtained from replicated trials (randomized block designs), and the variability parameters, such as phenotypic and genotypic coefficient of variation, heritability, and genetic advance, were calculated following standard methods (Lush, 1940; Burton, 1952; Johnson et al., 1955; Panse et al., 1961).

The mean data of IWP and WP-22-2 in different experiments were compared by Students *t*-test for significant differences in the R statistical program [(R Core-Team, 2018) and ggplot2 (Wickham, 2016)].

### Quantitative Real-Time PCR

The PCR efficiency was calculated with the default software itself (Applied Biosystems, Waltham, MA, United States). The transcript abundance of the qRT PCR, normalized to the endogenous control *OsActin*, was analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### Whole-Genome Sequencing Data

The raw reads were processed for adaptor removal and quality trimming using AdapterRemoval (version 2.2.0). A paired-end alignment of high-quality reads to the reference genome ("Nipponbare"v7.0; http://rice.plantbiology.msu.edu/) was performed using Bowtie2 (version 2-2.2.9) with default parameters, and variant calling was performed with default settings of SAMtools (version 0.1.18).

# RESULTS

In this study, IWP mutants were developed as explained in the previous reports (Ramchander et al., 2014, 2015a,b), and the mutant lines were phenotyped for several yield-contributing agronomic traits with emphasis on plant height and short duration. Significant variations in these traits were observed in $M_2$ and $M_3$ generations. Mutants exposed to 100 Gy of γ irradiation recorded the lowest 50% flowering (106.2 mean days), while 300 Gy recorded the lowest plant height of 138.7 cm (**Supplementary Material 1**). $M_3$ of 100, 200, and 300 Gy irradiation showed high heritability and genetic advance for the traits, namely the number of grains per panicle, primary culm length, secondary culm length, and first, second, third, and fourth internode lengths (**Supplementary Materials 2**, **3**).

**TABLE 1 |** Comparison of different traits between Improved White Ponni (IWP) and WP-22-2 in the generation of $M_6$.

| Traits | IWP | WP-22-2 |
|---|---|---|
| Days to 50% flowering | 110 | 99 |
| Plant height (cm) | 149.9 | 91.6 |
| Number of productive tillers | 21.65 | 21.25 |
| Panicle length (cm) | 26.3 | 24.2 |
| Grains per panicle | 224.5 | 264.25 |
| Single plant yield (g) | 40.79 | 54.55 |
| Thousand grain weight (g) | 15.6 | 12.45 |
| Milling (%) | 61.7 | 66.65 |
| Head rice recovery (%) | 53.05 | 57.6 |
| Grain length (mm) | 5.45 | 4.95 |
| Grain breadth (mm) | 2 | 1.85 |
| LB ratio | 2.73 | 2.68 |
| Grain length after cooking (mm) | 8.1 | 7.2 |
| Grain breadth after cooking (mm) | 2.75 | 2.5 |
| Linear elongation ratio | 1.49 | 1.45 |
| Breadthwise elongation ratio | 1.38 | 1.35 |
| Alkali spreading value | 3 | 3 |
| Chlorophyll content (SPAD units) | 32.96 | 31.97 |
| Average leaf area ($cm^2$) | 25.64 | 24.42 |

## Identification of Mutant Lines for Desired Phenotypes

Several mutants recorded lesser duration of flowering (ranging from 79 to 92 days), dwarf plants (85–105 cm plant height), and better tillering ability (32–44 tillers) with fine grains (**Supplementary Material 3**). The lengths of the first four internodes of the mutants showed significant variations. The first internode length of the mutants ranged from 16.6 to 42 cm, while it was 35.3 cm in IWP-control. The second internode length ranged from 11.5 to 40.1 cm in the mutants. At $M_6$ generation, WP-22-2 mutant showed superior phenotypes, including days to flowering (99 days), PH (91.6 cm), and SPY (54.55 g), and cooking quality traits similar to those of the IWP control (**Figure 1**, **Table 1**). The IWP control plants showed 110 days to flowering, 149.9 cm PH, and 40.79 g SPY. The average leaf area and the chlorophyll content were also similar in the WP-22-2 mutant and IWP. Thus, considering the agronomic superiority of WP-22-2 over IWP, WP-22-2 was chosen for

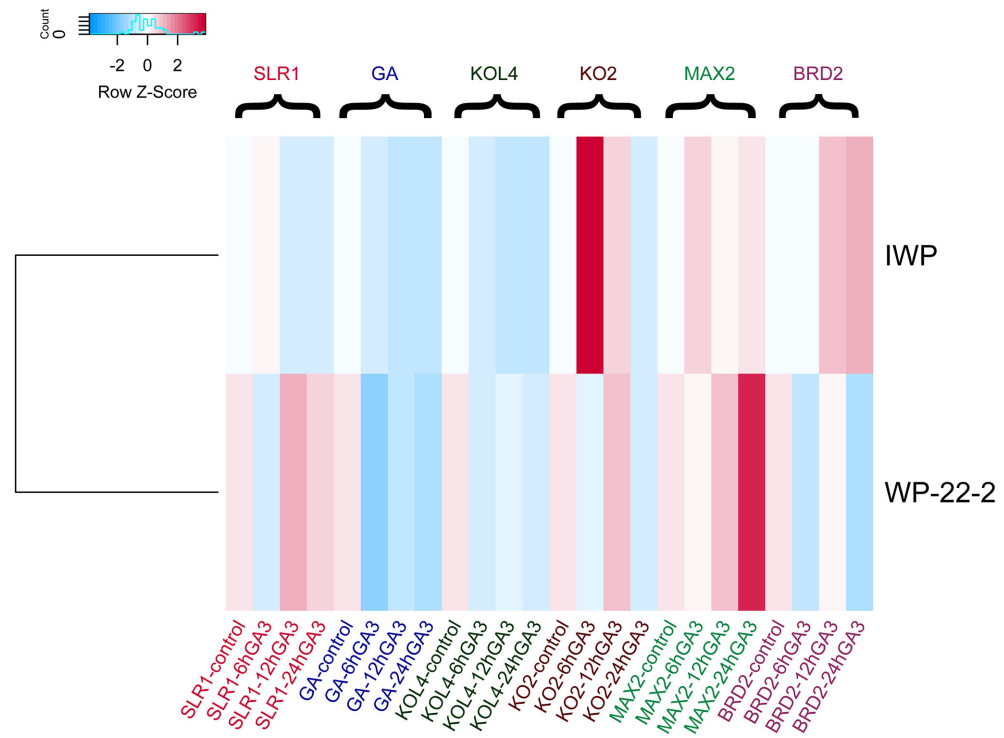


**FIGURE 2 |** Heatmap of the gene expression data of IWP and WP-22-2. $ddC_T$ values of the quantitative real time polymerase chain reaction (qRT-PCR) were plotted. Clear expression changes were observed for the genes KO2 (ent-kaurene oxidase2), MAX2 (Fbox/LRR repeat MAX2 homolog), and brassinosteroid deficient 2 (BRD2) between IWP and the mutant WP-22-2.

further characterization to understand the molecular basis of semi-dwarfism and earliness in flowering.

## Morphological and Molecular Response of WP-22-2 to Exogenous $GA_3$ Treatment

In the earlier study, we identified that WP-22-2 was highly sensitive to gibberellin (**Supplementary Material 4**) compared with IWP (Andrew-Peter-Leon et al., 2021) (**Figure 2**). Therefore, the effect of the exogenous application of $GA_3$ (50 μM) was studied through foliar spray on WP-22-2. Seedlings at the two-leaf stage (15 days old) had shorter internodes (1st and 2nd) than IWP; however, $GA_3$ application resulted in leaf elongation at these internodes to a level similar to the wild-type, IWP. To gain further insights into this, the expression of six genes playing roles in regulating plant height was studied in WP-22-2 and IWP treated with $GA_3$. Overall, the data showed a differential expression pattern of all the genes examined (**Figure 2**, **Supplementary Materials 5**–**7**). A notable upregulation of *OsSLR* was observed in the WP-22-2 12-h sample compared with IWP. However, there are no noticeable changes in the expression of *OsKOL4* throughout all the time points of WP-22-2 and IWP. The expression levels *OsKOL2* were similar in both WP-22-2 and IWP across the time points except in the 6-h sample of IWP, where a significant upregulation was observed. Similarly, *OsMAX2* was found to be upregulated in the 24-h sample of WP-22-2 compared with IWP. Interestingly, *OsBRD2*, a negative regulator of gibberellin metabolism, showed downregulated expression in WP-22-2 upon $GA_3$ application ($P < 0.01$) (**Figure 2**).

## Low-Throughput Genome Sequencing and Analysis of Sequence Data

The genomes of WP-22-2 and IWP were sequenced using the Illumina platform, and the sequence reads were compared to identify SNPs and indels. A total of 38 SNPs and a prominent 356-bp deletion were observed in the coding regions of B3 DNA binding domain (LOC_Os03g42290), *OsFBX267* (LOC_Os08g09460), and *OsGA20ox2* (LOC_Os01g66100) genes. Comparing sequence data of Nipponbare, IWP, and WP-22-2 showed 15 SNPs in the seven exons of the B3 DNA binding domain. However, a single SNP at the seventh exon (G/G/A at position 5231 bp of B3 DNA loci) was unique to WP-22-2 (**Table 2**, **Supplementary Material 8**). Similarly, the SNPs observed at 17 sites of the three exons of *OsFBX267* (LOC_Os08g09460) were unique to WP-22-2 (**Table 2**, **Supplementary Material 9**). Studying the nature of base change showed that changes in Cs (cytosines) and Ts (Thymines) were predominantly present. In *OsGA20ox2*, a large deletion of 356 bp was observed in WP-22-2, and this deletion included 262 nucleotides at the 3′ end of exon 1, complete intron 1 (103 nucleotides), and an 18-nucleotide deletion at the 5’ end of exon 2 starting from the 296th to 652nd bp of

**TABLE 2 |** Single nucleotide polymorphisms (SNPs) observed in the B3 DNA and OsFBX267 gene coding regions of Nipponbare, IWP, and WP-22-2 genotypes.

| Gene | Position | SNPs (Nipponbare/IWP/WP-22-2) | Base position in the gene (from the start of the codon "ATG") |
|---|---|---|---|
| B3 DNA (LOC_Os03g42290) | Exon 1 | G/A/A | 20 |
| | Exon 3 | G/A/A | 2358 |
| | | C/T/T | 2414 |
| | | A/G/G | 2532 |
| | | A/C/C | 2578 |
| | | T/C/C | 2670 |
| | | C/A/A | 2719 |
| | | A/C/C | 2732 |
| | Exon 5 | C/T/T | 3871 |
| | | G/T/G | 3828 |
| | Exon 7 | T/C/C | 4996 |
| | | G/T/A | 5132 |
| | | C/T/T | 5173 |
| | | G/G/A | 5231 |
| | | C/A/N | 5344 |
| OsFBX267 (LOC_Os08g09460) | Exon 1 | T/T/C | 36 |
| | | C/C/T | 41 |
| | | G/G/A | 44 |
| | Exon 2 | T/T/C | 457 |
| | | G/G/C | 464 |
| | | C/C/T | 475 |
| | | T/T/C | 489 |
| | | C/C/T | 516 |
| | | T/A/G | 523 |
| | | A/A/C | 535 |
| | | C/C/A | 543 |
| | | C/C/G | 622 |
| | | C/C/T | 624 |
| | | C/C/T | 636 |
| | | C/C/A | 677 |
| | Exon 3 | G/G/A | 1487 |
| | | T/T/C | 1530 |
| | | T/T/C | 1556 |
| | | T/T/C | 1579 |
| | | G/G/C | 2068 |

the gene. In addition, two SNPs at exon 2 (T/T/A at position 679 and C/C/T at position 756 bp: Nipponbare/IWP/WP-22-2, respectively) were found to be unique to WP-22-2 (**Figure 3**, **Table 3**).

## *In silico* Characterization of Mutations in *OsGA20ox2* and *OsFBX267*

The SNPs and deletions in *OsGA20ox2* and *OsFBX267* were further analyzed. The *OsGA20ox2* gene of WP-22-2 had a deletion of 97 amino acids. This deletion results in a stop codon that truncates the protein, and, therefore, the protein expressed by *OsGA20ox2* gene would be non-functional. The structural differences in the protein expressed by *OsGA20ox2* of WP-22-2 and IWP are shown in **Figure 4**. In the case of *OsFBX267*, a comparison of sequence data showed 20 nucleotide substitutions. Among these substitutions, T→ C and C → T transitions were prominent (six and five times, respectively, from IWP to WP-22-2) (**Figure 5**, **Table 2**). Of these 20 SNPs, 7 and 13 were synonymous and non-synonymous (12 missense and 1 nonsense) substitutions, respectively. Noteworthy, a G to A transition at 44th base in WP-22-2 converted a tryptophan codon to a stop codon, resulting in premature termination of translation. The effect of base changes in *OsGA20ox2* and *OsFBX267* were validated using SIFT (Ng and Henikoff, 2003) and PROVEAN (Choi et al., 2012; Choi and Chan, 2015) scores. The SIFT scores indicated that four amino acid substitutions affected the protein function, while the PROVEAN predicted that three amino acid substitutions were deleterious (**Table 4**). However, these scores have minimal relevance and correlation with the protein functions, as both *OsGA20ox2* and *OsFBX267* of WP-22-2 undergo a premature termination. Thus, while either of the genes does not produce functional proteins, this study suggests the implication of these genes in regulating early maturity and semi-dwarfism in WP-22-2 (**Figures 4**, **5**). Thus, the loss of function of *OsGA20ox2* and *OsFBX267* has evidently promoted early maturity and semi-dwarfism in WP-22-2.

## Editing and Analysis of *OsGA20ox2* (*SD1*) in Pusa Basmati 1

Rice cultivar Pusa Basmati-1 offers a highly responsive system for tissue culture compared with other *indica*-type cultivars. To validate the mutation in *OsGA20ox2* identified in WP-22-2, the same gene in PB-1 was subjected to targeted genome-editing using the CRISPR-Cas9 approach (**Figure 6**). Around 100 seeds were inoculated for callus, and 80 healthy calli were co-cultivated with an *Agrobacterium* harboring CRISPR-Cas9-SD1 construct. The transformation produced seven hygromycin-positive (*hptII*) plants (**Figure 7**) with a transformation efficiency of 8.75%. Targeted amplification of *SD1* in $T_0$ showed a deletion of several nucleotides, and that the plants were comparatively shorter (48 cm) compared with the control (PB-1; 95 cm). These preliminary data confirm the involvement of *SD1* in regulating plant height in rice; however, further functional characterization is necessary to delineate the precise mechanism underlying semi-dwarfism in *SD1* loss-of-function mutants.

# DISCUSSION

This study deployed the use of γ-irradiation to achieve genomic changes in IWP, a popularly grown rice variety in Southern India. In addition, this study shows that the frequency of semi-dwarf mutants was higher in lower doses of γ- irradiation (100 and 200 Gy) than higher doses. The higher doses of mutagens cause lethality, which reduces the survival of the mutants (Shadakshari et al., 2001; Singh et al., 2006; Nayudu et al., 2007; Anilkumar, 2008). In addition, most of the morphological mutants identified in $M_2$ generation failed to inherit in $M_3$ generation. These

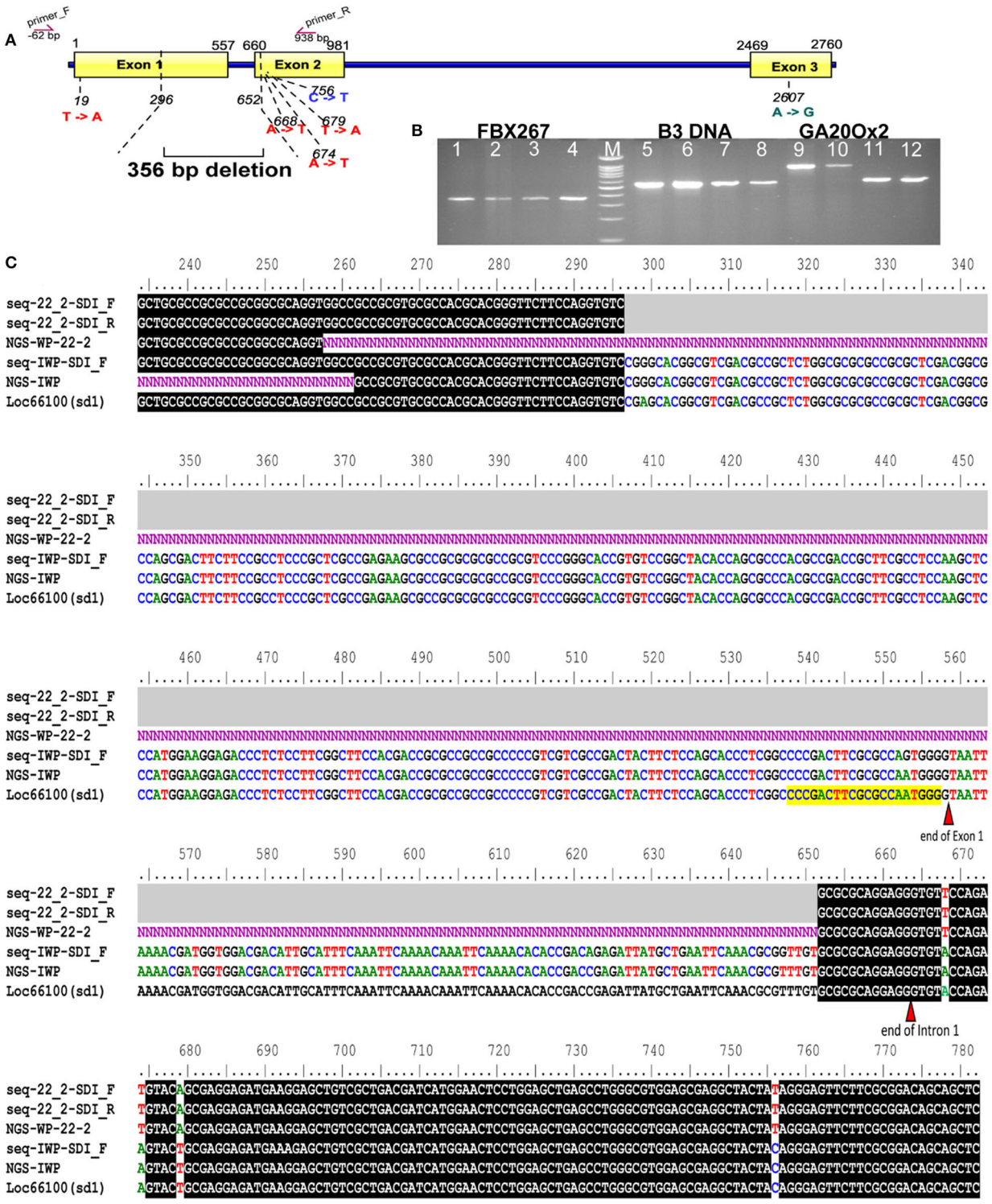


**FIGURE 3 |** Gene illustration and sequence alignment of SD1 locus. **(A)** Gene illustration showing the regions of deletion and other single nucleotide variations between IWP and WP-22-2; binding sites of the primer used for Sanger sequencing are marked as arrows above the gene diagram. **(B)** Cropped gel image of B3 DNA, FBX267, and GA20Ox2 gene regions of IWP and WP-22-2 (biological duplicates) amplified with PCR primers; IWP: 1 and 2, 5 and 6, 9 and 10; WP-22-2: 3 and 4, 7 and 8, 11 and 12; M: 100 bp ladder—each band represents a 100-bp increment from the lower band; a large deletion is observed between IWP and WP-22-2 in the GA20Ox gene (Full-length gel image is presented in **Supplementary Material 10**). **(C)** Sequence alignments showing single nucleotide variations. The horizontal gray bars indicate (in seq-22_2-SD1_F and seq-22_2-SD1_R) deletion. *Order of alignment: Sanger (WP-22-2_F&R)*, *NGS WP-22-2*, *Sanger IWP*, *NGS IWP and annotated Nipponbare LOC_Os01g66100*.

**TABLE 3 |** Details of mutations in the OsGA20ox2 gene of WP-22-2.

| S.No. | Gene location | Annotated gene characteristics (as per IRGSP 1.0) | Sequence variations in IWP | Sequence variations in WP-22-2 |
|---|---|---|---|---|
| | Deletions | | | |
| 1. | Exon 1 | 558bp length | Nil | 262 bp deletion at the 3′ end |
| 2. | Intron 1 | 102bp length | Nil | 93 bp deletion at the 5′ end |
| | **Single nucleotide variations** | | | |
| 1. | Exon 2 | 'A' at the 5th base of exon 2 | 'A' | A → T |
| 2. | | 'A' at the 11th base of exon 2 | 'A' | A → T |
| 3. | | 'T' at the 16th base of exon 2 | 'T' | T → A |
| 4. | | 'C' at the 93rd base of exon 2 | 'C' | C → T |
| 5. | Intron 2 | 'T' at the 82nd base of intron 2 | 'T' | T → G |
| 6. | | 'C' at the 206th base of intron 2 | C → G | 'C' |
| 7. | | 'A' at the 272nd base of intron 2 | A → G | 'A' |
| 8. | | 'T' at the 338th base of intron 2 | T → A | T → A |
| 9. | | 'G' at the 1100th base of intron 2 | G → A | G → A |
| 10. | | 'C' at the 1247th base of intron 2 | C → T | C → A |
| 11. | Exon 3 | 'A' at the 138th base of exon 3 | A → G | A → G |

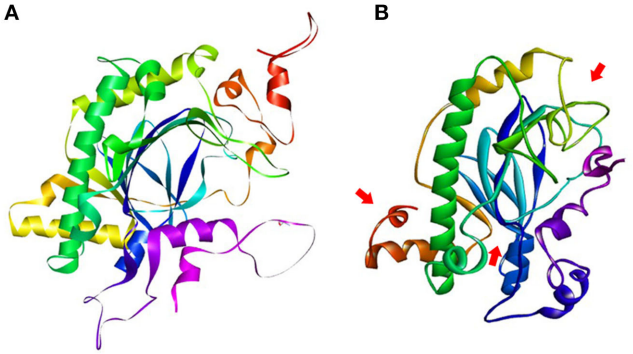


**FIGURE 4 |** Protein structure prediction for the *OsGA20Ox2* gene. **(A)** IWP; **(B)** WP-22-2-loss of amino acids and structural changes is clearly visible in the mutant. The arrows indicate the visible structural changes and amino acid composition in the mutant WP-22-2.

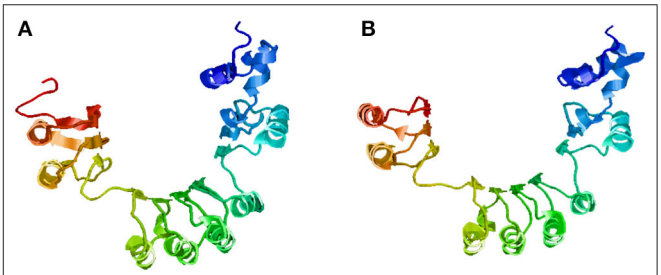


**FIGURE 5 |** Protein structure prediction for the *OsFBX267* gene. **(A)** IWP; **(B)** WP-22-2—premature terminations and single nucleotide variations have caused structural changes in the mutant.

characters may be controlled by recessive genes or are susceptible to the environment (Luo et al., 2012). The considerable shift in the means and variances between the $M_2$ and $M_3$ generations suggests the effects of recombination events in the mutants (Johnston, 2001; Siddiqui and Singh, 2010). The high heritability and high genetic advance observed in the $M_6$ generation of mutants indicated that the traits were fixed in the selected genotypes or that undesirable traits were screened out. Based on the overall morphological superiority, high yield, fine grain structure, and similar cooking quality traits, the WP-22-2 mutant of IWP was selected as the superior mutant (**Figure 1**, **Table 1**).

Intercalary meristem cell division and elongation are the major causes for internodal elongation in rice, and deficiencies in these processes severely affect plant height. The earlier studies proved that the seedlings of WP-22-2 are highly sensitive to external $GA_3$ application and this resulted in increase in seedling height similar to IWP. The gibberellin-sensitive mutants are primarily defective in genes controlling the gibberellin pathway of rice (Hedden and Sponsel, 2015). Semi-dwarfism in WP-22-2 is caused by the reduced elongation of internodes at the early stages of plant growth. In matured WP-22-2 plants, reduction in all four internodes was observed, resulting in sturdy plant architecture. After introducing the rice variety IR8, the OsGA20ox2 has been used as the semi-dwarfing gene for rice breeding throughout the world, and its role has been well studied (Tomita and Ishii, 2018). In plants, the GA20 oxidase converts GA intermediates into bioactive forms such as $GA_1$ and $GA_4$ (Yamaguchi, 2008); hence, loss of function may cause dwarfism in rice plants. The *OsGA20ox2* gene was downregulated in both IWP and WP-22-2. Genome sequence analyses show the presence of a 356-bp deletion in *OsGA20ox2*. The results confirm similar deletion mutations that have been reported earlier (Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). It is evident from the findings that the deficiency caused loss of function and reduced the expression of the *OsGA20ox2* gene in WP-22-2. Furthermore, the observed differences in the expression of the *BRD2* gene (**Supplementary Material 4**) between the IWP and WP-22-2 emphasize the deficient gibberellin metabolism in the mutant causing semi-dwarfism. Significant changes in expression

**TABLE 4 |** Sorting Intolerant From Tolerant (SIFT) and PROVEAN scores for mutations in the OsFBX267 gene of WP-22-2 and its predicted effects on the protein.

| S. No. | Base changes (IWP, [base position], WP-22-2) | Amino acid changes (IWP, [a.a. position], WP-22-2) | Type of mutation | SIFT | | PROVEAN | |
|---|---|---|---|---|---|---|---|
| | | | | SIFT score | Predicted effect | Provean score | Predicted effect |
| 1. | T[36]C | Gly[12]Gly | Silent mutation | N/A | N/A | - | - |
| 2. | C[41]T | Ser[14]Phe | Missense mutation | 0.00 | Affect protein function | −4.8 | Deleterious |
| 3. | G[44]A | Trp[15]STOP | Non-sense mutation | Functional protein (N/A) | N/A | −19.719 | Deleterious |
| 4. | T[97]C | Ser[33]Pro | Missense mutation | 0.30 | Tolerated | 1.358 | Neutral |
| 5. | G[104]C | Arg[35]Pro | Missense mutation | 0.03 | Affect protein function | 2.697 | Neutral |
| 6. | C[115]T | Pro[39]Ser | Missense mutation | 0.15 | Tolerated | −2.764 | Deleterious |
| 7. | T[129]C | Asp[43]Asp | Silent mutation | N/A | N/A | - | - |
| 8. | C[156]T | Leu[52]Leu | Silent mutation | N/A | N/A | - | - |
| 9. | A[163]G | Ile[55]Val | Missense mutation | 0 | Affect protein function | −0.292 | Neutral |
| 10. | A[175]C | Ile[59]Leu | Missense mutation | 1 | Tolerated | 1.317 | Neutral |
| 11. | C[183]A | Ala[61]Ala | Silent mutation | N/A | N/A | - | - |
| 12. | C[262]G & C[264]T | Leu[88]Val | Missense mutation | 0.27 | Tolerated | 1.989 | Neutral |
| 13. | C[276]T | Arg[92]Arg | Silent mutation | N/A | N/A | - | - |
| 14. | C[317]A | Ala[106]Glu | Missense mutation | 0.22 | Tolerated | −2.061 | Neutral |
| 15. | G[501]A | Prol[167]Pro | Silent mutation | N/A | N/A | - | - |
| 16. | C[539]T | Leu[180]Pro | Missense mutation | 0.06 | Tolerated | −0.008 | Neutral |
| 17. | T[565]C | Cys[189]Arg | Missense mutation | 0.75 | Tolerated | 2.089 | Neutral |
| 18. | T[588]C | Cys[196]Cys | Silent mutation | N/A | N/A | - | - |
| 19 | C[1087]G | His[363]Asp | Missense mutation | 0 | Affect protein function | 1.806 | Neutral |

levels of more than one locus suggest an epistatic interaction of genes.

The deletion and SNPs observed (Monna et al., 2002; Spielmeyer et al., 2002) suggest the γ-rays induced mutability of the *OsGA20ox2* exon 1 region. In addition, four SNPs observed in intron 2 and exon 3 of the gene were unique to IWP and WP-22-2. The mutations had no visible lethal effects on other phenotypic traits and were found to be similar to the other semi-dwarf rice cultivars developed with the *SD1* gene. The presence of a functional *SD1* wild-type allele in tall plants and in few rice cultivars and landraces has been validated. A similar allele in semi-dwarf landraces indicates that *GA20ox2* is the vulnerable locus that causes semi-dwarfism in rice (Han et al., 2019). It offers key information that silencing this gene through targeted dmutagenesis, especially by CRISPR/Cas9, could create semi-dwarf mutants in a short time without causing lethal effects. To state further, the allelic variations of this region can be analyzed from diverse germplasm sets to effectively use this finding to identify mutable regions and to explore existing variations. This method could reduce the time required by conventional mutation breeding to select the best mutant lines without lethal effects.

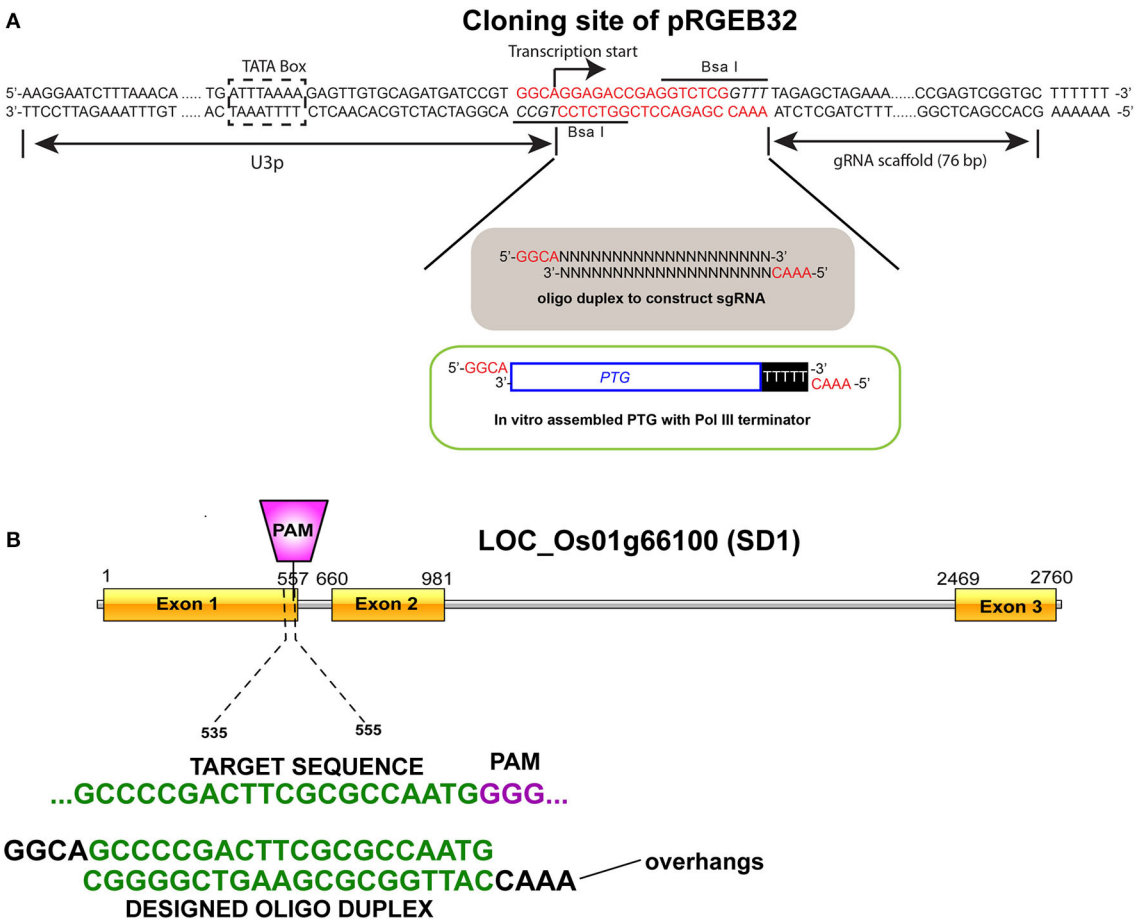


**FIGURE 6 |** Single guard ribonucleic acid (sgRNA) for targeted genome engineering. **(A)** Cloning site of pRGEB32 plasmid when digested with *BsaI* restriction enzyme leaves a four-base overhang on either side. **(B)** Target site for sgRNA binding at the exon 1 of the *OsGA20ox2* gene (LOC_Os01g66100). A 20-nt oligonucleotide complex was designed and ligated with the pRGEB32 plasmid.

Genome-wide searches to identify the gene(s) responsible for the early flowering in WP-22-2 were attempted with the NGS data. An F-box-containing protein, which is a potential target for early flowering, was identified in WP-22-2. F-box-containing proteins regulate photomorphogenesis, circadian clock and flowering time control in plants, and gibberellic acid signaling (Dill et al., 2004). Although the functions of only a few F-box-containing proteins were elucidated, many F-box-containing proteins are known to control flowering time and to cause gibberellin-insensitive dwarfs in rice (Sasaki et al., 2003). Multiple SNPs were identified in the *OsFBX267* (LOC_Os08g09460) gene of WP-22-2 from this investigation. Previous reports on the localized upregulation of this gene in floral parts similar to many heading date-related rice genes emphasize its role in earliness in flowering (Jain et al., 2007; Mohapatra et al., 2014). The multiple SNPs observed in the coding regions of *OsFBX267* resulted in a premature termination codon in exon 1 (**Figure 5**). The amino acid substitutions predicted were found to be highly deleterious to the normal function of this gene product. This information is the key finding in explaining the early maturity of WP-22-2 (**Supplementary Material 9**). However, the *in-silico* based analyses performed are preliminary, and further investigations with mapping populations could strengthen the prediction reported in this study.

As a proof of concept, we silenced the *OsGA20ox2* gene in the rice cultivar Pusa Basmati-1, a highly suitable variety for *Agrobacterium*-mediated transformation (Mohanty et al., 1999). The *Agrobacterium*-mediated transformation in PB-1 resulted in many hygromycin-positive plants (**Figure 7**). In one mutant, an allelic variation was observed in genome-edited PB-1 (CrSD1) than in control PB-1. This revealed the deletion affected by the CRISPR-Cas9-SD1 construct. In addition, the $T_0$ plant was dwarf (48 cm) compared with the control (90–110 cm) and showed normal panicle exertion (**Figure 7**). The morphological observations and the PCR analyses of the genomic region showed that the CRISPR editing was successful in PB-1. However, a further analysis of the genome-wide effects of CRISPR on non-specific targets is essential.

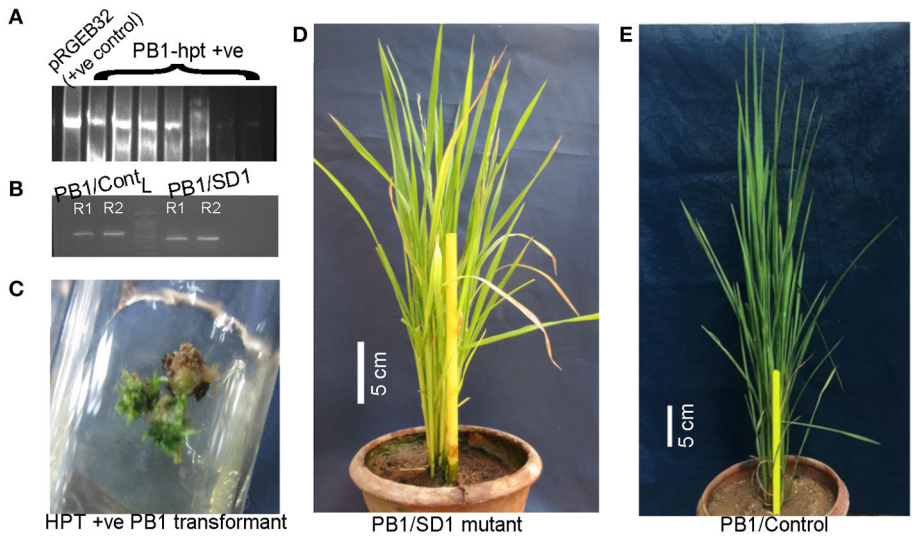


**FIGURE 7 |** *Sd1* mutants of cultivar Pusa Basmati-1 (PB-1) were developed using CRISPR-Cas9. **(A)** HPTII screening of PB-1/SD1 transformants (lane 1—pRGEB32 plasmid as positive control, other lanes—PB-1/hpt positive transformants); (full-length gel presented in **Supplementary Material 11**); **(B)** A PB-1/SD1 mutant was observed with a small deletion in the exon 1 of the *OsGA20ox2* gene; lanes 1 and 2: biological duplicate of PB-1/Control and lanes 3 and 4: biological duplicate of PB-1/SD1 (cropped gel image; full-length gel is presented in **Supplementary Material 12**). **(C)** Shoot initiation from the hygromycin positive calli. **(D,E)** PB-1/SD1-mutant and PB-1-control respectively; scale bars: 5 cm.

## CONCLUSIONS

This study suggests the role of two genes, viz., *OsFBX267* and *OsGA20ox2*, in negatively influencing the height and maturity of rice, wherein mutations in these genes result in semi-dwarf and early flowering phenotypes. As $M_6$ stable lines have been established, this could serve as a potential donor for developing new varieties for functional characterization and field trials. Mutation breeding has been a versatile tool being used for crop improvement. The advent of next-generation genomics has enabled the precise localization of mutations, which could be further exploited for targeted improvement of yield-contributing agronomic and climate-resilient traits. This study is an excellent example of coupling classical mutagenesis with NGS to identify two candidate genes, which were further studied. Also, targeted editing of *OsGA20ox2* in Pusa Basmati1 has been demonstrated to validate the findings. Altogether, the study has founded a base for further research on *OsFBX267* and *OsGA20ox2* to investigate their roles in regulating dwarfism and early flowering in this crop. This also opens the avenue for exploring these genes in other related grass species through comparative genome mapping approaches.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

MP conceptualized and designed the study. MA-P-L and RS performed the experiments. MA-P-L, RS, KK, JY, MM, and MP analyzed and interpreted the results. MA-P-L wrote the initial draft of the manuscript. JY and MM edited and revised the manuscript. All the authors read and approved the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.714066/full#supplementary-material

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# Toward Integrated Multi-Omics Intervention: Rice Trait Improvement and Stress Management

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Rice (*Oryza sativa*) is an imperative staple crop for nearly half of the world's population. Challenging environmental conditions encompassing abiotic and biotic stresses negatively impact the quality and yield of rice. To assure food supply for the unprecedented ever-growing world population, the improvement of rice as a crop is of utmost importance. In this era, "omics" techniques have been comprehensively utilized to decipher the regulatory mechanisms and cellular intricacies in rice. Advancements in omics technologies have provided a strong platform for the reliable exploration of genetic resources involved in rice trait development. Omics disciplines like genomics, transcriptomics, proteomics, and metabolomics have significantly contributed toward the achievement of desired improvements in rice under optimal and stressful environments. The present review recapitulates the basic and applied multi-omics technologies in providing new orchestration toward the improvement of rice desirable traits. The article also provides a catalog of current scenario of omics applications in comprehending this imperative crop in relation to yield enhancement and various environmental stresses. Further, the appropriate databases in the field of data science to analyze big data, and retrieve relevant information *vis-à-vis* rice trait improvement and stress management are described.

**Keywords: genomics, metabolomics, omics, proteomics, rice, stress, transcriptomics**

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## INTRODUCTION

Rice (*Oryza sativa*) is a staple crop for billions of the world population. World agriculture faces a daunting task to proportionally ramp-up rice production for meeting the enormous demand of human consumption (Khan et al., 2015). Concomitantly, adverse environmental conditions negatively impact rice production and cause significant yield loss. Biotic and abiotic stresses either in combination or individually prevent the attainment of full genetic potential for optimal rice growth and yield (Raza et al., 2019). According to the 2021 data of FAOSTAT (Food and

Agricultural organization), rice is one of the highest globally harvested crops[1]. According to the latest census by FAOSTAT in the year 2019, around 160 million hectares of land is planted with rice which cumulatively produces approximately 750 million tons of rice worldwide (see text footnote 1). Asia is the leading producer of rice and contributes to about 90.6% of the production share (see text footnote 1).

Apart from the immense economic importance, rice has also emerged as a model crop (genome size = 4.3MB) for monocots (Izawa and Shimamoto, 1996). The simple genome of rice led to easy and early genome sequencing of rice (Goff et al., 2002; Yu et al., 2002; Sasaki, 2005; Huang et al., 2013; Stein et al., 2018). For the two popular rice sub-species namely, *O. sativa* ssp. *Japonica* and *O. sativa* ssp. *Indica*, the pioneer draft genome was released in the year 2002 (Goff et al., 2002; Yu et al., 2002). Following the release of the draft genome, high-throughput technologies were employed to assemble the complete reference genome of rice (Sabot et al., 2011; Gao et al., 2013). Much recently, the genome availability of 13 domesticated and wild rice varieties has highlighted the genetic conservation across the genus *Oryza* (Stein et al., 2018).

In molecular biology and data science, the word "ome" refers to the study of special, temporal, and global changes occurring in an organism. Omics is a branch of science to gauge the functions and extract relevant biological information in a single or bunch of cells, tissues, or organs. The easy accessibility of whole-genome sequences from rice provides a platform for several omic studies like genomics, transcriptome, proteome, and metabolome (Delseny et al., 2001; Komatsu and Tanaka, 2005; Agrawal and Rakwal, 2011; Kyndt et al., 2012; Zheng et al., 2013; Chen et al., 2014; Lin et al., 2017; Li et al., 2018; Song S. et al., 2018; Zarei et al., 2018; Zhang et al., 2019; Peng Yuan et al., 2020). These techniques form the core components of omics technology. Over the years, substantial progress has been made in these methods in relation to almost all organelles, cells, tissues, and organs of rice. Rice genomics led to the discovery and functional characterization of pivotal genes that play crucial roles in improving rice productivity (Yano et al., 2016; Tang et al., 2019; Volante et al., 2020). The application of transcriptomics to rice has widened the understanding of complex molecular responsive mechanisms, differential gene expression, and regulatory pathways under varying conditions (Takehisa et al., 2012; Kumar and Dash, 2019; Sun et al., 2019). This information can be successfully processed for rice crop improvement. Similarly, proteomics and metabolomics has also contributed drastically for rice trait improvements (Oikawa et al., 2008; Agrawal and Rakwal, 2011; Calingacion et al., 2012; Kim S.T. et al., 2014; Baslam and Mitsui, 2020). The application of secretome for the identification of various novel secreted proteins and global mapping of phosphorylation sites is also worth mentioning (Cho and Kim, 2009; Agrawal et al., 2010; Que et al., 2012; Chen et al., 2016). Additionally, well-recognized proteomes have abetted in re-annotating the rice genome to unravel the proteins of unidentified functions. These relevant findings are implemented for genetic improvement in relation to agronomic traits and response to biotic/abiotic stresses. The major challenge ahead for functional genomics and system biology is to integrate genomics, transcriptomics, proteomics, and metabolomic information for a better understanding of cellular biology. The present review recapitulates the core omics techniques *viz.*, genomics, transcriptomics, proteomics, and metabolomics to emphasize the advances achieved in rice omics research. Further, this review is aimed to reiterate the existing rice-omics scenario and how the implication of data science is gaining significance for rice trait improvement and stress management across the scientific community.

## GENOMICS AND TRANSCRIPTOMICS: AN OVERVIEW

Genomics is defined as the study of structure, function, evolution, and interaction of genes which provides complete information about the genetic make-up of an organism. The core components of genomics include genetic engineering, DNA sequencing, and deep analysis of the functions of genome. Genetic code is considered the foundation of biological life. The prime resources for understanding the genome involve the sequencing of DNA code and studying the gene expression patterns. The complete genome sequencing of *Arabidopsis thaliana* (Kaul et al., 2000) ushered to the post-genomic era in plant research. In the year 2005, the rice genome was sequenced under International Rice Genome Sequencing Project (Sasaki, 2005). The neoteric advances in the DNA marker technologies for identifying Single Nucleotide Polymorphism (SNP) have resulted in uncovering desirable traits. Massive parallel sequencing commonly referred to as next generation sequencing (NGS) has revolutionized the research underlying plant sciences (**Figure 1**). NGS utilizing Illumina/Solexa, Ion Torrent Personal Genome Machine (PGM) and Pacific Biosciences (PacBio) techniques have completely transformed the genomic and transcriptomic studies through their accuracy and robustness (Dhondt et al., 2013; Heather and Chain, 2016). Genome-wide association studies (GWAS) and quantitative trait loci (QTL) mapping to comprehend the genetic variance and inheritance of complex quantitative traits have also gained considerable significance in the recent past (Bekele et al., 2013; Bao, 2014; Reig-Valiente et al., 2018). Thus, genomics delivers fast and accurate approaches for crop biotechnology by enabling methods for marker-assisted selection and molecular breeding.

Next, transcriptomics deals with the study of the entire transcriptome (sum of all RNA transcript) of an organism at a particular developmental stage or under a specific physiological condition (Blumenberg, 2019). Studying the transcriptomes of a variety of diverse populations aid in linking the genotype to a particular phenotype. Enormous population-wide transcriptome studies have been conducted on rice and other agronomic crops to understand the underlying mesh of networks in crop improvement (Kremling et al., 2018; Groen et al., 2020; Iqbal et al., 2020b). Nonetheless, due to the limitations associated with the sampling of below-ground tissues, the majority of transcriptomic studies are focused on above-ground tissues

[1]http://www.fao.org/

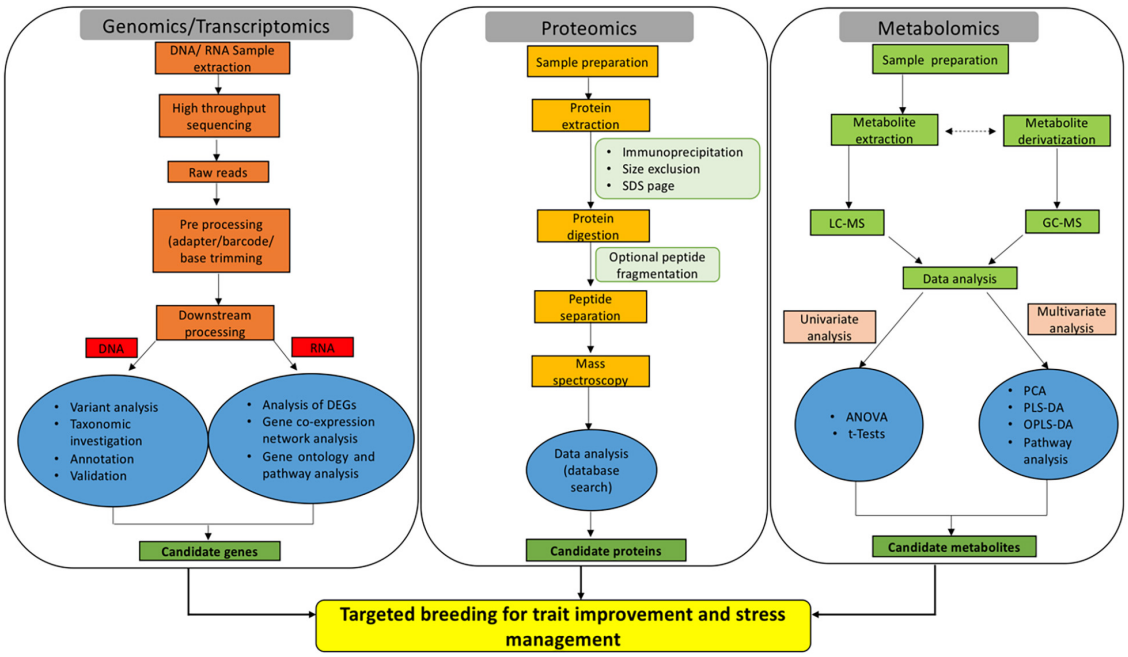


**FIGURE 1 |** Omics-based approaches are emerging as efficient tools for dissecting the key genes, proteins, and metabolites implicated in rice trait improvement and stress acclimation responses.

(Yoshino et al., 2019). In crux, transcriptomic studies allow the identification of mRNA, long non-coding RNAs, and small RNAs as well as the understanding of gene organizations and expression profiles (**Figure 1**; Wang et al., 2009). Generally, transcriptomic methods rely on sequencing [serial analysis of gene expression-SAGE (Moustafa and Cross, 2016), expressed sequence tags- ESTs (Parkinson and Blaxter, 2009) and RNA-seq (Wang et al., 2009)] or hybridization [suppression subtractive hybridization-SSH (Sahebi et al., 2015) and microarray (Hrdlickova et al., 2017)]. RNA-seq is considered as the best approach in comparison to other hybridization or sequencing-based methods, as far as the coverage and resolution are considered. ESTs (Zhou et al., 2003), SAGE (Bao et al., 2005), SSH (Chang et al., 2019), microarray (Wang et al., 2016), and RNA-seq (Ereful et al., 2020; Zainal-Abidin et al., 2020; Divya et al., 2021) have been linked extensively in the elucidation of complex mechanisms for rice trait improvement and stress management.

## Genomics and Transcriptomics in Rice Trait Improvement

The major challenge ahead of rice breeders is to enhance rice productivity and improve related agronomic traits. This task becomes difficult to accomplish using traditional breeding techniques. The difficulty is further aggravated due to epistatic interactions of yield contributing genes (Mei et al., 2006). Taking into account the quality of rice, genomics and transcriptomics have offered several breakthroughs. Generally, the characteristics linked with rice quality include taste, gel consistency, amylose content, texture, aroma, nutritional value, gelatinization temperature, and resistant storage. Genetically modified methods have been frequently utilized to improve the above-mentioned characteristics. One of the early examples includes the generation of golden rice that contains significant levels of beta-carotene (Ye et al., 2000). Golden rice was produced by transforming the exogenous genes *psy*, *crtI,* and *lcy*, along with their upstream elements. Beta-carotene is a known precursor in vitamin A synthesis and hence golden rice is considered nutritionally rich in comparison to white rice. Nonetheless, efficient technologies such as DNA markers for marker-assisted selection (MAS) of agronomic traits are pivotal to yield and quality improvement (Das et al., 2017). Minor and major QTLs for some yield components, *viz.,* plant height, spikelets per panicle, length of panicle, length of grain, weight of grain, yield per grain, and harvest index have been identified (**Table 1**; Septiningsih et al., 2003; Bernier et al., 2007). The QTLs contributing for grain length (*qGL3*), grain width and weight (*qGW2*), grain weight (*qgw3*), grain number (*qGn1*), grain length and weight (*qGS3*), and plant height (*Ph1*) are based on Mendelian factors (Li et al., 2004; Fan et al., 2006; Wan et al., 2006; Ashikari et al., 2007; Song et al., 2007; Qi et al., 2017). For example, the *GW2* gene (RING-type protein) is localized on chromosome 2 that modulates the width and weight of rice grains (Song et al., 2007). *GS3* (putative transmembrane protein) is localized on chromosome 3 that modulates grain width, weight, thickness, and length (Fan et al., 2006). Such important alleles and genes linked to DNA markers can be potentially utilized in MAS for improving rice yield and quality. The accessibility of sequenced rice genome accelerated the identification of polymorphic markers (Feltus et al., 2004; Shen et al., 2004).

Centromeres of rice chromosomes have also been successfully sequenced and assembled. This information can be applied for the construction of artificial rice chromosomes (Nagaki et al., 2004; Wu et al., 2004). Such data can be directly linked to phenotypic traits and successfully used for functional analysis. In recent times, gene cloning and functional analysis linked to yield and quality in important rice cultivars have been significantly improved by various scrupulous approaches. These encompass mutant screening, comparative genome analysis, production of cross populations, and identification of wild variates with better qualitative and quantitative traits (Shomura et al., 2008; Jiang et al., 2012).

One of the early examples from QTL mapping involves dense and erect panicle 1 (DEP1) accountable for governing the number, weight, and size of rice grain (Ashikari et al., 2005; Huang, Feng et al., 2009). Furthermore, QTLs are also linked to ideal plant architecture (IPA) and wealthy farmer's panicle (WFP) (Jiao et al., 2010; Miura et al., 2010). IPA and WFP contribute to a greater number of panicles branching and higher grain yield in rice. Later, recombinant inbred lines (RILs) population was used to detect 27 QTLs on 10 rice chromosomes (Yan et al., 2014). The RILs in this study were obtained from a cross of Huahui 3 (*Bt/Xa21*) and Zhongguoxiangdao. 12 of these QTLs contributed to rice grain shape and yield. Intriguingly, the two already known genes, *Bt* gene (insect-resistant) and *Xa21* gene (disease-resistant) were closely linked to QTLs responsible for grain shape and weight. In the Huahui 3 rice cultivar, *Bt* fragment insertion localized on chromosome 10. The *Bt* fragment insertion might disrupt grain-related QTLs which resulted in compromised yields in transgenics (Yan et al., 2014). The introgression of *Xa21* gene into Minghui 63 rice cultivar contained a donor linkage drag and affected QTL alleles to regulate the shape and yield of grain. This information can be utilized for breeding applications to recuperate rice grain shape and yield (Yan et al., 2014). Recently, another RIL population obtained from KRH-2 (IR58025A/KMR3R) was utilized to identify QTLs governing crop yield (Kulkarni et al., 2020). A genetic map of 294.2 cM with 126 simple sequence repeats (SSR) was made. Overall, 22 QTLs were recognized with phenotyping and genotyping data. The study reported a novel QTL linked to panicle length (*qPL3-1*). The other QTLs identified were total grain yield/plant (*qYLD3-1*), panicle weight (*qPW3-1),* plant height (*qPH12-1),* and flag leaf width (*qFLW4-1).* Moreso, considerable epistatic interactions were detected for the length of panicle and grain yield per plant. *In silico* analysis of the QTLs highlighted the functions of candidate genes linked with preferred traits (Kulkarni et al., 2020). The high-yielding RILs harboring the yield associated QTLs were recognized as restorers. This indicates their probable deployment in the generation of excellent rice hybrids. Further, single-nucleotide polymorphism (SNP) data provides a strong foundation for exploring rice diversity and gene–trait relationships that can be successfully implemented in crop improvement through linkage mapping (McNally et al., 2009; McCouch et al., 2010; Roy and Lachagari, 2017; Zainal-Abidin et al., 2019). SNP markers linked with rice grain yield have been well documented recently. GWAS was used for genotyping of 541

**TABLE 1 |** List of QTLs in rice trait improvement and stress management.

| QTL | Trait/stress | References |
|---|---|---|
| *qYLD3-1, qPW3-1, qPH12-1, qFLW4-1, and qPL3-1* | total grain yield, panicle weight, plant height, flag leaf width, panicle length | Kulkarni et al., 2020 |
| *qtl12.1/qDTY12.1* | decreased number of days to flowering, higher harvest index, increased biomass and plant height | Bernier et al., 2007 |
| *qDTY1.1* | Grain yield under drought and plant height under drought | Ghimire et al., 2012 Venuprasad et al., 2012b Vikram et al., 2016 |
| *qDTY2.1, qDTY3.1, qDTY2.2, qDTY9.1*, and *qDTY12.1* | grain yield under drought stress | Bernier et al., 2007; Venuprasad et al., 2009; Swamy et al., 2011; Mishra et al., 2013 |
| *qDTY1.1, qDTY1.3,* and *qDTY8.1* | grain yield under drought stress | Catolos et al., 2017 |
| *qRT9.1* and *qRT5.1* | Root trait under drought stress | Catolos et al., 2017 |
| *qDTY1.1*, *qDTY3.3,* and *qDTY6.3* | grain yield across the seasons under severe and moderate drought | Yadav et al., 2019 |
| *Saltol* | Salt stress | Gregorio, 1997; Bonilla et al., 2002; Niones, 2004; Thomson et al., 2010; Alam et al., 2011 |
| *qSKC-1* and *qSNC-1* | Shoot potassium concentration and shoot sodium concentration | Zhou et al., 2013; Deng et al., 2015b; Jing et al., 2017 |
| *qSL2*, *qRL2.1, qSIS2 qSDW2,* and *qRL2.2* | shoot length, root length, salt injury score, and shoot dry weight | Amoah et al., 2020 |
| *qCT3.12, qCT6.7, and qCT9.6* | QTL affecting cold tolerance associated with spikelet fertility (%) | Liang et al., 2018 |
| *qLTGR4d-9-1, qLTGR4d-9-2, qLTGR2d-9-1, qLTGI-9-1, qLTGR2d-9-2, and qLTGI-9-2* | QTLs associated with seed germination under cold stress in the RIL population of rice | Yang et al., 2020 |
| *qLTSR-9-2, qLTSR-9-1, qLTSR-9-1, qLTNSR-9, and qLTNSR-9* | QTLs associated with cold tolerance of the RIL population of rice at the bud stage | Yang et al., 2020 |
| *MQTL2.5, MQTL8.1, and MQTL9.1* | significant count of *R*-genes contributing to sheath blight, rice blast, and bacterial blight resistance | Kumar and Nadarajah, 2020 |
| *qBL3* | Leaf and neck blast resistance | Devi et al., 2020 |
| *SUB1* | Submergence stress | Xu et al., 2006; Fukao and Bailey-Serres, 2008; Chakraborty et al., 2021 |
| *AG1* and *AG2* | anaerobic germination under flooding | Mondal et al., 2020 |

rice accessions by 167470 SNPs. The study concluded 15 SNPs to be significantly related to grain yield (Pantalião et al., 2020). Additionally, trait-linked simple sequence repeat (SSR) markers were deployed to study an important rice agronomic trait-aroma (Jasim Aljumaili et al., 2018). The study quantified the genetic divergence using SSR markers in aromatic rice accessions. This led to the identification of promising accessions for introgression (Jasim Aljumaili et al., 2018). SSRs were also utilized to study colored rice germplasm (Black-Purple and Red Pericarp Color) (Park et al., 2019). Taking into account the nutritional quality, the genetic diversity of rice grain iron and zinc levels in the representative groups of local and exotic rice accessions was evaluated by SSR markers. Aromatic rice fine grain accessions contained high iron and zinc levels in brown rice in comparison to coarse grain accessions (Raza et al., 2020). Neoterically, GWAS and functional analysis of 520 rice accessions identified *OsZIP18* as the prime genetic determinant for regulating branched-chain amino acid levels (Sun et al., 2020). Thus, *OsZIP18* can be considered a potential gene for enhancing rice nutritional value. *OsZIP18* can be of significant importance as humans are unable to synthesize branched-chain amino acids.

Plant breeders have suggested the IPA that comprises many important agronomic traits such as low tiller counts, more grains per panicle, few or no unproductive tillers, and thick and strong stems (Jiao et al., 2010; Li et al., 2012). Back in 2010, semi-dominant QTL, *IPA1* (ideal plant architecture 1) was cloned and characterized. This QTL encodes a squamosa-promoter binding protein-like transcription factor (TF) named *OsSPL14*. *OsSPL14* can regulate few relevant genes such as *OsTB1* (negative regulator of lateral branching) (Takeda et al., 2003) and *DEP1* (grain yield-related protein) (Huang, Qian et al., 2009). Moreover, *OsSPL14* at the reproductive stage can facilitate higher grain yield and panicle branching at the reproductive stage (Jiao et al., 2010; Miura et al., 2010). Nonetheless, microRNA (*miR156*) negatively regulates *OsSPL14* (Xie et al., 2006). The *OsSPL14* mRNA is cleaved by *miR156* to suppress it functions. Transgenic rice with IPA characteristics was generated by incorporating point mutation at the *OsmiR156*-targeted site in *OsSPL14* (Jiao et al., 2010; Miura et al., 2010). Over-expressing *miR156* also leads to fast leaf/tiller initiation and advanced leaf maturation in rice (Xie et al., 2012). *GW8* gene positively regulates the yield and width of rice grains (Wang et al., 2012). A mutation in the promoter of *GW8* gene was found in the *indica* Basmati rice varieties. This mutation lowered the *GW8* expression and resulted in slender grain with a better appearance. Interestingly, *GW8* encodes OsSPL16 protein which is a target of *miR156*. In this case also, MAS was implemented to concomitantly upgrade the appearance and enhance the grain yield (Wang et al., 2012). Additionally, *OsmiR156* was reported to regulate the tillering-associated genes (*TB1, LAX1, and DWARF 53*) (Liu Q. et al., 2019). These studies demonstrated that *miR156* is a crucial regulator in rice. Another miRNA, *miR172* is associated with reduced seed weight, floral defects, and delayed transition of spikelet meristem to floral meristem in rice (Zhu et al., 2009). Furthermore, a miRNA/MADS/TCP/D14 (miMTD) regulatory system has also been reported to regulate tillering in rice (Guo et al., 2013). The expression of *OsMADS57* is negatively regulated by *OsMIR444a.* This in-turn negatively modulates the expression of *D14* to affect rice tillering. This mechanistic outline can be focused for high grain yield in rice breeding programs (Guo et al., 2013). *OsmiR397* is an endogenous rice miRNA that is expressed in seeds (Xue et al., 2009; Chen et al., 2011), undifferentiated, and differentiated calli (Luo et al., 2006). Over-expression of miRNA gene-*OsmiR397* is linked with grain size and panicle branching, eventually leading to increased rice grain production (Zhang et al., 2013). The *miR397* targets *OsLAC* gene (linked to brassinosteroid sensitivity) to cleave its mRNA and disrupt the overall function (Zhang et al., 2013). On similar lines, *miR159* targets *OsGAMYB and OsGAMYBL1* (*GAMYB-LIKE 1*) genes. The activity of mature *miR159* was hindered by STTM (Short Tandem Target Mimic). This resulted in enhanced expression of *OsGAMYB and OsGAMYBL1* with reduced size of organ, diameter of stem, length of flag leaf, size of grain panicle, and spikelet hulls (Zhao et al., 2017). On similar grounds, miRNA microarray profiling identified *miR319* expression has a suppressive effect on rice plant height (Liu et al., 2017). In a recent study, transgenic rice with disrupted *miR396*-targeting site in *OsGRF8*; and knockout of *miR396e* and *miR396f* exhibited improved panicle branching and grain size (Zhang J. et al., 2020). Likewise, down-regulation of *OsmiR1432* resulted in enhanced expression of *Acyl-CoA thioesterase (OsACOT)* to promote grain filling. The disruption of *OsmiR1432* lead to heavier grains with improved yield by atleast 17% (Zhao et al., 2019).

Early efforts to decipher the entire transcriptome began in the 1990s (Lowe et al., 2017). During the last decade, RNA-seq method that uses deep-sequencing technologies has gained huge popularity for various crop improvement programs. Generally, in RNA-seq, total or fractionated RNA is made into a library of cDNAs fragments. Either one or both the cDNA ends are ligated with adapters. Each molecule is then sequenced in a high-throughput manner to obtain a short stretch of sequences either from one end (single-end sequencing) or both ends (pair-end sequencing) (**Figure 1**). The read length may vary from 30 to 400 bp depending upon the sequencing technology. The sequenced reads are finally aligned either to a reference genome or assembled *de novo* to generate meaningful information (expression profile and transcriptional structure). As already discussed, transcriptomics aids in deciphering un-annotated genes and analyzing gene expression patterns (Lowe et al., 2017). RNA-seq was performed to gain insights into the genome-wide transcription patterns of *O. sativa japonica* and *indica* subspecies (Lu et al., 2010). Whilst most of the RNA-seq studies in rice are largely focused on stress management, few have also been conducted for improving agronomic traits. In this context, the RNA-seq approach was utilized to establish the involvement of alternative splicing in rice mineral nutrient homeostasis (Dong et al., 2018). This was further extended to large-scale GWAS and transcriptome studies to identify genes affecting the rice glycemic index (Anacleto et al., 2019). The glycemic index in rice is an important parameter for a large population of society suffering from Type II diabetes, obesity, and hypertension (Mohan et al., 2014). In a recent study, the rice genome annotation was improvised by RNA-seq experiments. The study resulted in the identification of 1584 new peptides and 101 new loci matched to novel peptides (Ren et al., 2019). The

identification of these novel peptides and loci in the near future can be linked to traits of agronomic importance.

## Genomics and Transcriptomics in Rice Stress Management

The prime objective of rice research is to improve crop yield and acclimatization to unfavorable environmental conditions. The rice genome had been sequenced years back, but high-quality genome annotation of rice is necessary for the researchers working in this arena. In this direction, the accomplishment of Rice Annotation Project (RAP) database[2] established on the new chromosome pseudomolecule Os-Nipponbare-Reference-IRGSP-1.0 (a joint version of IRGSP and MSU pseudomolecules) (Kawahara et al., 2013) was imperative. The preliminary response of plants toward stress is the induction of signal transduction pathways. Generally, the second messenger molecules in signal transduction cascades are responsible for the regulation of stress-responsive genes. The induction or suppression of stress-responsive genes in-turn generates an appropriate response.

## Abiotic Stress

The generation of transgenic plants for functional validation of genes associated with a particular trait heavily relies on genomics and transcriptomics. For instance, it had been shown that 5000 genes were upregulated and 6000 genes were downregulated upon drought exposure to rice (Bin Rahman and Zhang, 2016; Joshi et al., 2016). These genes are grouped into three main categories: membrane transport genes, signaling-related genes, and transcriptional regulatory genes (Upadhyaya and Panda, 2019; Kim et al., 2020). The expression of these genes in rice governs the biochemical, physiological, and molecular mechanisms under drought stress (Dash et al., 2018; Gupta et al., 2020). Further, considering the transgenic approach, numerous genes in rice are identified to be differentially expressed upon drought exposure (Kumar et al., 2017; Upadhyaya and Panda, 2019). The mode of regulation may be either ABA-dependent or ABA-independent (Du et al., 2018; Gupta et al., 2020). In this regard, *OsJAZ1* in an ABA-dependent manner attenuates drought tolerance in rice (Fu et al., 2017). Similarly, LEA proteins and osmoregulatory genes confer drought tolerance to rice plants (Dash et al., 2018; Upadhyaya and Panda, 2019). *OsPYL/RCAR5*, *EcNAC67* (Kim H. et al., 2014; Rahman et al., 2016), *OsDREB2B*, *CYP735A,* and *OsDREB1F* (Kim et al., 2020) are also involved in morphological adjustments of rice upon drought exposures. Additionally, the DREB2-like gene *OsDRAP1* has been reported in modulating drought tolerance (Huang et al., 2018). Recently, an allele of the flowering gene *OsMADS18* was shown to be a potential candidate in drought tolerance during breeding (Groen et al., 2020). An increase in rice grain yield upon drought exposure is also accomplished by transgenic approaches. This includes generation of transgenics with genes namely, *OsLEA3-1* (Xiao et al., 2007), *OsbZIP71* (Liu C. et al., 2014), *OsWRKY47* (Raineri et al., 2015), *OsbZIP46* (Tang et al., 2012), and *OsNAC10* (Jeong et al., 2010). In a similar vein, in response to salinity stress, *OsCOIN*, *OsDREB2A*, *OsMYB2*, *OsbZIP71*, *OsbZIP23* are reported as key players in the accretion of osmoprotectants and antioxidants, enhanced transporter activity for sodium and potassium ions (Liu et al., 2007; Xu et al., 2008; Sun et al., 2010; Takasaki et al., 2010; Yang et al., 2012; Gumi et al., 2018), regulation of other salt-responsive genes (Nakashima et al., 2007; Wang et al., 2008; Jan et al., 2013; Liu C. et al., 2014), improved fresh weight (Huang et al., 2007), stomatal closure (Hu et al., 2006), and high seedling survival (Hu et al., 2008; Mallikarjuna et al., 2011). The gain of function of these salt-responsive genes permits the transgenic rice plants to have adequate osmoregulation and less oxidative damage. A recent study advocates that *OsSTAP1* is an AP2/ERF transcriptional activator that positively controls salt tolerance. *OsSTAP1* works by reducing the sodium/potassium ratio and sustaining cellular redox homeostasis (Wang et al., 2020). Taking cold stress into consideration, *OsbHLH1* (Wang et al., 2003), *OsDREB1G* (Moon et al., 2019), *OsCTZFP8* (Jin et al., 2018), *OsICE1* and *OsICE2* (Deng et al., 2017) are few of the many rice genes implicated in cold acclimatization and tolerance. Furthermore in rice, methylation profiles and transcriptional responses to cold at the seedling stage have also been reported in the recent past (Guo et al., 2019).

As discussed in the previous section DNA markers and MAS are indispensable components of plant breeding (Das et al., 2017). Grain yield upon stress exposure is the chief trait associated with breeding programs (Bernier et al., 2007; Venuprasad et al., 2007; Kumar et al., 2008). Identifying QTLs linked with stress tolerance or susceptibility can assist breeders to choose desired genotypes with less yield compensation (**Table 1**; Shanmugavadivel et al., 2017). Grain yield itself is a complex trait and in combination with stress, becomes enormously challenging. Thus, the selection and determination of traits for QTL mapping under unfavorable environmental cues is crucial. A major QTL for grain yield upon drought exposure was identified in 2007 (Bernier et al., 2007). Under drought conditions, a sum total of 436 F3 derived lines from Vandana and Way Rarem were QTL mapped. A major QTL (*qtl12.1/qDTY12.1*) was identified between SSR markers, namely RM28048 and RM511. This QTL was linked with decreased number of days to flowering, higher harvest index, increased biomass, and plant height (Bernier et al., 2007). Later, in 2009 the influence of *qtl12.1* was evaluated under varied target population of environments (Bernier et al., 2009). The results were consistent with the same effect on grain yield upon drought across various environments. Nonetheless, the uniformity of major yield QTL under adverse conditions in different genetic backgrounds is equally important. Eventually, 3 rice populations (N22/IR64, N22/MTU1010, and N22/Swarna) were evaluated and mapped for a major grain yield QTL, *qDTY1.1* (Vikram et al., 2011). Across all the 3 populations, *qDTY1.1* was mapped on chromosome 1 and was considered appropriate for marker-assisted breeding. Moreso, bulk segregant analysis identified *qDTY1.1* in the genetic background of Swarna and IR64 rice cultivars (Ghimire et al., 2012). Upon drought exposure, *qDTY1.1* accounted for 32 and 9.3% of the phenotypic variation in Swarna and IR64 respectively for grain yield (Ghimire et al., 2012). Additionally, *qDTY1.1* was found to be associated with plant height (*sd1*) in Vandana/IR64 populations

[2]http://rapdb.dna.affrc.go.jp/

(Venuprasad et al., 2012b). Consequently, in large segregating populations recombinant alleles with un-associated *sd1* and *qDTY1.1* might generate drought-tolerant varieties with shorter height (Vikram et al., 2016). Similarly, *qDTY2.1, qDTY3.1, qDTY2.2, qDTY9.1*, and *qDTY12.1* are also reported for grain yield under drought stress (Bernier et al., 2007; Venuprasad et al., 2009; Swamy et al., 2011; Mishra et al., 2013). Another QTL, *qDTY6.1* mapped on chromosome 6 in the genetic backgrounds of Apo/Swarna, Apo/IR72, and Vandana/IR72. *qDTY6.1* explains the genetic variance (40–66%) for grain yield under aerobic conditions and enhanced the performance of Swarna and IR72 (drought-susceptible cultivars) under aerobic conditions (Venuprasad et al., 2012a). Catolos et al. (2017), further identified 3 major QTLs contributing to grain yield, namely *qDTY1.1, qDTY1.3,* and *qDTY8.1* as well as 2 major QTLs for root trait, namely *qRT9.1* and *qRT5.1*. The mapping population was produced by crossing Dular (drought-tolerant) and IR 64_21 (drought-sensitive). Neoterically, high-density linkage map of rice was constructed by genotyping-by-sequencing (Yadav et al., 2019). The linkage map was generated by employing two $BC_1F_3$ mapping populations namely Swarna*2/Dular and IR11N121*2/Aus196. The study identified six *qDTY* QTLs (three consistent effect QTLs) in Swarna*2/Dular and eight *qDTY* QTLs (two consistent effect QTLs) in IR11N121*2/Aus 196 mapping population. The relative analysis further identified four stable new QTLs, namely *qDTY2.4*, *qDTY3.3*, *qDTY6.3*, and *qDTY11.2* accounting for 8.62 to 14.92% phenotypic variance. Three QTLs (*qDTY1.1*, *qDTY3.3,* and *qDTY6.3*) were linked to grain yield across the seasons under severe and moderate drought (Yadav et al., 2019). Contrary to drought, submergence stress is a phenomenon associated with exposure of plants to excessive water for longer periods. An important QTL associated with submergence tolerance is *SUB1* (*submergence 1*) (Xu et al., 2006; Fukao and Bailey-Serres, 2008). A recent study affirmed that *SUB1* influences concomitant leaf gas film thickness and surface hydrophobicity (Chakraborty et al., 2021). Leaf gas film provides improved ethylene dissipation and decreased *in-planta* accumulation. This eventually results in the delay of ethylene-induced leaf senescence upon submergence stress (Chakraborty et al., 2021). Another flooding stress-related condition involves the exposure of plants to hypoxia. QTLs for hypoxia tolerance in rice were identified during the germination stage (Kim and Reinke, 2018). Genotypic data from Illumina 6K SNP chip was used to identify QTLs related to tolerance of anaerobic germination (AG). Rice lines with *qAG1b* + *qAG1a* + *qAG8* possessed 50%, *qAG1b* + *qAG1a lines possessed 36%, while qAG1b* + *qAG8* possessed 32% of survival rate under anaerobic conditions (Kim and Reinke, 2018). In yet another study, responses of *AG1* and *AG2* QTL ILs were assessed during anaerobic germination under flooding. The study revealed that genotypes with *AG1* and *AG2* had greater seedling emergence and faster elongation in flooded soils (Mondal et al., 2020).

Much alike drought, salinity tolerance is a genetically and physiologically complex trait that is governed by a distinctive set of QTLs (Moradi et al., 2003). It is well established that salinity tolerance is autonomous at the seedling stage and reproductive stage (Mohammadinezhad et al., 2010). The major salt-tolerant QTL identified is *Saltol* QTL which has been extensively deployed worldwide to generate better performing rice cultivars (Gregorio, 1997; Krishnamurthy et al., 2020; Yadav et al., 2020). *Saltol* QTL was identified in IR29 (sensitive variety) and Pokkali (tolerant variety) RIL population which mapped on chromosome 1. AFLP markers (P3/M9-8 and P1/M9-3) flanks the *Saltol* QTL resulting in 64.3–80.2% of the phenotypic variance. This QTL is associated with low sodium levels in plants. *Saltol* QTL has been further fine mapped between the SSR markers RM1287 and RM7075 (10.71 and 15.12 Mb) that comprise the SKC1 locus (Bonilla et al., 2002; Niones, 2004; Thomson et al., 2010; Alam et al., 2011). Several other QTLs had been identified for traits such as shoot sodium concentration (SNC), shoot potassium concentration (SKC), and shoot sodium/potassium ratio (Lin et al., 2004; Ren et al., 2005; Haq et al., 2010; Pandit et al., 2010; Zheng et al., 2015). Bimpong laboratory (Bimpong et al., 2014a,b) fine mapped QTLs for salinity stress tolerance deploying Hasawi as a salt-tolerant donor parent. They used SNPs for genotyping and linkage map preparation. Furthermore, the QTLs namely, *qSKC-1* and *qSNC-1* were mapped in F2 mapping populations derived from *rss2* and *rss4* (Nipponbare) as well as Zhaiyeqing8 (*indica*) (Zhou et al., 2013; Deng et al., 2015b). Later in 2017, *qSKC-1* was finely mapped between the markers RM578 and IM8854 within 45 kb region in F2 populations derived from Nipponbare/ZYQ8 and rss4/ZYQ8 (Jing et al., 2017). Similarly, *rst1* mutant (*rice salt-tolerant 1*) was used to reveal that *rst1* is regulated by a recessive gene (Deng et al., 2015a). QTL mapping was performed between *rst1* and Peiai 64 to identify the possible loci of the *rst1* gene, which was found on chromosome 6 (Deng et al., 2015a). Additionally, RILs obtained from IR29 (salt-sensitive) and Hasawi (salt-tolerant) were used by Bizimana et al. (2017) to identify the QTLs on chromosomes 1, 2, 4,6, 8, 9, and 12. None of the *Saltol* or QTLs were found near this position. This indicated that tolerance in the cultivar Hasawi is attributed to new QTLs which are different from *Saltol/SKC1* (Bizimana et al., 2017). Apart from QTLs/genomic regions linked to salt tolerance based on bi-parental mapping populations, an association panel following GWAS approaches to study marker-trait association has also been used (Emon et al., 2015; Kumar et al., 2015). 20 SNPs were identified to be significantly linked with sodium/potassium ratio (Kumar et al., 2015). Also, this study could identify the *Saltol* region, which accounts for salinity tolerance as a prime link with sodium/potassium ratio (Kumar et al., 2015). In an identical manner, Wn11463, an STS marker for *SKC1*, and RM22418 on chromosome 8 were identified at the seedling stage to be linked with salinity tolerance (Emon et al., 2015). Very recently, 308 $F_4$ families from Sahel 317/Madina Koyo were evaluated using SNPs for salt tolerance at the early seedling stage (Amoah et al., 2020). The genotypic data were regressed on to their phenotype to detect the QTLs, and a high-density genetic map was prepared with 3698 SNPs. Multiple interval mapping revealed 13 QTLs associated with shoot length, root length, salt injury score, and shoot dry weight on chromosomes 2, 3, 4, 6, 7, 10, and 12. On chromosome 2, three QTLs (*qSL2*, *qRL2.1,* and *qSIS2*) and two QTLs (*qSDW2* and *qRL2.2*) were tightly linked, while on chromosome 7, another two QTLs (*qSDW7*

and *qSL7*) were strongly associated (Amoah et al., 2020). Taking cold tolerance into account, RILs derived from Dasanbyeo (*indica*)/TR22183 (*japonica*) crosses in Yanji (high-latitude area), Kunming (high-altitude area), Chuncheon (cold water irrigation) and Suwon (normal) were used to study the influence of QTL and epistatic QTL (E-QTL) with respect to cold-related traits at the reproductive stage. In three different cold treatment locations, six QTLs for spikelet fertility were detected. Furthermore, 57 QTLs and 76 E-QTLs were identified for nine cold-associated traits; out of them 19 QTLs and E-QTLs had substantial interaction of QTLs with environments (QEIs). This study illustrated that epistatic effects and QEIs are imperative for QTLs linked with cold tolerance (Jiang et al., 2011). QTLs controlling cold tolerance were also studied at germination and early seedling stages with RILs derived from crosses between *japonica* and *indica* sub-species. Composite interval mapping revealed five QTLs at the germination stage with 5.7–9.3% phenotypic variance explained, while nine QTLs were found at the early seedling stage with 5.8–35.6% phenotypic variance explained. The study reported only one common QTL, probably indicative of growth-stage specificity of cold tolerance (Ranawake et al., 2014). Another study performed at the reproductive stage in rice involved 84 $BC_2$ cold tolerance introgression lines (ILs) that were generated through backcrossing. These cold tolerance ILs along with 310 random ILs were deployed for studying genetic networks fundamental to cold tolerance in rice. The segregation distortion method revealed seventeen major QTLs for cold tolerance in five selective introgression populations (Liang et al., 2018). Recently, RILs obtained from *indica* rice H335 (low temperature-tolerant) and *indica* rice CHA-1 (low temperature-sensitive) were used to detect QTLs linked with low-temperature tolerance at bud and germination stages. A high-density genetic map revealed 11 QTLs; among which six QTLs accounted for 5.13–9.42% phenotypic variation explained at the germination stage, while five QTLs accounted for 4.17–6.42% phenotypic variation explained at the bud stage (Yang et al., 2020).

Next generation sequencing that can robustly ascertain approximately all the RNAs in cells has been extensively deployed for miRNA analysis, particularly in identifying new or rice-specific stress-responsive miRNAs. A number of rice miRNAs are expressed upon encountering biotic and abiotic stresses (**Table 2**). The majority of stress responsive miRNAs are conserved and possess an analogous effect among rice and other plant species. For example, rice *miR398* modulate the expression of *Os-CSD1* and *Os-CSD2* (similar to its targets in *Arabidopsis thaliana* -Cu or Zn superoxide dismutases) as well as responses to abiotic and biotic stresses (Li et al., 2011). A prominent report of drought-induced miRNA in rice involves *miR169g*. *miR169g* is notably up-regulated upon drought exposure (Zhao et al., 2007; Jian et al., 2010; Zhou et al., 2010). Apart from the established role of *miR169g* in drought tolerance, it is also reported to be induced by salt stress to cleave mRNA of the NF-YA TF (Zhao et al., 2009). Moreso, *miR169g* negatively regulates rice immune responses against the blast fungus (Li et al., 2017). Similarly, *miR393* is also induced by both, salinity and drought conditions (Gao et al., 2011; Xia et al., 2012; Lu et al., 2018). In addition, *miR319* is down-regulated upon cold stress (Lv et al., 2010), however, when over-expressed it could increase cold tolerance after chilling acclimation in rice (Yang et al., 2013; Wang et al., 2014). Furthermore, the reproductive tissues of rice treated with drought, salt, and cold stresses were used to prepare small RNA libraries. The RNA libraries were sequenced to gain insights into the involvement of miRNAs is stress responses (Barrera-Figueroa et al., 2012). A number of stress-modulated miRNAs were identified by matching the expression patterns under control and stress conditions. This paved the discovery of new miRNAs that might play important roles in stress responses associated with rice (Barrera-Figueroa et al., 2012). Thus, a single miRNA can regulate the signaling cross-talk between various pathways related to environmental stresses and can be linked with several traits, indicating a pleiotropic effect. Contrary to the pleiotropic effect, distinct miRNAs might contribute to a common function. For instance, *miR169*, *miR397*, *miR528*, *miR827*, *miR1425*, *miR319a.2,* and *miR408-5p* are all linked with $H_2O_2$-oxidative stress (Li et al., 2011). In an identical manner, Illumina sequencing revealed 29 known and 32 novel miRNAs to be differentially expressed upon salt stress in *Oryza glaberrima* (Mondal et al., 2018). Nonetheless, small RNA libraries sequenced from rice seedlings subjected to cadmium stress revealed a set of miRNAs, all of which contributed to stress regulation (Huang S.Q. et al., 2009; Ding et al., 2011). A report by Zhang et al. (2018) suggested that the *miRNA166* knockout rice mutants exhibited higher drought tolerance and smaller xylem diameter (Zhang et al., 2018). A recent study also used small RNA sequencing to identify *osa-miR12477* (Parmar et al., 2020). The *osa-miR12477* regulates the expression of *LAO* (L-ascorbate oxidase) for salt tolerance in the plant.

## Biotic Stress

Taking biotic stress into consideration, 13 and 16 blast resistance QTLs were recognized in Jin23B/CR071 and Jin23B/QingGuAi3 rice populations, respectively. The study revealed major and minor QTLs interactions as the basic genetic mechanism for blast resistance in CR071 and QingGuAi3 rice lines (Jiang et al., 2020). Lately, *pi 66(t)* was recognized as one of the recessive genes governing rice blast (Liang et al., 2016). Furthermore, the status and diversity of 12 major blast resistance genes were studied amongst 80 different rice varieties (Yadav et al., 2017). Molecular markers for genes *Pi54, Pib, Piz, Piz-t, Pik, Pi-kh, Pik-p, PikmPik-h, Pita/Pita-2, Pi2, Pi9, Pi1,* and *Pi5* were utilized in this investigation. Recently in *Oryza glumaepatula*, characterization of a wide effect QTL showed *Pi68(t)* as a potential gene for field resistance and neck blast in rice (Devi et al., 2020). Another very recent study focused on the meta-analysis of QTL with multiple disease resistance in rice (Kumar and Nadarajah, 2020). The study revealed *MQTL2.5, MQTL8.1, and MQTL9.1* have a significant count of *R*-genes which denotes 10.21, 4.08, and 6.42% of the total genes respectively. The defense-related genes contribute approximately 3.70, 8.16, and 6.42% of the total number of genes in *MQTL2.5, MQTL8.1,* and *MQTL9.1*, respectively. The study further led to the recognition of QTL hotspots for sheath blight, rice blast, and bacterial blight resistance. The potential gene candidates within these regions might be implemented for rice crop improvement via the intervention of genetic engineering.

With the increasing advent of high-throughput technologies, researchers have used microarrays and NGS/deep sequencing to accomplish genome-wide expression analysis to identify stress-regulated miRNAs (**Table 2**; Baldrich and San Segundo, 2016; Nadarajah and Kumar, 2019; Kar and Raichaudhuri, 2021). Xu et al. (2014) deployed microarray to study miRNA expression profiles in black-streaked dwarf virus (SRBSDV)-infected rice. They uncovered 56 miRNAs and 24 target genes to be potentially linked with diseased conditions. NGS was used to study small RNA expression profiles of rice seedlings infested with rice dwarf virus (RDV) and rice stripe virus (RSV). Campo et al. (2013) relied on high-throughput RNA sequencing to unravel a novel *osa-miR7695*. This miRNA negatively controls an alternatively spliced transcript of *OsNRAMP6* (*natural resistance-associated macrophage protein 6*), while its over-expression improves the resistance to *Magnaporthe oryzae* (Campo et al., 2013). In yet another study for the blast fungus *M. oryzae, miRNA169* was shown to inhibit the expression of its target nuclear factor Y-A genes. This resulted in decreased rice immunity against the pathogen (Li et al., 2017). A comparable effect was detected against the blast fungus with Osa-*miRNA164a* that targets *OsNAC60* gene (Wang Z. et al., 2018).

**TABLE 2 |** List of miRNAs in rice trait improvement and stress management.

| miRNA | Function | References |
|---|---|---|
| *miR156* | fast leaf/tiller initiation and advanced leaf maturation in rice; regulate the tillering-associated genes (*TB1, LAX1 and DWARF 53*) | Xie et al., 2012; Liu Q. et al., 2019 |
| *miR172* | reduced seed weight, floral defects and delayed transition of spikelet meristem to floral meristem in rice | Zhu et al., 2009 |
| *miR397* | grain size and panicle branching, eventually leading to increased rice grain production | Zhang et al., 2013 |
| *miR159* | reduced size of organ, diameter of stem, length of flag leaf, size of grain panicle, and spikelet hulls | Zhao et al., 2017 |
| *miR319* | suppressive effect on rice plant height | Liu et al., 2017 |
| *miR396* | improved panicle branching and grain size | Zhang J. et al., 2020 |
| *miR1432* | enhanced expression of *OsACOT* to promote grain filling | Zhao et al., 2019 |
| *miR398* | modulate the expression of *Os-CSD1* and *Os-CSD2* as well as responses to abiotic and biotic stresses | Li et al., 2011 |
| *miR169g* | Drought, salt, and blast fungus | Zhao et al., 2007, 2009; Jian et al., 2010; Zhou et al., 2010; Li et al., 2017 |
| *miR393* | Drought, salinity, and cold | Lv et al., 2010; Gao et al., 2011; Xia et al., 2012; Lu et al., 2018 |
| *miR169*, *miR397*, *miR528*, *miR827*, *miR1425*, *miR319a.2* and *miR408-5p* | $H_2O_2$-oxidative stress | Li et al., 2011 |
| *miRNA166* | drought tolerance and smaller xylem diameter | Zhang et al., 2018 |
| *miR12477* | regulates the expression of *LAO* (L-ascorbate oxidase) for salt tolerance | Parmar et al., 2020 |
| *miR7695* | negatively controls an alternatively spliced transcript of *OsNRAMP6,* while its over-expression improves the resistance to *M. oryzae* | Campo et al., 2013 |
| *miRNA169* | decreased rice immunity against *M. oryzae* | Li et al., 2017 |
| *miRNA164a* | negatively regulates rice immunity against *M. oryzae* | Wang Z. et al., 2018 |

## PROTEOMICS AND METABOLOMICS: AN OVERVIEW

Proteomics is a robust and powerful discipline that involves large-scale identification and quantification of proteins including, their structure and physiological functions. Precisely, proteome denotes a set or the entire complement of proteins within a cell, tissue, or organism. Proteome provides a data-rich panorama of regulation of expressed proteins under specific conditions. The word proteomics is an amalgamation of two words (protein and genome) and was first coined in 1994 by Mark Wilkins (Shah and Misra, 2011). Proteomics appendages the other omics techniques *i.e.,* genomics, transcriptomics, and metabolomics to cognize the function and structure of the protein of interest. Proteomics has proven to be a forte for the rice research community. Proteogenomics (large-scale proteome information is processed for genome annotation refinement) has greatly assisted in this direction (Helmy et al., 2011). Proteomes are available for almost all rice tissues and organs under normal or stressed conditions (Agrawal and Rakwal, 2011; Kim S.T. et al., 2014). Proteomics-based techniques are used in different capacities for crop improvement and deciphering environmental stress mechanisms. Nonetheless, the field of proteomics is exceedingly dynamic in nature due to the intricate regulatory systems governing the protein expression levels. Mass spectrometry (MS) with liquid chromatography (LC-MS-MS) and matrix-assisted laser desorption/ionization (MALDI-TOF/TOF) are central to current proteomics. The classical techniques for protein purifications involve ion-exchange chromatography (IEC), affinity chromatography, and size exclusion chromatography (SEC) (Agrawal et al., 2010; Agrawal et al., 2013). Enzyme-linked immunosorbent assay (ELISA) and western blotting are used for studying selective proteins (Yang and Ma, 2009; Kim et al., 2013). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional differential gel electrophoresis (2D-DIGE), and two-dimensional gel electrophoresis (2-DE) techniques are routinely utilized for separation of complex protein mixtures (Choudhary et al., 2009; Pandey et al., 2010; Jaiswal et al., 2013). These techniques can be efficiently utilized to analyze a small set of proteins and are incapable of measuring protein expression levels. The technique 2-DE allows the study of differentially expressed proteins with the simultaneous detection and quantification of several protein spot isoforms, encircling post-translational modifications. Nevertheless, 2-DE based proteomics is biased

against low abundance and hydrophobic proteins. For high-throughput protein expression analysis protein microarrays or chips have been established. However, it cannot be utilized to determine the function of complete proteome (Han et al., 2014a)., Edman degradation, MS, isotope-coded affinity tag (ICAT) labeling, stable isotope labeling with amino acids in cell culture (SILAC), multidimensional protein identification technology (MudPIT), and isobaric tag for relative and absolute quantitation (iTRAQ) are the few techniques for quantitative proteomic (Dong et al., 2014; Han et al., 2014b; Li Y. et al., 2014; Liao et al., 2014; Zhang et al., 2014; Li et al., 2015). Likewise, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy are the main high-throughput technologies to determine the 3-D structure of a protein (Liao et al., 2014; Liu C.W. et al., 2014; Zhang et al., 2014). High-throughput data yields large quantities of proteomics data which is analyzed by various bioinformatics databases (**Figure 1**). Proteomics analysis in rice are grouped into gel-based (1-DE, 2-DE, and 2-DIGE), gel-free (LC-MS/MS, MudPIT, iTRAQ), and a coalescence of these two methods (Agrawal et al., 2013). Thus, proteomics enables to globally decipher the protein expression profiles and their analogous post-translational modifications.

Metabolomics is the systematic analysis of chemical processes including metabolites, substrates, intermediates, and products of cellular metabolism. Precisely, metabolomics involve the characteristic fingerprints that discrete cellular processes lay down resulting in a unique metabolic profile (Daviss, 2005; Gong et al., 2013). Genomics, transcriptomics, and proteomics reveal the expression pattern or cellular function of a gene within the cell. However, metabolomics offers a straight functional read-out of the physiological state associated with an organism (Daviss, 2005). The tools and techniques deployed for metabolomic data recording and processing have been proved to be more sophisticated than ever. The studies encircling the metabolome data have been long ramification of "hypothesis generator," which remains a subject of further evaluation (Hall, 2006). Taking rice metabolomics in particular, it is a high-throughput technique to profile metabolites implicated directly or indirectly in metabolic processes (**Figure 1**). Further, it is widely deployed to monitor and evaluate the cellular metabolic state and quality of rice (Okazaki and Saito, 2016). Generally, metabolomics involves optional separation of small metabolites by gas chromatography (GC), high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS); followed with MS to identify and quantify metabolites. Crude extracts are utilized to profile metabolites in a non-targeted approach; thus, chromatographic separation is often essential to analyze fractionated compounds (Fukushima and Kusano, 2014). GC-MS is particularly the method of choice for the study of low molecular-weight metabolites. The process of chemical derivatization makes low molecular-weight metabolites acquiescent to GC. The major advantage of GC-MS is capacity metabolite profiling and targeted metabolite quantitation. This aids in the study of several metabolites in a single GC-MS-MS multiple reaction monitoring (MRM) run. MS is a stand-alone technique that is highly sensitive and specific. Alternately, the sample material with no prior separation is directly infused into the mass spectrometer. MS itself imparts adequate selectivity to separate and detect metabolites. Advanced techniques such as NMR, LC-MS, GC-MS, inductively coupled plasma (ICP)-MS, HPLC, and direct flow injection (DFI)-MS have significantly contributed to metabolic profiling (Uawisetwathana and Karoonuthaisiri, 2019). Fourier transform infrared spectroscopy (FTIR) is also popular in metabolomics for its capacity to simultaneously analyze and characterize intricate building blocks (Junot and Fenaille, 2019). Shortly, integrating metabolomics with genomics and proteomics has assisted in a proficient dissection of genetic, phenotypic, and protein level information in rice. Thus, the rice metabolome generates a "fingerprint" of diverse rice samples to ascertain the varieties that are crucial to rice trait improvement and stress management (Wei et al., 2018).

## Proteomics and Metabolomics in Rice Trait Improvement

One of the major traits associated with rice is the aroma. Two genes, *betaine-aldehyde dehydrogenase* (Bradbury et al., 2008) and *glyceraldehyde-3-phosphate dehydrogenase B form* (Lin et al., 2014) are involved with fragrance in rice. Moreover, aromatic rice has a flavor compound, 2-acetyl-1-pyrroline (2AP). Proteomic analysis of two isogenic lines of Thai jasmine rice was performed to gain insights into the 2AP biosynthetic pathway. 2-DE was performed on both isogenic lines which identified aldehyde dehydrogenase, a key enzyme responsible for 2AP production (Wongpia et al., 2016). Rice grains are also known to contain low quantities of storage proteins (glutelins, prolamins, albumins, and globulins). Few of them are allergens (α-amylase/trypsin inhibitor, globulins, β-glyoxylase, and glutelins). Proteins from 4 different rice varieties were analyzed by 2D-GE. Further investigation revealed, few of the differentially abundant proteins as allergenic proteins. Particularly, a deletion in the 1000 bp upstream region of the *globulin* gene has been recognized, probably contributing to the varied abundance of the protein in the Karnak cultivar. This is useful for cultivar identification in commercial samples (Graziano et al., 2020). In another interesting study, three cytochrome P450 homoeologs (*Os03g0603100*, *Os03g0568400*, and *GL3.2*) and *OsBADH2* were edited with the CRISPR/Cas9 to produce novel rice mutants. Evidently, CRISPR/Cas9 has revolutionized the arena of plant sciences (Iqbal et al., 2020a). The mutants exhibited elevated yields and enhanced aroma. RNA-seq and proteomic analysis were done to unravel the underlying modifications. Mutants showed increased grain size, grain cell number, and high 2AP content. RNA sequencing and proteomic analysis showed the involvement of genes and proteins linked to the cytochrome P450 family, grain size and development, and cell cycle (Usman et al., 2020). Anthocyanin and proanthocyanin are flavonoids that are present in good quantities in black and red rice. To decipher the molecular pathways, a study was performed to understand the flavonoid biosynthetic pathway in red, black, and white colors rice cultivars. A comprehensive profile of mRNA and expressed proteins in diverse colored rice varieties was obtained by RNA sequencing of caryopsis and iTRAQ analysis. A total of 3417,

329, and 227 genes were distinctive for red, white, and black rice, respectively. Furthermore, the proteomes of black, white, and red rice contained 13,996 distinctive peptides corresponding to 3916 proteins. Interestingly, 32 genes were shown to be implicated in the flavonoid biosynthesis pathway. From those 32 genes, only *CHI*, *F3H*, *ANS*, and *FLS* were ascertained by iTRAQ (Chen et al., 2019). A similar study on two black rice cultivars (BALI and Pulut Hitam 9), two red rice cultivars (MRM16 and MRQ100), and two white rice cultivars (MR297 and MRQ76) using label-free liquid chromatography Triple TOF 6600 tandem mass spectrometry (LC-MS-MS) was conducted. The study profiled and ascertained the proteins associated with nutritional values (antioxidant, folate, and low glycemic index) and quality (i.e., aromatic) based on peptide-centric scoring from the Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) approach (Sew et al., 2020). Recently, the effect of germination (post 24 h) on nutrition-associated proteins in 4 rice cultivars was studied using shotgun proteomics. In-gel digestion coupled with tandem mass spectrometry (GeLC-MS/MS) was performed on 4 rice cultivars to analyze the total proteins from non-germinated seeds and 24 h germinated seeds. Total phenolic content was also measured post 0, 24, and 48 h of germination by Folin-Ciocalteu assay. The study revealed that seed nutrition-related proteins, particularly phenolic proteins increased post-germination. A 2.20 – 15.90 folds increment in the expression of phenylalanine ammonia-lyase, serine carboxypeptidase-like protein, isoflavone-7-O-methyltransferase, isoflavonoid glucosyltransferase, glycosyltransferase family 61 protein, and UDP-glucose flavonoid 3-O-glucosyltransferase was observed post-germination. The study supported the notion that rice germination for 24 h influences the enhanced nutrition of brown rice and the phenolic biosynthetic pathway (Maksup et al., 2020).

Genetic engineering intervention in the generation of genetically modified rice cultivars is well illustrated by "Golden rice" (Khan et al., 2015). Wild-type rice is devoid of vitamin A or its precursor-beta-carotene. Its deficiency affects the human population that consumes rice as a staple food. The rice genome was genetically engineered with a multi-gene biochemical pathway to synthesize beta-carotene that is eventually metabolized by humans to synthesize vitamin A (Baranski, 2013; Khan et al., 2015). Pleiotropic effects, mutation, and inactivation of endogenous genes are the basis for the generation of such cultivars with unintended phenotypes (Matsaunyane and Dubery, 2018). Genetic alteration of *phytoene synthase* (*Psy*) and *phytoene desaturase* (*crtI*) that leads to metabolic regulation and adaptation of "golden rice" has been extensively studied (Gayen et al., 2016). Transgenic and non-transgenic seeds of golden rice were collected for proteomic and metabolomic studies. HPLC analysis identified significantly high levels of carotenoids in the transgenics. The higher level of carotenoid in the transgenics is attributed to *Psy* and *crtI* expressions. Also, the GC-MS approach was deployed to detect the changes in the carbohydrate metabolism pathway in the transgenics (Decourcelle et al., 2015). The transgenics accumulated higher amounts of galactose, fructo furanose, D-glucoronate, and D-sorbitol. Surprisingly, the proteomic results were found to be in correlation with the metabolomic data as greater activities for enzymes (pullulanase and UDP-glucose pyrophosphorylase) were found in the transgenics. These enzymes are imperative to carbohydrate metabolism and are linked with the biosynthesis of carotenoids (Chen et al., 2007; Gayen et al., 2016). Additionally, the activity of pyruvate phosphate dikinase implicated in pyruvate biosynthesis (precursor of carotenoid) was also found to be higher in the transgenics (Gayen et al., 2016). Song J.M. et al. (2018) conducted an interesting study on rice leaves and grains to further accentuate the role of metabolomics in rice research (Song E.H. et al., 2018). The metabolic profile of two rice cultivars (early maturing rice cultivar- EMC and late maturing rice cultivar-LMC) was assessed by an NMR- based metabolomics ($^1$H NMR). Distinct metabolic profiles in leaves and grains at all growth stages of EMC and LMC were detected. For rice grains, significantly elevated levels of sucrose, amino acids, and fatty acids were observed in EMC than LMC. Thus, the nutritional value in EMC rice grains was higher than LMC rice grains (Song E.H. et al., 2018). In a recent study, phenolics, especially flavonoids and antioxidants in two rice varieties (*Oryza sativa*-Os and *Zizania latifolia*-Zl) were studied. A UHPLC-QqQ-MS-based metabolomics approach revealed that Zl possessed higher levels of phenolics, flavonoids, proanthocyanidins, and antioxidant activity. Out of 159 identified flavonoids, 78 showed differential expression (72 up-regulated and 6 down-regulated in the Zl). The majority of flavonoids in Z1 were related to anthocyanin biosynthesis owing to its better nutrition profile (Yu et al., 2021). A more holistic study on rice metabolomics involved 17 cultivars from 7 different countries (Zarei et al., 2018). The group of metabolites and metabolome significantly varied amongst the cultivars. On average, 411 metabolites per cultivar were annotated and 71 metabolites were different between them. Prior, a similar study depicting the disparities between *indica* and *japonica* sub-species had been conducted (Hu et al., 2014). Among the 92 significantly variable metabolites, 66 were up-regulated in *japonica* while 26 were up-regulated in *indica* cultivars. Asparagine had higher quantities in the *indica* sub-species and was regarded as the most variable of all the metabolites according to the Random Forest ranking. The metabolites of interest demarcating the two sub-species were associated with nitrogen metabolism, translocation, inorganic nutrition storage, and stress responses. Trait-associated metabolites with respect to biosynthetic and catabolic pathways will deepen the knowledge toward rice trait improvement (**Table 3**). Yet another study focused on the pathways related to the aroma in fragrant rice (Daygon et al., 2017). As stated earlier, 2AP in rice is a pivotal aroma compound. The analysis by Daygon et al. (2017) using GC × GC-TOF-MS showed 6-methyl, 5-oxo-2,3,4,5-tetrahydropyridine (6M5OTP), 2-acetylpyrrole, pyrrole and 1-pyrroline were related with the synthesis of 2AP in aromatic rice cultivars. Further, the GWAS indicated that all the above 4 compounds were linked with a single QTL that harbors the *FGR* gene linked with GABA production (Daygon et al., 2017). Recently, GC-MS based approach has also been used to assess rice grain quality through profiling of volatiles and metabolites in rice grains (Llorente et al., 2019). Thus, proteomics and metabolomics have contributed significantly in comprehending

the underlying pathways and compounds associated with rice trait improvement.

## Proteomics and Metabolomics in Stress Management:

Proteomics and metabolomics-based studies are expected to improve rice plant responses toward fluctuating environmental conditions. In the past few years, the contribution of omics sciences has been immense in rice research for studying the pathways, metabolites, and proteins involved in combating stress. Some case studies with respect to abiotic and biotic stresses are discussed below.

## Abiotic Stress

The application of proteomics in rice stress management includes the study of physiological and proteomic analysis of the rice mutant *coleoptile photomorphogenesis 2* (*cpm2*-disrupted in allene oxide cyclase). The study revealed negative regulation of jasmonic acid (JA) in drought tolerance (Dhakarey et al., 2017). Tandem mass tagging and Nano-LC-MS-MS was performed to comprehend the involvement of JA under drought at the molecular level. The histological, metabolite and proteome-based transcript analysis revealed the favorable adaptations and responses against drought stress, mainly coordinated by the absence of JA in the *cpm2* roots (Dhakarey et al., 2017). In a similar vein, proteomic analysis of drought-responsive proteins by LC-MS-MS revealed photosynthesis-related adaptations via NADP(H) homeostasis to drought (Chintakovid et al., 2017). Recently, 8 genotypes of *japonica* and *indica* sub-species at the late vegetative stage were studied with nano LC-MS-MS (nanoflow liquid chromatography-tandem mass spectrometry) for drought stress (Hamzelou et al., 2020). Label-free quantitative shotgun proteomic analysis of 8 rice genotypes subjected to drought unraveled 1253 non-redundant proteins under well-watered and drought conditions. In all the 8 genotypes, 8 proteins were induced under drought stress (Hamzelou et al., 2020). A more comprehensive study by Du J. et al. (2020) involved proteomics, metabolomics, and physiological analyses upon heavy nitrogen exposure before (NBD) and after drought (NAD) on rice (Du J. et al., 2020). The proteomic experiments were carried by tandem mass tagging of rice leaves subjected to NBD and NAD. The samples were analyzed by LC-MS-MS with the amount of qualitative protein and quantitative protein being 4254 and 3892 respectively. Upon drought exposure, NBD had higher chlorophyll content and photosynthetic rate, enhanced activities of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase, and catalase, and declined malondialdehyde (MDA) content (Du J. et al., 2020). Next, the application of multi-omics in salinity stress involves an analysis by Xu et al. (2017) utilizing the techniques 2-DE and MALDI TOF (Xu et al., 2017). The relative proteomic analysis was performed amongst the dry and imbibed seeds of salt-tolerant *japonica* landrace Jiucaiqing with 150 mM NaCl. A total of 14 proteins were identified to be implicated in seed imbibition. Many of the identified proteins were involved in energy supply and storage. Upon analysis, 2,3-bisphosphoglycerate-independent

**TABLE 3 |** List of proteomics and metabolomics techniques in rice trait improvement and stress management.

| Method | Key finding | References |
|---|---|---|
| 2-DE | proteins related with aroma compound biosynthesis | Wongpia et al., 2016 |
| 2D-GE | Development of a molecular marker useful for the analysis of commercial products | Graziano et al., 2020 |
| HPLC Fractionation and LC-MS/MS Analysis | cytochrome P450 family, grain size and development, and cell cycle | Usman et al., 2020 |
| iTRAQ analysis | flavonoid biosynthesis pathway | Chen et al., 2019 |
| LC-MS-MS and SWATH-MS | Nutritional and quality traits | Sew et al., 2020 |
| GeLC-MS-MS | enhanced nutrition of brown rice and the phenolic biosynthetic pathway | Maksup et al., 2020 |
| GC-MS | carbohydrate metabolism pathway | Decourcelle et al., 2015 |
| GC-MS | endosperm specific carotenoid pathways | Gayen et al., 2016 |
| $^{1}$H NMR | intrinsic physiology and potential eating quality | Song E.H. et al., 2018 |
| UHPLC-QqQ-MS | better nutrition profile | Yu et al., 2021 |
| UPLC-MS-MS | rice gene–bran metabolite relationships | Zarei et al., 2018 |
| UPLC-MS-MS and GC-MS | Metabolic variation between japonica and indica rice cultivars | Hu et al., 2014 |
| GC × GC-TOF-MS | Aroma in fragrant rice | Daygon et al., 2017 |
| GC-MS | rice grain quality through profiling of volatiles and metabolites | Llorente et al., 2019 |
| Tandem mass tagging and Nano-LC-MS/MS | involvement of JA under drought at molecular level | Dhakarey et al., 2017 |
| LC-MS-MS | photosynthesis-related adaptations via NADP(H) homeostasis to drought | Chintakovid et al., 2017 |
| nLC-MS-MS | Drought stress | Hamzelou et al., 2020 |
| LC-MS-MS | Rice growth and grain yield with heavy nitrogen application before and after drought | Du J. et al., 2020 |
| 2-DE and MALDI TOF | seed imbibition under salt stress | Xu et al., 2017 |
| iTRAQ | early salinity response | Lakra et al., 2019 |
| LC-MS-MS | Rice yield under salinity stress | Li et al., 2020 |
| 1D LDS-PAGE, in-gel digestion, and LC-MS-MS | cold tolerance response | Lee et al., 2017 |
| 2-DE and MALDI-TOF-MS | cold tolerance response | Ji et al., 2017 |
| nLC-MS-MS | cold tolerance response | Wang J. et al., 2018 |
| $^{1}$H NMR and GC-MS | drought tolerance via GABA biosynthesis, sucrose metabolism and antioxidant defenses. | Nam et al., 2016 |
| GC-MS | drought and heat stress in the field | Lawas et al., 2019 |
| GC-MS | metabolic pathways associated with photosynthesis upon drought exposure | Ma et al., 2016 |
| GC-MS | Salinity stress | Xie et al., 2020 |
| GC-MS | Salinity stress | Gupta and De, 2017 |
| GC-TOF-MS | Salinity stress | Wanichthanarak et al., 2020 |

*(Continued)*

**TABLE 3 |** (Continued)

| Method | Key finding | References |
|---|---|---|
| LC-MS-MS | rice metabolic network underlying *OsDRAP1*-mediated salt tolerance | Wang et al., 2021 |
| EESI-MS | Cold stress | Du S. et al., 2020 |
| 2D Nano LC MS-MS, 2-DE, In-gel digestion and MALDI-TOF MS | accumulation of photosynthesis and defense associated proteins by *Pseudomonas fluorescens* and *Sinorhizobium meliloti* | Kandasamy et al., 2009; Chi et al., 2010 |
| UHPLC-DAD/ESIQTOF | metabolomics signatures such as decreased alkylresorcinol quantities and the differential induction of N-p-coumaroylputrescine and N-feruloylputrescine (antimicrobial compounds) | Valette et al., 2020 |
| HPLC | exudation of salicylic acid | Kandaswamy et al., 2019 |
| LC-MS/MS | *Pseudomonas aeruginosa* is linked with the synthesis of SAR related compounds such as siderophores and antibacterial compounds | Yasmin et al., 2017 |
| iTRAQ | regulatory response to *M. oryzae* in durable resistant vs. susceptible rice genotypes | Ma Z. et al., 2020 |
| QTOF-UPHPLC | Bayogenin 3-O-cellobioside confers non-cultivar-specific defense against *Pyricularia oryzae* | Norvienyeku et al., 2021 |
| 2-DE and MALDI-TOF-MS-MS | accumulation of novel proteins potentially involved in defense against *Rhizoctonia solani* | Karmakar et al., 2019 |
| iTRAQ | rice immunity to infection by *R. solani* | Ma H. et al., 2020 |
| Tandem mass tagging with LC-MS/MS | interaction between rice and *X. oryzae* | Zhang F. et al., 2020 |
| iTRAQ | defense response to *M. oryzae* in rice with blast resistance gene *Piz-t* | Tian et al., 2018 |

phosphoglycerate mutase (*BPM*), glutelin (*GLU2.2* and *GLU2.3*), glucose-1-phosphate adenylyltransferase large subunit (*GAS8*), and cupin domain-containing protein (CDP3.1 and CDP3.2) were close to QTLs for seed dormancy, seed reserve utilization, and seed germination. Interestingly, *CDP3.1* co-localized with *qIR-3* for imbibition rate. The study further established the function of *CDP3.1* in regulating seed germination upon salinity stress (Xu et al., 2017). Later, iTRAQ was deployed to analyze the disparities in the proteome of salt-sensitive (IR64) and salt-tolerant (Pokkali) seedlings upon salt exposure (Lakra et al., 2019). Significantly higher levels of proteins implicated in photosynthesis (oxygen evolving enhancer proteins OEE1 and OEE3, PsbP) and stress tolerance (ascorbate peroxidase, SOD, peptidyl-prolyl cis-trans isomerases, and glyoxalase II) were found in the shoots of Pokkali. Upon salinity exposure, ribulose bisphosphate carboxylase/oxygenase activase and glutamate dehydrogenase were found to be highly induced in Pokkali (Lakra et al., 2019). Further, Li et al. (2020) performed a shotgun proteomic analysis of germinated rice under salinity conditions. Seven Thai rice cultivars (Pathumthani, Phitsanulok2, RD31- salt tolerant cultivars; RD29, RD41, Riceberry- moderately salt tolerant cultivars; and RD47- salt susceptible cultivar) were germinated under 200 mm NaCl for 96 h. Shotgun proteome analysis from all the seven cultivars identified 1339 proteins. A total of 51 proteins (involved in protein modification, signal transduction, stress response, transport, and transcription) were exclusively expressed only in salt tolerant cultivars (Li et al., 2020). Shotgun proteome analysis was also done on rice anthers from a cold-tolerant variety, Dianxi 4. Normal anthers and cold exposed anthers at the young microspore stage were compared for protein expression. A total of 3835 non-redundant proteins were detected, of which 441 proteins were expressed differentially. The study identified C2 domain proteins, and GRPs as promising signaling factors for cold tolerance response (Lee et al., 2017). A more holistic proteomic study on rice seedlings subjected to cold stress was performed using 2-DE and MALDI-TOF-MS on cold sensitive line 9311 and cold tolerant variety Fujisaka 5. In total, 59 proteins associated with cold resistance were observed in this study (Ji et al., 2017). Moreover, cold-sensitive cultivar 9311 and cold-resistant hybrid wild rice DC907 with a 9311 genetic background were utilized to perform quantitative proteomic analysis with tandem mass tags. In DC907, 366 distinct proteins were identified which were primarily implicated in ATP synthesis, photosystem, reactive oxygen species (ROS), stress response, cell growth, and integrity (Wang J. et al., 2018).

Nuclear magnetic resonance analysis was used to evaluate the metabolomic changes in watered and drought-exposed transgenic rice grains. A demarcating metabolic profile was observed under different watering conditions in transgenic and wild-type rice grains. Upon drought exposure, significantly elevated levels of GABA (244.6%), fructose (155.7%), glucose (211.0%), glycerol (57.2%), glycine (65.8%), and aminoethanol (192.4%) were found in the transgenics (Nam et al., 2016). GABA is one of the pivotal metabolites often linked to abiotic stresses in rice. It is known to induce oxidative injuries in rice arising due to various stresses such as osmotic, salinity, or senescence (Ansari et al., 2005; Sheteiwy et al., 2019). The role of GABA in stress regulation has been recently reviewed extensively (Ansari et al., 2021; Khan et al., 2021). Similar to the NMR-based approach, a GC-MS-based metabolomics approach was deployed to study the metabolite profile of rice cultivars at different developmental stages under drought and heat conditions. More than 50% of identified metabolites were different in two of the three cultivars (Anjali', Dular, and N22). The drought, heat, and combined drought and heat susceptible-Anjali; the drought, heat, and combined drought and heat tolerant- N22; the drought tolerant, heat and combined drought and heat susceptible-Dular were analyzed for drought and heat responses (Lawas et al., 2019). A GC-MS metabolomic approach along with transcriptome analysis was also used to study the key metabolic pathways associated with photosynthesis upon drought exposure. The study was designed on drought-sensitive cultivar IRAT109 and the drought-tolerant cultivar IAC1246 to determine the transcript and metabolic responses upon long-term drought exposure (Ma et al., 2016). For recent metabolic studies encircling salt stress in rice, GC-MS was utilized to profile metabolites in five rice varieties with a comparable genetic

background and varying growth performances under salt stress. The study showed enriched levels of amino acids in salt-tolerant lines (G58, G1710, and IR64) in comparison to salt sensitive lines (G45 and G52) under non-stress conditions. In all five varieties, the levels of Sorbitol, melezitose, and pipecolic acid were enhanced significantly upon salinity stress. This probably indicated that these compounds might be responsible to regulate salt stress responses in rice. Moreover, the sensitive varieties experienced more noticeable enhancement in metabolites levels during early stress treatment in comparison to the tolerant varieties (Xie et al., 2020). An analogous study by Gupta and De (2017) revealed similar results upon salt stress in rice. The study employed GC-MS for assessing the metabolic profile; and found serotonin and gentisic acid as the key metabolites (Gupta and De, 2017). In yet another study, metabolomics (GC-TOF-MS) and transcriptomics (RNA-seq) were jointly utilized to decipher pathways, metabolites, and metabolic hotspots in rice upon salinity stress (Wanichthanarak et al., 2020). A very recent report also combined metabolomic (LC-MS-MS) and transcriptomic (RNA-seq) approaches to study the rice metabolic network underlying *OsDRAP1*-mediated salt tolerance (Wang et al., 2021). Over-expressing *OsDRAP1* results in differential expression of intrinsic salt tolerance genes. Moreso, proline, valine glyceric acid, phosphoenolpyruvic acid, and ascorbic acid accumulated at higher concentrations in the over-expressing lines, depictive of their role in salinity tolerance. Much alike drought and salt stress, the implementation of metabolomics is also extended to cold stress (Zhao et al., 2013). A recent study in this context involves electrospray ionization mass spectrometry (EESI-MS) to profile the metabolic changes of Qiutianxiaoting (chilling-tolerant variety) and 93-11 (chilling-susceptible variety) under low-temperature stress (Du S. et al., 2020). The study revealed that phenylpropanoid biosynthesis, flavone, and flavonol biosynthesis pathways were activated in 93-11 upon low-temperature exposures. In Qiutianxiaoting, low-temperature exposures activated methyl jasmonate biosynthesis-associated genes, which probably mitigated the chilling damage making it the more tolerant cultivar (Du S. et al., 2020).

## Biotic Stress

Metabolomics and proteomics of rice biotic stress at their homeostasis or adverse environmental condition is used to extract system information. The underpinning mechanisms of biotic stress responses in rice are well elucidated by targeted biochemical, metabolic, and proteomic analysis of host-pathogen interactions (Ahuja et al., 2012; Liu Y. et al., 2019; Vo et al., 2021). In view of this several relevant studies have been made. Plant growth-promoting rhizobacteria (PGPR) aids plants in nutrient uptake and phytohormone synthesis. Early studies involving proteomics revealed photosynthesis and defense associated proteins accumulation by *Pseudomonas fluorescens* and *Sinorhizobium meliloti* (Kandasamy et al., 2009; Chi et al., 2010). Similarly, an early metabolomics study encircling PGPR was performed on two rice varieties infested with *Azospirillum lipoferum* 4B and *Azospirillum* sp. B510 (rice-associated *Azospirillum* species). The study found alterations in flavonoids and hydroxycinnamic derivatives which were predominantly dependent on the cultivar-PGPR strain interaction (Chamam et al., 2013). Moreover, 10 different PGPR strains inoculation of Nipponbare resulted in metabolomics signatures such as decreased alkylresorcinol [5-tridecyl resorcinol, 5-pentadecyl resorcinol, 5 (12-heptadecyl) resorcinol] quantities and the differential induction of N-p-coumaroylputrescine and N-feruloylputrescine (antimicrobial compounds) (Valette et al., 2020). Additionally, *Pseudomonas* is a known PGPR that acts as a bioagent to combat rice diseases. HPLC of rice roots infested by *Pseudomonas putida* revealed enrichment of salicylic acid (Kandaswamy et al., 2019). Likewise, *Pseudomonas aeruginosa* is linked with the synthesis of systemic acquired resistance (SAR) related compounds such as siderophores (1-hydroxy-phenazine, pyocyanin, and pyochellin) and antibacterial compounds (4-hydroxy-2-alkylquinolines and rhamnolipids) (Yasmin et al., 2017). The recent proteomics and metabolomics researches encircling rice response to disease causing pathogens have been intensively reviewed (Azizi et al., 2019; Meng et al., 2019). Metabolomics mostly highlighted the disparities of necrotrophic and biotrophic stages which included the accretion of metabolic photosynthetic compounds at biotrophic stage or phenolic compounds at necrotrophic stage (for review, see Azizi et al., 2019). A neoteric study for rice blast iTRAQ revealed that the pathogen-associated molecular pattern (PAMP)-triggered immunity might be induced at the transcriptome level but was suppressed at the protein level in susceptible rice varieties (Ma Z. et al., 2020). The study also revealed that probenazole-inducible protein 1 (PBZ1) and phenylpropanoid accumulated in both resistant and susceptible cultivars (Ma Z. et al., 2020). Intriguingly, a QTOF-UPHPLC based metabolomic study found a saponin, Bayogenin 3-O-cellobioside as a novel saponin identified in rice (Norvienyeku et al., 2021). Consequently, Bayogenin 3-O-cellobioside is well related with rice blast resistance against *Pyricularia oryzae.*

Sheath blight in rice is triggered by a necrotrophic fungus-*Rhizoctonia solani,* which is linked with cell death at the early stages of infection. Photosynthesis and sugar metabolism alters drastically upon *Rhizoctonia solani* infection (Lee et al., 2006). Further, two other metabolomic reports revealed the elevated levels of glycolysis and TCA cycle compounds (succinate, pyruvate, and aconitate), reduced levels of sugar (sucrose, glucose, fructose, glucosone, turanose, galactose, hexopyranose, maltose, and glucopyranose), accumulation of ROS, salicylic acid, jasmonic acid, aromatic aliphatic amino acids, phenylpropanoids, and suppression of myo-inositol (Suharti et al., 2016; Ghosh et al., 2017). Additionally, Karmakar et al. (2019) performed 2-DE and MALDI-TOF-MS-MS on control and *AtNPR1*-transgenics before and after *R. solani* infestation to study the proteome and metabolome profiles (Karmakar et al., 2019). Mitogen-activated protein kinase 6, probable protein phosphatase 2C1, probable trehalose-phosphate phosphatase 2, and heat shock protein were primarily recognized as the main compounds related to *R. solani* infection in rice. Moreover, the iTRAQ technique highlighted the difference in ROS modulation between the tolerant and susceptible varieties (Ma H. et al., 2020). The proteins were implicated in

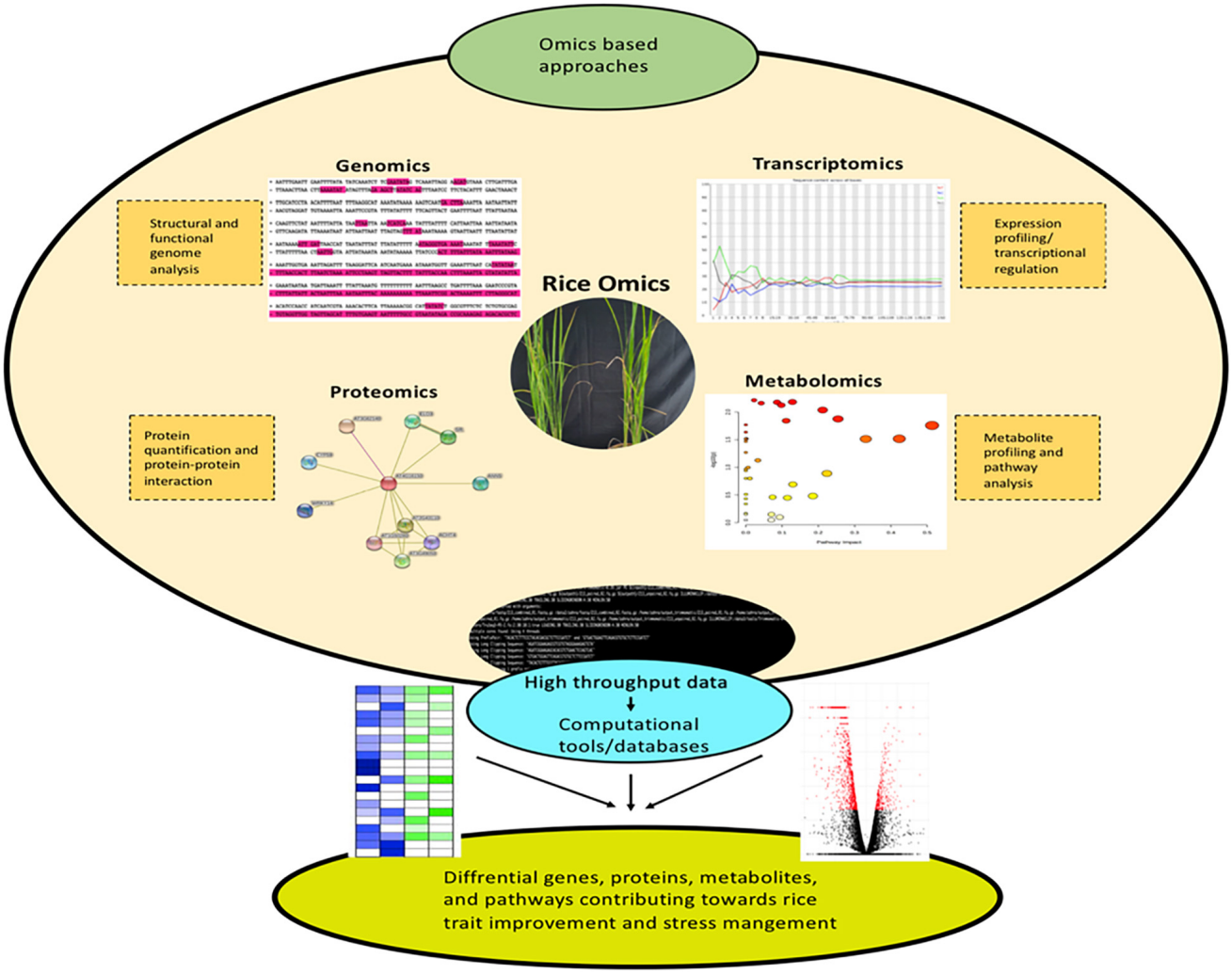


**FIGURE 2 |** Overview of omics techniques with respective databases and tools in rice trait improvement and stress management. Databases and tools used include: genomics-PlantCARE, transcriptomics-FASTQC, proteomics-STRING, metabolomics-MetaboAnalyst 5.0. For volcano plot R was used, while MAPMAN was used to generate the heatmap.

the regulation of glyoxylate and dicarboxylate metabolism, glycine, serine, and threonine metabolism, unsaturated fatty acid biosynthesis, and glycolysis/gluconeogenesis pathways. Several studies have investigated the differences in rice proteomes after challenging two major rice pathogens; *M. oryzae*, *Xanthomonas oryzae,* and/or their elicitors (Jha et al., 2007; Pandey and Sonti, 2010; Wu et al., 2016; Meng et al., 2019). For example, iTRAQ analysis was performed to study rice blast using *Piz-t* transgenic lines (*Piz-t*; rice blast *R* gene). Comparative proteome profiling on the *Piz-t* transgenic Nipponbare line (NPB-Piz-t) and wild-type Nipponbare (NPB) revealed differentially expressed proteins related to defense, stress, hormone, pathogenesis, and cytochrome P450 (Tian et al., 2018). Similarly, comparative proteomic profiling highlighted novel insights into the interaction between rice and *X. oryzae* (Zhang F. et al., 2020). The above examples constitute a few of the contemporary developments made by proteomics and metabolomics in response to abiotic/biotic stresses (**Table 3**).

## DATABASES FOR RICE OMICS RESEARCH

The omics data sources include whole-genome sequencing data, RNA-sequencing data, protein-protein interaction data, and whole metabolome analysis data. Systematic accessibility, retrieval, and storage of omics data is the fundamental prerequisite for rice research. Omics-based research generates massive volumes of data that coincides with bioinformatics for meaningful processing of biological information. Accordingly, the subject of prime importance in molecular biology is how proficiently large volumes of data can be processed to retrieve meaningful information. This underlines the extreme

need for molecular biology databases. Omics-based databases are not just the assembly of data in a system, but a platform from which information can be searched easily and quickly. Efficient molecular biology databases usually have the following functionality. First, data is linked to other meaningful information. For instance, sequence information linked to genetic resources can assist in genome-wide studies. Second, the search is intuitive and is key-word based. Third, large volumes of data can be downloaded easily without errors. Apart from the above features, open access is also an important requirement. Open access helps the users to browse and download the same data multiple times without any charges. Thus, data download, upload, and accessibility are essential for biological databases.

## Genomics and Transcriptomics Bioinformatics Tools and Databases

The availability of rice genome sequencing data from several species and cultivars has led to enormous research encircling the biological diversity of rice (Li J.Y. et al., 2014). Many tools and databases are established over the years to store, retrieve, and interpret big omics data. Extensive genome databases have been developed since the establishment of the Rice Genome Annotation Project (RGAP) (Ouyang et al., 2007) and Rice Annotation Project Database (Ohyanagi et al., 2006; Sakai et al., 2013). The functional genomics of rice is often studied with the OryGenesDB (Droc et al., 2006) and rice functional genomics express database (RiceGE). Both these databases utilize flanking sequence tag (FST) information for genome interpretation. Similarly, the RiceGE database provides relevant information on mutants. To gain access to genome data for various cultivars, the ricepan-genome browser (RPAN) (Sun et al., 2017) and Rice Information Gateway (RIGW) (Song J.M. et al., 2018) are prevalently used. Additionally, the Information Commons for Rice (IC4) database provides data regarding sequence variation and transcriptome profiles. For GWAS studies, HapRice- an SNP haplotype database (Yonemaru et al., 2014) and Ricebase-genome information platform for molecular markers such as SSRs (Edwards et al., 2016) are routinely deployed by bioinformaticians. Few other databases for SNP searches include OryzaGenome v2 (Ohyanagi et al., 2016), RiceVarMap (Zhao et al., 2015), and the SNP-Seek database (Alexandrov et al., 2015). GWAS data is often converted into a high-density rice array (HDRA) to cover 39,045 non-transposable elements in rice (McCouch et al., 2016). A Manhattan plot for the HDRA data is generated by GWAS viewer. This kind of analysis generally requires programming skills. However, graphic user interface (GUI) interface platforms such as Intelligent Prediction and Association Tool (iPat) (Chen and Zhang, 2018) and the rice imputation server (Wang D.R. et al., 2018) are also available for GWAS studies.

In the past few years, transcript-assembly algorithms have revolutionized the arena of rice transcriptomic research. Generally, the databases dedicated to transcriptome provides information regarding genome-wide expression profiles. An extremely important database in this context is OryzaExpress (Hamada et al., 2011). This database contains expression data from 1206 samples of 34 experimental series of GPL6864 (Agilent 4 × 44K microarray platform) and 2678 samples of 153 experimental series of GPL2025 (Affymetrix Rice Genome Array platform). In addition, Rice Oligonucleotide Array Database (ROAD) contains 1867 publicly available rice microarray data (Cao et al., 2012). Another database named Collections of Rice Expression Profiling database (CREP) provides access to data from 190 Affymetrix GeneChip Rice Genome Arrays from 39 tissues (Wang et al., 2010). The information regarding rice field/development, plant hormone, and cell/tissue type can be retrieved from the RiceXpro database (Sato et al., 2013a,b). Additionally, the uniformed viewer for integrated omics (UniVIO) database can be utilized to analyze 43 hormone-related compounds (Kudo et al., 2013). For biotic stress-related studies in rice, the plant expression database (PlexDB) (Dash et al., 2012) and EXPath database (Chien et al., 2015) are commonly used. Likewise, EXPath provides tissue/organ specific expression, gene ontology (GO), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for six model crops, including rice. The Rice eFP browser (Winter et al., 2007) intuitively displays expression values using color gradients. Large volumes of rice mRNA sequencing data in different conditions is also accessible via the Transcriptome Encyclopedia of Rice database (Kawahara et al., 2016). Moreso, the Rice Expression Database (RED) is a reservoir of gene expression profiles from different rice tissues under varying environmental conditions (Xia et al., 2017). Together with RED, Expression Atlas is often used to access the gene expression profiles from recent researches (Papatheodorou et al., 2018). Genevestigator is yet another database that allows curation, visualization, and analysis of microarray or RNA sequencing data (Hruz et al., 2008). Nonetheless, large volumes of transcriptome data from different tissues or under different conditions also allow co-expression analysis. PlantArrayNet (Lee et al., 2009), the plant co-expression database (Yim et al., 2013), and the CoP database (Ogata et al., 2010) are useful for rice co-expression studies. These web-based tools use a standard pipeline to offer useful knowledge about the genes co-expressed with a gene of interest. Apart from these tools, the ATTED-II database provides co-regulated gene relationships to deduce gene functions (Obayashi et al., 2018). Additionally, NetMiner is a standalone tool for exploratory analysis and visualization of network data (Yu et al., 2018). The transcriptome analysis is frequently coupled with promoter analysis for identifying the *cis*-regulatory elements. The representative databases to study the motif organization include plant *cis*-acting regulatory DNA elements (PLACE) (Higo et al., 1999), plant *cis*-acting regulatory elements (PlantCARE) (Lescot et al., 2002), plant promoter database (PPDB) (Yamamoto and Obokata, 2007), and plant promoter analysis navigator (PlantPAN) (Chow et al., 2016). A rice-specific promoter analysis database is the Osiris database (Morris et al., 2008). It is a repository for promoter sequences and probable TF binding sites for 24,209 rice genes. Although it has now become obsolete. However, the MEME suite (Bailey et al., 2009) is now generally the choice of researchers for performing web-based motif identification. Further, several databases have been developed lately to dissect

the gene expression patterns regulated by non-coding RNA (ncRNA). The pyrosequencing generated small-RNA sequences for rice and maize are routinely accessed via the Cereal Small RNA Database (Johnson et al., 2007). For miRNA-based studies, plant non-coding RNA database (PNRD) (Yi et al., 2015) and miRBase (Kozomara et al., 2019) are dedicated data resources. PNRD stores data from 166 plant species to generate valuable information regarding miRNAs, intronic long ncRNAs (lncRNA), and unknown ncRNAs. Likewise, the miRbase contains miRNAs information from 271 organisms. The rice miRNA information can be accessed on miRbase with a file named osa.gff3. On similar lines, annotations of 287 eukaryotic lncRNAs are provided by the Long Non-coding RNA database (lncrnadb) (Quek et al., 2015). The data for multiple miRNA variants from eight species, including rice is provided by IsomiR bank database (Zhang et al., 2016). Moreover, the plant ceRNA database (PceRBase) (Yuan et al., 2017) and plant circular RNA database (PlantcircBase) (Chu et al., 2017) cover information regarding competing endogenous RNA (ceRNA) and circular RNA (circRNA) respectively.

## Proteomics and Metabolomics Bioinformatics Tools and Databases

In context to systems biology, protein-protein interactions are pivotal to large complex networks (Rao et al., 2014). Several interactome datasets have been hosted to study the protein-protein interactions in rice. Such resources are distinct in relation to number of interactions, source of the embedded interactome, and accessible organisms. One of the methods for envisaging protein-protein interactions is the interolog approach. According to interolog approach, the function of a protein is conserved and passed through its orthologs in evolutionary-related species. Thus, the orthologs of interacting proteins in one organism conserve their interactions in a different organism. Based on the interolog approach, 37112 interactions amongst 4567 proteins are summarized approach by the Rice Interactions Viewer (RIV) database. Amongst these interactions, 1671 are self-interactions while 35441 are hetero-interactions (Ho et al., 2012). The predicted rice interactome network (PRIN) is yet another rice database that uses interolog approach (Gu et al., 2011). It annotates 76585 non-redundant rice protein interaction pairs amongst 5049 rice proteins. Meaningful interactions are validated by PRIN upon fetching the gene expression data, sub-cellular localization information, and GO annotation. Additionally, the database of interacting proteins in *Oryza sativa* (DIPOS) uses the interolog approach and domain-based predictions to depict the protein-protein interactions. This database hosts 14614067 pairwise interactions amongst 27746 proteins (Sapkota et al., 2011). Further to outspread the interactome, several approaches namely text-mining, neighborhood analysis, co-expression analysis, fusion analysis, and co-occurrence analysis are prevalently deployed (Szklarczyk et al., 2016). For extensive interactome coverage, the STRING database utilizes a broad range of sources available, from text-mining to computational predictions (Szklarczyk et al., 2016). STRING database provides both predicted and indirect interactions networks, where the nodes represent the proteins while the edges are the predicted functional association. The information for 2031 organisms is present on the STRING database. One of the latest versions of STRING (v10.5) supports network connections for 26428 *japonica* proteins and 18789 *indica* proteins. Interactions are based on combined scores which are calculated by combining the probabilities from different evidence channels. Moreso, protein-protein interactions are also deduced by the RiceNet database (Lee et al., 2015). This database offers gene prioritization based either on network direct neighborhood or context-associated hubs.

Apart from protein-protein interaction databases, the resources that host annotated proteomes are also crucial for proteome-wide studies. The protein sequences and their corresponding annotations are frequently updated by the UniProt database. For rice, annotations of 48916 *japonica* proteins are hosted on UniProt (Bateman et al., 2015; Consortium, 2019). Additionally, OryzaPG-DB based on the short-gun proteogenomics concept is a proteogenomics database for the annotation of rice proteome. It provides peptide-based expression profiles with corresponding genomic origin along with the annotation of novelty for each peptide (Helmy et al., 2012). Manually Curated Database of Rice Proteins (MCDRP) digitizes protein-related experiments. The process of digitization has overcome the limitations associated with text-based curation. MCDRP is periodically updated and currently contains data for approximately 1800 rice proteins (Gour et al., 2014). To study protein functions based on their structures, the plant protein annotation suit database (Plant-PrAS) is used. Various physio-chemical parameters, structural properties, novel functional regions, transmembrane helices, and signal peptides from the genomes of six model plants (including rice) are provided by Plant-PrAS (Kurotani et al., 2015).

Orthologous proteins provide valuable information about unannotated proteins. GreenPhyl DB v5 is a web-based tool for functional and comparative genomics for 27 reference genomes (including rice). It facilitates comparative analysis of species and protein domains. Metabolic pathway-related information can also be accessed via GreenPhyl DB. 44786 out of 60647 rice sequences present on GreenPhyl DB have an InterPro domain (Rouard et al., 2011). Another database that enables cross-species proteomic comparative analysis is the Putative Orthologous Groups 2 Database. This database supports three other species (*Arabidopsis thaliana, Zea mays*, and *Populus trichocarpa*) along with rice to integrate the data from predicted proteomes into putative orthologous groups. Interpro domain keyword or ID, gene model or transcript accessions, known or predicted intracellular location can be used to query the database. It provides information on probable protein localization, gene descriptions, and domain organizations (Tomcal et al., 2013). Similarly, the InParanoid database assesses the orthologs based on the InParanoid algorithm. For a specific protein, the orthologs can be searched by gene identifier, protein identifier, or by a blast search against InParanoid protein dataset (Sonnhammer and Östlund, 2015). Likewise, orthologous matrix (OMA) is a database to infer orthologs among complete genomes (Altenhoff et al., 2018). The PANTHER (Protein Analysis Through

**TABLE 4 |** Omics tools and databases.

| Database name | Omics | Description | URL |
|---|---|---|---|
| RiceGE | Genomics | It is a rice functional genomics express database | http://signal.salk.edu/cgi-bin/RiceGE |
| Oryza sativa genome Database | Genomics | It provides a sequence-centered genome view for *Oryza sativa (ssp. japonica)*, | http://www.plantgdb.org/OsGDB/ |
| MSU-RGAP | Genomics | It provides sequence and annotation data for the rice genome (Nipponbare subspecies) | http://rice.uga.edu/ |
| RAP-DB | Genomics | It facilitates the analysis of genome structure and function of rice on the basis of the annotation. | https://rapdb.dna.affrc.go.jp/ |
| Gramene | Comparative Genomics | It is an open-source data resource for comparative functional genomics in crops and model plants | http://www.gramene.org/ |
| Oryzabase | Genomics | It is a comprehensive rice database providing information of classical rice genetics to recent genomics | https://shigen.nig.ac.jp/rice/oryzabase/ |
| OryGenesDB | Genomics | It displays sequence information such as the T-DNA and Ds flanking sequence tags (FSTs) | https://orygenesdb.cirad.fr/index.html |
| TIGR rice genome annotation DB | Genomics | It is a group of databases for searching with the BLAST programs blastn, blastx, tblastn, or tblastx. | http://blast.jcvi.org/euk-blast/index.cgi?project=osa1 |
| MOsDB | Genomics | It is a resource for publicly available sequences of the rice | https://pgsb.helmholtz-muenchen.de/plant/rice/index.jsp |
| Rice mutant DB | Genomics | It contains the information of approximate 129,000 rice T-DNA insertion (enhancer trap) lines generated by an enhancer trap system. | http://rmd.ncpgr.cn/ |
| Information Commons for Rice (IC4R) | Genomics | It is a database contributing to rice genome sequences, updating rice gene annotations and integrating multiple omics data | http://www.ic4r.org/ |
| RiceVarMap | Genomics | It is a database for rice genomic variation and its functional annotation. | http://ricevarmap.ncpgr.cn/ |
| RiceFrend | Genomics | It is a gene coexpression database in rice | https://ricefrend.dna.affrc.go.jp/ |
| Rice Transcription Factor Phylogenomics DB | Genomics | It combines various data types present in public databases encircling the structural features, orthologous relationships, availability of mutants and gene expression patterns for TF families | http://ricephylogenomics-khu.org/tf/home.php |
| Rice transporter database | Genomics | It contains information for all putative rice transporters | https://ricephylogenomics.ucdavis.edu/transporter/genInfo.shtml |
| The Rice Information GateWay (RIGW) | Genomics | It provides the sequenced genomes and related information in systematic and graphical ways | https://rice.hzau.edu.cn/rice_rs3/ |
| Rice Pan-genome Browser | Genomics | It provides sequences and gene annotations for the rice pan-genome and Gene presence-absence variations (PAVs) of rice accessions | https://cgm.sjtu.edu.cn/3kricedb/ |
| SNP-Seek | Genomics | Provides information related to rice SNPs | https://snp-seek.irri.org/ |
| Ricebase | Genomics | It is a rice breeding and genetics platform integrating molecular markers, pedigrees, and whole-genome data | https://www.ricebase.org/ |
| Rice imputation server | Genomics | It utilizes genetically and geographically diverse accessions to impute rice datasets out to 5.2M SNPs using the IMPUTE2 software. | http://rice-impute.biotech.cornell.edu/ |
| Ensemble_rice | Comparative Genomics | It is used for genome assembly, comparative genomics, and gene annotation | http://plants.ensembl.org/Oryza_sativa/Info/Index |
| Rice Diversity: GWAS Viewer | Genomics | Comprehensively view GWAS data | http://rs-bt-mccouch4.biotech.cornell.edu/GWAS_Viewer/plot/ |
| PLAZA | Comparative Genomics | It is an access point for plant comparative genomics centralizing genomic data produced by different genome sequencing programs. | https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_monocots/ |
| PlantGDB | Comparative Genomics | It is used for sequence assemblies and annotation | http://www.plantgdb.org/ |
| Phytozome | Comparative Genomics | It is used for accessing, visualizing and analyzing sequenced plant genomes, as well as selected genomes and datasets | https://phytozome.jgi.doe.gov/pz/portal.html |
| ROAD | Genomics | It is a rice oligonucleotide array database | http://ricephylogenomics-khu.org/road/home.php |
| FIT-DB | Transcriptomics | It provides result of statistical modeling of transcriptomic dynamics in the field condition | https://fitdb.dna.affrc.go.jp/ |
| EXPath 2.0 | Transcriptomics | It is a database that collects and uses expression profiles derived from microarray under various conditions to infer metabolic pathways | http://expath.itps.ncku.edu.tw/ |
| Rice eFP | Transcriptomics | One of the major functions of Rice eFP is gene expression analysis | http://bar.utoronto.ca/efprice/cgi-bin/efpWeb.cgi |
| RiceXPro | Transcriptomics | It is a repository of gene expression profiles derived from microarray analysis of tissues/organs | https://ricexpro.dna.affrc.go.jp/ |

*(Continued)*

**TABLE 4 |** (Continued)

| Database name | Omics | Description | URL |
|---|---|---|---|
| RED | Transcriptomics | It is a repository of gene expression profiles derived from RNA-Seq data on rice tissues | http://expression.ic4r.org/ |
| TENOR | Transcriptomics | It is the transcriptome encyclopedia of rice | https://tenor.dna.affrc.go.jp/ |
| OryzaExpress | Transcriptomics | It is a sub-platform of PlantExpress for a single-species GEN analysis in rice. | http://plantomics.mind.meiji.ac.jp/OryzaExpress/ |
| UniVIO | Transcriptomics | It provides a data set of hormonome and transcriptome analyses in 14 organs of rice at the reproductive stage and in gibberellin-related mutants. | http://univio.psc.riken.jp/ |
| Genevestigator | Transcriptomics | It helps in analysis of deeply curated bulk tissue and single-cell transcriptomic data from public repositories with visualization tools | https://genevestigator.com/ |
| Expression Atlas | Transcriptomics | It helps to deduce gene expression across species and biological conditions | https://www.ebi.ac.uk/gxa/experiments?organism=Oryza+sativa+Japonica+Group |
| PLACE | Transcriptomics | It is a database of motifs found in plant cis-acting regulatory DNA elements | https://www.dna.affrc.go.jp/PLACE/?action=newplace |
| PlantCARE | Transcriptomics | It is a database for promoter analysis | http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ |
| PPDB | Transcriptomics | It is a database for promoter analysis | http://ppdb.agr.gifu-u.ac.jp/ppdb/cgi-bin/index.cgi |
| PlantPAN_V2 | Transcriptomics | It is an informative resource for detecting transcription factor binding sites | http://plantpan2.itps.ncku.edu.tw/index.html |
| NetMiner | Transcriptomics | An ensemble pipeline for high-quality RNA-seq-based gene co-expression network inference | https://github.com/czllab/NetMiner |
| PlantArrayNet | Transcriptomics | It generates co-expression information between genes based on correlation coefficients from accumulated microarray data of rice | http://bioinfo.mju.ac.kr/arraynet/ |
| COP DB | Transcriptomics | It is a database management system for understanding plant gene function by associating between co-expressed genes and biological processes | http://webs2.kazusa.or.jp/kagiana/cop0911/ |
| PODC | Transcriptomics | It is a database providing the information of gene networks (GENs) and knowledge-based functional annotations | http://plantomics.mind.meiji.ac.jp/podc/ |
| Plant rDNA database | Transcriptomics | It is an online resource providing information on numbers and positions of ribosomal DNA signals and their structures | https://www.plantrdnadatabase.com/ |
| PlantcircBase | Transcriptomics | It is a repository of publicly available back-splice junction sequences and their full-length sequences of circRNAs | http://ibi.zju.edu.cn/plantcircbase/ |
| PceRBase | Transcriptomics | It is a plant competing endogenous database | http://bis.zju.edu.cn/pcernadb/index.jsp |
| miRBase | Transcriptomics | the microRNA database | http://www.mirbase.org/ |
| IsomiR Bank | Transcriptomics | Database for microRNA isoforms. It is an integrative resource that contains the sequence and expression of isomers | https://mcg.ustc.edu.cn/bsc/isomir/ |
| PNRD | Transcriptomics | It is a plant non-coding RNA database | http://structuralbiology.cau.edu.cn/PNRD/index.php |
| CSRDB | Transcriptomics | It is a cereal small RNAs database | http://sundarlab.ucdavis.edu/smrnas/ |
| PlantRNA | Transcriptomics | The PlantRNA database contains tRNA gene sequences retrieved from fully annotated plant nuclear, plastidial and mitochondrial genomes. | http://plantrna.ibmp.cnrs.fr/plantrna/ |
| Rice Interactions Viewer | Proteomics | It is a database for protein-protein rice interaction | http://bar.utoronto.ca/interactions/cgi-bin/rice_interactions_viewer.cgi |
| STRING | Proteomics | It is used to study protein-protein interaction and functional enrichment analysis | https://string-db.org/ |
| PRIN | Proteomics | It is used to predict rice interactome network | http://bis.zju.edu.cn/prin/ |
| RiceNet | Proteomics | It is an improved network prioritization web server for rice | https://www.inetbio.org/ricenet/ |
| MCDRP | Proteomics | It is a manually curated database for rice proteins | http://www.genomeindia.org/biocuration/whatsnewver6.php |
| OryzaPG-DB | Proteomics | It is a rice proteogenomics database | https://github.com/MoHelmy/oryza-PG/ |
| Plant-PrAS | Proteomics | Database for physicochemical and structural properties | http://plant-pras.riken.jp/ |
| OMA browser | Proteomics | It is used for ortholog analysis | https://omabrowser.org/oma/home/ |
| POGsDB | Proteomics | It facilitates cross-species inferences about gene functions | http://pogs.uoregon.edu/#/ |
| InParanoid8 | Proteomics | To study ortholog groups with inparalogs | https://inparanoid.sbc.su.se/cgi-bin/index.cgi |

*(Continued)*

**TABLE 4 |** (Continued)

| Database name | Omics | Description | URL |
|---|---|---|---|
| GreenPhyl v4 | Proteomics | It is a database for species, genomes and pan-genomes | https://www.greenphyl.org/cgi-bin/index.cgi |
| Panther | Proteomics | It is used to perform Protein ANalysis THrough Evolutionary Relationships | http://www.pantherdb.org/ |
| PhosphoRice | Proteomics | It is a meta-predictor of rice-specific phosphorylation site | https://github.com/PEHGP/PhosphoRice |
| MassBank | Metabolomics | Metabolite annotation and MS-MS database | https://massbank.eu/MassBank/ |
| METLIN | Metabolomics | It is a collection of MS/MS data and incorporates the NIST MS/MS library and search software | https://www.sisweb.com/software/ms/wiley-metlin.htm |
| KNApSAcK | Metabolomics | Comprehensive Species Metabolite Relationship Database | http://www.knapsackfamily.com/KNApSAcK/ |
| MetaboLights | Metabolomics | It is a database for metabolomics experiments and derived information | https://www.ebi.ac.uk/metabolights/ |
| Plant Reactome | Metabolomics | Used to find reactions and pathways | https://plantreactome.gramene.org/index.php?lang=en |
| OryzaCYC | Metabolomics | To study plant metabolic network | https://plantcyc.org/databases/oryzacyc/6.0 |
| KEGG_rice | Metabolomics | To decipher pathway maps | https://www.genome.jp/kegg-bin/show_organism?menu_type=pathway_maps&org=dosa |
| RICECYC | Metabolomics | It is a catalog of known and/or predicted biochemical pathways from rice | http://pathway.gramene.org/gramene/ricecyc.shtml |
| MapMan | Metabolomics | It is used in pathway analysis | https://mapman.gabipd.org/home |
| Plant GSEA | Metabolomics | It is used for gene set enrichment analysis | http://structuralbiology.cau.edu.cn/PlantGSEA/index.php |
| AgriGo | Metabolomics | It is an ontology analysis tool | http://bioinfo.cau.edu.cn/agriGO/ |
| KEGG Mapper | Metabolomics | It is a tool for KEGG pathway mapping | https://www.genome.jp/kegg/mapper.html |
| MetaboAnalyst 5.0 | Metabolomics | It is a comprehensive platform dedicated for metabolomics data analysis | https://www.metaboanalyst.ca/ |

Evolutionary Relationships) tool allows the classification of proteins (and their corresponding genes) to facilitate high-throughput analysis. This tool classifies proteins according to family/sub-family, molecular function, biological process, or pathway (Thomas et al., 2003; Mi et al., 2012). Finally, PANTHER uses the library of trees to predict the orthologs. Moreover, an online orthology analysis and annotation visualization tool- plant orthology browser (POB) allows interactive pairwise comparison and visualization of genomic traits via gene orthology. It currently hosts 20 genomes, and syntenic blocks are recognized for a pair of genomes using strand orientation and physical mapping (Tulpan and Leger, 2017).

Plants produce numerous metabolic compounds to sustain growth under normal or adverse conditions. In this direction, databases that support rice metabolome studies accelerate functional genomics research. The online platform MetaboLights hosts curated metabolite information. It offers a single access point for a number of metabolomic studies. This is a cross-species, cross-technique analysis which covers metabolite structures and their reference spectra (Haug et al., 2013). For cross-species comparative analysis, the plant metabolic network (PMN) database contains data from 22 species. It contains information related to genes, enzymes, compounds, reactions, and pathways involved in primary and secondary metabolism in plants (Schläpfer et al., 2017). The PMN hosts one multi-species reference database-PlantCyc and 126 species/taxon-specific databases. The rice metabolic database of PMN is called OryzaCyc (V 6.0). The OryzaCyc (V 6.0) houses 569 pathways consisting of 3345 reactions and 2614 compounds for 6325 enzymes. In a similar vein, RiceCyc is a catalog of known and/or predicted biochemical pathways from rice. It is developed, maintained, and curated by the Gramene database (Jaiswal et al., 2006). Gramene is an integrated data resource for comparative functional genomics which hosts 93 reference genomes, including rice (Naithani et al., 2016; Tello-Ruiz et al., 2021). Gramene provides information on metabolic networks, transport, genetic, signaling, and developmental pathways. Recently, Plant Reactome which is a comparative plant pathway knowledgebase of the Gramene project has been updated (Naithani et al., 2020; Tello-Ruiz et al., 2021). It utilizes rice as a reference plant for manual curation of pathways and currently hosts 298 reference pathways, including metabolic, transcriptional, transports, hormone, and plant developmental pathways (Naithani et al., 2020). Kyoto Encyclopedia of Genes and Genomes (KEGG) is a platform for analyzing a broad range of high-throughput datasets, including metabolome data. It helps in deciphering high-level functions and utilities of the biological system. KEGG is frequently updated (last updated 2021) and four databases (pathways, genes, compounds, and enzymes) perform the major functionalities. Small molecules and metabolite-related information can be assessed from the KEGG compounds database (Kanehisa et al., 2017). Also, the KEGG mapper is used to map a set of genes, proteins, or small molecules on network databases *viz.,* KEGG pathways and KEGG modules. The four KEGG mapping tools include reconstruct, search, color, and join. Further, the MAPMAN tool is generally used for enrichment analysis or pathway mapping in rice. The tool consists of a scavenger module, the

ImageAnnotator module, and the PageMan module. Processed high-throughput datasets are fetched into the MAPMAN tool for visualizing the data (in the form of a heat map) in the context of metabolic pathways. Multiple testing correction using either benjamini hochberg, benjamini yekutieli or bonferroni is performed as a part of statistical analysis by MAPMAN (Usadel et al., 2009a,b). For GO analysis, agriGO is a popular web-based platform (Tian et al., 2017). It focuses on agricultural species and currently supports 394 species and 865 datatypes. It uses analysis tools namely Singular Enrichment Analysis (SEA), Parametric Analysis of Gene set Enrichment (PAGE), BLAST4ID (Transfer IDs by BLAST), and SEACOMPARE (Cross comparison of SEA). Custom analysis tools on agriGO include custom direct acyclic graph (DAG) tree and Scatter Plot (Tian et al., 2017). Nonetheless, it is challenging to increase the GO annotations and corresponding terms in constantly accruing datasets. Thus, the gene set enrichment analysis (GSEA) method was devised to overcome the issue of low coverage of GO-annotated genes. GSEA is a computational method that establishes the biological meaning of input genes. This is performed by measuring the overlap between an input gene list and a backend gene set. A GSEA server-PlantGSEA utilizes 20290 defined gene sets from varied resources. PlantGSEA enables the GSEA for rice and three other model plants using a unique ID (usually Affymetrix probe ID or gene locus ID) as input. The output provides enrichment analysis with statistical significance and better visualization (Yi et al., 2013). The previously discussed PANTHER tool also extends its functionality for GO analysis (Mi et al., 2017). Thus, omics tools and databases supplement in-depth rice research for a better understanding of underpinning molecular mechanisms (**Figure 2**). A summary of relevant omics tools and databases is provided in **Table 4**.

## CONCLUSION AND FUTURE PROSPECTS

Improving rice productivity mainly depends upon functional characterization and analyses of genes that are vital to agronomic traits. In rice research, high-throughput technologies had been employed for several years to gain insights into the mechanistic details of molecular pathways. Genomics provides information regarding the most dominant or recessive genes in rice varieties, while transcriptomics aids in elucidating complex expression networks of RNA in rice that can be imperative to yield or stress responses. Similarly, proteomics leads to ascertaining major proteins contributing to rice improvement, while metabolomics provides crucial signatures of metabolites related to rice quality and yield enrichment. Bioinformatics databases assimilate the data from omics sciences to generate the complete set of information about the factors contributing to the enhancement of quality, quantity, or stress responses in rice. Thus, the omics generated datasets can expedite gene discoveries and functional characterizations in rice for crop improvement. Also, plant system biology has deepened the understanding of metabolism, stress responses, and integrative omics research. Moreover, the advent of CRISPR/Cas9 genome editing technology and its combination with omics studies has widened the horizons of rice research. Rice omics research is the new avenue that offers great potential. An integrative omics platform offering access to complete bioinformatics data will help researchers to implement new techniques in forward/reverse genetics and breeding programs. Taken together, omics-based rice research along with the cutting-edge technologies holds great potential for rice yield enhancement and stress management.

## AUTHOR CONTRIBUTIONS

MIA conceptualized and designed the study. ZI, MSI, and MIRK compiled the data and wrote the manuscript. All authors have read the manuscript and agreed for publication.

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# Transcriptional Changes in the Developing Rice Seeds Under Salt Stress Suggest Targets for Manipulating Seed Quality

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Global sea-level rise, the effect of climate change, poses a serious threat to rice production owing to saltwater intrusion and the accompanying increase in salt concentration. The reclaimed lands, comprising 22.1% of rice production in Korea, now face the crisis of global sea-level rise and a continuous increase in salt concentration. Here, we investigated the relationship between the decrease in seed quality and the transcriptional changes that occur in the developing rice seeds under salt stress. Compared to cultivation on normal land, the japonica rice cultivar, Samgwang, grown on reclaimed land showed a greatly increased accumulation of minerals, including sodium, magnesium, potassium, and sulfur, in seeds and a reduced yield, delayed heading, decreased thousand grain weight, and decreased palatability and amylose content. Samgwang showed phenotypical sensitivity to salt stress in the developing seeds. Using RNA-seq technology, we therefore carried out a comparative transcriptome analysis of the developing seeds grown on reclaimed and normal lands. In the biological process category, gene ontology enrichment analysis revealed that the upregulated genes were closely associated with the metabolism of biomolecules, including amino acids, carboxylic acid, lignin, trehalose, polysaccharide, and chitin, and to stress responses. MapMan analysis revealed the involvement of upregulated genes in the biosynthetic pathways of abscisic acid and melatonin and the relationship of trehalose, raffinose, and maltose with osmotic stress. Interestingly, many seed storage protein genes encoding glutelins and prolamins were upregulated in the developing seeds under salt stress, indicating the negative effect of the increase of storage proteins on palatability. Transcription factors upregulated in the developing seeds under salt stress included, in particular, bHLH, MYB, zinc finger, and heat shock factor, which could act as potential targets for the manipulation of seed quality under salt stress. Our study aims to develop a useful reference for elucidating the relationship between seed response mechanisms and decreased seed quality under salt stress, providing potential strategies for the improvement of seed quality under salt stress.

**Keywords: climate change, *Oryza sativa*, reclaimed land, salt stress, seed quality, sea-level rise, transcriptional changes in developing seeds**

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## INTRODUCTION

During the 20th century, global sea-levels rose by approximately 12–22 cm. Satellite images reveal that the sea-levels are currently rising by approximately 3.4 mm per year (Walthall et al., 2012). Sea-level rise is affected mainly by climate change (Mimura, 2013), and the global average surface temperature has increased by approximately 0.74°C (0.56°C–0.92°C; Walthall et al., 2012). Between 1955 and 2010, the world ocean heat content also continuously increased (Mimura, 2013), and ocean temperatures as well as sea-levels are predicted to continue to rise indefinitely (Walthall et al., 2012). Sea-level rise directly affects the quality of water available on lands and in rivers near the coast, and the World Bank has predicted a reduction in rice production in Thailand and Vietnam, both rice exporters, due to an increase in the salinification of key river deltas caused by sea-level rise (Walthall et al., 2012). Around 80,000 hectares on the Gulf Coast in the United States are utilized for rice production; however, because of long-term subsidence, saltwater has intruded into rice production areas (Walthall et al., 2012). In the Republic of Korea (Korea), 81 reclaimed lands (RLs) were prepared mostly in southern and western coastal areas, comprising a total area of 186,639 hectares, corresponding to 11.7% of the total arable land and 22.1% of the land used for rice production (Lim et al., 2020). RLs in Korea, although critically important for rice production, are extremely vulnerable to sea-level rise and its accompanying increase in salt concentration because compared to normal land (NL), they already contain a significantly higher salt concentration and are located near the sea (Walthall et al., 2012; Lim et al., 2020). Accordingly, RLs are highly effective test sites for determining the effect of sea-level rise on rice cultivation and seed harvest.

Several studies of rice cultivation and production have reported that salt stress causes abnormal agronomic traits, resulting in a decrease in yield (Cheong et al., 1995; Choi et al., 2003; Baxter et al., 2011; Thitisaksakul et al., 2015), thousand grain weight (Peiris et al., 1988; Choi et al., 2003; Thitisaksakul et al., 2015), and a delay of heading (Cheong et al., 1995; Choi et al., 2003). Under salt stress, particularly, sodium (Na; Peiris et al., 1988; Cheong et al., 1995) and protein (Peiris et al., 1988; Choi et al., 2003; Thitisaksakul et al., 2015) contents were increased compared with those under normal conditions without salt stress. Moreover, Choi et al. (2003) reported that salt stress decreased the palatability of rice seeds. In rice seeds, the increase in protein content resulted in a decrease in eating quality (Juliano et al., 1965). Furthermore, the seed quality of rice is among the most important agronomic traits in rice breeding programs (Lau et al., 2015; Kobayashi et al., 2018).

**Abbreviations:** ABA, abscisic acid; ABA2, ABA deficient2, short-chain alcohol dehydrogenase; ASMT, N-acetylserotonin O-methyltransferase; GolS, galactinol synthase; NCED, 9-cis-Epoxycarotenoid dioxygenase; TDC, tryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; T6PP, trehalose-6-phosphate phosphatase; TRE, trehalase; ZEP, zeaxanthin epoxidase; AP2/ERF, APETALA2/Ethylene Responsive Factor; bHLH, basic helix–loop–helix; GRAS, GAI (Gibberellin-Acid-Insensitive), RGA (Repressor of GA1), SCL (SCARECROW); HSF, heat shock factor; NAC, NAM, ATAF, and CUC; PLATZ, plant AT-rich sequence and zinc binding; SOS, salt overly sensitive.

Therefore, we focused on the abnormalities observed in the agronomic trait of seed quality under salt stress. To understand the decrease in seed quality of rice under salt stress during its entire life cycle, we investigated how transcriptional changes occur in the developing rice seeds under salt stress through RNA-sequencing. To date, investigations on the global transcriptome changes occurring in the developing rice seeds under salt stress are limited. Therefore, we aimed to provide a useful reference for exploring molecular mechanisms to improve or maintain the quality of rice seeds under salt stress.

## MATERIALS AND METHODS

### Growth of Rice Plants and Investigation of Agronomic Traits

In 2018, we transplanted Samgwang seedlings (*Oryza sativa* subsp. *japonica*) and cultivated them on NL, the experimental paddy field of the Chungcheongnam-do Agricultural Research and Extension Service (CARES, latitude: 36.74°N, longitude: 126.82°E), and RL, the Seosan reclaimed land Section B (latitude: 36.66°N, longitude: 126.34°E). We cultivated three replicates by a completely randomized design using the standard rice cultivation method of CARES (**Supplementary Figure 1**). We further investigated agronomic traits, including days until heading, culm length, panicle length, spikelet numbers per panicle, thousand grain weight, and yield. Mature seeds were harvested at 146 days after transplanting from 100 plants per replicate.

### Analysis of the Chemical Properties of Topsoil Samples Collected From NL and RL

We collected topsoil samples from NL and RL in three replicates and sent them to the National Instrumentation Center for Environmental Management (NICEM), Seoul, Republic of Korea, for chemical analysis. The NICEM, using its in-house methods, performed the following chemical analyses: pH; electrical conductivity (EC); and total nitrogen (N), phosphorus (P), sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), and sulfur (S) contents. Based on the summary of the analysis methods performed in the NICEM, we analyzed pH and EC using a pH meter (HM-30R, DKK-TOA, Japan) and an EC meter (CM-25R, DKK-TOA, Japan), respectively. In addition, we analyzed the total nitrogen content using a Kjeltec 2400/8400 Kjeldahl Analyzer (FOSS Tecator AB, Sweden) and quantified P, Na, K, Mg, Ca, and S using inductively coupled plasma atomic emission spectroscopy (ICP-1000IV, Shimadzu, Japan; An et al., 2018; Lim and Yi, 2019).

### Investigation of Palatability and Amylose, Total Nitrogen, P, Na, K, Mg, Ca, S, and Trehalose Contents in the Rice Seeds of Samgwang Grown on NL and RL

We analyzed the palatability and amylose, N, P, Mg, K, Ca, and S contents of the brown or milled rice of Samgwang, a

japonica rice cultivar, at the Department of Southern Area Crop Science, National Institute of Crop Science. We examined the palatability of the Samgwang seeds grown and harvested on NL and RL as per the method described by Lee et al. (2020a) using a TOYO mido meter (MA-90B, TOYO, Japan). All analyses were performed for two of the three replicates owing to limited research funds. We measured amylose content in milled rice using Juliano's method (Juliano, 1971) and quantified total nitrogen (N) in brown rice using the Macro Determinator (CN928, LECO Corp., U.S.A.) using Lee's methods (Lee et al., 2020a). We analyzed the P, K, Mg, Ca, and S contents of brown rice through an energy dispersive X-ray fluorescence spectrometer (NEX CG, Applied Rigaku Technologies, Inc., U.S.A.) using the methods published in the journal, Plant Breeding and Biotechnology (Lee et al., 2020b).

We quantified sodium (Na) content in brown rice using inductively coupled plasma atomic emission spectroscopy (ICP-1000IV, Shimadzu, Japan) according to the methods presented by Lim and Yi (2019).

We quantified trehalose content in brown rice using the methods developed in the Center for University-Wide Research Facilities at the Jeonbuk National University, which employs liquid chromatography/mass spectrometry–mass spectrometry (LC/MS-MS; Kim et al., 2021). We purchased standard trehalose from Sigma-Aldrich (Saint Louis, MO, United States).

## RNA-Sequencing of the Developing Seeds of Samgwang Grown on NL and RL Through Illumina Sequencing and Further Analyses of RNA-Sequencing Data

We collected the developing seeds of Samgwang grown on NL and RL in two replicates. The developing seeds were sampled from one panicle per plant and six panicles per replicate at 8 and 15 days after heading (DAH), respectively. Next, we extracted total RNA from frozen and ground samples of the developing seeds using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. We sent the total RNA samples to Macrogen, Inc (Seoul, Republic of Korea) for RNA-seq using the Illumina technology (the RNA-seq raw data obtained by paired-end type sequencing with 101-bp read length are available at: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-10774).

We carried out the cleaning procedure for raw sequence reads by removing low-quality nuculeotides (Phred score < 20) and short sequence reads (read length < 20 nt). Next, we mapped the processed reads on the Nipponbare reference genome sequence obtained from the Rice Genome Annotation Project Database[1] (version 7.0) using the HISAT2 software with default parameters (Kim et al., 2019a). Furthermore, we quantified the raw read counts in the BAM file using featureCounts software (Liao et al., 2013). We also conducted an analysis of differentially expressed genes (DEGs) using the DESeq2 package (Love et al., 2014) and selected genes with $p < 0.05$ and $|\mathrm{Log}_2(\text{fold change})|$ of >1 (i.e., |fold change| > 2).

[1]http://rice.plantbiology.msu.edu

Gene ontology (GO) enrichment was performed according to the method described by Hong et al. (2021). We retrieved GO terms for the biological function of DEGs from the Rice Oligo Array Database.[2] The enrichment value was calculated by dividing the query total with the query expected value, and we chose GO terms having a Hyper value of $p < 0.05$ and an enrichment value of >2 (Hong et al., 2021). For the functional classification of DEGs, we used MapMan software to group genes into distinct functional categories and visualize the data through various overview diagrams. Finally, we uploaded the RGAP Locus IDs to MapMan to obtain their functional classifications (Usadel et al., 2009).

## Gene Expression Analysis by Quantitative Real Time-Polymerase Chain Reaction

We synthesized cDNA from 1 μg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, United States). The primer sets used for qRT-PCR are presented in **Supplementary Table 1**. We used the rice actin gene (*OsACT*; *LOC_Os11g06390*) as a reference gene (**Supplementary Table 1**). We performed qRT-PCR in the Rotor-Gene Q system (QIAGEN, Hilden, Germany) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, United States). In addition, we determined the relative expression of genes presented in **Supplementary Table 1** using the Pfaffl method (Pfaffl, 2004; Kim et al., 2019b; Moon et al., 2019).

## Generation of Heatmap

We generated the heatmap data to graphically express DEGs detected in developing rice seeds under salt stress using Mev software[3] (Moon et al., 2020).

## Statistical Analysis

We performed the *t*-test and correlation analysis using SAS 9.4 TS Level 1M5 (Ver.1.0.19041; SAS Institute Inc., Cary, NC, United States).

# RESULTS

## Topsoils Collected From NL and RL Showed Different Chemical Properties

Both NL (the experimental paddy field of CARES) and RL (section B of the Seosan reclaimed land) used for field experiments were located in Chungcheongnam-do, Republic of Korea, and share very similar latitudes (**Supplementary Figure 1**). We compared the chemical properties of the topsoil samples collected from NL and RL (**Table 1**). Of the chemical properties analyzed, we found no significant differences in terms of pH and P content between the two locations. N and Ca contents in RL were significantly lower than those in NL; however, other minerals, such as Na, K, Mg, and S, and EC in RL were significantly higher than those in NL. The topsoil of RL

[2]http://ricephylogenomics-khu.org/road/go_analysis.php
[3]https://mev.tm4.org

exhibited significantly high amount of Na, while that of NL exhibited scant amounts of Na (**Table 1**).

## Rice Grains Developing Under Salt Stress Exhibited Reduced Seed Quality and Enhanced $Na^+$ Content

We selected Samgwang, a japonica rice cultivar, with high palatability for this study, because Samgwang is one of the 18 premium rice cultivars registered in Korea and has the largest cultivation area in Chungcheongnam-do (Cho et al., 2020). We then examined the agronomic traits of Samgwang, grown on NL and RL (**Figure 1**) and confirmed the apparent damage caused by salt stress in unhulled and brown rice of Samgwang grown on RL, but not in those on NL. The heading date of Samgwang was significantly delayed on RL compared to that on NL, and the thousand grain weight and yield were decreased more significantly on RL than that on NL. However, no significant difference in culm length between NL and RL was observed in Samgwang. In addition, Samgwang exhibited a significant difference in terms of panicle length and spikelet numbers per panicle on RL, compared to those on NL (**Figure 1**).

We further analyzed the quality of seeds of Samgwang harvested on both NL and RL by using palatability and amylose, N, and mineral contents, including P, Na, K, Mg, and S contents (**Table 2**). Of these minerals, seeds of Samgwang grown on NL contained only a small amount of Na, but those grown on RL accumulated huge amounts of Na. For seeds of Samgwang grown on RL compared to those grown on NL, amylose content and palatability were significantly decreased, and N contents was significantly higher in Samgwang seeds grown on RL, but no significant difference between NL and RL was observed in P contents on Samgwang seeds. However, the seeds of Samgwang grown on RL exhibited a significant increase in the accumulation of minerals, including Na, K, Mg, and S compared to those grown on NL (**Table 2**).

## Transcriptional Changes in the Rice Developing Seeds Under Salt Stress

We performed RNA-seq of Samgwang using the total RNA of developing seeds at 8 and 15 DAH by Illumina sequencing for the investigation of the transcriptional changes occurring in the developing seeds under salt stress using RNA-seq analysis. After the raw data of RNA-seq for the developing seeds of Samgwang were obtained, we combined the raw data obtained for the developing seeds grown on NL and RL at 8 and 15 DAH. The objective was to obtain universal DEGs throughout seed development with four biological replicates. We obtained DEGs from further analysis of raw data (see "Materials and methods"; **Figure 2A**; **Supplementary Table 2**). We selected DEGs under both two-fold change and adjusted value of $p < 0.05$, thereby arriving at the selection of 253 upregulated and 238 downregulated DEGs (**Figure 2A**; **Supplementary Table 2**). To confirm the reliability of DEGs generated from the combined raw data, correlation analysis was then performed for the expression values of DEGs obtained at 8 and 15 DAH seeds grown on NL and RL (**Supplementary Table 3**). For seeds grown on NL and RL, the expression values of DEGs at 8 DAH highly correlated with those at 15 DAH (i.e., 0.91802, $p < 0.0001$ for seeds grown on NL and 0.94980, $p < 0.0001$ for seeds grown on RL), indicating that reproducibility between biological replicates obtained at 8 and 15 DAH for each treatment is very high (**Supplementary Table 3**).

## GO Enrichment Analysis Suggests a Close Association Between Salt Stress During the Seed Ripening Stage and Metabolism and Stress Responses

We performed GO enrichment analysis to classify DEGs into GO terms corresponding to the biological process (**Figure 2B**). We found upregulated DEGs under salt stress to be closely associated with metabolisms of several biomolecule-related GO terms, including the cellular amino acid and derivative metabolic process (GO:0006519), the carboxylic acid metabolic process (GO:0019752), the lignin catabolic process (GO:0046274), the trehalose biosynthetic process (GO:0005992), the polysaccharide catabolic process (GO:0000272), and the chitin catabolic process (GO:0006032) as well as stress-related GO terms, including response to oxidative stress (GO:0006979) and response to stress (GO:0006950). DEGs downregulated under salt stress were closely associated with GO terms different from those of DEGs upregulated under salt stress, and included protein polymerization (GO:0051258), the cellulose biosynthetic process (GO:0030244), the regulation of translation (GO:0006417), base-excision repair (GO:0006284), the microtubule-based process (GO:0007017), and the negative regulation of catalytic activity (GO:0043086; **Figure 2B**).

## Significance of Abscisic Acid, Melatonin, and Carbohydrate Biosynthesis Pathways in Seed Development Under Salt Stress Was Emphasized Through MapMan Analysis and Verified by qRT-PCR, Supporting the Reliability of RNA-Seq Data

To perform additional functional classification of the selected DEGs found in developing rice seeds in response to salt stress, we performed MapMan analysis (**Supplementary Figure 1**). Our data from MapMan analysis confirmed the putative relationship to drought and/or salt stress of DEGs involved in the biosynthetic pathways of biomolecules—including ABA (Mizrahi et al., 1971; Most, 1971; Arad et al., 1973), melatonin (Mukherjee et al., 2014), trehalose (Garcia et al., 1997; Vasquez-Robinet et al., 2008), raffinose (Pattanagul and Madore, 1999; Bellaloui et al., 2013), and maltose (Fougère et al., 1991; Rizhsky et al., 2004), and we therefore chose these biomolecules for further analysis (**Figures 3–5**).

We detected 9-cis-Epoxycarotenoid dioxygenase (*NCED*, *LOC_Os03g44380* and *LOC_Os07g05940*; Qin and Zeevaart, 1999) and short-chain alcohol dehydrogenase (*ABA2*, *LOC_Os04g33240*; Schwartz et al., 1997), involved in the biosynthetic pathway of ABA, as DEGS upregulated by salt stress (**Figure 3**) in addition to ABA-responsive genes, including *LOC_Os04g44500*, *LOC_Os10g34730*, and *LOC_Os12g29400*.

**TABLE 1** | The chemical properties of topsoil for NL and RL, respectively, in 2018.

| Location | pH | EC (ds/m) | N (%) | P (mg/kg) | Na (mg/kg) | Mg (mg/kg) | K (mg/kg) | S (mg/kg) | Ca (mg/kg) |
|---|---|---|---|---|---|---|---|---|---|
| NL | 5.2 ± 0.36 | 0.35 ± 0.038 | 0.142 ± 0.0067 | 15.85 ± 3.62 | 46.03 ± 9.71 | 97.22 ± 21.78 | 108.98 ± 17.21 | 267.61 ± 2.67 | 856.96 ± 149.83 |
| RL | 5.2 ± 0.20 | 4.17 ± 0.787 | 0.127 ± 0.0038 | 13.62 ± 0.31 | 1344.45 ± 198.65 | 330.52 ± 35.71 | 286.17 ± 34.41 | 1460.23 ± 137.56 | 302.74 ± 54.18 |
| Values of $p^{\#}$ | 1.0000 | 0.0011 | 0.0246 | 0.3473 | 0.0003 | 0.0006 | 0.0013 | 0.0001 | 0.0038 |

*Data represent mean ± standard deviation (SD). #The value of p for each chemical property was determined by analysis of t-test between NL and RL.*

Additionally, zeaxanthin epoxidase (*ZEP*, *LOC_Os04g37619*), although not included in DEGs with 2-fold change, did however exhibit an approximately 1.6-fold change with adjusted value of $p < 0.05$, indicating its trend toward a high salt stress response. Next, we performed qRT-PCR to verify RNA-seq data for the aforementioned genes through relative expression data from qRT-PCR for all genes except for *LOC_Os12g29400*, one of the ABA-responsive genes, due to difficulties in the design of the proper primer set for PCR reaction (**Figure 3**).

Similar to the biosynthetic pathway of ABA, the biosynthetic pathway of melatonin was also upregulated under salt stress (**Figure 4**). In **Figure 6** we present upregulated DEGs, including tryptophan decarboxylase (*TDC*, *LOC_Os08g04540* and *LOC_Os08g04560*; Noé et al., 1984; De Luca et al., 1989; Songstad et al., 1990), tryptamine 5-hydroxylase (*T5H*, *LOC_Os12g16720*; Kang et al., 2007; Fujiwara et al., 2010), and N-acetylserotonin O-methyltransferase (*ASMT*, *LOC_Os09g17560*; Kang et al., 2011). We also verified these DEGs through qRT-PCR, thereby confirming the corresponding RNA-seq data (**Figure 4**).

In terms of the metabolic pathways of several carbohydrates, including trehalose, raffinose, and maltose, related to drought and/or salt stress, we confirmed the salt stress-induced upregulation of DEGs including trehalose-6-phosphate phosphatase (*T6PP*, *LOC_Os26910* and *LOC_Os04g46760*), trehalase (*TRE*, *LOC_Os10g37660*; Müller et al., 2001), galactinol synthase (*GolS*, *LOC_Os03g20120* and *LOC_Os07g48830*; Taji et al., 2002), and β-amylase (*LOC_Os03g04770* and *LOC_Os10g41550*; **Figure 5**). Furthermore, we performed the verification of these selected DEGs through qRT-PCR, to confirm their correspondence with RNA-seq data.

## LC/MS–MS Analysis Reveals the Reduction of Trehalose in Seeds Grown on RL

Interestingly, of the above carbohydrates, *T6PP* (*LOC_Os26910* and *LOC_Os04g46760*) and *TRE* (*LOC_Os10g37660*), both involved in the metabolic pathway of trehalose, catalyze trehalose 6-phosphate into trehalose and convert trehalose into glucose, respectively (**Figure 5A**). We analyzed the trehalose content of the brown rice of the japonica rice cultivar, Samgwang, grown on NL and RL using LC/MS–MS to investigate whether the quantity of trehalose corresponded to the expression of *T6PP* (*LOC_Os26910* and *LOC_Os04g46760*) and *TRE* (*LOC_Os10g37660*). Our results indicated that the quantity of trehalose was significantly decreased in the brown rice of the cultivars grown on RL compared to that of those grown on NL, explaining seed damage caused by salt stress in RL (**Figures 1, 5**).

## Transcriptional Regulation and the Response of Storage Proteins in Developing Seeds Under Salt Stress Are Important

From DEGs in developing seeds of Samgwang both up- and downregulated under salt stress, we searched transcription factors (TFs) to unearth more transcriptional regulation for responses of developing rice seeds by salt stress (**Figure 6**;

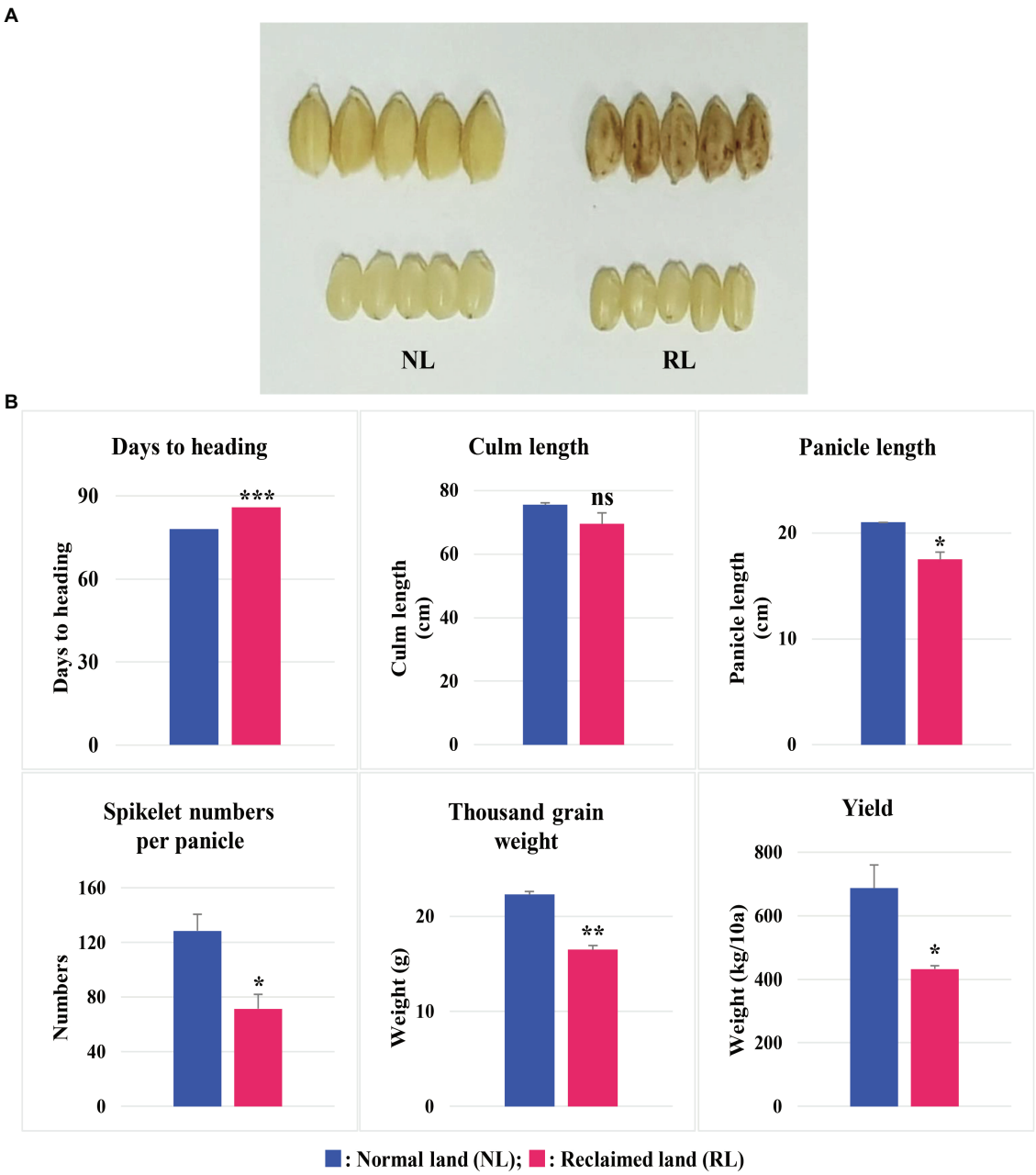



**FIGURE 1 |** Agronomic traits of Samgwang grown on normal land (NL) and reclaimed land (RL) in 2018. **(A)** Rice seeds harvested on NL and RL. **(B)** Agronomic traits, including days until heading, culm length, panicle length, spikelet numbers per panicle, thousand grain weight, and yield. Data represent mean ± SD. ns: not significant; *$0.01 < p < 0.05$; **$0.001 < p < 0.01$; ***$p < 0.001$. Significant difference for each dataset was determined by *t*-test.

**TABLE 2 |** Palatability, amylose, total nitrogen and minerals, including P, Na, Mg, K and S, analyzed from mature seeds of Samgwang harvested in NL and RL in 2018.

| Location | Palatability | Amylose (%) | N (%) | P(mg/kg) | Na (mg/kg) | Mg (mg/kg) | K (mg/kg) | S (mg/kg) |
|---|---|---|---|---|---|---|---|---|
| NL | 76.9 ± 0.6 | 18.01 ± 0.06 | 1.16 ± 0.05 | 2830.0 ± 438.4 | 17.73 ± 1.07 | 2380.0 ± 70.7 | 3085.0 ± 106.1 | 901.0 ± 1.4 |
| RL | 67.8 ± 1.1 | 14.75 ± 0.22 | 1.81 ± 0.01 | 3737.5 ± 208.6 | 729.28 ± 77.93 | 3227.5 ± 194.5 | 3,525 ± 77.8 | 1390.0 ± 42.4 |
| Value of $p^{\#}$ | 0.0095 | 0.0025 | 0.0031 | 0.1183 | 0.0059 | 0.0285 | 0.0419 | 0.0037 |

*Data represent mean ± SD. #The value of p for each chemical property was determined by analysis of t-test between NL and RL.*

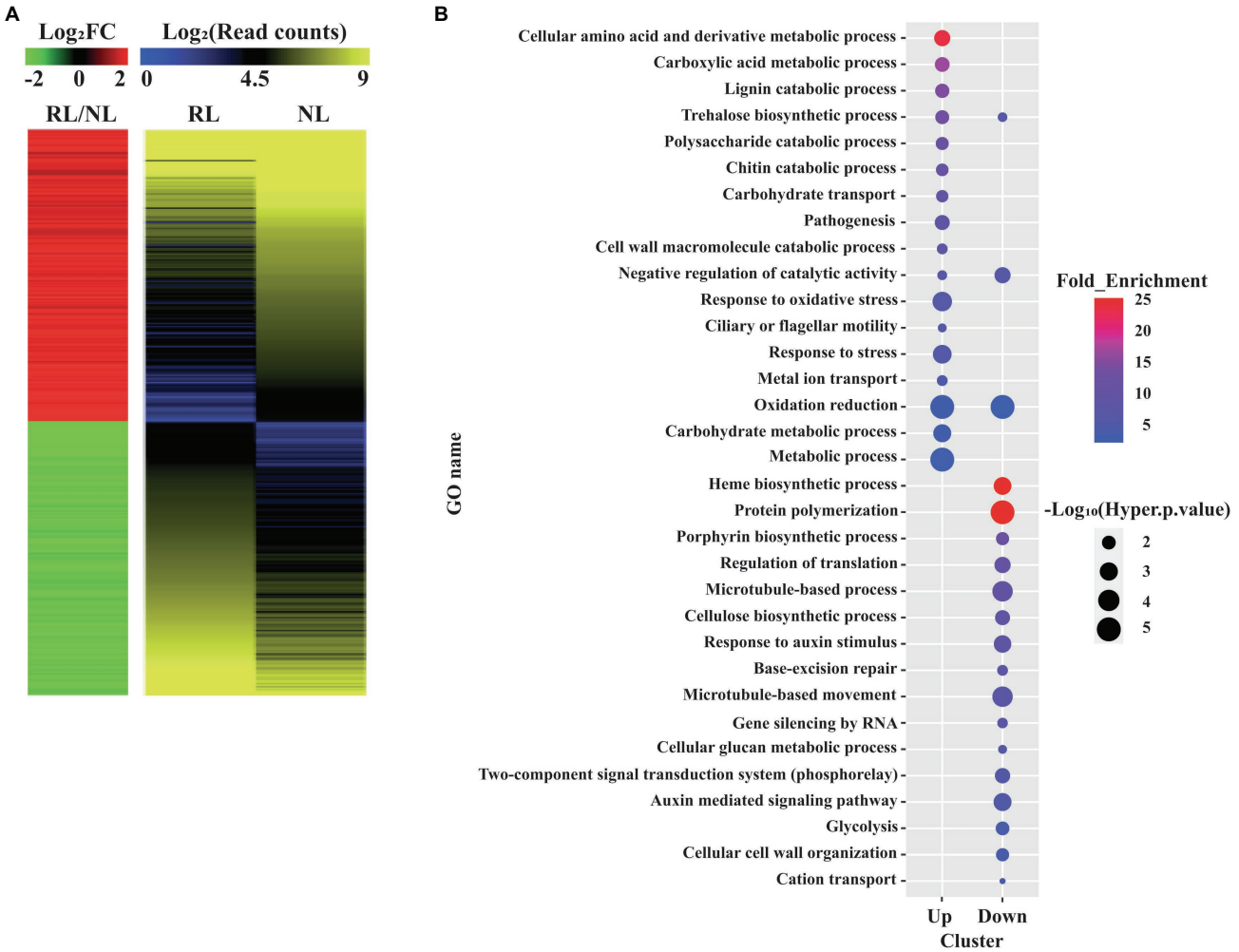
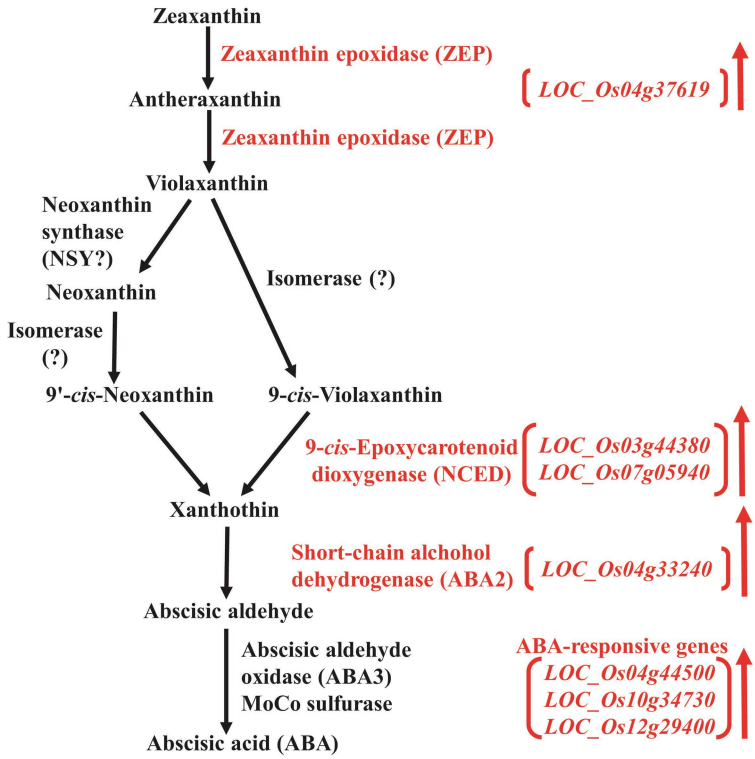



**FIGURE 2 |** Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) detected in the developing seeds of Samgwang grown on reclaimed land (RL). **(A)** Heatmap analysis of the DEGs. A total of 491 up or downregulated DEGs between normal land (NL) and RL were visualized. Both heatmaps were generated by using average values of four replicates in the transcriptome analysis. The red and green colors in the left panel indicate up and downregulated DEGs, respectively. The $Log_2FC$ (FC, fold change) and $Log_2$(Read counts) indicate the $Log_2$ transformed value of normalized read counts and fold change (RL/NL), respectively, which was generated by RNA-seq. **(B)** GO enrichment analysis of the 491 DEGs. GO terms based on the biological process were visualized as the dot plot. Dot color and size represent fold enrichment value (query number/query expected value in selected GO term) and $-Log_{10}$ (Hyper value of *p*). The redder and larger dots indicate statistically significant enriched GO terms.

**Supplementary Table 4**). We categorized 33 TFs detected in DEGs data as AP2/ERF (APETALA2/Ethylene Responsive Factor; Licausi et al., 2013), GRAS [GAI(Gibberellin-Acid-Insensitive), RGA(Repressor of GA1), SCL(SCARECROW); Pysh et al., 1999], bHLH (basic helix-loop-helix; Hu et al., 1996; Kiribuchi et al., 2004, 2005), MYB (Dai et al., 2007), NAC (NAM, ATAF, and CUC; Ohnishi et al., 2005; Hu et al., 2006), basic region/leucine zipper (bZIP; Kusano et al., 1995; Gupta et al., 1998), zinc finger (von Arnim and Deng, 1993; Lippuner et al., 1996; Song et al., 1998), WRKY (Zhang et al., 2004; Wei et al., 2008), plant AT-rich sequence and zinc binding (PLATZ; Wang et al., 2018, 2019), and heat shock factor (HSF; Hübel et al., 1995; Lee et al., 1995; Xiang et al., 2013). In developing rice seeds under salt stress, DEGs for GRAS, WRKY, and PLATZ were downregulated while DEGs for bHLH, MYB, and HSF were upregulated. Furthermore, in AP2/ERF, NAC, bZIP, and zinc finger, we identified genes showing both up- and down-regulation. Specifically, for zinc finger, we detected a total of ten DEGs in developing rice seeds under salt stress, nine of which were upregulated. Interestingly, in GO analysis data (biological process) of upregulated DEGs for TFs, two bZIPs (*LOC_Os02g43330* and *LOC_Os09g21180*) and two zinc fingers (*LOC_Os02g52210* and *LOC_Os09g26780*) were grouped into "response to stress," and two bHLHs (*LOC_Os02g45170* and *LOC_Os09g28210*) and one NAC (LOC_Os05g34310) were classified as "anatomical morphogenesis" and "multicellular organismal development," respectively. All DEGs for MYB, including *LOC_Os01g09640*, *LOC_Os01g64360*, *LOC_Os01g74410*, and *LOC_Os04g42950*, were



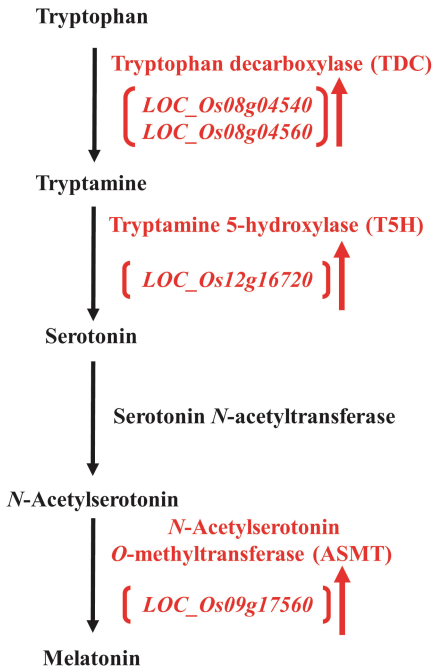

| Gene name | Gene ID | RNA-seq | | qRT-PCR | |
|---|---|---|---|---|---|
| | | $Log_2FC$ | Adjusted *p* values | $Log_2FC$ | *p* Values |
| *ZEP* | *LOC_Os04g37619* | 0.69 | 0.0447 | 0.58 | 0.0007 |
| *NCED1* | *LOC_Os03g44380* | 2.24 | 2.92E-10 | 1.14 | <0.0001 |
| *NCED2* | *LOC_Os07g05940* | 2.87 | 3.37E-07 | 1.06 | 0.0047 |
| *ABA2* | *LOC_Os04g33240* | 1.93 | 0.0402 | 1.60 | <0.0001 |
| ABA-responsive gene1 | *LOC_Os04g44500* | 1.29 | 0.0363 | 1.31 | <0.0001 |
| ABA-responsive gene2 | *LOC_Os10g34730* | 2.48 | 0.0062 | 1.36 | <0.0001 |
| ABA-responsive gene3 | *LOC_Os12g29400* | 1.02 | 0.0341 | N.A. | N.A. |

**FIGURE 3 |** Biosynthetic pathway of abscisic acid (ABA) in the developing seeds of Samgwang grown on reclaimed land (RL) and verification of upregulated genes through qRT-PCR. ↑ indicates upregulation. The $Log_2FC$ (FC, fold change) expresses the $Log_2$ value of a ratio of expression value of RL to that of normal land (NL; RL/NL), which was generated by RNA-seq and qRT-PCR, respectively, from the developing seeds of Samgwang. The significant difference for each dataset was determined by *t*-test.

categorized by the program as "biosynthetic process" (**Figure 6**; **Supplementary Table 4**).

In addition, we found, unsurprisingly, that genes for seed storage proteins, including glutelin and prolamin, were upregulated by salt stress (**Supplementary Table 5**). DEGs for seed storage proteins comprise 10 glutelin genes and 17 prolamin genes, indicating that seed storage proteins might be affected by osmotic stress mediated by salinity.

We further generated DEGs dataset for the developing seeds of Samgwang at 8 and 15 DAH, respectively, to exhibit the information of DEGs according to the seed developmental stages (**Supplementary Table 6**). Under salt stress, DEGs from 8 DAH seeds of Samgwang contained 460 upregulated and 335 downregulated genes, while, in 15 DAH seeds of Samgwang, 243 upregulated and 98 downregulated genes were detected (**Supplementary Table 6**). Several transcription factors, including NAC (*LOC_Os05g34310*), HSF (*LOC_Os08g43334*), bZIP (*LOC_02g43330*, *LOC_Os07g08420* and *LOC_Os09g21180*) and zinc finger (*LOC_Os03g49730* and *LOC_Os09g26780*), were selected in **Figure 6**; **Supplementary Table 4** to verify newly generated DEGs data of the developing seeds of Samgwang at 8 and 15 DAH, respectively, using qRT-PCR (**Supplementary Table 7**). The expression patterns of tested transcription factor genes detected at 8 and/or 15 DAH seeds through RNA-seq are well matched with qRT-PCR data, but some genes showed significant expression at 8 or 15 DAH in the qRT-PCR data in spite of no expression at a specific developmental stage in the RNA-seq data (**Supplementary Table 7**). For further confirmation, 5 heat shock protein genes detected in DEGs data of the developing seeds of Samgwang at 8 and 15 DAH, respectively, were selected (**Supplementary Tables 6, 8**). As expected, the RNA-seq data correspond to the data of qRT-PCR, with the exception for some genes at a specific developmental stage (**Supplementary Table 8**). These results indicate that




| Gene name | Gene ID | RNA-seq | | qRT-PCR | |
|---|---|---|---|---|---|
| | | $Log_2FC$ | Adjusted *p* values | $Log_2FC$ | *p* Values |
| *TDC1* | *LOC_Os08g04540* | 2.62 | 0.0015 | 1.61 | <0.0001 |
| *TDC2* | *LOC_Os08g04560* | 3.02 | 0.0017 | 1.45 | <0.0001 |
| *T5H* | *LOC_Os12g16720* | 1.19 | 5.23E-05 | 1.47 | <0.0001 |
| *ASMT* | *LOC_Os09g17560* | 1.49 | 4.61E-06 | 1.40 | <0.0001 |

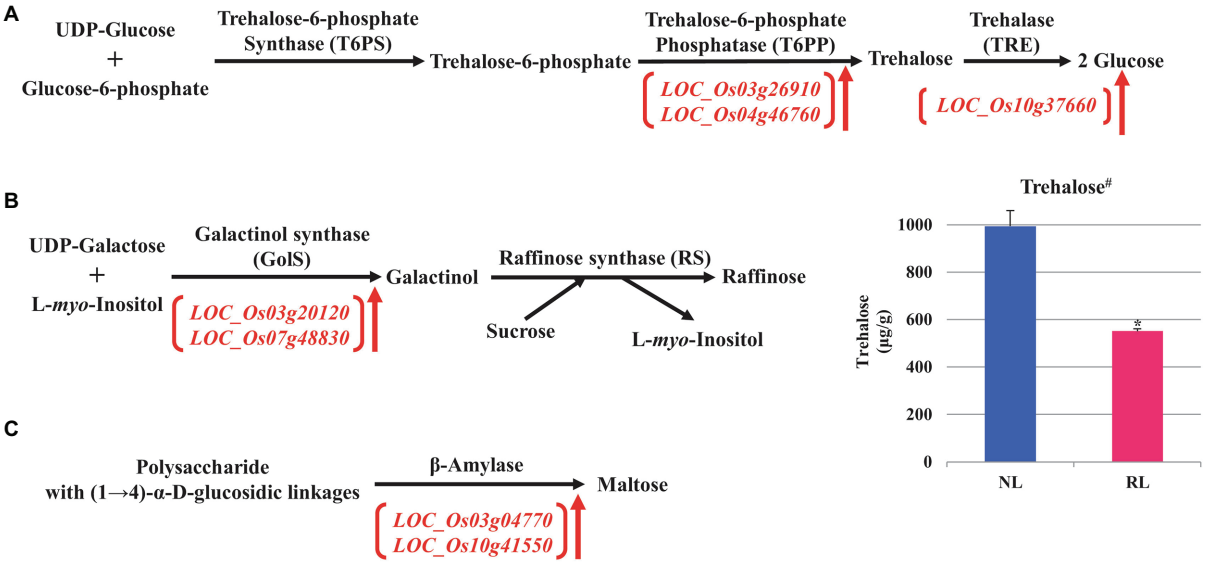
**FIGURE 4 |** Biosynthetic pathway of melatonin in the developing seeds of Samgwang grown on reclaimed land (RL) and verification of upregulated genes through qRT-PCR. ↑ indicates upregulation. The $Log_2FC$ (FC, fold change) expresses the $Log_2$ value of a ratio of expression value of RL to that of normal land (NL; RL/NL), which was generated by RNA-seq and qRT-PCR, respectively, from the developing seeds of Samgwang. The significant difference for each dataset was determined by *t*-test.

DEGs data from each developmental stage of Samgwang seeds might be useful for further studies.

## DISCUSSION

Global sea-level rise, which is an effect of climate change, has continued to occur throughout the entire 20th century, and is currently ongoing, resulting in saltwater intrusion (Walthall et al., 2012; Mimura, 2013). The RLs, corresponding to 11.7% of the total arable land and 22.1% of that used for rice production in Korea face a crisis of sea-level rise, which directly causes an increase in salt concentrations (Mimura, 2013; Lim et al., 2020). As reported by Cheong et al. (1995), topsoil samples of some selected RLs in the western coastal areas of Korea contain significantly higher levels of minerals, including Na, K, and Mg, than those of NLs, apart from the Ca levels, which are higher in topsoils of NL, as shown in **Table 1**. Calcium deficiency is known to occur in alkaline soils. However, little is known about Ca deficiency in the topsoils of RL (Bower and Turk, 1946). In plants, Ca functions as a structural component that stabilizes the cell wall, a secondary messenger of physiological, developmental, and stress-related processes, and also as an element in plant immune signaling (Thor, 2019). Regarding the growth and development of plants, the relatively different Ca content between NL and RL is a promising topic for further research.

The growth of rice plants of Samgwang, the japonica rice cultivars, was negatively affected by a high salt condition, i.e., among the agronomic traits examined in this study, salt stress significantly decreased yield and thousand grain weight and significantly delayed heading date (**Figure 1B**); this was consistent with the results of previous studies (Peiris et al., 1988; Cheong et al., 1995; Choi et al., 2003; Baxter et al., 2011; Thitisaksakul et al., 2015). Similar to the research data reported by Peiris et al. (1988) and Cheong et al. (1995), we confirmed a significant accumulation of minerals such as Na, K, Mg, and, S in the seeds of Samgwang grown on RL (**Table 2**), which was caused by saline soil containing considerably higher levels of several minerals than normal soil (**Table 1**). Under salinity, however, palatability and amylose content were significantly decreased in the seeds of Samgwang (**Table 2**), indicating a decrease in seed quality as shown by Choi et al. (2003). Moreover, it is still largely unknown how the mineral content of soil is related to




| Gene name | Gene ID | RNA-seq | | qRT-PCR | |
|---|---|---|---|---|---|
| | | $Log_2FC$ | Adjusted *p* values | $Log_2FC$ | *p* Values |
| *T6PP1* | *LOC_Os03g26910* | 1.52 | 2.14E-05 | 1.60 | <0.0001 |
| *T6PP2* | *LOC_Os04g46760* | 1.30 | 0.0038 | 1.38 | <0.0001 |
| *TRE* | *LOC_Os10g37660* | 1.31 | 2.67E-07 | 1.38 | <0.0001 |
| *GolS1* | *LOC_Os03g20120* | 1.77 | 0.0008 | 1.66 | <0.0001 |
| *GolS2* | *LOC_Os07g48830* | 1.30 | 0.0401 | 1.48 | <0.0001 |
| β-Amylase gene1 | *LOC_Os03g04770* | 1.04 | 0.0050 | 1.76 | <0.0001 |
| β-Amylase gene2 | *LOC_Os10g41550* | 1.41 | 0.0250 | 1.59 | <0.0001 |

**FIGURE 5 |** Metabolic pathways of carbohydrates putatively related to salt stress in the developing seeds of Samgwang grown on reclaimed land (RL) and verification of upregulated genes through qRT-PCR. **(A)** Trehalose; **(B)** Raffinose; **(C)** Maltose. ↑ indicates upregulation. #indicates that trehalose was quantified in the brown rice of Samgwang grown on normal land (NL) and RL by LC/MS–MS. The $Log_2FC$ (FC, fold change) expresses the $Log_2$ value of a ratio of expression value of RL to that of NL (RL/NL), which was generated by RNA-seq and qRT-PCR, respectively, from the developing seeds of Samgwang. The significant difference for each dataset was determined by *t*-test. *$0.01 < p < 0.05$.

the accumulation of minerals in seeds and various agronomic traits. Therefore, further investigation is necessary to determine the mechanism underlying abnormal agronomic traits related to the transcription of genes in developing rice seeds and provide a better understanding of the effect of salinity on rice seeds, facilitating a more successful rice breeding program to overcome the decrease in seed quality under salt stress. This is of critical importance because, in rice breeding programs, after yield, seed quality is considered to be one of the most important agronomic traits (Lau et al., 2015; Kobayashi et al., 2018).

In plants, the salt overly sensitive (SOS) pathway has been known as the system for the removal of $Na^+$ through $Ca^{2+}$ signaling because salt stress causes osmotic stress and the accompanying accumulation of ions, resulting in a decrease in plant growth; however, as we show in **Table 2**, in RL, minerals—including Na, K, Mg, and S—accumulated significantly in the mature seeds of Samgwang compared to those accumulating in seeds grown on NL (Lamers et al., 2020). The GO enrichment analysis of DEGs, selected from RNA-seq data of the rice developing seeds under salt stress, demonstrated the close association between some upregulated DEGs and stress-related GO terms, including response to oxidative stress (GO:0006979) and response to stress (GO:0006950; **Figure 2B**). Furthermore, data from our MapMan analysis confirmed that the biosynthetic pathway of ABA was upregulated in the rice developing seeds under salt stress (**Figure 3**; **Supplementary Figure 2**; Mizrahi et al., 1971; Most, 1971; Arad et al., 1973). It has been established that ABA regulates osmosis, ions, and reactive oxygen species under salt stress (Lamers et al., 2020). Moreover, salt stress is related to the biosynthesis of melatonin (Zhan et al., 2019; Ahn et al., 2021), which is upregulated by it (**Figure 4**). As we show in **Figure 2B**, we identified GO terms related to the metabolic pathways of several biomolecules, and further MapMan analysis exhibited that the biosynthetic pathways of trehalose (Garcia et al., 1997; Vasquez-Robinet et al., 2008), raffinose (Pattanagul and Madore, 1999; Bellaloui et al., 2013), and maltose (Fougère et al., 1991; Rizhsky et al., 2004), all related to drought and/or salt stress, were also upregulated by it (**Figure 5**; **Supplementary Figure 2**). The upregulated biosynthesis of these metabolites, related to abiotic stress, could be closely connected to a decrease in both thousand grain weight and amylose content in the seeds of Samgwang (**Figure 1;**

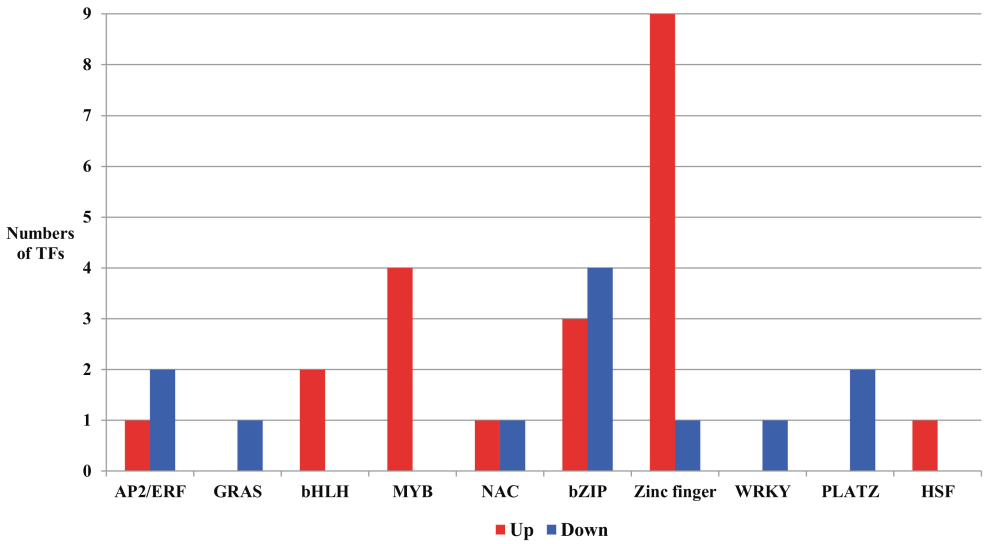



**FIGURE 6 |** Transcription factors (TFs) up or downregulated due to salt stress in the developing seeds of Samgwang. AP2/ERF: APETALA2/ethylene responsive factor; GRAS: GAI (Gibberellin-Acid-Insensitive), RGA (Repressor of GA1), SCL (SCARECROW); bHLH, basic helix-loop-helix; NAC: NAM, ATAF, and CUC; bZIP, basic region/leucine zipper; PLATZ, plant AT-rich sequence and zinc binding; HSF, heat shock factor.

**Table 2**), resulting in an excessive use of energy to protect their own seeds from osmotic and ionic stress. In addition, the expression of seed storage protein genes, including glutelin and prolamin, showed an increase under salinity (**Supplementary Table 5**) and, as outlined by Baxter et al. (2011), corresponded to an increase in the glutelin content of rice seeds; however, prolamin content under salinity varies according to each rice cultivar. Interestingly, elevated ozone concentration caused an increase in protein content (Frei et al., 2012; Zhou et al., 2015) and a decrease in yield and starch concentration in rice (Frei et al., 2012), thereby corresponding to our data presented in **Figure 1; Table 2**; and **Supplementary Table 5**. This indicates that rice plants might optimize their metabolism in seeds to survive abiotic stress.

We detected TFs involved in transcriptional regulation of responses in developing rice seeds to salt stress, and categorized them as AP2/ERF (Licausi et al., 2013), GRAS (Pysh et al., 1999), bHLH (Hu et al., 1996; Kiribuchi et al., 2004, 2005), MYB (Dai et al., 2007), NAC (Ohnishi et al., 2005; Hu et al., 2006), bZIP (Kusano et al., 1995; Gupta et al., 1998), zinc finger (von Arnim and Deng, 1993; Lippuner et al., 1996; Song et al., 1998), WRKY (Zhang et al., 2004; Wei et al., 2008), PLATZ (Wang et al., 2018, 2019), and HSF (Hübel et al., 1995; Lee et al., 1995; Xiang et al., 2013; **Figure 6**; **Supplementary Table 4**). Of these TFs, all bHLHs, MYBs, zinc finger, and HSF genes (apart from one zinc finger gene) were upregulated in developing seeds under salt stress (**Figure 6**; **Supplementary Table 4).** Under salt stress, previous studies reported the upregulation of bHLH (*LOC_Os09g28210*; Li et al., 2015), MYB (*LOC_Os01g64360*; Zhou et al., 2016), and zinc finger (*LOC_Os03g60560*; Zhou et al., 2016), but the exact biological functions for these genes are not known (Li et al., 2015; Zhou et al., 2016). Furthermore, the overexpression of *LOC_Os09g26780*—a Zinc finger protein gene—enhanced salt-tolerance in rice (Peethambaran et al., 2018). Drought induced another MYB, *LOC_Os04g42950* (Yu et al., 2020). Salt stress induced bZIP (*LOC_Os02g43330*), an upregulated DEG (Zhou et al., 2016). Interestingly, the overexpression of AP2/ERF (*LOC_Os05g49700*) resulted in a reduction of rice plant height (Ma et al., 2020). The focus of these previous studies was the TFs of shoot and/or root and the whole plant body, but not developing seeds (Li et al., 2015; Zhou et al., 2016; Peethambaran et al., 2018; Ma et al., 2020). This indicates the likelihood that unlike the genes of other organs, such as shoot and root, seed-specific genes are expressed in developing rice seeds.

As noted above, we investigated Samgwang, a premium rice cultivar registered in Korea, with high palatability for a case study of transcriptional changes occurring in developing seeds under salt stress. However, to obtain more robust data concerning the response to high salinity, more transcriptome analysis might be required to examine other rice cultivars with different genetic background, resulting in more reliable scientific data on the response of seeds developing under salt stress.

## CONCLUSION

Overall, our study represents a case study on the responses of rice plants, focused on seeds, to salt stress. Particularly, we discussed phenotypic and transcriptional changes related to seed quality here. Many remaining questions should be addressed through further studies on the responses of developing rice seeds to salt stress. The maintenance of a sustainable balance between survival under salinity and the improvement of seed quality remains to be addressed through manipulation and a combination of DEGs in rice breeding programs.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

CL, H-JK, and K-HJ conceived and designed the experiments. CL analyzed the data. CL, C-TC, W-JH, Y-SL, and J-HL conducted the experiments. CL wrote the manuscript. CL, W-JH, Y-SL, H-JK, and K-HJ contributed to the manuscript revision. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.748273/full#supplementary-material

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# Identification of Key Genes in 'Luang Pratahn', Thai Salt-Tolerant Rice, Based on Time-Course Data and Weighted Co-expression Networks

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Salinity is an important environmental factor causing a negative effect on rice production. To prevent salinity effects on rice yields, genetic diversity concerning salt tolerance must be evaluated. In this study, we investigated the salinity responses of rice (*Oryza sativa*) to determine the critical genes. The transcriptomes of 'Luang Pratahn' rice, a local Thai rice variety with high salt tolerance, were used as a model for analyzing and identifying the key genes responsible for salt-stress tolerance. Based on 3' Tag-Seq data from the time course of salt-stress treatment, weighted gene co-expression network analysis was used to identify key genes in gene modules. We obtained 1,386 significantly differentially expressed genes in eight modules. Among them, six modules indicated a significant correlation within 6, 12, or 48 h after salt stress. Functional and pathway enrichment analysis was performed on the co-expressed genes of interesting modules to reveal which genes were mainly enriched within important functions for salt-stress responses. To identify the key genes in salt-stress responses, we considered the two-state co-expression networks, normal growth conditions, and salt stress to investigate which genes were less important in a normal situation but gained more impact under stress. We identified key genes for the response to biotic and abiotic stimuli and tolerance to salt stress. Thus, these novel genes may play important roles in salinity tolerance and serve as potential biomarkers to improve salt tolerance cultivars.

**Keywords: salt tolerant rice, 3' Tag Seq, time-series data, weighted co-expression network, two-state co-expression network, network-based analysis**

## INTRODUCTION

Rice is the most important food crop in the world and is predominantly grown in South, Southeast, and East Asia. Rice is produced in a wide range of locations environments, including salinity and drought. Salinity is a limiting factor in rice production, particularly in Southeast Asia, where many regions have experienced decreasing rice yields due to increased soil salinity (Pattanagul and Thitisaksakul, 2008). To solve this problem, it is important to understand the molecular underpinnings of salt tolerance, which is controlled by multiple genes and involves several mechanisms [for reviews: (Chen et al., 2021a; Liu et al., 2021; Ponce et al., 2021)], including osmotic adjustment (Sripinyowanich et al., 2013; Nounjan et al., 2018) for review: (Rajasheker et al., 2019), ion homeostasis [for review: (Hussain et al., 2021)], reactive oxygen species (ROS) scavenging (Chutimanukul et al., 2019; Parveen et al., 2021), membrane repairs and photosynthesis adaptation (Udomchalothorn et al., 2017; Chutimanukul et al., 2018; Lekklar et al., 2019; Chaudhry et al., 2021). These mechanisms are regulated through various sensors and signaling cascades, including the calmodulin signaling pathway (Yuenyong et al., 2018) with interaction with abscisic acid signaling (Saeng-ngam et al., 2012) and protein kinase cascade [for review: (Chen et al., 2021a)].

Many studies have examined salt tolerance variation in rice and its use in breeding programs, seeking to identify salt-tolerance genes (Ganie et al., 2019). Salt tolerance is a polygenic trait. Although some salt tolerance genes have been identified in rice, a full understanding of tolerance gene networks and the connected cellular mechanisms is still missing. Identification of salt-tolerant germplasm and the causal genes is important for the development of new rice cultivars (Flowers, 2004). With the development of sequencing techniques, the identification of candidate genes that are related to biological phenotypes was mainly based on comparing their gene expression levels among different experimental groups.

Nowadays, the study of molecular interactions in a dynamic network of biological factors has been widely used to simplify and analyze the complexity of biological systems (Marshall-Colon and Kliebenstein, 2019). One of the most widely used networks is a gene co-expression network (Charitou et al., 2016). Co-expression analysis is a classical and powerful method for reconstructing a gene functional interaction network using transcriptomic data. This network can be used to describe the relationships among cellular components or molecules based on the hypothesis that genes with similar expression patterns are often functionally related (Dam et al., 2018). Thus, many analysis pipelines and tools for a gene co-expression network have been developed recently (Fuller et al., 2007; Langfelder and Horvath, 2008; Usadel et al., 2009; Movahedi et al., 2012; Chen et al., 2017; Liu et al., 2017, 2018; Suratanee et al., 2018; Zhang et al., 2018). For example, R package like 'DCGL' (Liu et al., 2010; Yang et al., 2013) and 'DiffCorr' (Fukushima, 2013) are useful methods for identifying differentially expressed genes directly based on the data set as well as on the co-expression networks. A common tool is weighted gene co-expression network analysis (WGCNA), an R package developed by Langfelder and Horvath (2008). This tool has been extensively applied for constructing a gene co-expression network to identify novel candidate biomarkers in many species such as *Escherichia coli* (Liu et al., 2018), mouse (Fuller et al., 2007), human (Liu et al., 2017), and plants (Zhang et al., 2018). WGCNA aims to construct a weighted co-expression network using the correlation coefficient between the expression profiles of two genes and then identify significant modules or groups of the genes having similar expressions or dense connections in the network. Recently, we developed a pipeline for analyzing a two-state gene co-expression network of 'Khao Dawk Mali 105' (KDML105) rice to prioritize and select the genes responding to salinity. The graph structures of both normal and salinity state networks showed the difference in the number of connections and dense clusters. Significantly, the salinity-state network demonstrates higher density in KDML105. The key genes responding to salinity were then identified as the genes with few partners under normal conditions but highly co-expressed with many more partners under salt-stress conditions (Suratanee et al., 2018).

This study developed an analysis pipeline to investigate a gene co-expression network for a local Thai rice variety with salt-tolerance ability, the so-called 'Luang Pratahn', to prioritize key genes regulating the salt-tolerance response processes. We collected the time-course transcriptomic data from leaf RNAs sequenced using 3′ Tag RNA-seq. The weighted co-expression networks of global view, normal-state, and salinity-state conditions were constructed to identify modules of highly co-expressed genes as well as to identify key genes in each module based on their network topology and centralization.

## MATERIALS AND METHODS

### Determination of Thai Salt Tolerance Cultivar

Two cultivars of local Thai rice, 'Mayom' and 'Luang Pratahn' were selected for the comparison of the phenotype under salt-stress conditions at seedling stages with 'Pokkali' and IR29, which are the salt-stress tolerance and salt-stress susceptible standard cultivars, respectively (Pattanagul and Thitisaksakul, 2008; Li et al., 2018). Rice seedlings were soil-grown under normal conditions for 14 days. Then, they were irrigated with 115 mM NaCl for 9 days. Regarding the salt-responsive phenotypes, the following were collected on days 0, 3, 6, and 9 after salt-stress treatment: salt injury score (SIS; Gregorio et al., 1997), leaf greenness determined by SPAD 502 chlorophyll meter (Minolta Camera Co. Ltd., Osaka, Japan), PSII efficiency (Fv/Fm) determined by Pocket PEA chlorophyll fluorimeter (Hansatech Instrument, King's Lynn, United Kingdom), shoot fresh weight (FW), shoot dry weight (DW), cell membrane stability (CMS) and relative water content (RWC). The plants grown with supplemented water instead of NaCl solution were used as controls.

## Transcriptomes of 'Luang Pratahn' Rice With 3'-Tag Seq

To investigate the mechanisms of salt tolerance, 'Luang Pratahn' rice was planted under control and salinity conditions at three biological replications. Continuous gene expression profiling of rice was performed at 0, 3, 6, 12, 24, and 48 h after salt-stress treatment. Salt-stress treatment started at 8: 00 am. Transcriptomes of rice were explored by using 3′ Tag RNA-seq. The tissue of 36 cDNA libraries was immediately stored in liquid nitrogen at −80°C, then total RNA was extracted using PureLink™ plant RNA purification reagent (Thermo Fisher Scientific Inc., Massachusetts, United States), and the genomic DNA was eliminated by DNase I (RNase-free; New England Biolabs Inc., Massachusetts, United States). Next, total RNA extracts were purified using 1.8X MagBind® TotalPure NGS (Omega Bio-Tek Inc., Georgia, United States), and gel electrophoresis was used to examine the quality of RNA. The cDNA library for 3′ Tag RNA-seq was prepared using QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen Inc., New Hampshire, United States). After that, the amount of cDNA was measured with a Qubit™ dsDNA BR Assay Kit (Thermo Fisher Scientific Inc., Massachusetts, United States).

## Data Preprocessing and Screening

From the experimental data, we sequenced the cDNA library using a Hiseq4000 sequencer (Illumina Inc., California, United States) and then using Spliced Transcripts Alignment to a Reference (STAR) with its default parameters. An ultrafast universal RNA-seq aligner was used to map sequencing reads to the genome of *Oryza sativa* var. japonica based on MSU Rice Genome Annotation Release 7 (MSU 7.0). Next, we used the htseq-count to count reads per gene in each library. The significant differentially expressed genes were determined by using DESeq2 (Love et al., 2014). From the raw data of 55,987 genes, the genes with a read count of zero for more than 80% of the total 36 libraries were removed, as they are considered unreliable genes. There were 20,435 genes left whose expression values were normalized using DESeq2 and tested for significant differences in signals between control and salt stress with hypothesis testing using the Wald test. The significantly differentially expressed genes at $p < 0.05$ were selected for co-expression network analysis. This procedure corrects for library size and RNA composition bias, the counts of a gene expression in each sample divided by size factors determined by the median ratio of gene counts relative to geometric mean per gene.

## Construction of Gene Co-expression Networks

### Global Co-expression Network

We constructed a global co-expression network from the experimental data following the principle of the WGCNA package in R (Langfelder and Horvath, 2008). Briefly, it started with the calculation of the Pearson correlation of the gene expressions for all gene pairs. Next, the similarity matrix was transformed into the adjacency matrix using a power adjacency function (Zhang and Horvath, 2005) for screening many pairwise correlations without considering the direction of the relationship. It applied an adjacency function to weight edges between two different genes; the best threshold parameters were selected to meet the criteria of the approximate scale-free topology network (Barabasi and Bonabeau, 2003). In our case, the soft threshold was determined as the lowest possible value according to a scale-free topology. To build a scale-free network, we chose power 10, which is the lowest power for which the scale-free topology fit index curve flattens out upon reaching a high value ($R^2 = 0.9010$).

### Two-State Co-expression Networks

To better understand the gene co-regulation and co-expression changes between normal and salt-stress conditions, the co-expression networks were built separately for the salinity and normal states to investigate the differences between the two situations (Suratanee et al., 2018). This analysis identifies the pairs of genes that have their interaction changed during such a transition. A transition from a normal state to a salt stress state is related to the perturbation of a set of genes that can propagate through the network and affect other connected genes. To investigate the role of key genes in the state transition of a biological system, the two networks were first constructed in the same manner as performing a global co-expression network prior. In detail, the first network is a so-called 'normal-state network' constructed from our control data set and using all time points. The second network is a 'salinity-state network' constructed from our salt-stress data of all time points. After that, all network properties and centrality measures were applied to both networks. The comparison of both networks was analyzed in terms of network properties.

To identify the list of genes that respond to the change of co-regulation and co-expression levels in these two different conditions, the key gene score was calculated for each network as we had done for the global network. Then, the less important genes (low score) in the normal state but of higher importance (high score) in the salinity network were selected as our key genes in this content as well.

## Module Detections

After a co-expression network has been constructed, the next step is to detect modules of co-expressed genes. Modules are defined as clusters of highly co-expressed groups. We compute the topological overlap matrix (TOM) that provides a similarity measure. TOM is high if genes have many shared neighbors, and a high TOM implies that genes have similar expression patterns and then turn it into a dissimilarity measure (topological overlap measure dissimilarity). Next, we perform hierarchical clustering of genes to group genes into modules using average linkage hierarchical clustering coupled with the TOM-based dissimilarity. Based on a hierarchical cluster tree, modules were defined as branches, and close modules merged. The outputs' module colors were related to time points as module-trait relationships (MTRs). Each module at different time points

implied that its gene members were either upregulated or downregulated at different time points after treatment with salt.

## Node Properties Based on Centrality Measures

Central nodes are known to play an important role in the structure, community, and real-world meaning in the essentiality of the network (Koschutzki and Schreiber, 2008). Many centrality measures have been developed in graph and network theory (Pavlopoulos et al., 2011). To estimate our genes in terms of network-based contents, we applied three commonly used centralities known as degree, betweenness centrality, and closeness centrality, and one local density measure known as clustering coefficient. These four measures are well-known and simple to be calculated and understood their meaning to the biological networks. They are very useful to key players in biological processes such as metabolites, genes, proteins, miRNAs, or transcription factors in many complex biology systems (Koschutzki and Schreiber, 2008; Jalili et al., 2016; Suratanee et al., 2018; Liu et al., 2020). These properties can all be achieved using Cytoscape (version 3.7.1.; Shannon et al., 2003). The descriptions of all measures are as follows.

Degree (DG) is a simple centrality measure that counts the number of neighbors of a node. If a node has many neighbors, it is important in the network because the higher the degree, the more central the nodes (Tang et al., 2014). Mathematically, the degree centrality of a vertex $v$ can be defined as $C_{DG}(v) = \deg(v)$, where $\deg(v)$ is the number of direct connections a node has with other nodes (Pavlopoulos et al., 2011).

Betweenness centrality (BW) is one of the most important centrality indices, which shows important nodes that lie on a high proportion of paths between other nodes in the network (Goh et al., 2003). The BW is defined as $C_{BW}(v) = \sum_{s \neq n \neq t} \left(\sigma_{st}(v) / \sigma_{st}\right)$, where $s$ and $t$ are nodes in the network different from $v$, $\sigma_{st}$ denotes the number of shortest paths from $s$ to $t$, and $\sigma_{st}(v)$ is the number of shortest paths from $s$ to $t$ that $v$ lies on. Then, the betweenness value for each node $v$ is normalized by dividing by the number of node pairs excluding $v$: $(N-1)(N-2)/2$, where $N$ is the total number of nodes in the connected component that $v$ belongs to. Thus, the betweenness centrality of each node is a number between 0 and 1.

Closeness centrality (CN) detects nodes that can spread information very efficiently from a given node to other reachable nodes in the network. Nodes with a high closeness score have the shortest distances to all other nodes. The CN of a node $v$ is defined as $C_{CN}(v) = 1/avg\left(L(u,v)\right)$, where $L(u,v)$ is the length of the shortest path between the two nodes $u$ and $v$. The CN of each node is a number between 0 and 1 (Li et al., 2020).

Clustering coefficient (CC) is the measurement of the degree to which nodes in a graph tend to cluster together. The CC of a node $v$ is defined as $C_{CC}(v) = 2e_v / \left(k_v(k_v - 1)\right)$, where $k_v$ is the number of neighbors of $v$ and $e_v$ is the number of true connected pairs between all neighbors of $v$. The CC of a node is always a number between 0 and 1. If the neighborhood is fully connected, the CC is 1, and 0 means that there are hardly any connections in the neighborhood (Barabasi and Oltvai, 2004).

After applying these procedures, all genes in the network or module were ranked based on each measure in descending order. The use of centralities should flag genes that are important for the network structure and thus are key candidate genes.

## Functional and Pathway Enrichment Analysis

We used gene ontology enrichment analysis (GO) from RiceNetDB,[1] a genome-scale comprehensive regulatory database on *O. sativa*, to investigate the biological process, cellular component, and molecular function of genes in each network. In addition, we tested the significant enrichment of annotation in our gene set by downloading two files of the gene ontology annotation list of the GOSlim file from the Rice Genome Annotation Project,[2] one for 20,435 genes and the other for genes in each module. Then, the significance was tested by Fisher's exact test and, after Benjamini-Hochberg correction, a value of p of 0.05 was used as the threshold for significant enrichment.

Pathway enrichment analysis helps researchers identify biological pathways that are enriched in a gene list. The gene list of the *O. sativa* japonica (Japanese rice) pathway was downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa et al., 2021). Then we used the same method as GO to evaluate the statistical significance and identify the most significantly associated KEGG pathway.

# RESULTS

## 'Luang Pratahn' Rice Is a Salt-Tolerant Variety in Thailand

Several varieties of local Thai rice were evaluated for salt tolerance, and 'Luang Pratahn' rice is one of the salt-tolerant varieties. Therefore, its phenotype responding to salt stress was investigated in comparison with the responses of 'Pokkali' and 'IR29' rice, the standard for salt tolerance and susceptibility, respectively, together with 'Mayom', a local Thai rice variety susceptible to salt stress. Based on the stability index of the SIS, 'Mayom' and 'IR29' displayed salt susceptibility, while 'Pokkali' and 'Luang Pratahn' clearly demonstrated the tolerance phenotype (**Figure 1**).

The relevant traits of these four cultivars under salt stress are illustrated in **Figure 2**. 'Pokkali' and 'Luang Pratahn' could maintain leaf greenness (indicated by the soil plant analysis development (SPAD) value, **Figure 2A**), PSII efficiency (Fv/Fm; **Figure 2B**) and FW (**Figure 2C**) under salt stress conditions, while 'Mayom' and 'IR29' could not. Moreover, 'Pokkali' rice showed significantly higher DW (**Figure 2D**), CMS (**Figure 2E**), and RWC (**Figure 2F**) than other cultivars tested after 9 days of salt-stress treatment. However, 'Luang Pratahn' showed higher

[1] http://bis.zju.edu.cn/ricenetdb/

[2] http://rice.plantbiology.msu.edu/downloads_gad.shtml

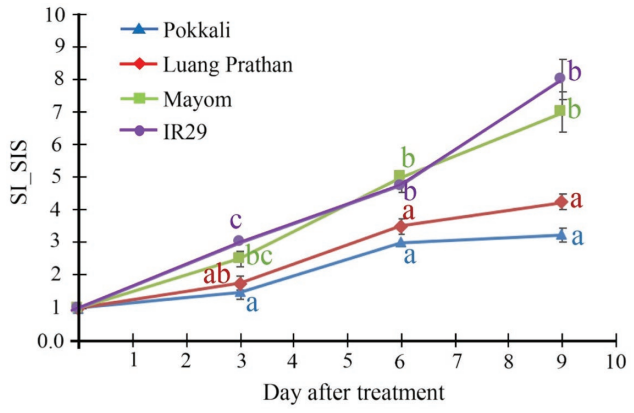


**FIGURE 1 |** Tolerance phenotype. Stability index (SI) of SIS of 'Pokkali', 'Luang Pratahn', 'Mayom' and 'IR29' rice at various seedling stages during a 9-day salt stress treatment. The different letters represent the significant differences in the means, based on Duncan's multiple range test, $p < 0.05$. The standard errors of the means are represented as bars.

DW, CMS, and RWC than 'Mayom' and 'IR29', confirming the salt tolerance phenotype of this cultivar. Therefore, 'Luang Pratahn' was selected for transcriptome analysis in the next step.

## Overview of Our Analysis Pipeline

Our analysis pipeline starts by collecting gene expression profiles of 'Luang Pratahn' in various time courses under control and salt-stress conditions, as shown in **Figure 3**. Then, the standard pipeline of data preprocessing and screening of this data set was performed by DESeq2. After that, the data were analyzed to construct a weighted co-expression network. Initially, a global co-expression network was constructed using all-time courses and conditions of the gene expression profiles. This global network was then used to identify important modules of significantly differentially expressed genes between control and salt-stress conditions. To identify which genes might be important for the significant modules, we built up two more co-expression networks separately under salt-stress conditions and control conditions. Network topologies, such as DG, BW, CN, and CC, were then investigated for each gene in these two networks to determine by the centrality measures which genes might be important under salt-stress conditions. The qualification of node centrality was also applied for each network and compared to find key genes that may be master regulators that orchestrate the salt-stress response. The identified modules from the global network and the resulting key genes from analyzing the node centralities from the two-state network were then tested with GO and pathway enrichment analysis. Finally, the selection of key genes and functional modules was reported, discussed, and validated by a literature search.

## Network Properties of the Global Network, Normal-State Network, and Salinity-State Network

The properties of the three weighted networks were compared across experimental conditions to investigate interacting genes. We first constructed a global gene co-expression network using the expression data from the 3′ Tag RNA-seq technique of all conditions and all-time points (0, 3, 6, 12, 24, and 48 h after treating salt in rice). Next, we constructed the two-state co-expression networks using control and salt-stress conditions and all time points. For all networks, we required the edge weight to be 0.1 or heavier (see **Materials and Methods**). **Table 1** summarizes the important structural properties of these three networks. The number of significant nodes in the global network was 1,183, while those of normal and salinity networks were 1,385 and 1,386, respectively. The number of significant co-expression relationships between two nodes were 94,797 edges for the global network, 181,121 edges for the normal-state network, and 186,083 for the salinity-state network. This indicated that the network of salinity state was slightly more complex than those of the normal state network, while the numbers of edges in the global network were roughly half those of both networks. The network diameters of both normal-state and salinity-state networks were quite different from those of the global network. This might imply that the global network had fewer links or connections, although the power of detecting connections may decrease when using the expression data of both salinity and normal conditions are combined to calculate the correlations. Moreover, the average clustering coefficients also suggested that these networks were quite dense. All follow the power-law distribution. Consistent with the hypothesis of decreased discovery power, the global network contained many more low-degree nodes than the other two networks, as shown in **Figure 4**.

## Functional Modules Over Time-Course Detection

To identify functional modules related to each time-course expression sampled in 'Laung Pratahn' rice, we clustered the global co-expression network into different groups of highly co-expressed genes, corresponding to the various time points. We evaluated a total of eight module-detection algorithms from WGCNA (see **Materials and Methods**). The result is shown in **Figure 5A** as the gene dendrograms or clustering trees with different colors. Each module contained 43–472 significant genes. The gray classification is reserved for genes outside of all modules. Module-trait associations were estimated using the correlation between the eigengene of each module and time point (see **Materials and Methods**) as a result of MTRs (**Figure 5B**). Each block shows the correlation coefficient values and the value of p between the gene expression level of the eigengenes in the detected modules on the y-axis and the time point on the x-axis. The color intensity describes the strength of the correlation. The results indicated that some modules were positively or negatively correlated with the expression profiles between control and salt conditions under different time points. Based on MTRs, we selected modules showing $|\text{correlation}| > 0.50$ and value of $p < 0.05$ as significant trait-related modules. Therefore, we obtained six significant modules highly related to salt stress timepoints 6, 12, and 48.

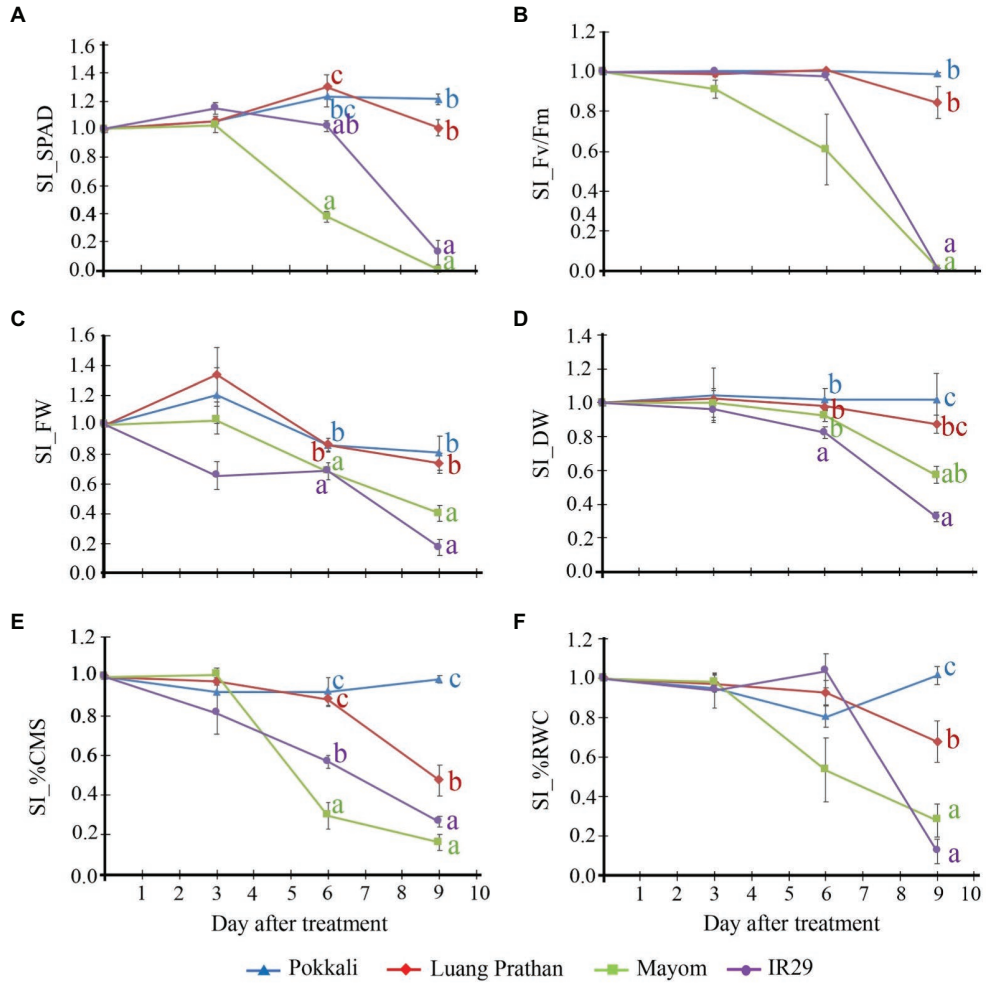


**FIGURE 2 |** Relevant traits of these four cultivars under salt stress. Stability index (SI) of leaf greenness (SPAD; **A**), PSII efficiency (Fv/Fm; **B**), shoot FW **(C)**, shoot DW **(D)**, CMS **(E)**, and RWC **(F)** of 'Pokkali', 'Luang Pratahn', 'Mayom' and 'IR29' rice at various seedling stages during a 9-day salt stress treatment. The different letters represent the significant differences in the means, based on Duncan's multiple range test, $p < 0.05$. The standard errors of the means are represented as bars.

Five modules were upregulated at different time points after the salt treatment. Yellow module was upregulated after treating with salt at 6 h (MTRs = 0.79, value of $p$ = 2e-08). Blue module (MTRs = 0.65, value of p = 2e-05), black module (MTRs = 0.77, value of $p$ = 8e-08), and green module (MTRs = 0.97, value of $p$ = 4e-21) were also up-related at 12 h. Red module (MTRs = 0.53, value of $p$ = 9e-04) was upregulated at 48 h. Only turquoise module (MTRs = −0.65, value of p = 2e-05) was downregulated at 12 h. This suggests that after increasing $Na^+$ and $Cl^-$ accumulation, rice is susceptible to salinity and develop the regulatory mechanisms to improve rice tolerance to salt stress both of activation and inhibition of the gene expression profile at different time points.

## Go and Pathway Enrichment Analysis

GO, and pathway enrichment analysis for each module was explored in three groups, i.e., biological process, cellular component, and molecular function, significantly enriched in all modules shown in **Supplementary Table S1**. Focusing on stress signaling pathways on these six modules, at 6 h, most of the genes in the yellow module were regulated after salt stress. These genes were significantly enriched in the process of response to stress, response to abiotic stimulus, and response to extracellular stimulus (**Figure 6A**). At 12 h, genes in the turquoise module were downregulated after salt stress. Genes in this group were enriched in response to abiotic stimulus (**Figure 6B**). The genes in blue, black, and green modules were strongly upregulated. In the blue module, genes were enriched in the ribosome, structural molecule activity, translation, and response to abiotic stimulus (**Figure 6C**). The black module with genes involved in thylakoid, photosynthesis, and membrane (**Figure 6D**). The green module was enriched with genes involved in response to abiotic stimulus, response to stress, and metabolic process (**Figure 6E**). At 48 h, the red module consisted of strongly upregulated genes enriched for response

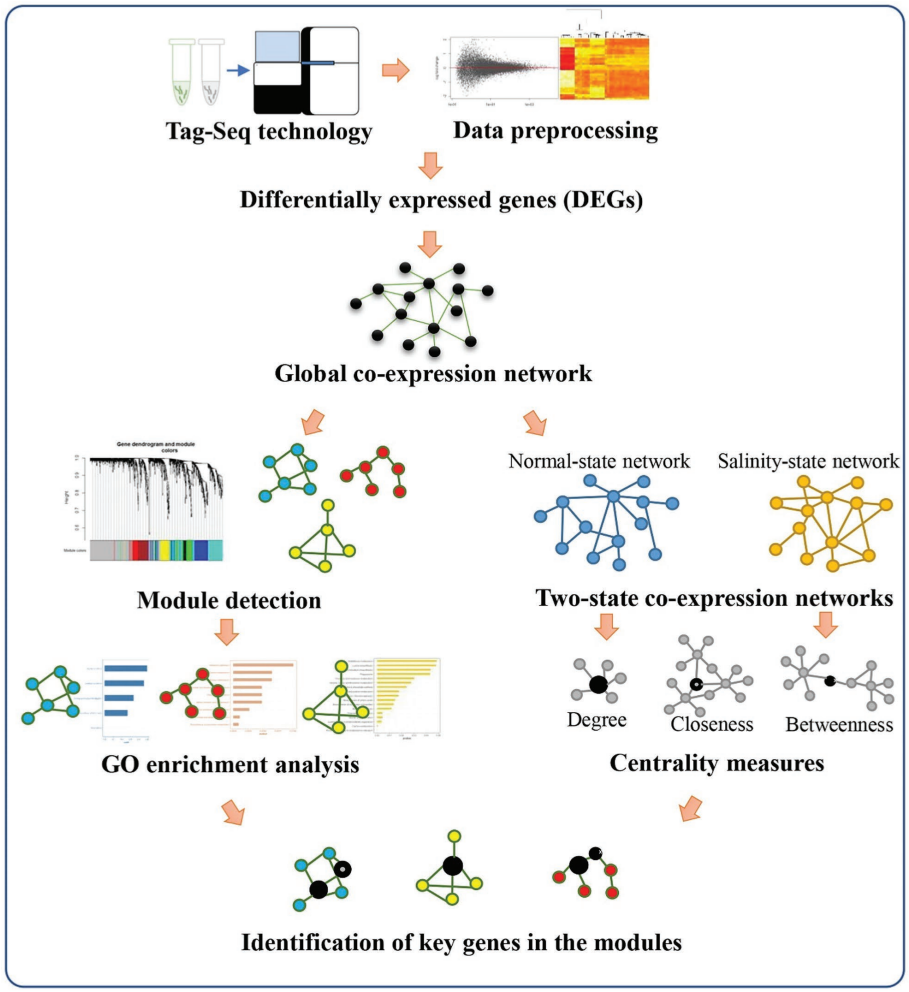


**FIGURE 3 |** Overview of our analysis pipeline. Expression data from Tag-Seq technology is processed to find out differentially expressed genes and construct a global co-expression network. The module-trait relationship detection is also performed by the WGCNA package. In parallel, the normal-state network and salinity-state network are constructed to calculate centralities for each gene in the networks. The comparison of the change in the centrality values for each gene is performed to identify key genes for each module.

**TABLE 1 |** Node and network properties of three co-expression networks.

| Network properties | Global network | Normal-state network | Salinity-state network |
|---|---|---|---|
| Number of nodes | 1,183 | 1,385 | 1,386 |
| Number of edges | 94,797 | 181,121 | 186,083 |
| Average degree | 160.265 | 261.547 | 268.518 |
| Diameter | 11 | 6 | 5 |
| Average clustering coefficient | 0.632 | 0.630 | 0.622 |
| Number of connections per node | 80.132 | 130.77 | 134.25 |

to stress, response to biotic stimulus, and metabolic pathways (**Figure 6F**). Based on the GO enrichment analysis, the genes involved with these six modules were enriched in the salinity response.

To investigate which pathways are involved in salinity stress responses, we performed KEGG pathway analysis in all modules, as shown in **Supplementary Table S2**. Based on the six significant modules, KEGG pathway enrichment results indicated some important pathways for regulating plant adaptation salt stress. Briefly, at 6 h, protein processing in the endoplasmic reticulum, carbon metabolism, biosynthesis of secondary metabolites, glycine, serine, and threonine metabolism, and metabolic pathways were pathways enriched in the yellow module (**Figure 7A**). At 12 h, in the turquoise module, biosynthesis of secondary metabolites, spliceosome, and RNA transport was significantly enriched (**Figure 7B**). The genes in the blue module were enriched for the ribosome, ribosome biogenesis in eukaryotes, and glycine, serine, and threonine metabolism (**Figure 7C**). In the black module, we found photosynthesis, photosynthesis-antenna proteins, and nicotinate and nicotinamide metabolism (**Figure 7D**). In the green module, the biosynthesis

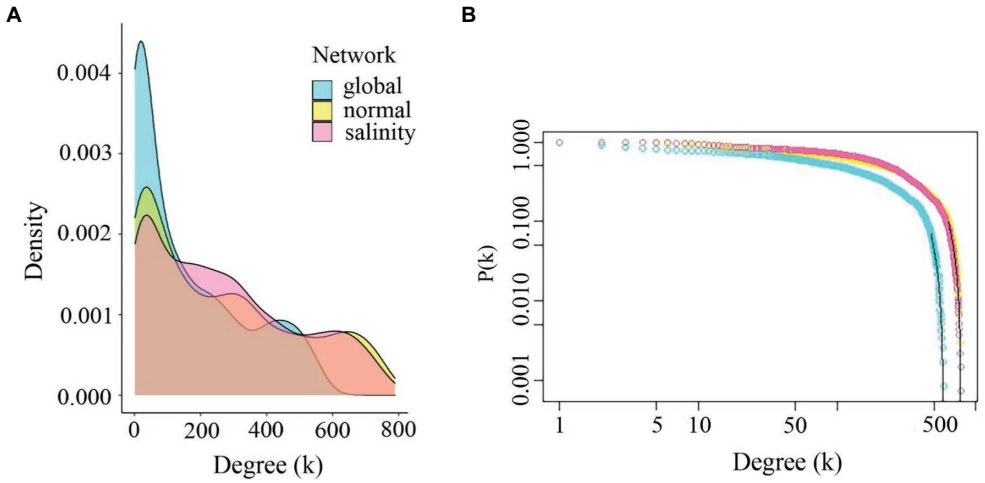


**FIGURE 4 |** Comparison of degree distributions and power-law responses. **(A)** Degree distributions for global, normal-state, and salinity-state networks. **(B)** Power-law plots of the global network, normal-state network, and salinity-state network. All networks follow the power-law distribution. The global network shows high low-degree genes compared to the other networks.

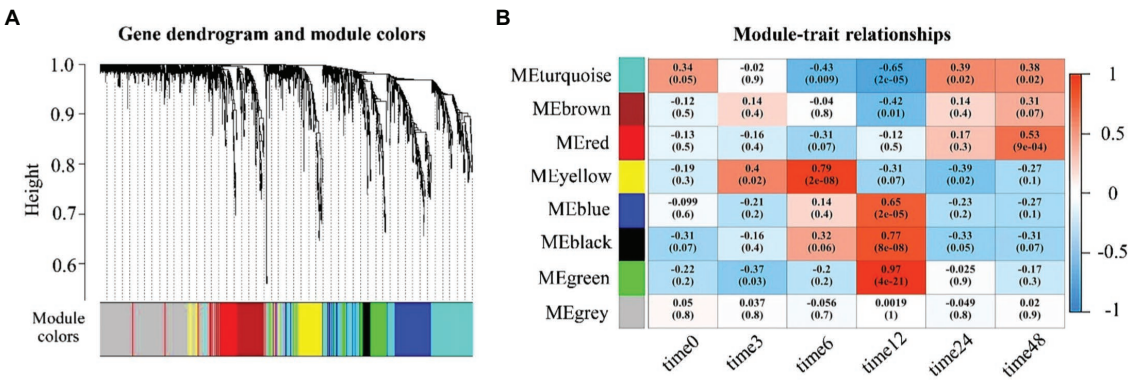


**FIGURE 5 |** Module-trait relationship results. **(A)** Clustering dendrograms of genes by WGCNA, with dissimilarity based on the topological overlap. The color row indicated the corresponding module colors which each colored represents a module that contains a group of highly connected genes. **(B)** Heatmap plot of the correlation of WGCNA modules with time points shows a Module-Trait Relationships (MTR). Each row corresponds to a module (labeled by color) and each column to a time result. MTRs are colored based on their correlation: red indicates a strong positive correlation, and blue indicates a strong negative correlation.

of secondary metabolites, ascorbate, and aldarate metabolism, and glycine, serine, and threonine metabolism were enriched (**Figure 7E**). At 48 h, the genes in the red module were enriched in the phosphatidylinositol signaling system, biosynthesis of unsaturated fatty acids, glutathione metabolism, biosynthesis of secondary metabolites, and metabolic pathways (**Figure 7F**). For all significant modules, most of the genes were enriched in the same KEGG pathway, including biosynthesis of secondary metabolites, glycine, serine, and threonine metabolism, glutathione metabolism, carbon metabolism, and metabolic pathways (Hasanuzzaman et al., 2017; Muthuramalingam et al., 2018; Wang et al., 2018). Genes in the black module (**Figure 7C**) were enriched in different pathways compared to other modules such as nicotinate and nicotinamide metabolism, carotenoid biosynthesis, and arginine biosynthesis (Noctor et al., 2006).

## Identification of Key Genes in Each Module

To identify key genes responding to salt stress in each module, we examined each gene in the module with the change in the centrality measure values between normal and salinity states. Genes that are less important (having low score) in the normal network but get high importance (having high score) in the salinity network are then selected as our key genes. In total, 107 key gene candidates identified by these criteria for all modules are shown in **Supplementary Table S3**. Forty-seven genes in the list are found related to salt stress or involved in various stress responses or belong to a gene family that plays a role in stress response with literature support (**Supplementary Table S3**). **Figure 8** shows the interaction networks of genes found in each module and the key genes.

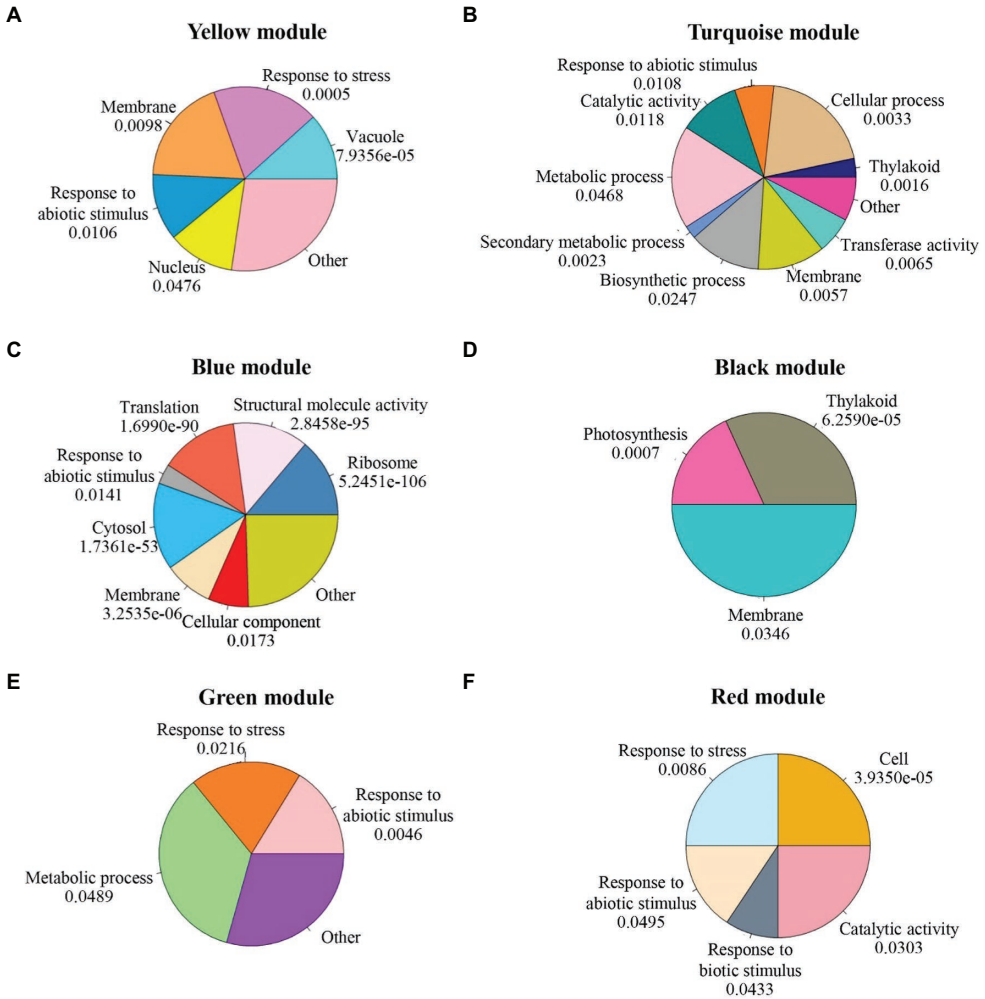


**FIGURE 6 |** GO enrichment analysis results for each module. The *p*-values were adjusted by Benjamini-Hochberg correction. **(A)** yellow module; **(B)** turquoise module;**(C)** blue module; **(D)** black module; **(E)** green module; and **(F)** red module.

Genes from all modules, together with the information from gene functions in other plant species, can be proposed for their role and interactions, as shown in **Figure 9**. To highlight biological significance for each module, its genes with the potential to involve in salt stress tolerance are noted as follows.

## Yellow Module

In this module, nine key genes were identified, and three out of those genes, which are *LOC_Os10g04860* (*OsOAA*), *LOC_Os12g43640* (*OsHAIKU2*), and *LOC_Os01g56460* (encoding mitochondria glycoprotein), were identified by more than one centrality marked blue in **Figure 8A**. These genes have the most edges directly connected and are indirectly connected with the shortest path length to other nodes in the network. They are important nodes in various circumstances. In **Figure 8A**, *LOC_Os01g56460*, *LOC_Os02g21009* (*OsCAX1*), *LOC_Os06g48960* (*AIG2- like* gene), and *LOC_Os02g57620* (citrate transporter) are detected important by betweenness centrality. This means that these three genes are global loading hubs that the community in the module highly contact or transmit a signal heavily passing through these genes in the salinity-state network than the normal-state network. The rest are *LOC_Os08g43560* (*OsAPX4*), *LOC_Os01g15270* (expressed protein), and *LOC_Os09g29380* (expressed protein), which are indicated by high transitivity or clustering coefficient. This means their neighboring genes had more connections or co-regulated to function in salt stress more than in normal situations.

Taken together, a proposed mechanism for this module can be captured, as shown in the yellow module in **Figure 9**. Receptor-like protein kinase HAIKU2 precursor, encoded from *LOC_Os11g12560*, was reported to be upregulated in the drought-tolerant cultivar., Douradão, under drought stress. Moreover, they also found that two peroxidase genes were upregulated (Silveira et al., 2015). This was consistent with our study that both receptor-like protein kinase HAIKU2 and *OsAPx4* (*LOC_Os08g43560*) were predicted as hub genes in

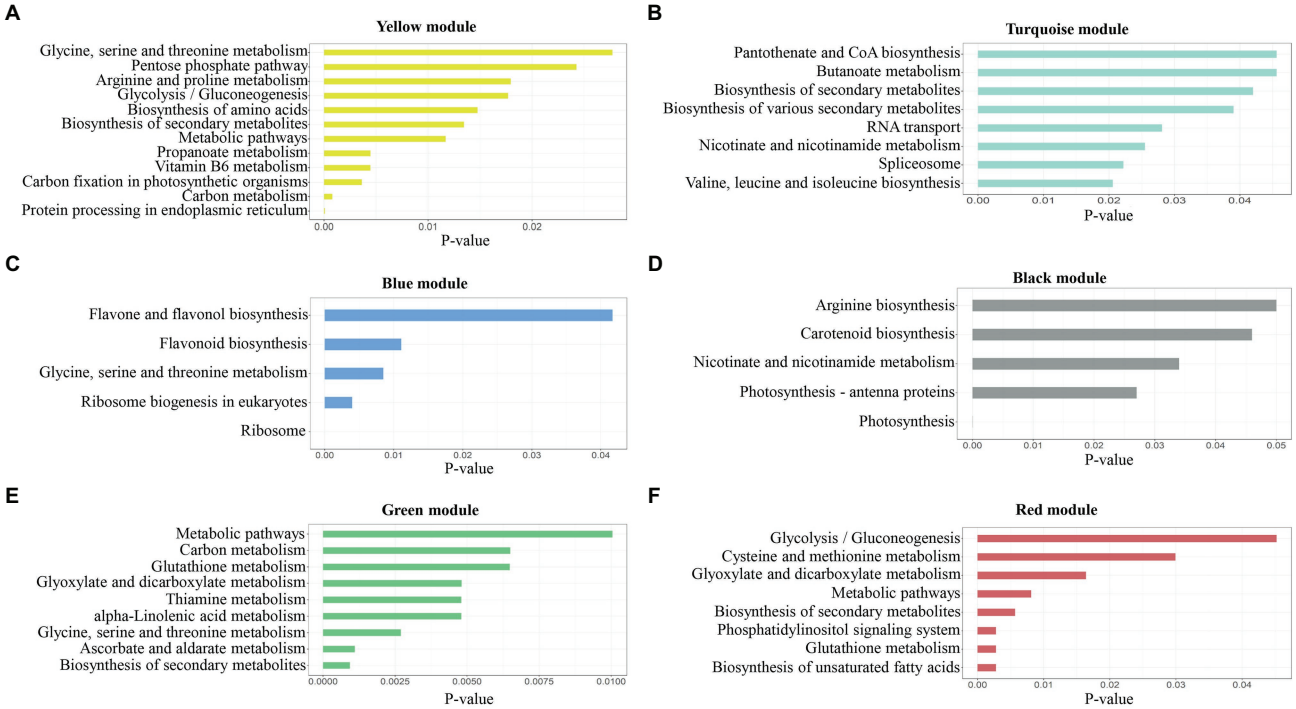


**FIGURE 7 |** Pathway enrichment analysis results for each module. The p-values were adjusted by Benjamini-Hochberg correction. **(A)** yellow module; **(B)** turquoise module; **(C)** blue module; **(D)** black module; **(E)** green module; and **(F)** red module.

the yellow module. In Arabidopsis, it was reported that two receptor-like kinases, LRR1, and KIN7 are targeted by PLANT U-BOX PROTEIN 11 (PUB11), an E3 ubiquitin ligase, and lead to their degradation during drought stress. This is regulated through abscisic acid (Chen et al., 2021c). The receptor-like protein kinase HAIKU2 is a type of leucine-rich receptor-like protein kinase (LRR-RLK). Therefore, the linkage among LRR-RLK, ABA signaling, and ROS scavenging process should be explored in the rice system to validate this module. Moreover, *OsCAX1* (*LOC_Os02g21009*) was also predicted as the major node in this module. In Arabidopsis, *CAX1* was reported to play a role in $Ca^{2+}$ cellular homeostasis in response to oxidative stress (Baliardini et al., 2016). Thus, in this module, we proposed that salt stress triggers the HAIKU2 receptor and the signal passed through ABA and oxidative stress, leading to the activation of *OsCAX1* to maintain $Ca^{2+}$ cellular homeostasis during salt stress conditions.

## Turquoise Module

The genes in this module were downregulated under the salt stress condition. Twenty-nine key genes have been detected with high values of thse centralities in the salinity state, as shown in **Figure 8B**. *LOC_Os01g02900* (*Glycosyltransferase*), *LOC_Os02g37090* (*Hydrolase*), *LOC_Os03g22380* (*OsSRp32*), *LOC_Os07g08440* (*OsPIF3*), *LOC_Os08g05960* (*OsDR10*), *LOC_Os09g30180* (F-box containing protein), and *LOC_Os12g03260* (*OsMATE53*) were detected for more than one measure. It means that they are quite important for the salinity-state network as a highly connected hub, heavily loading nodes, and/or close to the other nodes. Thirteen genes which are *LOC_Os01g05490* (*Triosephosphate isomerase*), *LOC_Os01g53060* (encoding peroxisomal membrane protein), *LOC_Os01g57030* (expressed proteins), *LOC_Os01g63810* (encoding starch binding domain-containing protein), *LOC_Os03g57110* (expressed protein), *LOC_Os05g48050* (a ribosomal protein), *LOC_Os06g06170* (expressed protein), *LOC_Os06g24730* (*OsNYC3*, Pheophytinase), *LOC_Os07g12110* (*OseIF3e*), *LOC_Os07g42714* (expressed protein), *LOC_Os08g43170* (*HMG-CoA synthase*), *LOC_Os10g42040* (*OsRIR1b*), *LOC_Os11g07916* (*nifU*) were detected with more loads by the betweenness centrality in the salinity-state network. Two genes, *LOC_Os03g63074* (*OsPAP15*) was located near the others closer under the salt stress detected by the closeness centrality. Among the neighbors for each of *LOC_Os02g27220* (*OsPP2C14*), *LOC_Os02g44940* (*OsRALFL8*), *LOC_Os03g63590* (metallo-beta-lactamase), *LOC_Os04g31030* (nitrate-induced NOI protein), *LOC_Os06g08640* (transferase), *LOC_Os08g10080* (*OMTN6, OsNAC104, ONAC104*), and *LOC_Os08g42400* (*OsNAC5, ONAC5*) were highly connected to each other which were detected by the clustering coefficient.

Several mechanisms are predicted in the turquoise module. One mechanism is the chloroplast response. *LOC_Os07g08440*, one of the nodes with high centrality, encodes basic helix–loop–helix protein 8 (bHLH8), which is similar to phytochrome-interacting factor 3 (PIF3) in Arabidopsis. The expression of

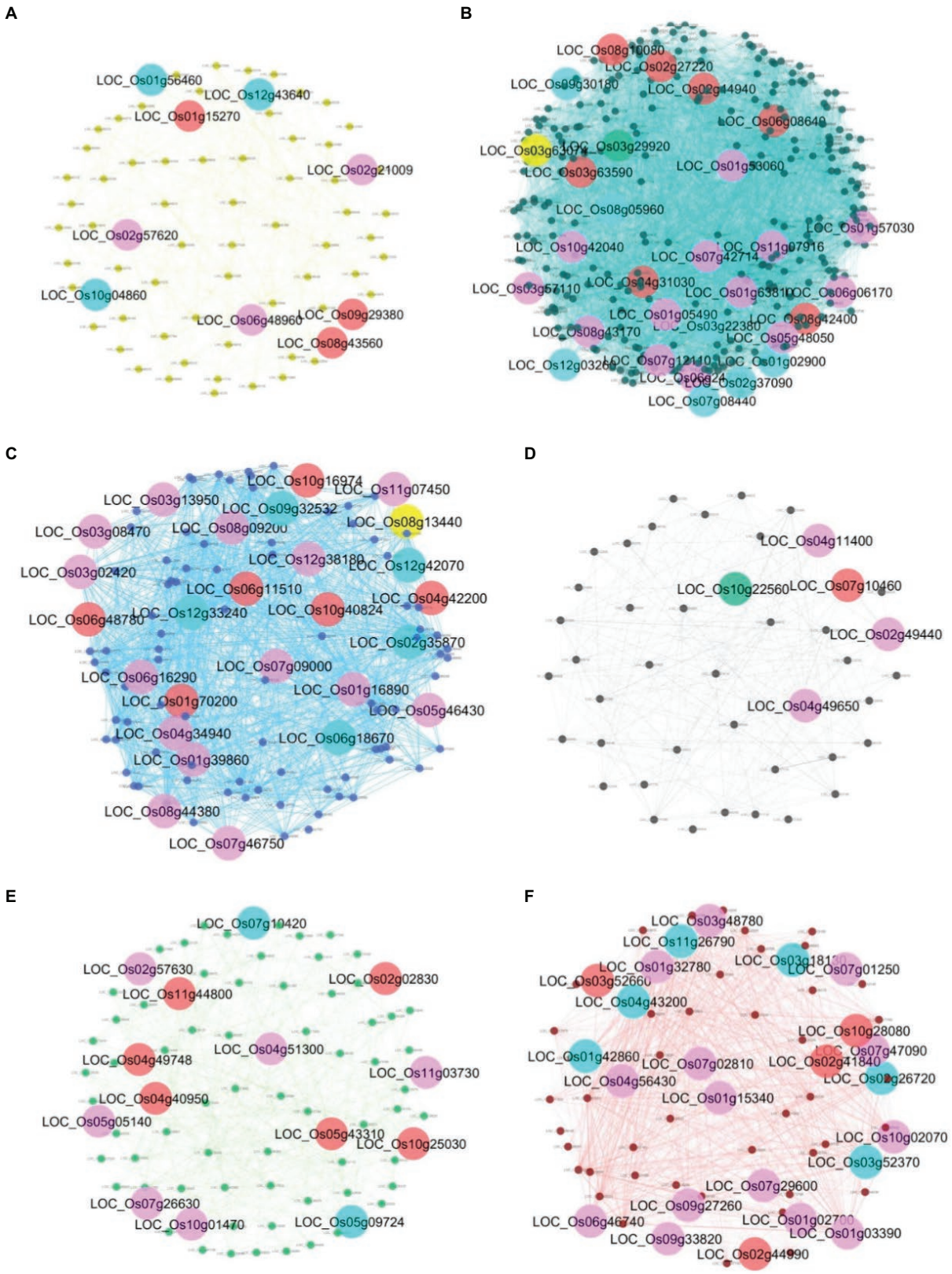


**FIGURE 8 |** Gene networks of each module and its key genes. **(A)** yellow module; **(B)** turquoise module;**(C)** blue module; **(D)** black module; **(E)** green module; and **(F)** red module. The big circle nodes with green, purple, yellow, and red represent the key genes from DG, BW, CN, and CC, respectively. The key genes that are identified by more than one centrality are marked as blue nodes.

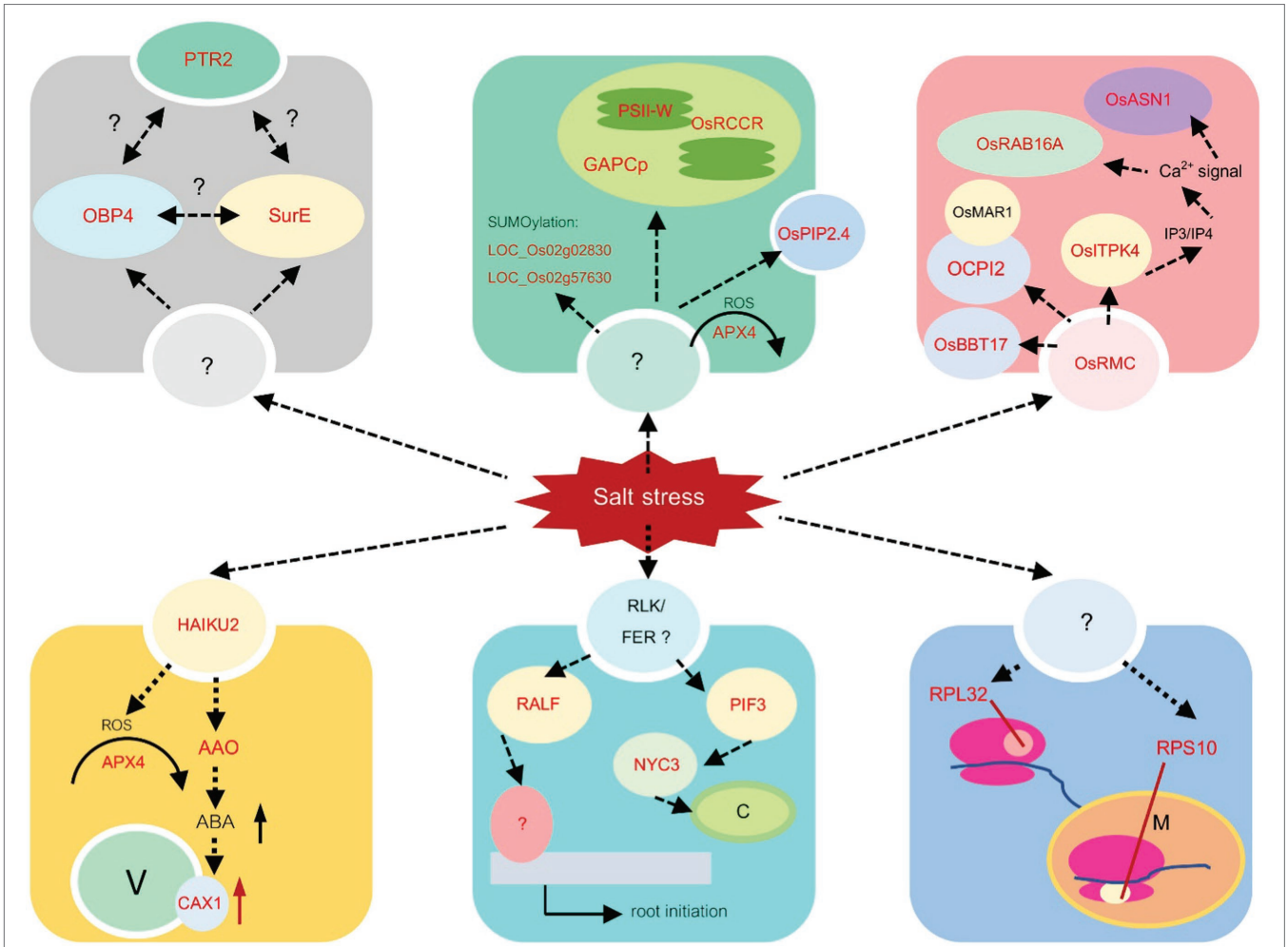


**FIGURE 9 |** Salt stress response mechanisms. The mechanisms based on the information from yellow, turquoise, blue, black, green, and red modules are proposed. The identified genes *via* transcriptome analysis in this study are shown in red letters, while the information from other research is written in black letters.

maize PIF3 (ZmPIF3) in rice under the ubiquitin promoter led to drought and salt tolerance (Gao et al., 2015). PIF3, however, was reported to be a repressor of chloroplast development (Stephenson et al., 2009). It regulates chlorophyll biosynthesis genes and ROS-responsive genes (Chen et al., 2013). Within this module, *LOC_Os06g24730* encoding pheophytinase (non-yellow coloring 3, *OsNYC3*) was also predicted. This enzyme functions during chlorophyll degradation (Morita et al., 2009). Therefore, the role of *OsPIF3* in salt tolerance should be investigated in the future. The second mechanism found in the module is post-transcription regulation. *OsSRp32* (*LOC_Os03g22380*) is one of ARGININE/SERINE-RICH SPLICING FACTORs reported by Isshiki et al. (2006). This gene has a high centrality value, suggesting regulation of other genes during salt stress. It contains 2 RNA recognition motifs (RRMs) in the N-terminus and an Arg/Ser-rich (SR) domain for protein–protein interaction in the C terminus. The plant SR proteins may contribute to constitutive and alternative splicing of rice pre-mRNA (Lopato et al., 1996; Isshiki et al., 2006). The connection between the function of SR proteins in RNA splicing as a post-transcriptional control and abiotic stress tolerance has been reported in various species (Morton et al., 2019; Kishor et al., 2020), including Arabidopsis, rice, and *Physcomitrella patens* (Melo et al., 2020).

Two additional genes with high centralities values are related to biotic stress response, *OsDR10,* and *OsMATE53*. *OsDR10* is a rice tribe-specific gene responsible for disease tolerance. *OsDR10* suppression in transgenic rice could enhance the resistance to bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (Xiao et al., 2009). The link between disease resistance and abiotic stress resistance is still unclear at this moment. *LOC_Os12g03260* (*OsMATE53*) encodes multi antimicrobial extrusion (MATE) protein. *OsMATE1* and *OsMATE2* were shown to suppress disease resistance (Tiwari et al., 2014). However, the MATE protein has not been previously implicated in the abiotic stress response. According to Huang et al. (2019), *LOC_Os12g03260* (*OsMATE53*) is one of the tandemly duplicated MATE genes on chromosome 12. Based on the phylogenetic analysis of the MATE gene family, *OsMATE53* is on the same clade of *OsMATE9* (*OsMATE1*), which is involved

in disease and stress tolerance (Tiwari et al., 2014). The involvement of *OsMATE53* in salt stress response should be explored in the future.

The last mechanism detected in the turquoise module suggests the root development due to salt stress. *LOC_Os02g44940*, encoding the Rapid Alkalinization Factor (RALFL8) family protein precursor. RALFL8 may participate in a new salt tolerance pathway (Zhao et al., 2021). The interaction between the receptor-like kinase (RLK) FERONIA (FER) and RALF1 controls root growth in Arabidopsis (Yu et al., 2020). Therefore, the turquoise module may control root growth during salt stress, as proposed in **Figure 9**. We detected that OsRALFL8 is connected to two NAC transcription factors, OsNAC5 and OsNAC104. Overexpression of *OsNAC5* improved salt tolerance under high salt stress (Takasaki et al., 2010), while OsNAC104 or OMTN6 was identified as the negative regulator for drought tolerance in rice (Fang et al., 2014). Therefore, it will be worthwhile to investigate the role of OsRAFL8, OsNAC5, and OsNAC104 roles in salt tolerance in rice.

## Blue Module

In this module, 26 genes were implicated in the salinity-state network, as shown in **Figure 8C**. Four genes, *LOC_Os02g35870* (*Avr9 elicitor response protein*), *LOC_Os06g18870* (*anthocyanidin 3-O-glucosyltransferase*), *LOC_Os09g32532* (*OsRPL32*), and *LOC_Os12g33240* (*mitochondrial ribosomal protein S10*), were predicted to be important during salt stress by more than one centrality. *OsRPL32* and *mitochondrial ribosomal protein S10* are hub genes in the salinity-state network since they have high degree connectivity and high closeness values. Most of the genes in this module have a high degree in both the normal-state and salinity-state networks. Therefore, no genes were detected by hub degree centrality in the salinity-state network. Fourteen genes were detected by the betweenness centrality. Interestingly, most of them are members of the ribosome pathway such as *OsRPL32*, *LOC_Os01g16890* (*60S ribosomal protein L30*), *LOC_Os05g46430* (*60S ribosomal protein L28-1*), *LOC_Os06g16290* (*ribosomal protein L7Ae*), *LOC_Os08g44380* (*L1P ribosomal protein*), and *LOC_Os06g48780* (*60S acidic ribosomal protein*). One transporter gene, *LOC_Os07g09000* (Phosphate transporter traffic facilitator 1, *OsPHF1*), and one transcription factor, *LOC_Os03g08470* (*OsERF1*), were also found by the betweenness centrality in the salinity-state network. One gene detected by closeness centrality was *LOC_Os08g13440* (*OsGLP8-12*), and the other six genes were detected by the clustering coefficient (**Supplementary Table S3**).

Ribosomal proteins have been investigated for their potential involvement in stress tolerance. Moin et al. (2016) generated enhancer-based activation-tagged plants and found that the 60 S ribosomal genes, *RPL6* and *RPL23A,* were activated. Constitutive expression of *RPL23A* in transgenic rice led to the increase in fresh weight, root length, proline, and chlorophyll contents under drought and salt stresses (Moin et al., 2017). Moreover, overexpression of *RPL6* enhanced salt tolerance (Moin et al., 2021), while the knockdown of *RPL14B* in cotton led to susceptibility in drought and salt stress (Linyerera et al., 2021). Therefore, *OsRPL32*'s role in salt tolerance should be validated. Mitochondria ribosomal protein, RPS10, was also predicted with a high centrality value, supporting the hypothesis that ribosome activities in both cytoplasm and organelles play an important role in salt tolerance in rice (**Figure 9**).

## Black Module

Five genes were detected as important in the salinity-state network when compared to the normal-state network (**Figure 8D**). One key gene, *LOC_Os07g10460* encodes 5′-nucleotidase SurE, which is an enzyme in the nicotinate and nicotinamide metabolism pathway (osa00760), acts among dense local communities detected by the clustering coefficient. It may represent a close group of co-expressed genes belonging to the same family or domain protein. The peptide transporter gene *LOC_Os10g22560* (*OsPTR2*) was found by the high degree connectivity in the salt stress condition. *LOC_Os04g11400* (expressed protein), *LOC_Os02g49440* (*OsOBF4*), and *LOC_Os04g49650* (DUF581 domain-containing protein) were detected by the betweenness centrality. Among these genes, *OsOBF4* is a transcription factor that has higher loads in the salt stress condition than the normal situation based on the betweenness centrality. Notably, the genes predicted in this module have never been reported to be involved in salt stress or other abiotic stress. Therefore, we plan to investigate how these genes, peptide transporter *OsPTR2*, *OsOBP4,* and *OsSurE*, function in salt stress response (**Figure 9**).

OBF (OCS element binding factors) is a class of basic-region leucine zipper (bZIP) transcription factors. In Arabidopsis, AtOBF4 binds to transcription factors with AP2/EREBP (ethylene-responsive element binding proteins) domain, suggesting that OBF4 responds to ethylene (Büttner and Singh, 1997). Moreover, OBF4 was shown to bind the promoter of *FLOWERING LOCUS T* (*FT*) and regulate its expression by forming a complex with CONSTANS (CO), the positive regulator for floral induction (Song et al., 2008). Further investigation of *OsOBF4* function in salt stress response and the involvement in floral induction is recommended.

## Green Module

As shown in **Figure 8E**, 14 genes were detected as key genes important in the salinity state compared to the normal state. *LOC_Os02g57630* (ubiquitin carboxyl-terminal hydrolase; *OsUCH2*), *LOC_Os04g51300* (ascorbate peroxidase; OsAPX), *LOC_Os11g03730* (Arabinofuranosidase 3; *OsARAF3*), *LOC_Os05g05140* (expressed protein), *LOC_Os07g26630* (Aquaporin PIP2.4; *OsPIP2.4*), and *LOC_Os10g01470* (homeobox associated leucine zipper; *OsHOX15*) were detected by the betweenness centrality. *OsAPX* is involved with ascorbate and aldarate metabolism (osa00053), glutathione metabolism (osa00480). *LOC_Os11g03730* is found related to amino sugar and nucleotide sugar metabolism (osa00520). *OsPIP2.4* is a transporter gene, while *OsHOX15* is a transcription factor. Five genes which are *LOC_Os11g44800* (expressed protein), *LOC_Os04g49748* (purine permease, *OsPUP6*), *LOC_Os04g40950* (Glyceraldehyde-3-phosphate dehydrogenase; *OsGAPDH*), *LOC_Os05g43310*

(Photosystem II reaction center W protein), and *LOC_Os10g25030* (red chlorophyll catabolite reductase; *OsRCCR1*) were detected by clustering coefficient, and two genes, *LOC_Os07g10420* (expressed protein) and *LOC_Os05g09724* (HAD superfamily phosphatase) are expected to be important because they were detected by more than one centrality. *OsGAPDH* (*LOC_Os04g40950*) is involved in glycolysis/gluconeogenesis (osa00010) and carbon fixation in photosynthetic organisms (osa00710) in metabolic pathways, while *LOC_Os02g02830* (Ubiquitin-conjugating enzyme 13; *OsUBC13*) may play a role in hormone-mediated stresses responses (E et al., 2015). *OsPUP6* (*LOC_Os04g49748*) is annotated as PUP-type cytokinin transporter 6.

*LOC_Os05g09724*, encoding HAD superfamily phosphatase, is the only annotated gene in the green module that was detected by DG and CN. One of the Haloacid Dehalogenase (HAD) superfamily, phosphoserine phosphatase from *Brassica juncea* L, was upregulated in salt stress (Purty et al., 2017) and the *HAD1* gene in soybean (*GmHAD1*) was shown to be involved in low phosphorus stress tolerance (Cai et al., 2018). In saline soil, P solubility is decreased due to high $Na^+$ concentration and soil pH (Martinez et al., 1996). The upregulation of HAD superfamily phosphatase (*LOC_Os05g09724*) gene may be related to the adaptation to P homeostasis under salt stress conditions. The responsive mechanism detected in the green module is located in plastids. Three genes involved in plastidial activity were predicted to be the major node genes, *LOC_Os04g40950* (*GAPCp*), encoding glyceraldehyde-3-phosphate dehydrogenase function in plastidial glycolytic pathway, *LOC_Os05g43310* encoding photosystem II reaction center W protein, and *LOC_Os10g25030* or *OsRCCR*, encoding red chlorophyll catabolite reductase. Plastidial glyceraldehyde-3-phosphate dehydrogenase was reported to be important to abiotic stress response in wheat. Overexpression of wheat *GAPCp* (*TaGAPCp*) enhanced chlorophyll accumulation and *TaGAPCp* could be induced by ABA and $H_2O_2$ (Li et al., 2019). Moreover, another *OsGAPDH* located on the same chromosome, *LOC_Os04g38600*, was identified as the major hub gene in drought tolerance (Chintakovid et al., 2017). *AtRCCR* involves chlorophyll catabolism (Wuthrich et al., 2000), supporting the involvement of the response in chloroplast detected in the green module. Moreover, in this module, genes involved in the ubiquitination process, *OsUBC13* and *OsUCH2*, were reported. Protein degradation by SUMOylation, the post-translational process *via* Small Ubiquitin-like Modifiers (SUMO) has been reported to regulate the rapid defense against environmental stresses, including drought, cold, heat, nutrient deficiency, and salt stresses (Ghimire et al., 2020). *OsUBC13* belongs to UBC class I and is induced by drought and salt stress (Zhiguo et al., 2015). Therefore, the regulation in the green module may involve post-translation control *via* SUMOylation to control the activity in chloroplast during salt stress as shown in **Figure 9**.

In this module, the gene encoding transcription factor (TF), *OsHOX15*, was predicted to be the key gene. *OsHOX15* is a homeobox-associated leucine zipper TF. It belongs to the HD-Zip II subfamily as it has a conserved 'CPSCE' motif downstream of the leucine zipper (Chan et al., 1998). Homeobox-associated leucine zipper TFs are involved in the regulation of plant development and stress-responsive mechanisms (Sharif et al., 2021). Two transporters are predicted in this module. *LOC_Os07g26630* encoding *OsPIP2.4* is identified as one of the key genes in the green module. It was reported to be predominantly expressed in roots with diurnal fluctuation. It also shows the high-water channel activity in the yeast system (Sakurai et al., 2005). Overexpression of *OsPIP2.4* in different rice cultivars resulted in different responses to drought stress due to the different physiological attributes (Nada and Abogadallah, 2020). The other transporter is *LOC_Os04g49748* encoding purine permease 6 (*OsPUP6*). It is predicted to have a role in cytokinin transport. Although there have been no reports on *OsPUP6* involved stress response, T-DNA insertion at the c-terminus of *OsPUP7* led to the increase in sensitivity to drought and salt stress. The mutant also showed an increase in plant height and grain size (Qi and Xiong, 2013). Moreover, the activation of purine permease, encoded by the *BIG GRAIN3* gene, potentially involved in cytokinin transport and led to salt tolerance in rice (Yin et al., 2020). Moreover, intermediate in purine catabolism, allantoin has been shown to have a role in abiotic stress tolerance (Kaur et al., 2021). *LOC_Os11g03730*, encoding Arabinofuranosidase 3 (*OsARAF3*), is another key gene in the green module. Downregulation of Arabinofuranosidase was detected in rice anther under salt stress conditions. It was thought to be involved in cell wall assembly and reorganization that led to cell wall loosening. The changes in Arabinofuranosidase activity in anthers may affect another dehiscence, leading to male sterility under salt stress in rice (Sarhadi et al., 2012).

Many of the key gene families in the green module are reported to be involved in salt tolerance. However, the connection among them has not been reported. The exploration of the interaction among genes in the module is recommended.

## Red Module

There are 23 genes identified as key genes for this module (see **Figure 8F**). Six genes, LOC_Os02g26720 (Inositol 1, 3, 4-trisphosphate 5/6-kinase; *OsITPK4*), LOC_Os03g18130 (asparagine synthetase; *OsASN1*), LOC_Os03g52370 (Proteinase inhibitor II; *PIII4*), LOC_Os01g42860 (*O. sativa* chymotrypsin protease inhibitor 2; *OCPI2*), LOC_Os04g43200 (caleosin related protein; *OsClo5*) and LOC_Os11g26790 (dehydrin; *OsRAB16A*) were detected by more than one centrality. Interestingly, *LOC_Os02g26720* is an inositol 1, 3, 4-triphosphate 5/6 kinase 4 (*OsITPK4*) involved in the inositol phosphate metabolism and phosphatidylinositol signaling system. This gene was detected by degree centrality, betweenness centrality, and closeness centrality in the salinity-state network compared to the normal network. Betweenness key genes were *LOC_Os03g48780* (Oxalate oxidase 4; *OsOXO4*), *LOC_Os11g26790* (*OsRAB16A*), *LOC_Os01g15340* (flowering-promoting factor-like 1, *OsFPFL1*), *LOC_Os07g01250* (*OsENODL18*), *LOC_Os07g47090* (*KIP1*), *LOC_Os10g02070* (Peroxidase A, class III peroxidase 126; *OsPrx126*), *LOC_Os06g46740* (early nodulin-like protein 18; *OsENODL18*), *LOC_Os09g33820* (Phospholipase A1),

*LOC_Os07g29600* (Zinc finger, RING/FYVE/PHD-type domain-containing protein, *OsRFPH2-17*), *LOC_Os01g02700* (protein kinase domain-containing protein), and *LOC_Os01g03390* (Bowman-Birk type bran trypsin inhibitor precursor, *OsBBT17*). *LOC_Os03g52660* (ATP synthase F1), *LOC_Os10g28080* (glycosyl hydrolase), *LOC_Os02g41840* (DUF584 domain-containing protein), and *LOC_Os02g44990* (F-box and DUF domain-containing protein; *OsFBDUF13*) were detected by clustering coefficients.

In this module, *OsITPK4* was detected. It was reported to be involved in drought and salt stress response (Du et al., 2011). Its expression is induced by salt stress up to 25-fold, compared to control. The high *OsITPK4* expression was detected in embryonic tissues. The function of this protein is to phosphorylate $IP_3$ to form $IP_4$. Both $IP_3$ and $IP_4$ are secondary messenger molecules responsible for mediating $Ca^{2+}$ levels to maintain $Ca^{2+}$ homeostasis (Wilson et al., 2001). Also, in this module, *LOC_Os04g56430*, which encodes cysteine-rich receptor-like protein kinase, *OsRMC*, was identified as a key gene. It is reported as the negative regulator of salt stress response in rice. Its expression can be induced by salt treatment *via* the regulation of the *OsEREBP2* transcription factor (Serra et al., 2013). Therefore, we propose that *OsRMC* may function as the receptor and other genes in this module *via* the IP3/IP4 and $Ca^{2+}$ levels. The functional genes for salt tolerance are also detected; *LOC_Os03g18130* encoding asparagine synthetase 1 (*OsASN1*) and *LOC_Os01g42860* encoding *O. sativa* chymotrypsin protease inhibitor 2 (*OCPI2*). The mutation in *OsASN1* resulted in a decrease in plant biomass and asparagine level in plant tissues (Luo et al., 2018). It is required for grain yield and grain protein content (Lee et al., 2020). In wheat, TaASN1 is induced by osmotic stress, salt stress, and ABA (Wang et al., 2005). Asparagine and proline are increased during salt stress in Arabidopsis. The *AtASN2* mutation resulted in susceptibility to salt stress (Maaroufi-Dguimi et al., 2011). OCPI2 has a role in salt tolerance in rice. *OCPI2* interacts with RING E3 ligase encoded by *O. sativa microtubule-associated RING finger protein 1* (*OsMAR1*) gene, which is highly expressed during salt and osmotic stress. The binding between *OCPI2* and RING E3 ligase leads to protein degradation *via* 26S proteasome. The overexpression of *OsMAR1* in Arabidopsis led to hypersensitivity to salt stress, suggesting the negative regulatory role of *OsMAR1 via OCPI2* interaction under salt stress conditions (Park et al., 2018). On the other hand, overexpression of *OCPI2* in Arabidopsis led to NaCl, PEG, and mannitol stress tolerance (Tiwari et al., 2015). Other proteinase inhibitors were also predicted as key genes in this module, namely, *LOC_Os01g03390* (*OsBBT17*) and *LOC_Os03g52370* (*PIII4*). *LOC_Os01g03390* encodes Bowman-Birk type bran trypsin inhibitor (*OsBBT17*) precursor. Bowman-Birk inhibitors (BBI) are a family of serine-type protease inhibitors, which are reported to involve not only in biotic stress response but also in abiotic stress response (Malefo et al., 2020; Xie et al., 2021). Proteinase inhibitor from maize (ZmMPI) was shown to interact with CBL-interacting protein kinase 42 (ZmCIPK42), which was identified to play a role in salt stress signaling. Overexpression of *ZmCIPK42* enhanced salt tolerance in maize and Arabidopsis (Chen et al., 2021b). Moreover, *LOC_Os11g26790* (*OsRAB16A*), encoding dehydrin, was detected in this module. Dehydrin is a group of proteins responding to osmotic stress and ABA to stabilize biomolecules and membranes (Tiwari and Chakrabarty, 2021). Therefore, the proposed mechanism related to salt stress is shown in **Figure 9**. The connection between these genes in the module from sensing to salt tolerance genes in different activities should be explored in the future.

## DISCUSSION

Salinity is the main agriculture problem in many areas in Southeast Asia, impacting rice growth and grain yield. 'Luang Pratahn' rice is one of the salt-tolerant varieties in local Thai rice. Our experiments demonstrated its tolerance phenotype (**Figures 1, 2**). To investigate salt tolerance mechanisms, the transcriptomic data of 'Luang Pratahn' rice were extracted using 3′-Tag RNA-seq technology to obtain gene expression profiles at different time points. Based on the transcriptomic data, the analysis pipeline (**Figure 3**) was conducted by first identifying differentially expressed genes and then constructing the global, salinity-state, and normal-state co-expression networks. These three gene sets follow the power-law distribution (**Figure 4**) in which they contain small high-degree genes and large low-degree genes. This indicates that some genes can be detected by network analysis techniques and are more important for the whole network structure than the other genes. The relative MTR was performed on the global co-expression networks with WGCNA to find groups of genes that work together at different time points (**Figure 5**). The benefit of a global network is the broader view of how genes are co-regulated in such a module, while the analysis of the two-state network helps us identify key genes that gained importance in the salinity-state networks compared to the normal-state network based on the centrality measures. Degree connectivity is highly connected in the salt-stress condition, while betweenness centrality detects higher loads passing key genes under the salt treatment. Closeness centrality indicates key genes in the salt condition display a short signal path to other genes in the network. The clustering coefficient gives the local connectivity among the partners of key genes. Different aspects of these measures help us to understand the role of each predicted key gene in each module. These four centralities have been applied because they are easy to interpret and provide different aspects covering local connectivity (degree and clustering coefficient), close commination (closeness centrality), and loading behavior (betweenness centrality) for a node in the network. Other centrality measures could be applied as well; however, the topological meaning might lead to some ambiguous interpretations.

By considering the gene information from all modules, we identified the key genes participating in the salinity sensing process, signal transduction, gene regulation at transcriptional, translational, and post-translational processes. The overall model is shown in **Figure 10**. Receptor-like protein kinase genes,

*OsHAIKU2* and *OsRMC* were predicted in the yellow and red modules, respectively. Within the yellow module, the signal transduction *via* ABA was proposed according to the existence of AAO and APX4 was predicted as key genes in this module. Moreover, *CAX1*'s regulation of $Ca^{2+}$ homeostasis supported the involvement of ABA signaling through the calmodulin pathway and upregulation of *AAO* gene expression (Saeng-Ngam et al., 2012). In the red module, $IP_3/IP_4$ signaling was predicted because OsITPK4 regulates $IP_3$ and $IP_4$ levels in the cell. Both $IP_3$ and $IP_4$ are secondary messenger molecules responsible for mediating $Ca^{2+}$ levels to maintain $Ca^{2+}$ homeostasis (Wilson et al., 2001). This signaling can mediate gene expression of other genes, including dehydrin (*OsRAB16A*), *OsASN1*, proteinase inhibitors (e.g., *OCPI2*, *OsBBT17*), and transporter genes regulating water (*OsPIP2.4*), phosphate (*OsPHF1*), and cytokinin (*OsPUP6*) transport. The expression of these genes was reported to affect abiotic stress tolerance (Chourey et al., 2003; Ganguly et al., 2012; Nagaraju et al., 2019; Luo et al., 2018; Park et al., 2018; Lee et al., 2020; Malefo et al., 2020; Nada and Abogadallah, 2020; Yin et al., 2020; Xie et al., 2021).

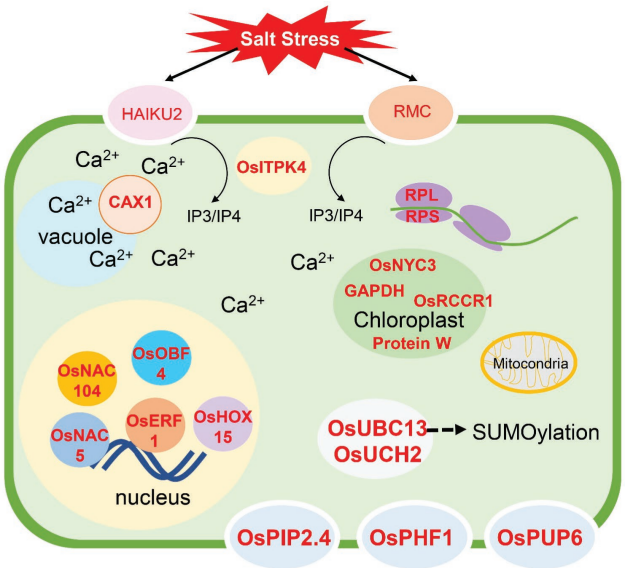


**FIGURE 10 |** Overall model for salt stress responses in 'Luang Pratahn' rice obtained from the transcriptomic analysis. The genes written in red represent the key genes identified in this research. Briefly, after receiving the salt stress by two receptor-like protein kinases, HAIKU2 and RMC, $IP_3/IP_4$ generated by OsITPK4 can trigger other responses through $Ca^{2+}$ signaling, and CAX1 is one of the transporters that participate in this process. Various transporters, water channel protein (OsPIP2.4), phosphorus transporter (OsPHF1), and cytokinin transporter (OsPUP6) are shown to be the key genes responding to salt stress. The transcription regulation through transcription factors such as OsNAC5, OsNAC104, OsOBF, OsERF1, and OsHOX15, and the translational regulation involved the expression of RPL and RPS genes function in cytoplasm and mitochondria are also reported to have the key functions in salt stress response. Moreover, the response in the genes functioning the chloroplast by encoding OsNYC3, GAPDH, OsRCCR1, and Protein W in the photosystem is detected. OsUBC13 and OsUCH2 are evidence of the salt stress response *via* the protein degradation through the SUMOylation mechanism.

Based on our studies, the response to salt stress also occurred in chloroplasts and mitochondria. In the chloroplast, *OsGAPDH*, encoding the W protein in photosystem II, pheophytinase (*OsNYC3*), and red chlorophyll catabolite reductase (*OsRCCR1*) were identified as key genes. This suggests a chloroplast role during salt stress and is consistent with previous findings that the ability to maintain chloroplast activity leads to salt tolerance in rice (Udomchalothorn et al., 2017; Boonchai et al., 2018; Chutimanukul et al., 2018). The participation of mitochondrial genes was detected with *RPS10*, which encodes a ribosomal protein subunit and *ATP synthase F1*. This suggests that the appropriate gene regulation in mitochondria contributes to salt tolerance in rice.

The regulation at transcriptional, translational, and post-translational levels is reported by this study. The transcriptome analysis method used in this study identified transcription factors previously reported to contribute to salt tolerance by different methods. Overexpression of *OsNAC5* could enhance salt tolerance in rice (Takasaki et al., 2010), while *OsNAC104* was playing a role as a negative regulator in drought stress (Fang et al., 2014). Homeobox-associated leucine zipper transcription factor, *OsHOX15*, was identified as a key gene in salt stress in this study. Sharif et al. (2021) reported that the *HOX* gene family is involved in stress response. Moreover, *OsERF1* and *OsOBF4* were also predicted as key genes, supporting the involvement of ethylene response during salt stress in rice. In Arabidopsis, *AP2/ERF* transcription factors are involved in ABA and ethylene responses and regulate abiotic stress tolerance (Xie et al., 2019), while *OsOBF4* was reported to respond to ethylene (Büttner and Singh, 1997). The involvement of these transcription factors should be validated. The effect of translation on salt stress response was predicted in the blue module. Several genes encoding the ribosome components were identified as the key genes in this module. Overexpression of *RPL23A* (Moin et al., 2017) and *RPL6* (Moin et al., 2021) enhanced salt tolerance in rice. Therefore, the detection of *RPL32*, *RPL30*, and *RPL28-1* in the blue module should be investigated for their role in salt tolerance. Protein degradation *via* SUMOylation was reported to regulate salt tolerance in rice (Srivastava et al., 2016, 2017; Mishra et al., 2018). In this study, genes involved in SUMOylation were predicted as key genes; *OsUBC13* and OsUCH2. *OsUBC13* was previously reported to be induced by salt stress (Zhiguo et al., 2015). The function of these genes and SUMOylation process should be investigated in 'Luang Pratahn' rice.

## CONCLUSION

In summary, we investigated salt tolerance responses in 'Luang Pratahn' rice, a local Thai rice cultivar with salinity tolerance. The experiments employed two conditions: control (optimal growth conditions) and salt stress, each featuring three biological replicates. Gene expression was measured by Tag-Seq at 0, 3, 6, 12, 24, and 48 h after the salt shock, producing 36 libraries. We investigated a co-expression network among 55,987 measured genes to identify those associated with salt tolerance. We found

significant expression differences between control and salt stress, identifying 1,386 genes. We built weighted co-expression networks in two channels using WGCNA. The first channel yielded a global co-expression network from all time points and conditions. Then, we identified important modules of differentially expressed genes between control and salt stress. The second channel yielded two weighted co-expression networks for the normal and salinity states, respectively. After that, centrality measurements, including DG, BW, CN, and CC, were applied for each gene in the network to rank their importance. We discovered 107 significant genes, involving in various mechanisms in salt responses, ranging from sensing of salt stress, signal transduction, hormonal response, and gene regulations *via* transcription, translation, and post-translation. This set of genes constitutes an important resource for improving the stress tolerance of rice in saline-affected areas.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/ under the BioProject ID: PRJNA747995.

## AUTHOR CONTRIBUTIONS

PS, KP, and SC: conceptualization. PC, LC, TB, and SC: conceived and designed the experiments. PC and PS: data curation. PS, AS, and KP: formal analysis. PS, PC, SC, AS, and KP: methodology. PS and KP: writing-original draft. AS, TB, KP, SC, and LC: writing-review and editing. All authors have read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.744654/full#supplementary-material

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# Metabolic Disturbance Induced by the Embryo Contributes to the Formation of Chalky Endosperm of a Notched-Belly Rice Mutant

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Grain chalkiness is a key quality trait of the rice grain, whereas its underlying mechanism is still not thoroughly understood because of the complex genetic and environmental interactions. We identified a notched-belly (NB) mutant that has a notched-line on the belly of grains. The line dissects the endosperm into two distinct parts, the upper translucent part, and the bottom chalky part in the vicinity of the embryo. Using this mutant, our previous studies clued the negative influence of embryo on the biochemical makeup of the endosperm, suggesting the need for the in-depth study of the embryo effect on the metabolome of developing endosperm. This study continued to use the NB mutant to evolve a novel comparison method to clarify the role of embryo in the formation of a chalky endosperm. Grain samples of the wild-type (WT) and NB were harvested at 10, 20, and 30 days after fertilization (DAF), and then divided into subsamples of the embryo, the upper endosperm, and the bottom endosperm. Using non-targeted metabolomics and whole-genome RNA sequencing (RNA-seq), a nearly complete catalog of expressed metabolites and genes was generated. Results showed that the embryo impaired the storage of sucrose, amino acid, starch, and storage proteins in the bottom endosperm of NB by enhancing the expression of sugar, amino acids, and peptide transporters, and declining the expression of starch, prolamin, and glutelin synthesis-related genes. Importantly, the competitive advantage of the developing embryo in extracting the nutrients from the endosperm, transformed the bottom endosperm into an "exhaustive source" by diverting the carbon (C) and nitrogen (N) metabolism from synthetic storage to secondary pathways, resulting in impaired filling of the bottom endosperm and subsequently the formation of chalky tissue. In summary, this study reveals that embryo-induced metabolic shift in the endosperm is associated with the occurrence of grain chalkiness, which is of relevance to the development of high-quality rice by balancing the embryo–endosperm interaction.

**Keywords:** **seed development, embryo–endosperm interaction, chalkiness, metabolome, transcriptome, rice physiology**

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## INTRODUCTION

High population pressure in rice-consuming regions always demands an increase in production, but the pursuit for a concomitant increase in grain quality is also increasing due to the enhanced quest for high-quality food in the developing countries like China (Bandyopadhyay et al., 2019; Song J. M. et al., 2019; Guo et al., 2020). Major quality traits of rice grain include the physical appearance, cooking, and sensory and nutritional properties. Among them, chalkiness, the opaque part of rice grain, has been set up as one of the main goals in rice breeding, for it affects not only the visual appearance quality but also the milling recovery of intact grains (Xi et al., 2014). In addition, high temperature occurring at the grain-filling stage has been reported as the most influential environmental factor inducing chalkiness, indicating that grain chalkiness would be a major threat to the rice industry in the scenario of global warming (Shi et al., 2017; Wada et al., 2019; Chang et al., 2021; Park et al., 2021; Yang et al., 2021). Therefore, it is urgent to elucidate the mechanisms responsible for chalkiness formation in rice grain.

Grain chalkiness is a complex trait governed by multiple genes and their interactions with the variable environments, as reflected by the slow advances in elucidating its genetic and physiological foundation. Though a large number of quantitative trait loci (QTLs) had been mapped, only fewer genes had been cloned and characterized (Liu et al., 2010; Sreenivasulu et al., 2015; Zhu et al., 2018; Xie et al., 2021). Carbon (C) or nitrogen (N) metabolism are the most important biochemical processes for grain filling, and their products, the starch, and storage proteins form the foundation of rice quality. Previously, incomplete accumulation of starch was widely accepted as the distinct histochemical property of the chalky endosperm tissue (Lisle et al., 2000; Singh et al., 2003). Later, a microscopic observation revealed that the chalky tissue is loosely packed with protein bodies as well as starch granules, indicating the importance of a balance between C and N metabolism when dealing with grain chalkiness (Xi et al., 2014). Concerning the physiological mechanisms, mounting evidences show that within the endosperm, disturbance either in C or N metabolism can result in chalky tissue occurrence (Yamakawa et al., 2007; Yamakawa and Hakata, 2010; Lin et al., 2017a,b; Wada et al., 2019). The C and N metabolisms are interdependent, and they interact across various levels in plant metabolism, indicating that they have to be subtly balanced to produce a proper proportion of starch and proteins and thereby avoid the formation of chalkiness. It should be noted that most of the previous studies on grain chalkiness were centered on the physiological aspects within the endosperm. On the other hand, fewer studies have been conducted to clarify the mechanism of the formation of chalkiness from the perspective of exogenous factors like the embryo.

Rice grain contains three distinct components including diploid embryo, triploid endosperm, and diploid maternal tissues. Growing evidence in rice, *Arabidopsis,* and maize suggest the importance of a communication between these compartments (Nowack et al., 2006; Widiez et al., 2017; Song W. et al., 2019; Zheng et al., 2019; Doll et al., 2020a,b; Xiong et al., 2021). The endosperm supports and nurtures the developing embryo and in return, the embryo transmits the signal to allocate the nutrients and thus influence the biochemical makeup of the endosperm (Zheng et al., 2019; Doll et al., 2020a; Song et al., 2021; Zhang et al., 2021). It is therefore suggested that embryo–endosperm interaction and their mutual metabolic signaling influence the rice quality like grain chalkiness.

We identified a notched-belly (NB) rice mutant that has a dichotomous appearance of endosperm, with opaque tissue in the lower basal parts, proximate to the embryo, and the translucent appearance in the upper part of the endosperm (Lin et al., 2014). Using this mutant, we explored the mechanism of chalkiness formation through biochemical, proteomic, and transcriptomic analysis. Notably, the embryo exhibited a substantial influence on the biochemical makeup of the endosperm and had a substantial negative impact on the overall storage of proteins, amino acids, and minerals in the chalky endosperm (Lin et al., 2016). Such modifications in the endosperm composition were mainly associated with the changes in the expression levels of the genes involved in the regulation of metabolites transporters and hormonal signaling in the chalky endosperm (Lin et al., 2017a). Altogether, these findings suggested that the embryo is involved in the formation of grain chalkiness by allocating nutrient distribution to the endosperm.

Metabolomics is a powerful tool to narrow the knowledge gap between the genotype and phenotype by monitoring and sensing the metabolic state in plant cells under various conditions. The advancement in mass spectrometry has made it more efficient and convenient to explore the metabolic dynamics and the underlying signaling mechanisms for seed development (Yamakawa and Hakata, 2010; Matsuda et al., 2012; Lin et al., 2017b; Li et al., 2019; Chen et al., 2020). In addition, it is applicable to breeding programs because of the link between sensory traits and metabolic profiles (Oikawa et al., 2008). Formerly, we performed an untargeted metabolic analysis of the NB mutant, and found that the chalkiness formation was associated with a metabolic shift from primary C and N metabolism to secondary metabolism like reactive oxygen species scavenging, osmoregulation, and cell wall synthesis, thus extending the understanding of the metabolomic mechanism underlying grain chalkiness (Lin et al., 2017b).

Considering that the recent studies focus highly on the endosperm, with no consideration of the role of embryo–endosperm interaction, we continued to use the NB mutant to explore the physiological foundation of grain chalkiness, by comparing the metabolite accumulation and gene expression profiles of the developing embryo and endosperm. Furthermore, a novel comparison method was invented to interpret the role of embryo in regulating the metabolic process in the endosperm, with the following objectives: (i) uncovering the metabolic dynamics of endosperm and embryo; (ii) manifesting and quantifying the embryo effect on endosperm development; and (iii) elucidating the mechanism of chalkiness formation from the perspective of embryo–endosperm interaction. The findings obtained here could offer insights into the metabolic

processes of grain formation, thus providing a valuable resource for the manipulation of molecular and physiological processes responsible for rice quality.

## MATERIALS AND METHODS

### Plant Materials and Sampling

The notched-belly mutant was obtained by treating the wild-type (WT) Wuyujing-3 with the mutagenic compound, ethyl methanesulfonate (EMS) (Lin et al., 2014). The experiment was conducted in 2018; rice seedlings were transplanted into a plastic pot of 29 cm in height and 30 cm in diameter. The pot was filled with 10 kg clay loam that has a pH of 6.41, 17.20 g $kg^{-1}$ of organic matter, 0.95 g $kg^{-1}$ of total N, 0.55 g $kg^{-1}$ of total P, 11.79 g $kg^{-1}$ of total K, 20.44 mg $kg^{-1}$ of Olsen-P, and 91.10 mg $kg^{-1}$ of exchangeable K. The plants were grown under natural environment condition at the vegetative stage. To ensure the consistency and stability of the environment during seed development, we translocated the plants with an identical flowering date into a growth chamber 2 days before anthesis. The growth conditions were kept similar to those mentioned by Lin et al. (2017b), with the modifications of day and night temperature to 31 and 24°C, respectively. The light intensity and the relative humidity were 600 μmol photons $m^{-2}$ $s^{-1}$ and 70 ± 5%, respectively. Middle rachis with a higher ratio of white-belly grains was sampled in three biological repeats for the NB and WT, at 10, 20, and 30 days after fertilization (DAF). Samples were immediately frozen by liquid nitrogen and stored at -80°C. For metabolomic and RNA-seq analysis, the developing grains were dehusked on the dry ice, and then manually dissected by the scalpel into two parts, the embryo and the endosperm. Subsequently, the endosperm was cut into the upper part (defined as the upper part of the endosperm, EnU) and the bottom part (EnB) along the notched line. For the WT, we divided the endosperm into two parts along the midline of the endosperm (**Figure 1**). It is to be noted that all the subsamples contained the outer layers of the grains, such as the seed coat and pericarp, due to technical difficulty.

### Metabolite Profiling by Multiple Analytical Platforms

Six biological replicates were used for metabolic analysis. An automated, robotic system MicroLab STAR (Hamilton, Reno, NV, United States) was used to prepare the embryo and endosperm samples for metabolite profiling. To ensure the quality, recovery standards were added for the quality check. The extraction was carried out with methanol by a vigorous shaking of 2 min. Subsequent centrifugation helped to recover the individual metabolites, and the evaporator was used to remove the organic solvent. Before the metabolomic investigation, the extracted samples were kept in nitrogen overnight. The resulting extract was divided into five fractions: two for analysis by two separate reverse phases (RP)/UPLC-MS/MS methods using positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS using the negative ion mode ESI, another for analysis by HILIC/UPLC-MS/MS using the negative ion mode ESI, and one was reserved for backup.

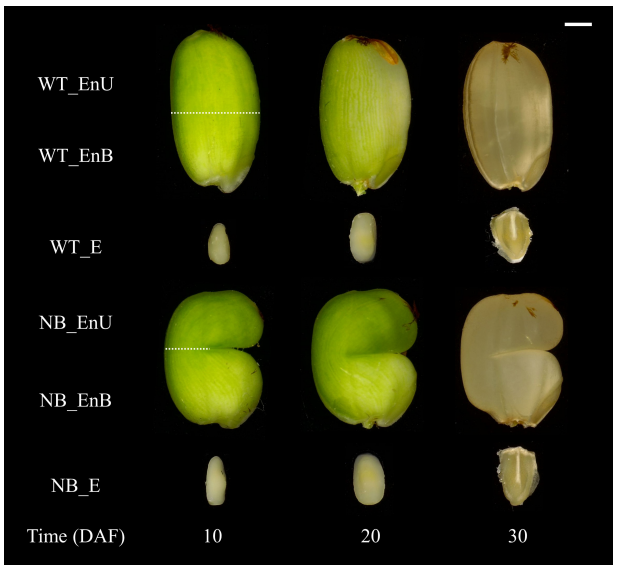


**FIGURE 1 |** Overview of the time series samples of embryo and endosperms. Dotted line indicates the manual dissection of endosperm, cutting it into upper and bottom endosperms. DAF, days after fertilization; E, embryo; En, endosperm; EnB, bottom part of endosperm; EnU, the upper part of endosperm; NB, notched-belly rice mutant; WT, wild-type of Wuyujing-3. Bar = 1 mm.

### Data Analysis and Construction of the Metabolic Atlas

The raw data from the three analytical platforms were extracted and processed in Metabolon Laboratory Information Management System. A reference library was created through approximately 1,500 authentic standards in Metabolon and used as a reference to identify the metabolites by an automated comparison. The quantification and data normalization of the significantly changing metabolites were performed according to previously used methods (Lawton et al., 2008; Rao et al., 2014). A brief data normalization step was employed to correct any instrument-based variation due to tuning differences across experimental days. The quantitative values of the metabolites were obtained from the counts of the integrated raw detector mass spectrometers. To preserve the variation with the data, all compounds from the extensively variable raw data were normalized directly on an identical graphical scale, with the normalized intensities scaled by their median values for each compound. Tests of significance were performed by Welch's two-sample *t*-test using the R program. The database of Plant Metabolic Network (PMN[1]) was used to target the metabolic pathways. The metabolic atlas was constructed through the Kyoto Encyclopedia of Genes and Genomes) pathway (KEGG[2]).

[1]http://www.plantcyc.org/

[2]http://www.genome.jp/kegg/pathway.html

Principal component analysis (PCA) was performed to classify the data from 108 samples, using Omicshare tools.[3]

## Gene Expression Profiling Using RNA Sequencing

Three biological replicates were used for RNA sequencing (RNA-seq) analysis. About 0.1 g tissue sample was used to extract the RNA by a kit from Invitrogen (Carlsbad, CA, United States). The extract was diluted with 100 μL of RNAase-free water. The RNA concentration was quantified by Nanodrop, with its quality evaluated using LabChip from Agilent (Santa Clara, CA, United States). Two evaluation systems, namely Agilent 2100 Bioanalyzer and StepOnePlus Real-Time PCR System (Applied Biosystems, Waltham, MA, United States) were used for the qualification and quantification of the sample library. Nibbonbare (IRGSP-1.0[4]) and BGISEQ-500 (BGI, Shenzhen, China) were referenced for reading and mapping analysis, and library sequencing, respectively. Only clean-read was processed further by HISAT2 (V2.1.0) (Kim et al., 2015) to ensure effective and high-quality mapping (Chen et al., 2018). Eventually, the expression level of different genes was estimated using RSEM (V1.2.8) software (Li and Dewey, 2011). A gene is considered as expressed only if its value of fragments per kilobase of transcript per million mapped reads (FPKM) is either equal to or exceeds 1. Notably, genes with an absolute value of $\log_2$ ratio $\geq 1$ compared with an FDR corrected value of $P \leq 0.001$ were designated as differentially expressed genes (DEGs) (Wang et al., 2010).

## Validation of Transcriptome Data Using Quantitative Reverse Transcription-PCR

For the credibility of RNA-seq data, it was verified by the quantitative reverse transcription-PCR (qRT-PCR). Twelve genes that are involved in C and N metabolism were selected for the verification of their expression pattern. Primers were designed through Primer 5.0 (An et al., 2009), as in **Supplementary Table 1**. Comparison of both the results revealed a similar pattern, verifying the validity of the RNA-seq data (**Supplementary Figure 1**).

## Metabolites and Genes Coexpression and Functional Enrichment Analysis

The coexpression analysis to study the ontology of metabolites and genes in the embryo and the endosperm was carried out using the MeV (Howe et al., 2010). Values of *Z*-score were used as input for MeV, and clustered by using the *K*-means and Pearson's correlation coefficients among the metabolites and genes. Metabolites were enriched in metabolic pathways using MBROLE online enrichment software (V2.0[5]) (López-Ibáñez et al., 2016). The *P*-value was used to identify the significant KEGG categories ($P$-value $< 0.05$). The annotation of genes into functional categories was carried out using the KEGG annotation. The hyper function in R software (R Core Team, 2013) was used to perform the functional enrichment of genes, with a default setting.

## Starch and Protein Content Analysis

For starch content analysis, the samples were subjected to ethanol extraction, followed by digestion in perchloric acid. Contents of starch were quantified by using an anthrone agent and measuring the absorbance at 620 nm (Hansen and Møller, 1975). The fractions of storage proteins including albumin, globulin, prolamin, and glutelin were extracted and measured according to the method of Ning et al. (2010).

## Statistical Analysis

The concentration data of the starch and proteins in the study are averages of triplicate observations. The SPSS statistics package version V19.0 (SPSS Inc., Chicago, IL, United States) was used for the statistical analysis, and Duncan's multiple range test ($P$-value $< 0.05$) was used for multiple comparisons for the significant difference.

# RESULTS

## General Description of the Metabolomics in the Developing Embryo and Endosperm

A total of 634 distinctly annotated metabolites were identified from all samples. Among them, 484 metabolites were accumulated in both the embryo and the endosperm, while only 109 and 41 metabolites were specifically accumulated in the embryo and the endosperm, respectively (**Figure 2A**). The metabolites identified in the embryo mainly covered the central metabolism pathway and partially the secondary metabolism pathways, including 175 amino acids, 73 carbohydrates, 175 lipids, 38 CPGEC (cofactors, prosthetic groups, and electron carriers), 64 nucleotides, 16 peptides, 43 secondary metabolites, 6 phytohormones, and 3 xenobiotics (**Figure 2B**). Within the endosperm, the identified metabolites consisted of 160 amino acids, 65 carbohydrates, 160 lipids, 33 CPGEC, 49 nucleotides, 17 peptides, 34 secondary metabolites, 5 phytohormones, and 2 xenobiotics (**Figure 2C**). As a whole, the general mapping of metabolites revealed almost a similar division of metabolites in the embryo and the endosperm.

However, further classification by PCA analysis shows that the identified metabolites and genes can be divided into two primary groups (the first component), each being associated with the embryo (I) and the endosperm (II), respectively (**Figure 2D**). The second component of PCA discriminated the early, middle, and late stages of development within the embryo and the endosperm (**Figure 2D**). Such a categorization indicated that the accumulation of metabolites during rice seed development is both tissue- and stage-specific (**Figure 2D**). Subsequently, to uncover the correlation between the upper and bottom endosperms across the developmental stages, we carried out another PCA that classified the metabolome data of two endosperm parts (**Figure 2E** and **Supplementary Figure 2**). Results showed that

[3]https://www.omicshare.com

[4]https://www.ncbi.nlm.nih.gov/genome/?term=IRGSP-1.0

[5]http://csbg.cnb.csic.es/mbrole2/analysis.php

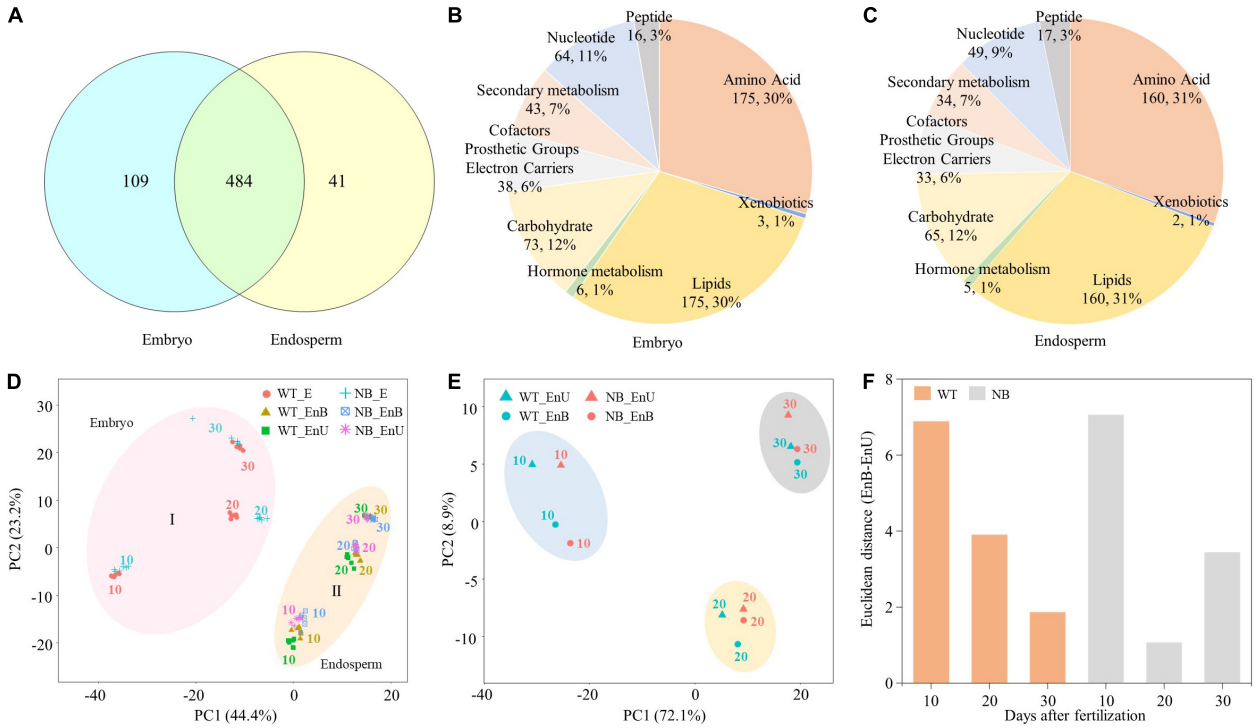


**FIGURE 2 |** Summary of metabolome data of embryo and endosperm. **(A)** Venn diagram of the 634 metabolites accumulated in the embryo and endosperm. **(B,C)** Classification and distribution of the identified metabolites in the developing embryo **(B)** and endosperm **(C)**. **(D)** PCA of the seed tissue metabolites populations. PCA plot shows two distinct groups of embryo and endosperm metabolites populations: Group I for embryo and Group II for endosperm. **(E)** PCA showing global metabolome relationships of time-series samples from the upper part and bottom part of the endosperm. **(F)** The distance between the upper part and the bottom part of the endosperm in PCA at each stage. Numbers of 10, 20, and 30 represent the sampling time points (days after fertilization, DOF).

the pattern of metabolomic development of endosperm parts varied between WT and NB. For instance, the difference between the upper endosperm and the bottom endosperm decreased linearly along with the seed development for the WT. On the contrary, NB showed a "V-type" pattern regarding this difference, being the largest at 10 DAF, the lowest at 20 DAF, and recovered at 30 DAF (**Figure 2F**). Collectively, the PCA analysis clearly bifurcated the metabolites across the developmental stages and suggested a disturbance in the developmental process of the bottom endosperm of NB due to its proximity to the embryo.

## Coexpressed Metabolite Sets of Embryo and Endosperm Development

To gain further insight into the metabolic changes during the rice seed development, we divided all tissue-annotated metabolites into six clusters based on their accumulation patterns using the *K*-means clustering algorithm (**Figure 3**). We identified the metabolites of three modules (DP4–DP6) that were enriched at more than one stage in the embryo and the endosperm, respectively, indicating some common cellular metabolic processes across several stages. In addition, clustering also helped to map out the compounds whose contents were more prevalent at one out of the three developmental stages (DP1–DP3), indicating the specific function of these modules at the corresponding stage (**Figure 3**). The coexpression clustering as well as the KEGG enrichment classification, both verified the PCA grouping and confirmed the stage and tissue specificity of the metabolites within the embryo and the endosperm (**Figures 3**, **4**). Notably, the amount and species of metabolites in the embryo and the endosperm were similar between the two genotypes (**Supplementary Table 2**). Therefore, we took WT as an example to describe the metabolic dynamics of the developing embryo and endosperm.

## Metabolic Processes in the Developing Embryo

Cellular metabolic processes characterizing each developmental stage were identified by KEGG terms that were overrepresented at particular coexpression modules (**Figures 3A**, **4A**). The early-stage was best represented by the module, dominant pattern-1 (DP1), as typified by the overrepresentation of the KEGG terms for the citrate cycle, oxidative phosphorylation, and amino acid metabolism. This is consistent with the high requirement of energy during embryo formation. The middle stage featured the module, DP2 that was enriched in KEGG terms including starch and sucrose metabolism, inositol phosphate metabolism, and galactose metabolism, which coincides with the maturation of the embryo. The DP3 presented an upregulation of metabolites involved in phenylalanine metabolism, and taurine and hypotaurine metabolism, suggesting the dormancy of the

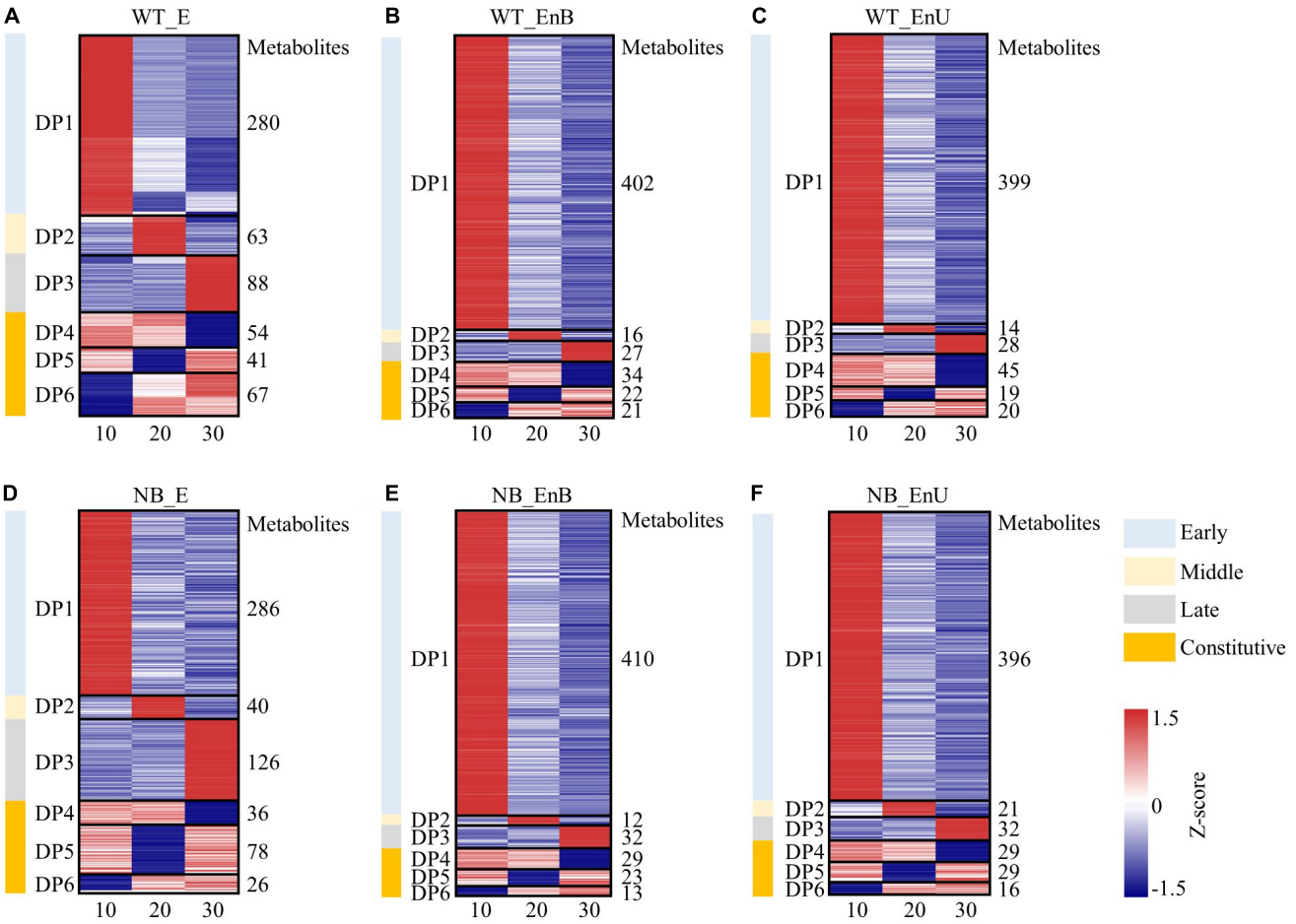


**FIGURE 3 |** Metabolite dynamics of the developing embryo and endosperm for WT **(A–C)** and NB **(D–E)**. Dynamic patterns of metabolites in different coexpression modules are shown for the embryo **(A,D)**, the bottom part of the endosperm **(B,E)**, and the upper part of the endosperm **(C,F)**. Expression data were *Z*-score standardized from –1.5 to 1.5; the number of metabolites in each module is shown on the right. Numbers of 10, 20, and 30 represent sampling time points (days after fertilization, DOF).

embryo. In addition, the metabolites formed modules, DP4–DP6 accumulated broadly in the embryo across the sample time points, involving amino acid metabolism, purine and pyrimidine metabolism, and nicotinate and nicotinamide metabolism.

## Metabolic Processes in the Developing Endosperm

Compared with the embryo, the majority of the metabolites decreased during endosperm development. This may be due to the gradual transformation of C and N metabolites into starch, protein, and lipid (**Figures 3B,C**, **4B,C**). Metabolites involved in the citrate cycle and in the metabolism of amino acid, glutathione (GSH), purine, and pyrimidine were enriched in the early stage (DP1). This may be related to the cell differentiation of endosperm. The proportion of metabolites in the middle stage (DP2) was the least, and the KEGG terms of endocytosis were enriched, which may coincide with the desiccation and program cell death of endosperm cells. The content of metabolites associated with endosperm maturation and those involved in the tryptophan metabolism increased significantly in the late stage (DP3). Notably, metabolites responsible for arginine and proline metabolism, nicotinate and nicotinamide metabolism, pantothenic acid and coenzyme A biosynthesis, and linoleic acid metabolism were overrepresented in DP4–DP6 and broadly expressed throughout the development.

## A Novel Method to Quantify the Effect of Embryo on Endosperm Development

The marked difference between the NB and WT in the metabolomic profiling and its changing pattern across the developmental stages indicated that the embryo could have a substantial influence on the metabolic processes in the endosperm (**Figures 2E,F**). Therefore, to quantify the embryo effect, we utilized the NB mutant to develop a novel comparison system, by comparing the upper and bottom endosperms of the NB, with those of the WT as reference (**Figure 5**).

Briefly, the comparison system employed in the present study comprises three components. (1) Position effect: it indicates the

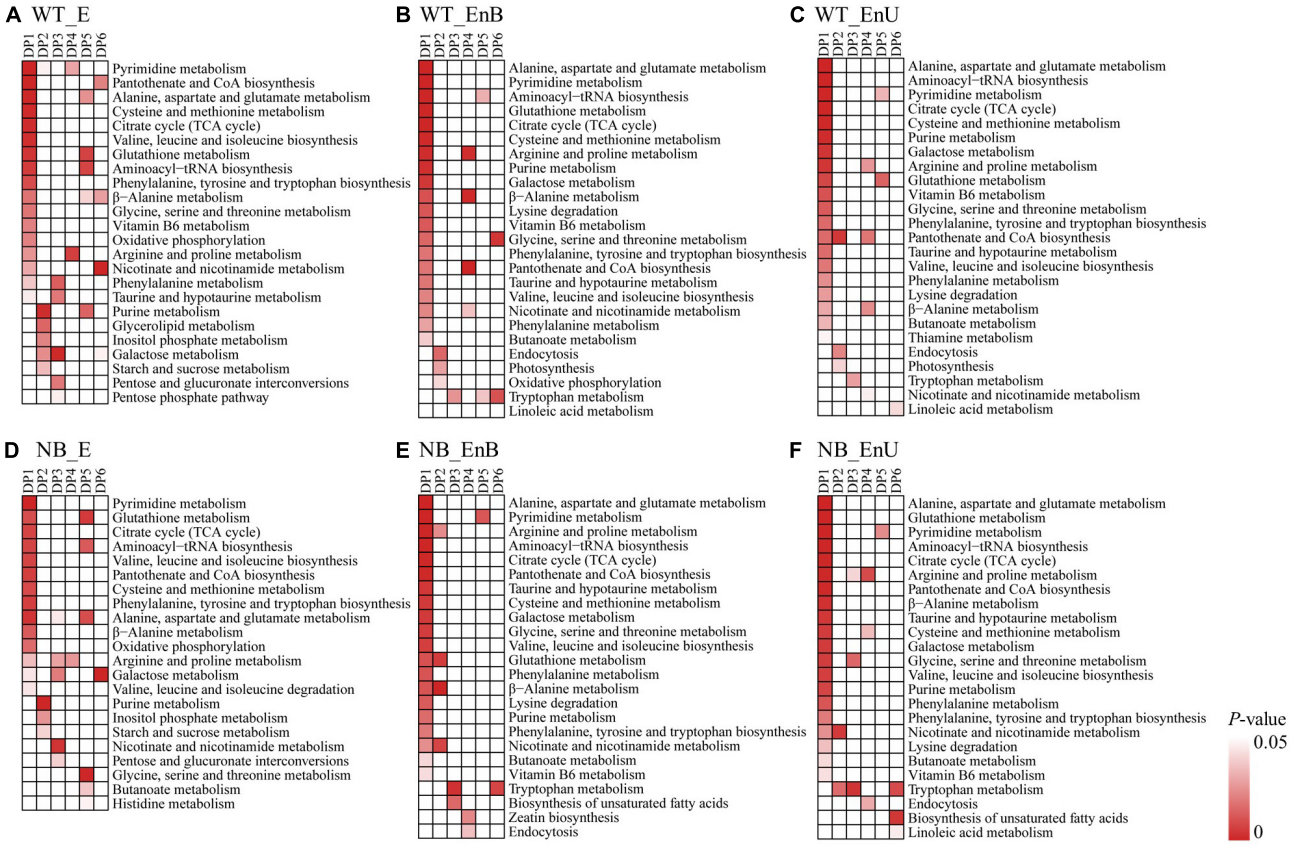


**FIGURE 4 |** Kyoto Encyclopedia of Genes and Genomes (KEGG) enriched in different coexpression modules of embryo and endosperm for WT **(A–C)** and NB **(D–F)**. Only significant categories (*P*-value < 0.05) are displayed.

difference in two parts of the endosperm based on their position within the WT grain, where both the nutrients and the signals can easily travel between their upper endosperms (WT_EnU) and bottom endosperms (WT_EnB) (**Figure 5A**). (2) Compound effect: comparison between the bottom (NB_EnB) and the upper (NB_EnU) endosperm of the NB mutant shows the combined effect of embryo and position (**Figure 5B**). Notably, the notched lines severely cut off the nutrient supply and signal movement to the upper endosperm. As a result, the influence of embryo is trapped inside the proximate part of the endosperm (NB_EnB). (3) Embryo effect: eventually, after quantifying both the position and compound effects, we can roughly evaluate the effect of embryo on the endosperm, by eliminating the position effect from the compound effect (**Figure 5C**).

To clearly explain the working principle of the comparison method, we employed three key metabolites, lysine, phosphoenolpyruvate (PEP), and maltose, which are involved in amino acid metabolism, energy metabolism, and starch metabolism, respectively (**Figure 5**). First, the position effect significantly downregulates the maltose content, but has no marked effect on those of the lysine and PEP. In contrast, the compound effect of the position and embryo increases the PEP and maltose whereas it reduces lysine. Finally, when the position effect is expunged, it uncovers the promotion of maltose and PEP and the inhibition of lysine under the embryo effect. Taken together, the metabolomic anomaly observed within the seed tissues of WT and NB during the metabolomic profiling was eventually verified by utilizing the novel comparison method. In addition, it also verified that the "V-shaped" pattern observed between the upper and bottom endosperm was caused by the altered metabolism under the embryo effect. Eventually, the influence of embryo on endosperm metabolism was confirmed.

## Embryo Effect on the Metabolic Dynamics of the Developing Endosperm

After verifying the metabolic disturbance in endosperm under the embryo effect, we further used the proposed comparison method to uncover the detailed consequences of the metabolic alteration in the endosperm and the relevant underlying mechanisms. By performing the complementary analysis of the metabolic and gene expression profiling, we not only assured that the embryo can disturb the metabolic processes in the endosperm, but also identified some key pathways in the endosperm that are induced by the embryo. Importantly, the identified pathways have clear relevance to the formation of chalky tissue, suggesting the embryo effect as one of the causes behind the chalky belly

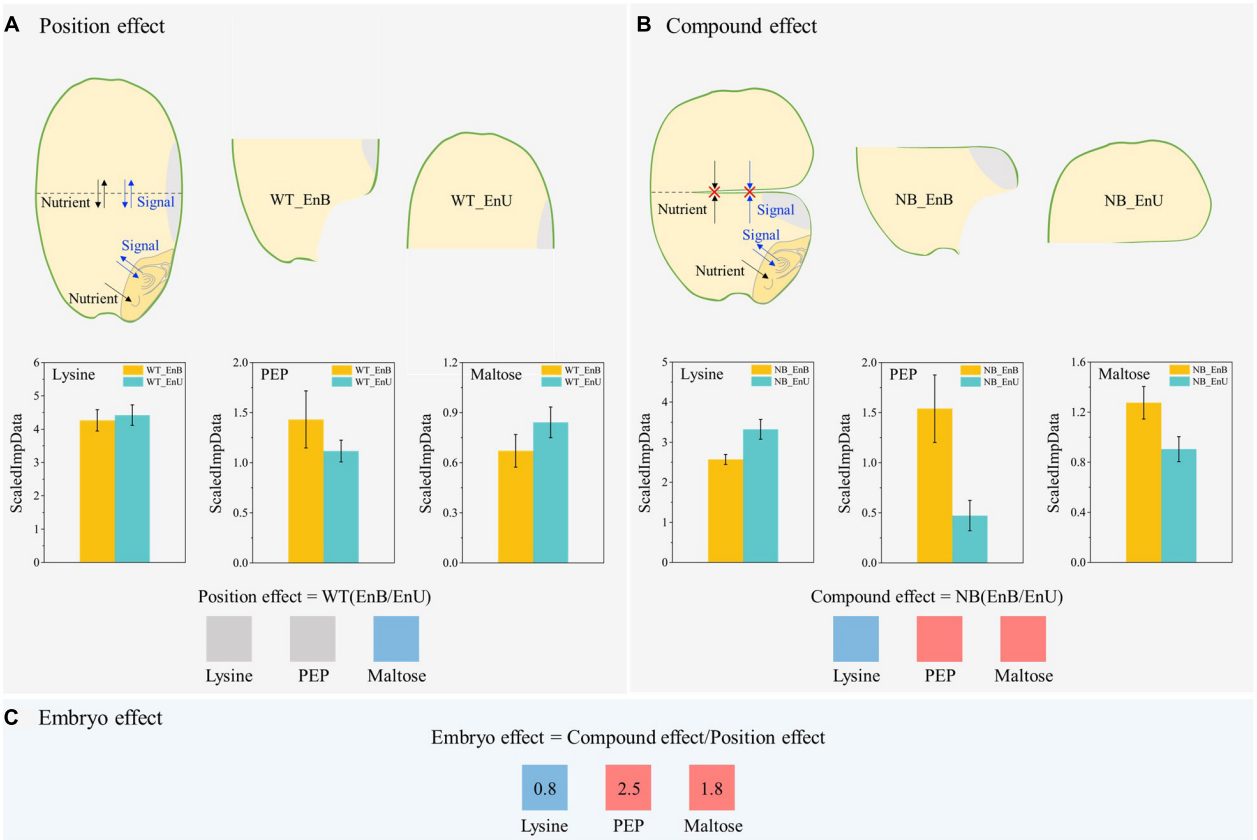


**FIGURE 5 |** Overview of the new comparison method for quantification of the embryo effect on endosperm development. This method comprises three key components: **(A)** Position effect: it is estimated by the comparison of upper endosperm (WT_EnU) and bottom endosperm (WT_EnB) of wild-type (WT), indicating the difference in two endosperm parts. Note that within this grain, both nutrients and signals could easily travel between the upper and bottom parts. **(B)** Compound effect: it is estimated by the comparison of the bottom (NB_EnB) and upper (NB_EnU) endosperm of the NB, showing the combined effect of embryo and position. Within the grain of NB, the notched line severely cut-off the nutrient supply and the signal movement to the upper endosperm. As a result, the major impact of embryo is trapped inside the proximate part of the endosperm (NB_EnB). **(C)** Embryo effect: after quantifying both the position and compound effects, we can roughly estimate the effect of embryo on endosperm, by the comparison of $NB_{(EnB/EnU)}/WT_{(EnB/EnU)}$. Three metabolites are used as examples to demonstrate the working principle of this method, as explained in detail in the main text. Three metabolites are used as examples to demonstrate the working principle of this method, as explained in detail in the main text. Red, navy blue, and gray boxes indicate upregulation and downregulation, and no marked differences of lysine, phosphoenolpyruvate, and maltose in the bottom endosperm as affected by position, compound, and embryo effect, respectively. For comparisons of position and compound effect, a marked difference is recorded according to Duncan's multiple range test ($P < 0.05$). For comparisons of embryo effect, a marked difference is defined as those $\leq$0.95 (downregulation) or $\geq$1.05 (upregulation). The numbers in boxes of panel **(C)** represent the calculated value for the embryo effect. PEP, phosphoenolpyruvate.

in the NB mutant. These pathways are explained in detail in the next section.

## The Ability of Metabolites Transport in the Bottom Chalky Endosperm Was Enhanced by the Embryo

The embryo had a significant influence on the expression pattern of transporters for amino acids, peptides, and sugars, resulting in the differential accumulation of relevant substrates (**Figure 6**). In general, most transporters were enhanced in the endosperm by developing the embryo at the early stage (10 DAF) with the exceptions of three amino acid transporters, such as *OsGAT1*, *OsLHT1,* and *Os02g0768300* (**Figure 6A**), and one sugar transporter *OsMST3* that were downregulated (**Figure 6C**). This increased expression of transporters in the endosperm was accompanied by a decline in the relevant substrates, such as amino acids (tyrosine, lysine, methionine, proline, leucine, threonine, and histidine; **Figure 6A**), peptides (glycylleucine, alanylleucine, threonylphenylalanine, leucylalanine, leucylglycine, valylleucine, leucylglutamine, and valylglutamine; **Figure 6B**), and sugars (sucrose, glucose (Glu), and fructose; **Figure 6C**). Overall, the upregulated trend of the transporters suggested the excavation of nutrients from the endosperm, likely due to the developing embryo, that acted as a "sink" within the developing seed at the early stage of embryo development.

At the middle and the late stages (20 and 30 DAF), most of the transporter genes still showed higher activity (**Figure 6**). However, the content of the substrates showed an opposite trend

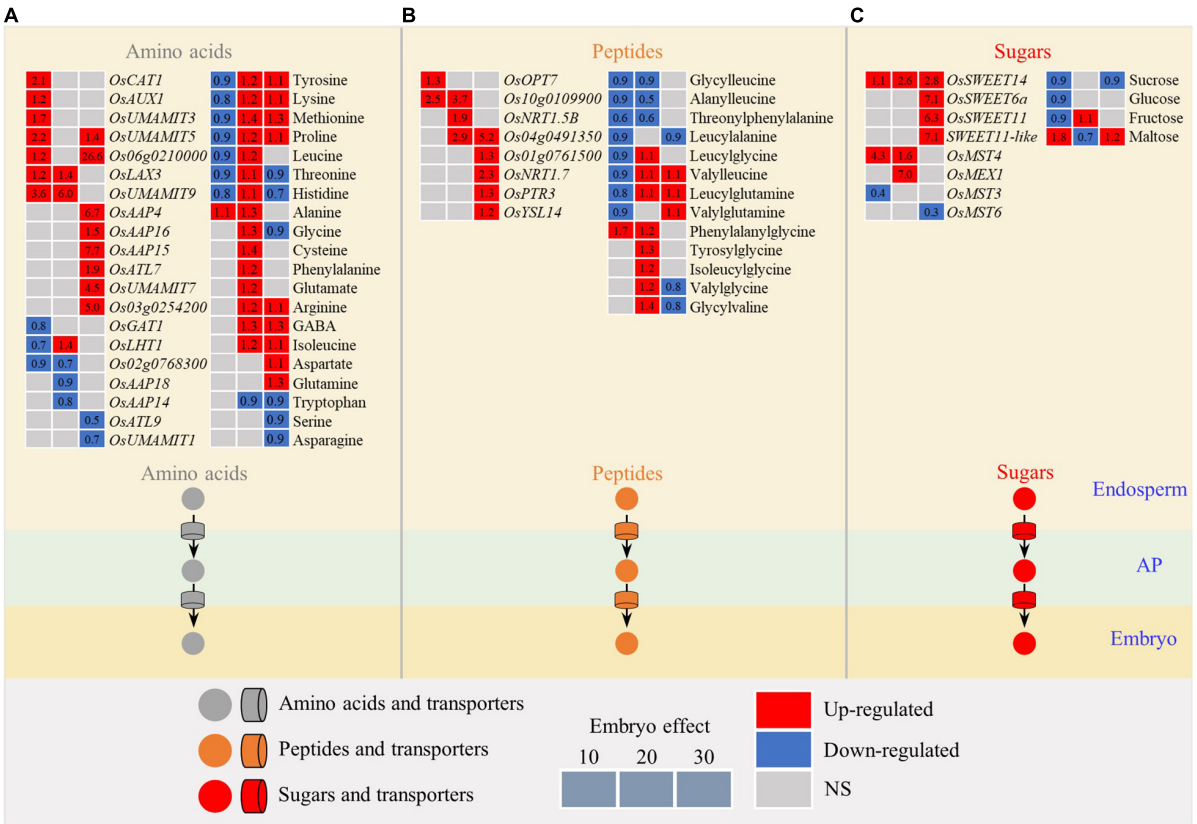


**FIGURE 6 |** Expression patterns of metabolites and genes involved in the transport of amino acids **(A)**, peptides **(B)**, and sugars **(C)** in the chalky endosperm as affected by the embryo. Gray, red, and navy-blue boxes denote non-significant, significant upregulation, and significant downregulation by the embryo, respectively. Data in boxes are the calculated values of the embryo effect. Numbers of 10, 20, and 30 above the gray boxes represent the sampling time points (days after fertilization, DOF). AP, apoplasm; NS, non-significant.

relative to that at 10 DAF. For example, with the exception of threonine, histidine, glycine, tyrosine, serine, and asparagine, the contents of other amino acids increased significantly (**Figure 6A**). Likewise, except glycylleucine, alanylleucine, threonylphenylalanine, and leucylalanine, the other nine dipeptides showed an overall upward trend (**Figure 6B**). In addition, fructose markedly increased at 20 DAF, but sucrose and Glu did not change (**Figure 6C**). At 20 DAF, the embryo enters the dormancy stage, not competing for nutrients with the endosperm. Nutrients, such as amino acids, peptides, and sugars, still continue to input into the bottom endosperm for accumulating storages, resulting in the simultaneous increase in the amino acids and peptides and their transporters. This may partly explain the inconsistency of the changing pattern of the nutrients between the early stage and the middle and late stages of embryo development.

## Catabolism of Starch in the Bottom Chalky Endosperm Was Induced by the Embryo

Genes and metabolites involved in carbohydrate metabolism were differentially expressed under the influence of the developing embryo (**Figure 7**). At the early stage (10 DAF), the embryo had a negative effect on sucrose, Glu, and fructose levels in the endosperm, probably due to its nutrient consumption (**Figure 7A**). Notably, despite the upregulation of *OsTPP2* gene that is related to the trehalose biosynthesis, the content of this sugar utilization related to disaccharide, decreased simultaneously with the other important sugars (**Figure 7**). Consequently, the insufficiency of the sucrose resulted in the decreased activity of starch synthesis genes, including *OsAGPL4*, *OsSSSIVa*, *OsSSIVb*, *OsSSSIIIa*, and *Os02g0807100* (**Figure 7B**). Meanwhile, maltose, the product of starch breakdown, and its precursors (Mal-hex, Mal-pen, Mal-tet, and Mal-tri) showed a significant increase, implying an accelerated breakdown of starch (**Figure 7A**). Similarly, *AMY3E*, an α-amylase gene for starch catabolism, also demonstrated a decreasing trend at 10 DAF (**Figure 7B**). Collectively, the deficiency of sucrose and the enhancement of starch degradation led to the reduction in starch content. Conversely, at the middle stage (20 DAF), sucrose metabolism and starch synthesis were enhanced in the endosperm, as evidenced by the increased accumulation of starch and the decrease of maltose and its precursor. Meanwhile, fructose, the decomposition product of sucrose, increased at 20 DAF and trehalose also showed the same

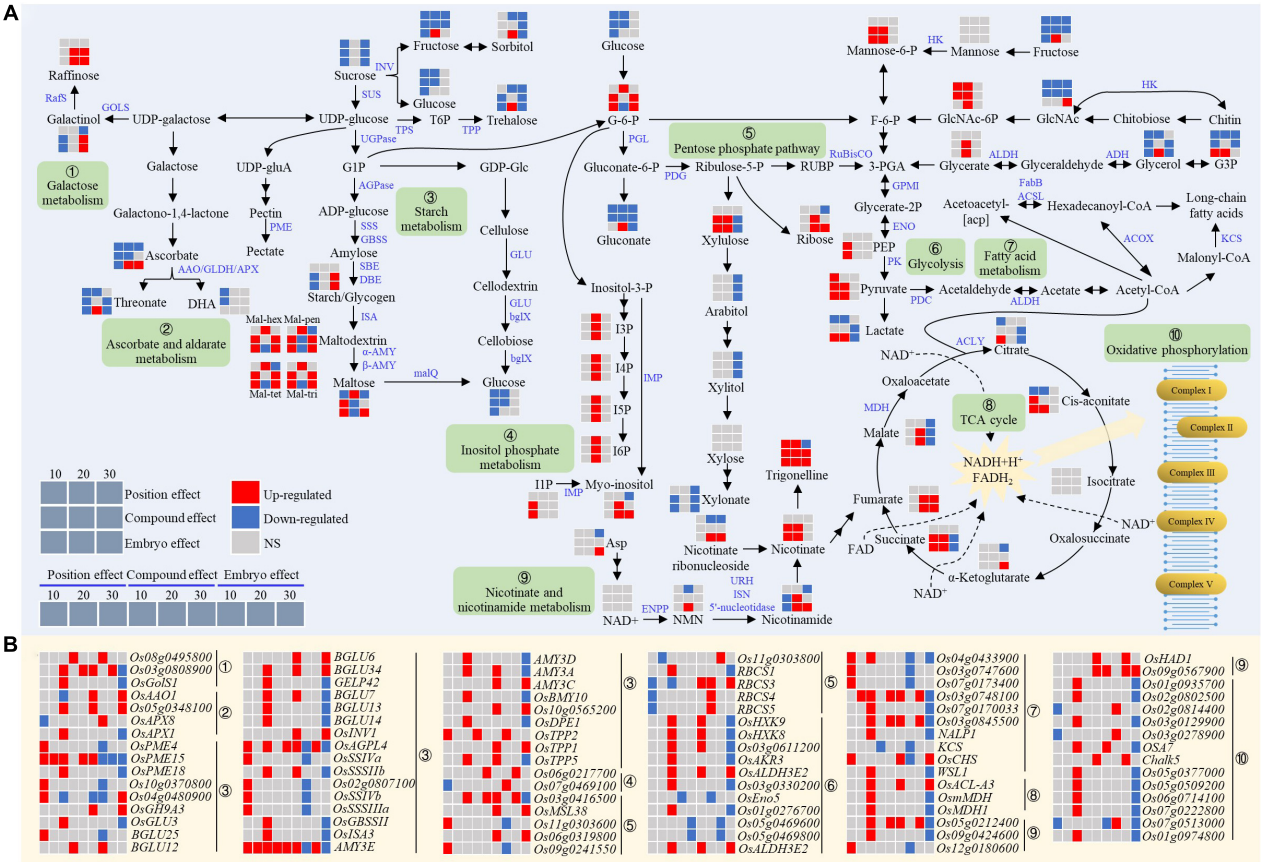


**FIGURE 7 |** Expression patterns of metabolites **(A)** and genes **(B)** for carbohydrates metabolism in the chalky endosperm as affected by the embryo. Gray, red, and navy-blue boxes denote non-significant, significant upregulation, and significant downregulation by the position, compound, and embryo effect, respectively. Numbers of 10, 20, and 30 above the gray boxes represent the sampling time points (days after fertilization, DOF).

trend (**Figure 7A**). In addition, the starch synthesis gene (*OsAGPL4*) and the degradation gene (*AMY3E*) were upregulated and downregulated, respectively (**Figure 7B**). Altogether, the metabolic pattern suggested that the developing embryo at the early stage induced the starch catabolism which might cause the impaired endosperm filling.

## Carbon Metabolism Is Shifted to Secondary Metabolisms in the Chalky Endosperm by the Embryo

The embryo had a positive effect on the inositol phosphate metabolism (**Figure 7**). At the early stage, myo-inositol and I1P were mainly accumulated, while during the later stages of endosperm development, phytic acid (I6P) and its precursors I5P, I4P, and I3P were significantly overrepresented in the chalky endosperm (**Figure 7A**). For example, I1P was significantly increased at 10 DAF, and myo-inositol and I6P and its precursors were upregulated at 20 or 30 DAF. In addition, the myo-inositol synthesis genes, *Os07g0469100* and *Os06g0217700* also showed the same trend (**Figure 7B**).

Ascorbate significantly decreased in the early stage but increased in the later stage (**Figure 7A**). Similarly, its downstream product, threonate also decreased at 10 DAF. In the metabolic pathway of ascorbate, AAO, GLDH, and APX participate in the synthesis of dehydroascorbic acid (DHA). The expression of *OsAAO1*, *GLDH* (*Os05g0348100*), and *OsAPX8* genes increased at 10 DAF or 30 DAF, while *OsAPX1* significantly decreased at 30 DAF (**Figure 7B**). However, there was no significant change in the DHA content during endosperm development (**Figure 7A**).

The content of raffinose increased significantly at the 20 and 30 DAF, while its precursor galactinol, first decreased at 10 DAF, and then increased at 30 DAF (**Figure 7A**). Galactinol synthase (GOLS) and raffinose synthase (RafS) catalyze the biosynthesis of raffinose (Falavigna et al., 2018; Li et al., 2020). *OsGolS1* was downregulated at 30 DAF, while the expressions of two RafS genes (*Os08g0495800* and *Os03g0808900*) increased at 10 DAF and 20 DAF, respectively (**Figure 7B**). In addition, the contents of other osmotic substances, such as erythritol, mannitol, and trigonelline, showed an increasing trend in the 20 DAF (**Figure 7A**).

Taken together, these trends of C metabolism clearly suggested the diversion of metabolic activities from the central storage-based metabolism to other secondary metabolic pathways that might limit the nutritional storage capacity of the endosperm, resulting in chalkiness formation.

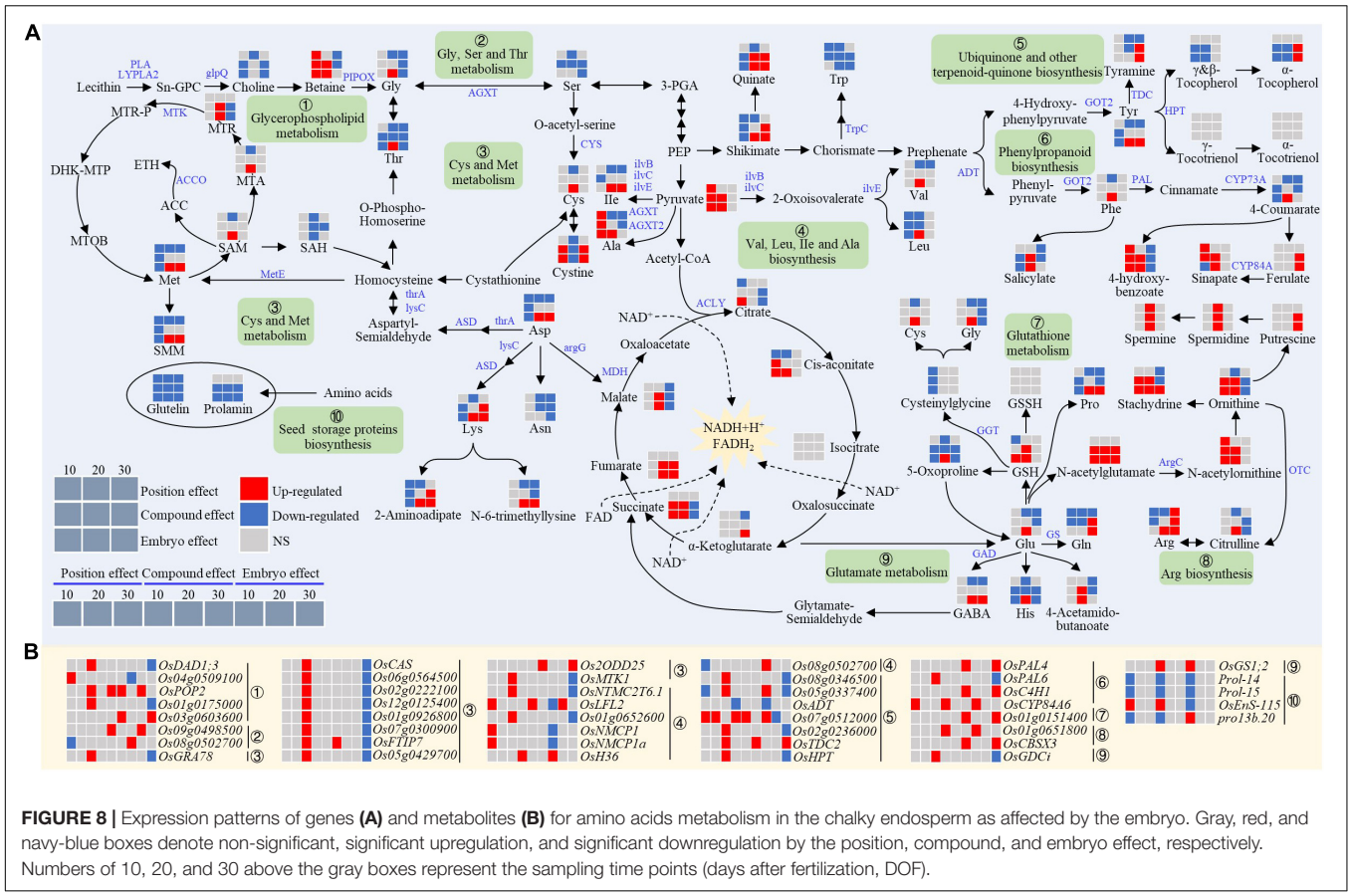


**FIGURE 8 |** Expression patterns of genes **(A)** and metabolites **(B)** for amino acids metabolism in the chalky endosperm as affected by the embryo. Gray, red, and navy-blue boxes denote non-significant, significant upregulation, and significant downregulation by the position, compound, and embryo effect, respectively. Numbers of 10, 20, and 30 above the gray boxes represent the sampling time points (days after fertilization, DOF).

## N Metabolism Switched From Storage Proteins Biosynthesis to Secondary Metabolism in the Chalky Endosperm by the Embryo

At the early stage, the embryo had a negative effect on amino acid levels in the endosperm (**Figure 8A**). The decrease of amino acids in the endosperm was accompanied by a decline in the expression levels of prolamin (*Prol-14* and *Prol-15*) and glutelin (*OsEnS-115*) synthesis genes (**Figure 8B**), which in turn inhibited the biosynthesis ability of storage proteins and resulted in a lower content of prolamin and glutelin (**Figure 8A**).

Arginine (Arg) metabolism in the chalky endosperm was markedly affected by the embryo (**Figure 8**). At the early and middle stages, ornithine, citrulline, and Arg showed an increasing trend (**Figure 8A**). The synthesis of Arg was first induced by the acetylation of Glu to produce *N*-acetylglutamate, which caused a subsequent increase of N-acetylornithine (NAO), ornithine, citrulline, and Arg. Moreover, in the arginine metabolism process, ArgC (*Os01g0651800*) and OTC (*OsCBSX3*) responsible for generating NAO and citrulline, exhibited upward trends at the early and late stages (**Figure 8B**). In addition, the increase of ornithine content in the endosperm was accompanied by the increased stachydrine, an important osmotic regulator in the plant cell. Betaine, another key osmoprotectant, showed a similar trend with stachydrine (**Figure 8A**).

Another remarkable effect of the embryo was detected in the glutamate metabolism. The GSH showed an increasing trend at the early and middle stages, which may be attributed to the altered glutamate metabolism (**Figure 8A**). For example, Glu content had no change at the early stage, but the contents of its derivatives (histidine and proline) decreased, indicating that the metabolic process was tilted toward the GSH synthesis pathway. In addition, the Glu level was upregulated at the middle stage, which in return enhanced the accumulation of GSH, histidine, and proline (**Figure 8A**).

In summary, it was observed that similar to C metabolism, the embryo also influenced the N metabolism of the NB endosperm, limiting the storage ability of endosperm and causing chalkiness at the bottom part of the endosperm.

## DISCUSSION

Embryo–endosperm interaction considerably affects the seed development and consequently the physico-chemical properties of rice grain, such as the chalkiness. This study took advantage of the NB mutant to devise a novel comparison method for quantifying the impact of embryo on metabolic processes in the developing endosperm, unraveling the fact that the embryo has a noticeable effect of diverting C and N metabolism from the biosynthesis of storage compounds to secondary pathways

in the chalky endosperm. This finding reveals new aspects on the role of embryo in the formation process of rice quality, and helps to map out effective strategies for high-quality rice breeding.

## Metabolic Signatures for Embryo and Endosperm Development

Metabolomics, combined with other "omics" methodologies, has emerged as an effective method for the studies of functional genomics and systems biology (Ghatak et al., 2018). Rice seed is a delicate biological system, mainly consisting of two genetically distinct tissues, the diploid embryo, and triploid endosperm (Wu et al., 2016a). So far, only few studies have studied both the embryo and endosperm in the same experiment (Galland et al., 2017). In the present study, using non-targeted metabolomics, we represented the distinct metabolic details about the embryo and the endosperm during seed development. The results revealed that both the embryo and endosperm samples were well distinguished by PCA, demonstrating a significant difference in the metabolism between two compartments. By *K*-means clustering algorithm and KEGG enrichment analysis, we integrated the cellular metabolic processes across the development phases of embryo and endosperm. Metabolic signatures of the embryo and endosperm at the major stages of seed developments are detailed as follows.

At the early stage (10 DAF), most of the morphogenetic events in the embryo have already occurred at this stage (Itoh et al., 2005), and the endosperm has finished tissue differentiation, forming two subregions, aleurone, and starchy endosperm, and starts storing starch and proteins (Wu et al., 2016b). At this phase, the metabolites related to glycolysis, TCA cycle, and oxidative phosphorylation were overrepresented both in the embryo and endosperm, indicating that the two compartments share common metabolic processes, which is consistent with the report by Belmonte et al. (2013).

At the middle stage (20 DAF), the embryo greatly grows to its maximum volume and starts to mature (Itoh et al., 2005). Meanwhile, the galactose, starch, sucrose, and inositol phosphate metabolism were active, which may play an important role in the transformation of embryo development from organ formation to maturity. For example, phytic acid (inositol 6-phosphate, I6P) is the principal storage form of phosphorus (P) in cereal grains and is considered a key compound in inositol phosphate metabolism (Perera et al., 2018). Previously, the phytic acid accumulation was observed during the rice seed development which was enhanced particularly at the middle stage (20 DAF) (Lin et al., 2017b). Similarly, in the present study, the rapid accumulation of phytic acid was observed, which can be taken as a sign of embryo maturation. Simultaneously, the endosperm starts to store the nutrients at the maximum rate. Heat map display of all data showed a clear overall decline in the relative levels of the vast majority of compounds as the seeds matured (**Figure 3**). This pattern is more obvious at this stage as C and N start to incorporate into macromolecules (starch, proteins, and lipids), which was in line with the finding of Lin et al. (2017b). In addition, the metabolites and genes related to starch and sucrose metabolism were also overrepresented (**Supplementary Figures 3, 4**).

At the late stage (30 DAF), the embryo becomes tolerant of desiccation after completion of reserve accumulation and undergoes a developmentally programmed dehydration event leading to dormancy and a quiescent state (Manfre et al., 2009). The starchy endosperm cells die completely upon seed maturation and desiccation (Zeeman, 2015). Low internal oxygen levels may be advantageous during seed desiccation by alleviating the oxidative damage of membranes and enzymes, which would otherwise lead to defects in seed vitality and longevity (Angelovici et al., 2010). In this study, genes related to peroxisome were commonly enriched in both the embryo and the endosperm at this stage, indicating that the antioxidant process is essential for seed desiccation.

## Effect of Embryo on Metabolic Transition of Developing Endosperm

As a major source of human calories, the rice seed is mainly composed of the two fertilization products, embryo and endosperm, and their surrounding maternal tissues. The formation of a viable rice seed requires a close cooperation between the embryo and endosperm (Lafon-Placette and Köhler, 2014; Robert, 2019; Anderson et al., 2021; Xiong et al., 2021). Knowledge of the embryo–endosperm interaction is essential for dissecting the mechanisms responsible for resource allocation within the developing seed, which is critical for the formation of both grain yield and quality. In comparison to another important cereal crop of maize (*Zea mays*), fewer studies have been conducted on this bidirectional communication in rice seeds (An et al., 2020). Maize is an excellent cereal model for research on the interaction between two compartments because of the relatively larger size of the embryo that accounts for 20% of the grain volume (Nagasawa et al., 2013; Chen et al., 2014). In contrast, the embryo tissue makes up only 2–3% of rice grain on the base of dry matter (Juliano and Tuaño, 2019), which may be a limitation for in-depth analysis of this interaction (Lafon-Placette and Köhler, 2014).

As interpreted above, the use of NB mutant made an interesting finding that the embryo has a role in regulating the accumulation of starch and protein in the chalky endosperm (Lin et al., 2016). Based on this, the current study continued to use the NB and WT as materials, thereby designing a new comparison system that shows the effect of the embryo on endosperm development. Our results found that the embryo impairs the storage of sucrose, amino acid, starch, and storage proteins in the bottom endosperm of NB by enhancing the expression of sugar, amino acid, and peptide transporters and declining the expression of starch, prolamin, and glutelin synthesis-related genes. The results were consistent with the previous studies and confirmed the transporters as an important "switch" to regulate nutrients allocation between the embryo and the endosperm (Lin et al., 2017a,b). Furthermore, the competitive

advantage of the developing embryo in translocating nutrients from the endosperm transforms the bottom endosperm into a "superior source" by shifting the C and N metabolism from the anabolism of storages to other metabolic pathways, such as central metabolism and osmotic and antioxidant regulation, verifying the speculation that embryo exerts a "counter-acting regulation force" on the endosperm during the seed development by influencing its metabolic processes and gene expression (An et al., 2020). However, considering its insignificant proportion in the rice grain, the significant impact of the mini-size embryo on the endosperm development remains to be further verified.

## Mechanism Underlying Chalkiness Formation From the Perspective of Embryo–Endosperm Interaction

Grain chalkiness is highly undesirable for the rice industry because of its negative effect on the appearance quality as well as the sensory and milling quality of rice. Though a number of chalkiness-related QTLs were mapped, only few genes were isolated and functionally characterized, with the formation and regulatory mechanism of rice chalkiness still being one the most formidable challenges for rice geneticists (Liu et al., 2010; Sreenivasulu et al., 2015; Zhu et al., 2018; Xie et al., 2021). From the viewpoint of crop physiology, the hampered work on the genetic dissection of grain chalkiness may be associated with the intricate nature of the physiological aspects of chalkiness formation. As explained in section "Introduction," the occurrence of chalky tissue is a result of unbalanced accumulation of starch and protein. Therefore, any disturbance in C or N metabolism can cause the formation of this opaque tissue due to the loose packing of starch granules or/and protein bodies. As shown in the current study, compared with the upper translucent endosperm, the levels of sucrose, amino acids, starch, and storage proteins in the bottom chalky endosperm are all downregulated. This finding agrees with our previous study (Lin et al., 2016, 2017a,b), supporting the critical role of C and N metabolism in chalkiness formation. However, most of the related studies are confined to the endosperm itself; fewer studies have been conducted to examine the role of the embryo. As a result, the signals that regulate the physiological processes in the endosperm are still unclear.

In this study, we successfully applied a novel comparison method to show the direct effect of embryos on the metabolic processes of the endosperm. Using WT as a reference, a comparison of the bottom and upper parts of NB grains confirmed the findings of our previous work as well as identified new clues responsible for grain chalkiness formation. Our results found that the embryo impairs the storage of sucrose, amino acid, starch, and storage proteins in the bottom endosperm of NB, by enhancing the expression of sugar, amino acid, and peptide transporters and declining the expression of starch, prolamin, and glutelin synthesis-related genes. In particular, the embryo effect was more evident at the critical stage of chalkiness formation (10 DAF). This stage is critical both for embryo morphogenesis and endosperm development, as evidenced by the highly expressed *Chalk5* gene that is responsible for chalkiness formation (Li et al., 2014). Furthermore, taking advantage of the altruistic behavior of endosperm toward embryo development, the developing embryo attempted to deplete its "adjacent source," the endosperm, by the excessive excavation of nutrients. And this could alter the endosperm metabolism, causing chalkiness formation due to the poor accumulation of storage compounds. In the previous study, the source-limitation was considered a key reason for chalkiness formation, reflected by the insufficiency of nutrients from photosynthetic organs, such as leaves that begins to decrease in the biosynthesis ability of storage compounds (starch and proteins) in the endosperm (Wang et al., 2008; Hu et al., 2020). In the present study, we uncovered the mechanism of chalky endosperm formation from the perspective of the embryo–endosperm interaction, showing that the chalky endosperm formation is not only caused by source limitation, but also by nutrient competition between the embryo and the endosperm. This finding indicates that the interaction between the embryo and the endosperm plays an important role in the formation of rice quality.

# CONCLUSION

This study unraveled the metabolic signatures across the development phases of the embryo and the endosperm, and demonstrated a direct effect of the embryo on the transition of metabolic patterns in the endosperm. A novel comparison method based on the NB mutant revealed a substantial influence of the embryo on the metabolic processes in the endosperm. The embryo imposed an inhibitory effect on the accumulation of sucrose, amino acid, starch, and storage protein in the endosperm, which might be related to the increased expression of sugar, amino acid, and peptide transporters and decreased the expression of starch, prolamin, and glutelin synthesis genes. Importantly, the embryo–endosperm interaction, particularly, the competitive advantage of the embryo in extracting the nutrients from the endosperm during the embryo development, transformed the C and N metabolism of the endosperm from storage biosynthesis to secondary metabolic pathways. Consequently, the impaired endosperm filling resulted in the formation of chalkiness. In summary, this integrative study highlights the role of the embryo in the endosperm chalkiness formation, with the underlying metabolic processes being identified. Hopefully, the results obtained here should help rice breeders to work out new pipelines for nurturing high-quality rice cultivars. Future studies should be centered on the genetic control of the reprogramming of metabolic pathways in the developing endosperm, and clarify the molecular mechanism involved in the communication between the embryo and the endosperm.

# DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA722833.

## AUTHOR CONTRIBUTIONS

YT had the main responsibility for data collection and analysis. YT and AM wrote the manuscript. LA, HC, GL, and YD revised the manuscript. ZL had the overall responsibility for experimental design, project management, and manuscript preparation. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.760597/full#supplementary-material

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REVIEW
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# Physiological and Multi-Omics Approaches for Explaining Drought Stress Tolerance and Supporting Sustainable Production of Rice

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Drought differs from other natural disasters in several respects, largely because of the complexity of a crop's response to it and also because we have the least understanding of a crop's inductive mechanism for addressing drought tolerance among all abiotic stressors. Overall, the growth and productivity of crops at a global level is now thought to be an issue that is more severe and arises more frequently due to climatic change-induced drought stress. Among the major crops, rice is a frontline staple cereal crop of the developing world and is critical to sustaining populations on a daily basis. Worldwide, studies have reported a reduction in rice productivity over the years as a consequence of drought. Plants are evolutionarily primed to withstand a substantial number of environmental cues by undergoing a wide range of changes at the molecular level, involving gene, protein and metabolite interactions to protect the growing plant. Currently, an in-depth, precise and systemic understanding of fundamental biological and cellular mechanisms activated by crop plants during stress is accomplished by an umbrella of -omics technologies, such as transcriptomics, metabolomics and proteomics. This combination of multi-omics approaches provides a comprehensive understanding of cellular dynamics during drought or other stress conditions in comparison to a single -omics approach. Thus a greater need to utilize information (big-omics data) from various molecular pathways to develop drought-resilient crop varieties for cultivation in ever-changing climatic conditions. This review article is focused on assembling current peer-reviewed published knowledge on the use of multi-omics approaches toward expediting the development of drought-tolerant rice plants for sustainable rice production and realizing global food security.

**Keywords: QTL, global food security, multiomics, rice, drought, abiotic stress, proteome, metabolome**

## INTRODUCTION

Rice (*Oryza sativa* L.) is the topmost grain consumed as a staple food by humans worldwide, most prominently in Asian countries (Asia, 2021; Samal et al., 2021). It belongs to the family *Poaceae* and tribe *Oryzeae* and presently comprises 22 wild species with only two species cultivated at a global level. Rice, with a genome size of 430 Mb, is an important model crop plant, and it has been reported that Asia ranks the highest in global rice production (Manavalan et al., 2012; Asia, 2021). As rice requires more water, up to 4000 liters per kilogram production, the plant is more prone to drought stress than other environmental stressors. Among all these stressors, drought stress is a major factor that can hinders the growth, yield and productivity of rice crops (Mumtaz et al., 2020). Drought is a multi-faceted stress and may affect rice production in various ways. In rainfed areas, it affects directly by further drying of the paddy soils, but in irrigated areas it hits in the form of declining underground water tables and become an issue for water sustainability. In the field, drought effects can be compounded by interactions with other biotic and abiotic stresses. Further, conventional cultural practices in rice require a season-long flooding, but in today's circumstances, it is unsustainable, and there is competition for the utilization of surface water (rivers, etc.) with increased urbanization; additionally, low underground water availability due to the continually declining water tables of aquifers is also becoming common. All these combinations have increased a strong need for understanding and increasing drought tolerance in rice crop.

The negative implications of drought stress on the physiological functioning of plants are mainly due to its reduced water potential and turgor pressure that suppress plant growth and metabolism (Lisar et al., 2012). Drought is a period in crop's growing season during which the soil moisture reaches to a certain level that reduces its yield or quality resulting from either limited irrigations or a below average rate of precipitation and higher evapo-transpirations, which causes a decline in plant growth and productivity (Rollins et al., 2013; McClung et al., 2020). Drought affects plants in numerous ways by impairing normal molecular, metabolic and physiological networks to reduce growth and metabolism (Zu et al., 2017). A typical implication of drought includes a decline in the expansion of leaves and an overall decrease in the stomatal conductance and rate of photosynthesis (Avramova et al., 2016; Barnaby et al., 2019). Furthermore, a reduction in growth is also reported to be induced by the inhibition of cell elongation, cell expansion and impairment in mitosis (Potopova et al., 2016). Against these consequences, plants respond at the morphological, physiological and molecular levels. These responses include maximum uptake of water by a dense and deep root system, reduction in water loss by closure of stomata, adjustments in osmosis, reducing the leaf area, altering the elasticity of cell wall and several more physiological adjustments (Saha et al., 2016). Physiological traits are critical to selecting drought-tolerant germplasm of crop plants, even though the response to drought stress by crop plants is dependent on the environment and diverges across genotypes and genetic interactions (Lonbani and Arzani, 2011).

At present, freshwater shortage is major issue on global scale and could be more critical in the near future as per climate fluctuation projections. In developing sustainable solutions to overcome water scarcity, there is a constant need for scouting strategies that could be possible alternatives to ensure the availability and accessibility of freshwater to crop plants (Noemi et al., 2014). Rice, among other cereal crops, demands access to freshwater, i.e., water-intensive cultivation. Moreover, half of humankind relies on rice as a staple food, and at the global level, a total of 160 million hectares (Mha) of cultivated land comprises this crop, most of which (around 40%) lies in Asian countries (Prasad et al., 2017). The rice plant is cultivated in flooded paddy fields since it requires an adequate water supply of almost 2 to 3 times more than dryland cereals.

Consequently, researchers are now faced with a challenge to increase the adaptation of rice cultivars to water-scarce conditions and to reduce the dependency on a large quantity of water to support their cultivation in almost dry land cultivation systems (Sandhu et al., 2014). Major adaptations include adjustments in physiological, anatomical and morphological characteristics of root and shoot traits (Kadam et al., 2015; Sandhu et al., 2016) (**Tables 1, 2**). An irrigated rice system is the most commonly cultivated practice, comprising up to 55% of the 158 Mha cultivated land area, whereas 34% of the 54 Mha land cover is shared by rainfed lowland systems, and rice grown in flood-prone areas shares 7% of the 11 Mha land area (Bouman et al., 2007). Approximately 37% of global rice production is from South Asia, among which 50% is rainfed (Dawe et al., 2010). Moreover, rainfed rice is also cultivated in sub-Saharan Africa, accounting for up to 84% of the total area of rice cultivation (Gauchan and Pandey, 2012). Since the global production of rice is grossly dependent on rainfed ecosystems, drought stress becomes a major factor behind decreased productivity from 13 to 35%. Thus, one can witness an enormous global loss in rice productivity compared to other crops due to its higher dependency on water and hence proneness to drought stress. All the evidence suggests that climate induced fluctuating rainfall and drought result in heavy losses to rice farming systems (Fahad et al., 2019).

A sustainable approach for rice production is to minimize irrigation, which is being realized in commercial production by the introduction of alternate wetting and drying (AWD) irrigation management (de Avila et al., 2015; Chen M.-H. et al., 2021; see also[1]). Currently, farmers are practicing safe-AWD levels because most current varieties are bred for season-long flood irrigation management under both lowland and upland rice cultivations. In AWD, there is a drying phase, and rice is susceptible to drying conditions, because they affect yield and grain quality (McClung et al., 2020). Shaibu et al. (2015) compared Nunkile and NERICA 4 rice varieties, which are adapted to upland and lowland irrigated conditions, under continuous flood and three different AWD schemes. The results showed that water productivity for continuous flood treatment was superior to all three AWD schemes. Hence,

[1]http://www.knowledgebank.irri.org/training/fact-sheets/water-management/saving-water-alternate-wetting-drying-awd

**TABLE 1 |** Drought tolerance genes that have been tested on rice.

| Sr. No | Cellular mechanism | Gene | Promoter | Genetic transformation method | Targeted phenotype | References |
|---|---|---|---|---|---|---|
| 1 | Abscisic acid metabolism | CAMV35SP | DSM2 | Agrobacterium | Oxidative and drought stress tolerance | Du et al., 2010 |
| 2 | ROS scavenging | OSSROIC | Ubi 1 | Agrobacterium | Oxidative stress tolerance and stomata closure regulation | You et al., 2013 |
| 3 | Protoporphyrinogen oxidase | PPO | – | Agrobacterium | Less oxidative damage and drought tolerance | Phung et al., 2011 |
| 4 | Ubiquitin ligase | OSSDIR1 | CAMV35 Ubi1 | Agrobacterium | Stomata regulation under drought stress | Gao et al., 2011 |
| 5 | Abscisic acid sensitivity | OSSAPK2 | – | – | Abscisic acid sensitivity and drought tolerance | Lou et al., 2017 |
| 6 | DNA damage repair and defense response | OsNAC14 | OsRAD51A1 | | DNA damage repair and defense response resulting in improved tolerance to drought | Shim et al., 2018 |
| **7** | Multiple stress tolerances in rice plants during both seedling and panicle development stages | OsAHL1 | $P_{AHL1}$ | – | Regulates root development under drought condition to enhance drought avoidance | Zhou et al., 2016 |
| 8 | Enhanced resistance to a bacterial pathogen | *Os*WRKY11 | CHIT 2 | – | Enhanced resistance to a bacterial pathogen | Lee et al., 2018 |
| 9 | Reactive oxygen species scavenging | OsLG3 | *OsLG3-OE* | – | Reactive oxygen species scavenging and drought tolerance | Xiong et al., 2018 |
| 10 | Increased moderate susceptibility to the pathogens | *OsMADS26* | ubiquitin1 | – | Increased moderate susceptibility to the pathogens and drought tolerance | Khong et al., 2015 |
| 11 | Induced a variety of environmental stresses and plant hormones | OsDRAP1 | CaMV35S | Agrobacterium | High expression in response to drought | Huang et al., 2018 |
| 12 | Increases ABA sensitivity and enhances osmotic tolerance in rice | *OsEm1* | LEA | – | ABA sensitivity and enhances osmotic tolerance promising for engineering drought tolerance in rice | Yu et al., 2016 |
| 13 | Reactive oxygen species (ROS)-scavenging | OsCML4 | CaMV35S | – | Reactive oxygen species (ROS)-scavenging and drought tolerance | Yin et al., 2015 |
| 14 | Control of tiller outgrowth | *OsIAA6* | PGD1 | Agrobacterium | Drought stress responses and the control of tiller outgrowth. | Jung et al., 2015 |
| 15 | Conversion of aspartate amino acid to glutamate was found to be associated with drought tolerance | *OsDREB1F* | CaMV35S | | The categorization of all the significant SNPs with H5 drought tolerant haplogroup supports their role in drought tolerance in rice | Singh et al., 2015 |

for significant water savings, farmers need improved varieties that can withstand drier soils without decreasing yield and grain quality. Thus, to develop improved rice cultivars that can produce profitable yields with significant water savings, researchers need a better understanding of drought tolerance mechanisms and of the tools available to them. Furthermore, the situation is compounded by the use of susceptible rice varieties by farmers in subtropical and tropical cultivated areas (Zu et al., 2017). Thus, it is important to develop new rice cultivars that possess traits such as higher yield and resilience to drought stress. The evolution and production of drought tolerant rice varieties are developed by understanding the molecular mechanisms and signal responses initiated by tolerant cultivars under water deficit conditions. This approach does not mean that the traditional germplasm or breeding line selection process does not play an important role in this process of developing new rice varieties, some of which could have desirable traits such as short duration with higher yield (Mehana et al., 2021). However, the underlying molecular mechanism must be understood at a holistic level, and therein omics tools are needed and are prominent.

The past few decades have witnessed extensive research based on application of omics technologies paving way to identify a large number of candidate genes, proteins and pathways to generate drought tolerant varieties of rice plants (Panda et al., 2021; **Figure 1**). Omics-based high throughput techniques facilitated unbiased studies on the genome, epigenome (epi)transcriptome, metabolome and proteome (Oliver et al., 1998; Hasin et al., 2017; Yu et al., 2021). Furthermore, technologies are employed to comprehend the underlying molecular mechanisms to resolve complex cellular responses and effects on the phenotypes of the crop (Manzoni et al., 2018; Upadhyaya and Panda, 2019). Considerable molecular data has been created by advancements in genomic and transcriptomic techniques but researchers are still behind in correlating the data with the proteomes due to limited depth of quantitative proteomic data inputs with respect to post-translational modifications especially (Molina et al., 2011; Ghatak et al., 2017). To date, gene expression analyses through applications of high-throughput technologies have revealed the transcription levels in cells. In addition, several investigations were conducted to correlate the transcriptomic

and proteomic information to highlight the critical impact of post-transcriptional modifications on cellular subtleties (Foss et al., 2011; Ghazalpour et al., 2011). Recent advancements in drought stress-related data support the role of omics technologies in understanding and determining the mechanistic make-up of plants, to develop crop varieties that are resilient to drought stress. Most of these studies have demonstrated the critical role of post-transcriptional and post-translational modifications of proteins in defensive mechanisms that allow the plant to adapt to a diverse range of abiotic stressors (Matsuura et al., 2010; Sormani et al., 2011; Juntawong and Bailey-Serres, 2012; Liu et al., 2012).

The quantification of biomolecules, such as genes and proteins, is now easier due to the advent of high-throughput technologies, for studying the transcriptomes and proteomes. For instance, the E3-Ubiquitin Ligases proteins abundantly expressed in rice help in modulation of abiotic stressors such as drought, salinity, nutrient deprivation and radiation (Melo et al., 2021). Drought tolerance genes and their cellular processes, such as signaling-controlled gene expression and cellular modifications, have been deeply investigated by multi-omics high-throughput technologies (Huynh et al., 2018; Yadav et al., 2019; Singh et al., 2021). Keeping in perspective the importance of a multi-omics approach to better understand abiotic stress tolerance mechanisms in crops and developing novel stress mitigation strategies, the present article comprehensively summarizes the state-of -the-art knowledge on the molecular aspects of drought stress. Moreover, the current review also presents insights gleaned thus far on drought stress using various omics approaches with a special focus on rice, given its global significance as a major food crop.

**TABLE 2 |** QTLs identified for drought tolerance related traits in rice.

| S. No | Targeted trait | Number of QTL'S | References |
|---|---|---|---|
| 1 | Grain yield | 1 (Qdty2.1) | Mishra et al., 2013 |
| 2 | Grain yield | 1 (Qdty3.2) | Yadaw et al., 2013 |
| 3 | Grain yield | 14 | Wang et al., 2014 |
| 4 | Filled grain number per panicle | 23 | Wang et al., 2014 |
| 5 | Panicle number per plant | 14 | Wang et al., 2014 |
| 6 | Grain yield | 1 (Qdty2.3) | Palanog et al., 2014 |
| 7 | Grain yield | 1 (Qdty2.2) | Palanog et al., 2014 |
| 8 | Grain yield | 4 | Saikumar et al., 2014 |
| 9 | Grain yield | 7 | Singh et al., 2015 |
| 10 | Grain yield | 1 (qDTY 12.1) | Swamy et al., 2018 |
| 11 | Flowering time | 1 | Prince et al., 2015 |
| 12 | Flowering time | 5 | Saikumar et al., 2014 |
| 13 | Flowering time | 1 | Sandhu et al., 2014 |
| 14 | Canopy temperature | 6 | Prince et al., 2015 |
| 15 | Biomass | 8 | Prince et al., 2015 |
| 16 | Biomass | 4 | Saikumar et al., 2014 |
| 17 | Drought index | 3 | Prince et al., 2015 |
| 18 | Grain weight | 2 | Zhou et al., 2013 |
| 19 | Grain yield | 24 | Verma et al., 2014 |
| 20 | Seed setting rate | 6 | Prince et al., 2015 |

## APPROACHES IN USE FOR CONFERRING DROUGHT TOLERANCE IN RICE

The improvement of rice varieties could be accomplished by mining genes and superior alleles capable of better signal perception, signal transduction, and functional roles in drought tolerance. The repository of resilient genes and traditional donors of these genes includes wild accessions, landraces and varieties, such as Aus 276, Birsa gora, Dhagaddeshi, Dular, Kali Aus, Nagina 22 (or N22), and Vandana. Considering genetic improvement, several varieties have been developed through the selection of resilient landraces for a specific trait. For example, the landrace "Rajbhog" found in the foothills of Nepal was used to develop Nagina 22 through selection processes and is also a well-known drought tolerant variety of rice (Vikram et al., 2011). Furthermore, a drought tolerant Kataush landrace from the Nepal Tarai region was characterized (Puri et al., 2010). Landraces also resulted in the development of Laloo-14, a drought tolerant Indian rice variety cultivated in rainfed areas of Madhya Pradesh state of India, which validates the immense potential of landraces for improving rice cultivars for cultivation in rainfed areas (Akshaya et al., 2017). The rich repository of resilient genes found in landraces makes them suitable candidates to look for adaptation related genes or superior alleles important for resiliency for production in limited irrigation management. Various drought tolerant Indian landraces have been reported to possess drought tolerant genes (Vanniarajan et al., 2012). For further improvement of rice crop, it is pertinent to also identify and characterize drought tolerant genes by modern molecular breeding technologies.

Apart from the landrace varieties and wild germplasm accessions, natural hybrids and genetic stocks also act as rich genetic sources for the improvement of rice cultivars (Biswas et al., 2020). This wide range of rice landraces can be used to identify genes by diverse approaches, including omics-based techniques (**Figure 2**), for their subsequent transfer into elite breeding lines.

Only a few traits that pertain to roles in water use efficiency (WUE) and tolerance to drought have been implemented in the field, even though numerous crop species have been screened by mining drought tolerant genes (Langridge and Reynolds, 2015). A wide range of techniques, such as omics tools, molecular breeding and precise phenotyping, augment the identification of candidate genes that pertain to metabolic and signaling pathways and that are pivotal for drought tolerance in crops. Besides, the workflows for initiating the in-depth cellular phenotyping of diverse crop plants through applications of multi-omics technologies will further augment the quest of unraveling the resilience mechanisms (Zivy et al., 2015). In addition, various studies, such as functional genomics and expression profiling of genes, are available to comprehend the complexity of drought tolerance mechanisms in crop plants (Delphine et al., 2010; Li et al., 2018). Differential gene expression analysis has also been used to identify drought responsive genes in crop plants (Guo et al., 2008; Gray et al., 2010).

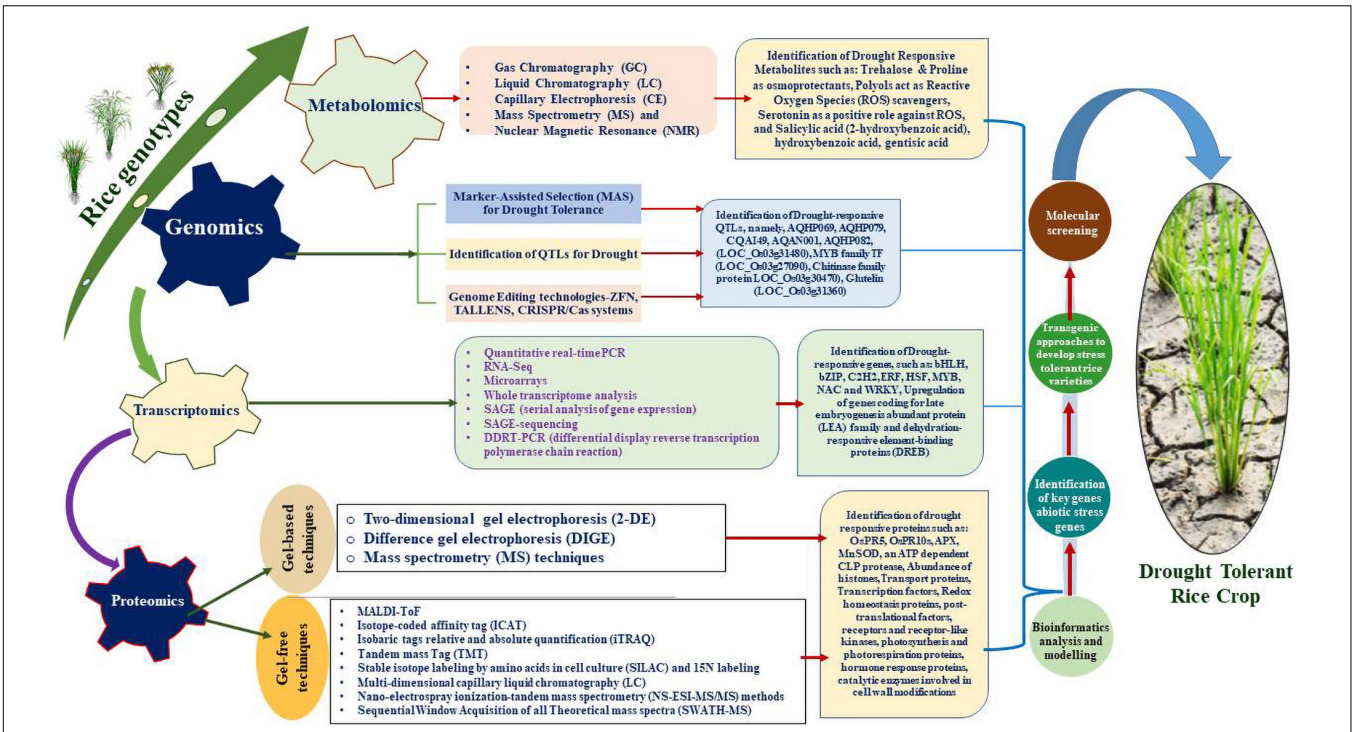


**FIGURE 1 |** Diagrammatic representation of OMICS approaches employed to unravel the genes/proteins to produce transgenic rice plants.

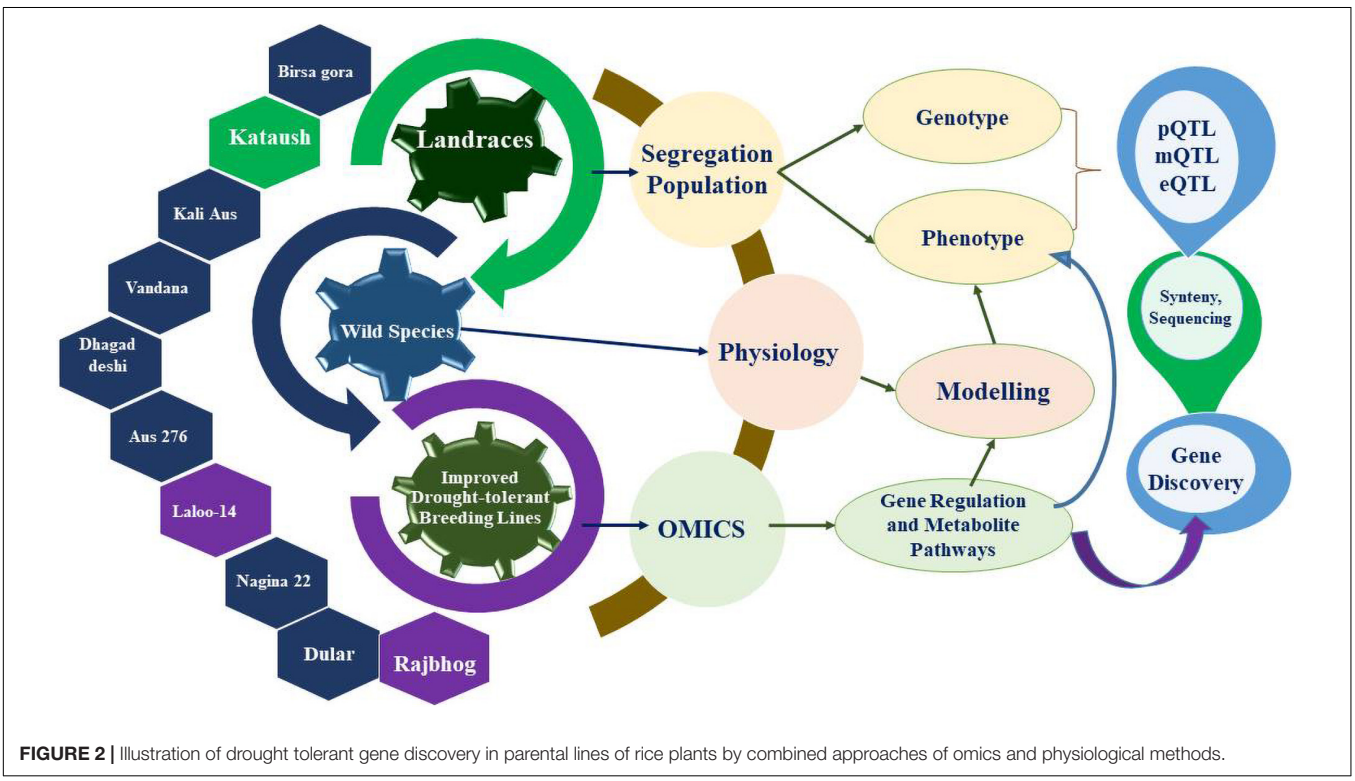


**FIGURE 2 |** Illustration of drought tolerant gene discovery in parental lines of rice plants by combined approaches of omics and physiological methods.

Even though realistic progress through omics technologies such as metabolomics, proteomics, and genomic methods compared with epigenomics methodologies is scant, these approaches have provided much needed and useful information pertaining to genetic and physiological attributes linked to drought tolerance. We aim to combine efforts in the application of omics approaches

in conjunction with plant breeding methods to extend the development of resilient rice cultivars aimed at cultivation in rainfed ecological niches or save significant amounts of water under irrigated field managements.

## Plant Traits

Physiological adjustments established by crop plants against drought stress enable them to regulate their water requirements for normal physiology, growth and metabolism (**Figure 3**). The following sub-sections detail the important physiological adjustments accomplished by crop plants against drought stress:

## Shoot Traits

Reductions in growth and metabolism are primarily induced by drought in rice plants. Moreover, the initiation of new aerial organs, such as the stems, leaves and pre-existing organs, in aerial parts of plants are considerably affected by drought stress (Chaves et al., 2002). Central to the reduced growth of these aerial parts is the decline in cell division in plants and modulation of the physio-chemical properties of cell walls due to an increase in cell wall rigidity, to reduce expansion and hence the growth (Granier et al., 2000; Cosgrove, 2005). A significant decline in the leaf expansion rate was observed when water stress of 80% transpirable water soil moisture for 20 days was observed in the vegetative phase in rice (Farooq et al., 2009). Thus, adaptations result in limited loss of water by transpiration through leaf surfaces. A reduction in the rate of photosynthesis in rice prevails based on the change in leaf color and conductance of mesophyll layer to $CO_2$ during the drought stress period (Lauteri et al., 2014). The change in green color is due to the relative chlorophyll content in rice leaves and can be measured by SPAD meter under drought conditions in comparison to the irrigated conditions (Ndjiondjop et al., 2010; Barnaby et al., 2019). Zinolabedin et al. (2008) reported contradictory observations regarding water and nutrients uptake by the plant root system, which in turn caused a reduction in the chlorophyll concentration and subsequent yellowing of the leaves. Under drought stress, plants roll their leaves to reduce the transpiration rate, to maintain water levels (Sié et al., 2008). Leaf rolling capacity is now considered to be one of the major physiological adaptation strategies by crop plants to maintain their water status during drought stress periods (Fukai and Cooper, 2002). Moreover, a direct correlation was observed between grain yield and leaf rolling attributes of crop plants. Henderson et al. (1995) reported that leaf tip drying acts as a good indicator of the level of drought stress. The rolling of leaves is a reversible physiological adaptation, even though leaf tip rolling induced by drought stress is irreversible. Other abiotic stress factors, such as freezing and salinity, are also linked to a decline in the availability of water to plants. The decline in water availability is quantified as a decrease in the water potential. The indicator of dehydration avoidance is recognized by the leaf water potential (LWP) (Pantuwan et al., 2002). The stomata are closed when the water level goes beyond the water deficit in leaves to lower the rate of transpiration by minimizing the damage caused by water loss. Moreover, the higher LWP is regulated by varietal differences in the stomatal response and the closure of stomata (Jongdee et al., 1998). Higher photosynthetic capability possessed by certain genotypes helps plants to protect from the onset of senescence under stress conditions. This so-called stay green trait possessed by plants also helps in assimilating nitrogen and helps in optimizing the photosynthetic capability of plants under drought stress (Borrell et al., 2001). Other physiological adaptations possessed by plants include modifications in water uptake, loss of water potential, enhancing accumulation of compatible solutes, overexpression of tolerant proteins, prevention of cell damage and enhancing repairing by cell division.

## Root Traits

Imperative to the growth and metabolism of plants is the absorption of water and nutrients, which is grossly dependent on modifications in the morphological and physiological characteristics of roots (Ghosh and Xu, 2014). Various characteristics such as, type of root system, its properties, structures and wide distribution of roots helps to uptake water from soil to maintain productivity under water stress conditions (Comas et al., 2013). Under drought stress, several root traits such as, long specific root length; root length density and small fine root diameters maintain the productivity and regulate water levels in rice crops (Comas et al., 2013). For example, under drought stress periods, it was reported that rice tolerant varieties such as, Chuanguyou208 and Deyou4727 showed larger length in roots, higher root number and weight under 30–50 cm depth soil layer and in addition these varieties also displayed higher activities of peroxidase (POD) and superoxide dismutase (SOD) (Wang X. et al., 2019). Moreover, the hydraulic properties of roots in rice under drought stress were enhanced by developing deep and thick root systems (Lipiec et al., 2013). These attributes helped rice plants to develop tolerance to drought through absorption of water from deep layers of soil (Gowda et al., 2011; Uga et al., 2013a). Moreover, supplementation of rice seedlings with $NH_{4+}$ resulted in higher root growth and an increased number of root tips to enhance water uptake under water stress conditions (Jia et al., 2020). In addition, rice seedlings supplemented with $NO^{3-}$ induced restriction in water uptake resulted in the induction of root aerenchyma formation (Yang et al., 2012). Later studies revealed that $NH4^{+}$ helped in higher water uptake compared with $NO^{3-}$ due to enhanced root growth.

## Inflorescence Traits

Rice plant is susceptible to water stress during reproductive stage, and the severity of yield and quality losses depend on magnitude and duration of the stress (Yang et al., 2019; Chen M.-H. et al., 2021). Shaibu et al. (2015) compared continuous flood with two AWD schemes focused on two different flowering stages: AWD up to the start of flowering (AWD1), and AWD up to the start of grain filling (AWD2). The results showed that both AWD schemes had significant yield loss compared with the continuous flood treatment. The results emphasize the need for robust inflorescence traits for drought tolerance. Under drought conditions, it is interesting to note that generally early morning period is cooler compared to late morning and afternoons. Hirabayashi et al. (2015) identified a novel QTL, qEMF3, for early-morning flowering trait from wild rice, *Oryza*

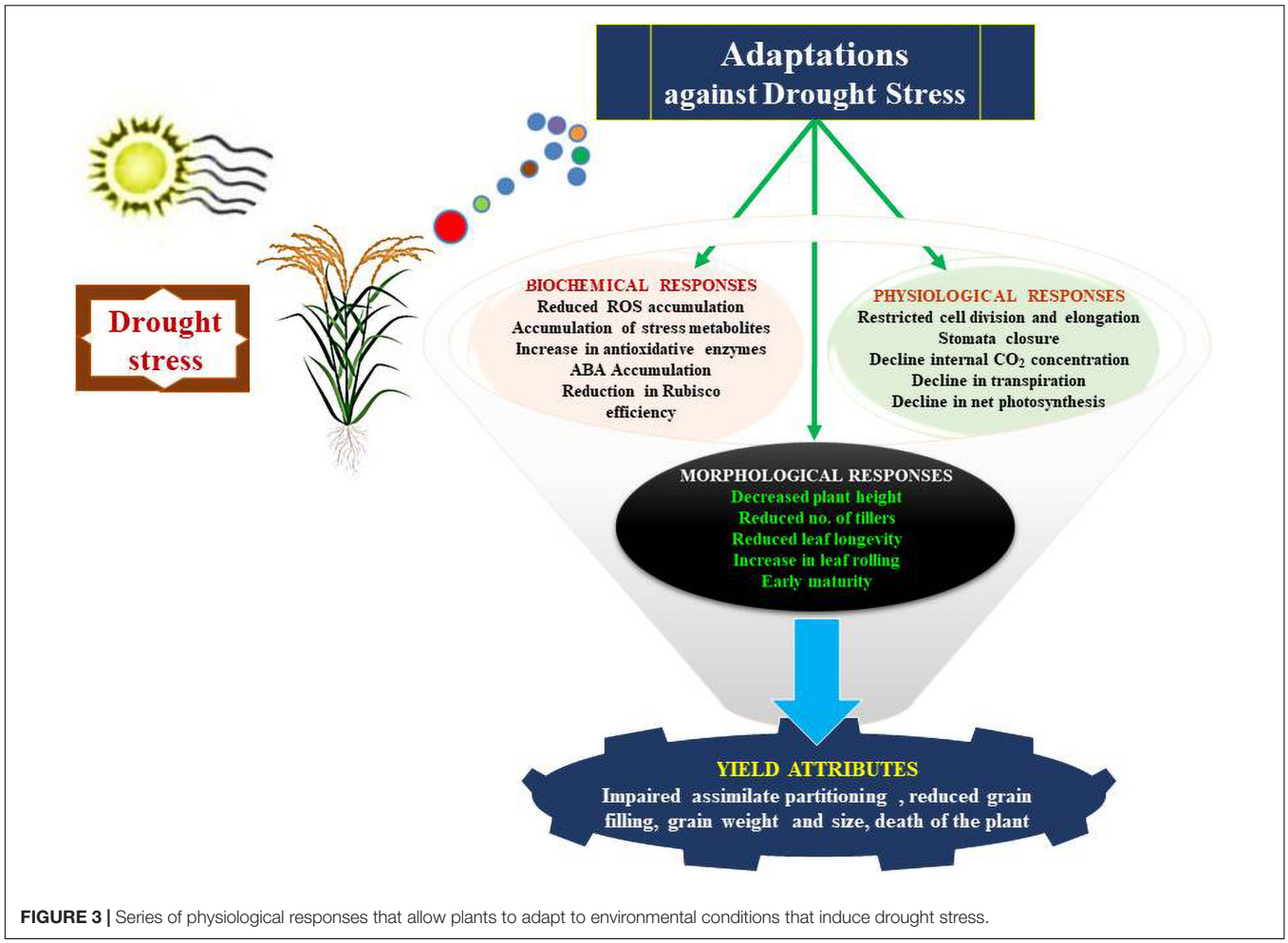


**FIGURE 3 |** Series of physiological responses that allow plants to adapt to environmental conditions that induce drought stress.

*officinalis*. The qEMF3 shifted flower opening time by 2 h earlier and improved spikelet fertility under stress environment. Similarly, studying pistil's role is another interesting inflorescence trait that has not been explored in detail to unravel molecular basis of stress tolerance (Wang et al., 2021). Rice is one of the best models to study the development of inflorescence through molecular analysis of inflorescence mutants for investigating architecture of inflorescence (Zhang and Yuan, 2014). The critical factors to decipher the complexity of rice traits include the total number of fertile tillers, and the development and architecture of the inflorescence (Ikeda et al., 2004). The rachis, primary and secondary branches, including the spikelet, are critical physiological features of rice inflorescences. The spikelet consists of one fertile floret, two sterile florets and two rudimentary glumes. Spikelet differentiation, the determining factor of seeds per panicle, depends on the establishment, transition and activity of axillary and apical meristems. The development of rice inflorescences is well explained by the ABCDE model for the specification of floral organs in addition to the role of genes such as OsMADS3, OsMADS14, OsMADS15, and OsMADS58 (Yoshida and Nagato, 2011). The inflorescence in rice is controlled by cytokinin and auxin signaling pathways, CLV-WUS signaling and expression of MADS box genes. In rice, the Drought and Salt Tolerance (DST) protein, a member of zinc-finger transcription factor, induces overexpression of OsCKX2 in the reproductive meristem for induction of inflorescence (Li et al., 2013). The depth in understanding the molecular mechanism underlying the regulatory responses against drought and that influence the inflorescence development will further reveal deep comprehensions regarding enhancing yield and thus food security in the changing climatic scenario.

## OMICS APPROACHES FOR UNDERSTANDING DROUGHT TOLERANCE

The methodologies studied under the omics approaches are categorized as genomics, transcriptomics, proteomics, metabolomics, epigenomics and comparative genomics.

### Genomic Approaches

Genomic studies are technical approaches for investigating the gene structure and functional dynamics of coding and non-coding sequences applicable to augmenting crop improvement. Important resources in structural and genomics studies include

mutant libraries, cDNAs, expression profiles, sequence data sets and quantitative trait loci (QTLs) (Jiang et al., 2011). For instance, Vikram et al. (2012) cloned several genes and identified QTLs that were important for drought tolerance in rice. Large numbers of QTLs were identified to resolve the complex nature of drought stress and alleviating mechanism for tolerating drought stress (Fleury et al., 2010). In addition, several QTLs are identified to be responsible for enhanced grain yield and for other secondary characteristics that allow withstanding drought stress in rainfed uplands and lowlands (Bernier et al., 2007; Venuprasad et al., 2009). Differential performance of QTLs has been observed in upland and lowland ecosystems under drought conditions. Finally, the best suitable QTLs are selected as per the ecosystems, genetic background and environmental conditions. Moreover, for developing high yielding varieties, marker assisted back crossing (MABC) was efficaciously applied experimentally in crop plants. For example, MABC has been employed to increase the productivity of KDML105 rice variety in north-eastern regions of Thailand (Kanjoo et al., 2012). It is expected in near future that applications of molecular breeding techniques such as, marker-assisted selection (MAS), SNP marker applications and genome wide assisted selection (GWAS) will pave way to understanding the molecular mechanisms underlying the resilience in crop plants against wide range of environmental challenges such as drought stress.

## Comparative Genomics

The evolutionary history of organisms is clearly revealed by carrying out comparative account on structure and functions of genes across the species. For example, the phylogenetic relationships of Poaceae family (members of grasses) are now well understood by investigated species-specific DNA markers. Further, the comparative studies of genomes have led to identify the syntenic regions of different species. For instance, it is found that two thirds of genes located on chromosome number 11 in wheat are distributed in 06 homologues of grass genomes (Singh et al., 2004). The QTL1.1 is classical example of syntenic relationship; this QTL is found in chromosome 3 in maize, chromosome number 4B in wheat and chromosome 6 in barely (Swamy et al., 2011). The rice drought grain yield QTL was shown to be homologues of with maize counterparts (Swamy et al., 2011). Comparative analyses showed the role of OsCPK9 and AtCPK10 genes in augmenting the ABA dependent drought responsive mechanism (Zou et al., 2010; Wei et al., 2014). Whereas, OsCPK10 played pivotal role in defending cellular membranes against ROS and drought stress (Kumar et al., 2014; Nakabayashi et al., 2014; Fang et al., 2015; Yin et al., 2015). Large numbers of stress responsive genes are reported to play critical role in development and growth of plants. In addition, a network of genes pertaining to seed development and stress response has been reported in rice (Cooper et al., 2003). Molecular chaperons have been found to play critical role in circumventing the drought response in crop plants. Upon 2DE-MS and GC/MS based proteomic and metabolomic techniques, it was reported that Bip (molecular chaperon binding protein) was overexpressed in transgenic soybean plant (Coutinho et al., 2021). Moreover, transgenic rice over expressing *OsSKIPa* gene performed well under drought conditions due to its critical role in expression of series of stress related genes including, PP2C, CBF2, RD22 and SNAC1 and also enhancing the ROS scavenging ability in rice plants (Hou et al., 2009). Furthermore, *OsbZIP46CA1* transcription factor gene, a modified product of OsbZIP46 was found to be overexpressed in transgenic rice under drought stress, was found to enhance the expression of stress related genes, most of which in turn downregulate the ABF/AREBs (Ning et al., 2012). The transgenic approaches thus lead to unraveling the repository of genes critical for regulating plant metabolism under drought and other stress conditions, thus paving a way toward global food security (Details regarding genes conferring drought tolerance in rice have been presented in **Table 1**).

# STRESS-INDUCED REGULATORY GENES AND QUANTITATIVE TRAIT LOCI FOR DROUGHT TOLERANCE

Against the stress induction, crop plants initiate a coordinated series of cellular and signaling processes. A great deal of physiological and morphological changes is actually the outcome of cellular responses regulated by large number of genes. It is reported that in rice, about 6,000 genes are downregulated and 5000 genes are upregulated under drought stress conditions (Maruyama et al., 2014). In addition, genome wide expression analysis led to identification of about 5,284 differentially expressed genes under drought conditions (Wang et al., 2011). For instance, the CO-like gene, *Ghd2* (grain number, plant height, and heading date2) is attributed to enhance the yield potential in rice. The *Ghd2* is almost prototypical to *Ghd7*, which helps in leaf senescence and drought tolerance. Contrary to this, it is reported that *Ghd2* overexpression considerably decreased the drought tolerance and once knocked-out it resulted in opposite effects (Liu et al., 2016). Another gene OsbZIP42 encoding a transcription factor is positive regulator of ABA dependent signaling pathway, hence critical for drought tolerance was found activated by stress or SAPK4 (ABA-activated protein kinase 4) (Joo et al., 2019). The OsbZIP42 genes, a member of EbZIP are found to be critical for established tolerance against the abiotic stress. In addition, large number of signaling cascades have been reported to be activated by ABA to activate adaptive response against abiotic stress conditions (Jiang and Zhang, 2002; Salazar et al., 2015; Sah et al., 2016).

In rice, SNAC1 and DST transcription factors (TFs) are activated by ABA mediated signaling to regulate stomatal movement (Hu et al., 2006; Huang et al., 2009). Furthermore, SNAC1 (stress responsive NAC1), a TF, is a member of the NAC family of TFs, which includes ATAF, CUC, and NAM, and approximately 149 members have been identified in rice plants. SNAC1 in particular is abundantly expressed in guard cells under drought stress conditions. Apart from ABA mediated activation of drought tolerant pathways, DREB (drought responsive element binding) is activated to act as frontline TFs to withstand drought stress (Yamaguchi-Shinozaki and Shinozaki, 2005). Moreover, the TF $C_2$-$H_2$-type zinc finger-containing protein helps in drought and salt tolerance and

mediates $H_2O_2$ induced pathways to mediate stomatal closure (Huang et al., 2009).

The ABA phytohormone is activated by drought stress, which in turn causes stomatal closure and expression of diverse genes. The concentration of ABA in guard cells directly influences the closure of stomata, and its concentration is increased during drought stress. The pyrabactin tolerance (PYR)/PYL (PYR1-like)/regulatory receptors that belong to ABA response receptors sense ABA to initiate the downstream signaling cascade (Ma et al., 2009; Park et al., 2009). Upon ABA binding, its receptor undergoes conformational changes, which enable binding and inactivation of PP2Cs (protein phosphatase 2C), a negative regulator, including ABA insensitive 2 (ABI2), ABA insensitive 1 (ABI1), PP2CA and homology to ABI1 (HAB1) (Geiger et al., 2009). Since the negative regulator of the signaling pathway PP2C is inhibited, downstream signaling molecules are released their by switching the signaling cascade. The Ser/Thr kinase OST1 (open stomata1/SnRK2.6/SRK2E) is the main target of PP2C and is an important junction for the regulation of transcriptional responses, including ABA and $CO_2$ responses. Upon release of OST1, it activates several TFs and phosphorylation of several plasma membrane proteins, which results in the closure of stomata through promoters such as AtRBOHF and SLAC1 (Fuji et al., 2009; Sirichandra et al., 2009; Klingler et al., 2010). In rice, protein complexes such as RCAR5/OsPYL, OsPP2C30, OREB1, and SAPK2 act as signaling units regulated by ABA (Kim et al., 2012). Among these proteins, OsPYL/RCAR5 were reported to be positive regulators of abiotic stress and are attributed to enhanced drought tolerance when overexpressed in transgenic rice plants. In rice, both reverse genetics and forward genetics have led to the identification of hundreds of stress responsive genes and respective QTLs (Wang et al., 2016).

A large number of kinases, such as OsMAPK5, which are functionally characterized as stress responsive MAPK (mitogen-activated protein kinase), have been found to be involved in regulating a diverse range of abiotic stresses, such as drought, cold and salt stress, even though they have been found to negatively regulate biotic stress, including bacterial and fungal infections (Xiong and Yang, 2003; Rohila and Yang, 2007). It has been reported that overexpression of TFs such as, SKIP, bZIP, and NAC helps to enhance drought tolerance in rice. In particular, NAC TFs, such as OsNAC5, OsNAC6, OsNAC9, OsNAC10, OsbZIP16, OsbZIP23, OsbZIP46, and OsbZIP71 are involved in enhancing drought tolerance in crop plants (Fukao and Xiong, 2013). In addition, WRKY TFs are also involved in drought tolerance and a large number of growth and developmental processes. For example, 97 WRKY genes identified in *O. nivara* (OnWRKY) and 89 WRKY genes in *Japonica* were mapped and identified in plants that have diverse range of functions (Sahebi et al., 2018).

A large number of QTLs have been identified in different populations linked to drought tolerance (Khowaja and Price, 2008). Large effect QTLs have also been identified for grain yield under drought conditions (Bernier et al., 2007). Approximately, 77 QTLs for grain yield and other aspects important for drought tolerance were identified by crossing two rice cultivars (Lanceras et al., 2004). These QTLs were identified to possess several physiological attributes, such as 05 QTLs for days to flowering, 6 QTLs for harvest index, 7 QTLs for grain yield, 7 QTLs for percent spikelet sterility, 8 QTLs for biological yield, 10 QTLs for total spikelet number, 11 QTLs for plant height and 23 QTLs for panicle number. Approximately 10 QTL components and high grain yield were identified by using a recombinant inbred population of rice, such as IR64 and Cabacu, under drought stress (Trijatmiko et al., 2014). The QTL, *qDTY12.1* was the first report linked to grain yield in the upland reproductive stage under drought conditions. This QTL was identified from the 436 F3 populations derived from Vandana and Way Raren (Bernier et al., 2007). The *qDTY2.1* and *qDTY3.1* identified in the backcross between Swarna and Apo are two other large effect QTLs known to affect grain yield under drought conditions at the lowland reproductive stage. Both QTLs showed pleiotropic effects on a few traits, such as PHT (plant height) and DTF (days to flowering) (Venuprasad et al., 2009). Moreover, Venuprasad et al. (2012a) reported *qDTY6.1* QTL has had a strong effect on aerobic drought stress. Grain yield under severe drought stress in lowland reproductive stage QTLs was identified in F3 populations derived from the crosses of Swarna × N22, IR64 × N22 and MTU1010 × N22 (Vikram et al., 2011). Later, QTLs were also identified in the Apo/IR64 and CT9993-5-10-1-M/IR62266-42-6-2 populations (Kumar et al., 2008; Venuprasad et al., 2012b). Other QTLs reported include *qDTY2.2, qDTY4.1, qDTY9.1*, and *qDTY10.1*, which were linked to grain yield and identified in populations obtained from backcrosses between IR64 and Aday Sel rice varieties (Swamy et al., 2013) (Details related to QTLs conferring drought tolerance in rice have been presented in **Table 2**).

## ROOT ARCHITECTURE FOR DROUGHT TOLERANCE

Water deficit is overcome by a series of genes responsible for root architecture and hence higher yields are observed in crop plants (Jeong et al., 2010; Iwata et al., 2013; Cheng et al., 2015). Specifically, the NAC family of TFs was found to be overexpressed, to regulate root architectures (Zheng et al., 2009). For instance, OsNAC9 is a member of the NAC TF that is responsible for root architecture under drought tolerance and grain yield (Redillas et al., 2012). Later, this gene was reported to be overexpressed when controlled by a constitutive or root specific promoter in transgenic rice under both drought and well-watered conditions. It was reported that recombinant lines of maize have 144% more yield than control plants under drought conditions (Zhan et al., 2015). In rice, the overexpression of the *OsDHODH1* gene, encoding a putative cytosolic dihydroorotate dehydrogenase (DHODH), enhanced drought and salt tolerance (Liu et al., 2009).

Enormous putative genes controlling physio-morphological traits confer drought tolerance in rice (Deivanai et al., 2010). The appropriate water and nutrient status of plants is largely supported by the root architecture (Kato et al., 2006). The rooting depth, root thickness, root density and distribution pattern of roots are various traits studied under root system architecture to regulate water and nutrient uptake to address adverse conditions of drought (Lilley and Fukai, 1994; Fukai and Cooper, 1995; Pantuwan et al., 1996; Wade et al., 1996). Furthermore, in

rice populations, various QTLs related to morphology and root index penetration have been identified (Champoux et al., 1995; Ray et al., 1996; Zhang et al., 2001; Henry et al., 2014; Kijoji et al., 2014). Important traits adapted by crop pants to retain water and nutrients during drought stress include the ratio of deep rooting (RDR), root growth angle (RGA) and direction of root elongation. In rice plants, overexpression of *EDT1/HDG11* genes resulted in enhanced root development and reduced stomatal density under drought conditions (Yu et al., 2013). For instance, squalene synthase (SQS), a key enzyme located in the endoplasmic reticulum is capable of catalyzing first reaction, i.e., conversion of two farnesyl pyrophosphates into squalene, a step in the isoprenoid metabolic pathway finally directed to synthesize sterols in plants (Tansley and Shechter, 2001). Under drought conditions, it was reported that the disruption of squalene synthase (SQS) function by RNAi led to enhanced root length, a higher number of lateral roots and a decline in the stomatal conductance (Manavalan et al., 2012).

Approximately 675 QTLs for root traits were identified by meta-analysis of 12 populations of crop plants (Courtois et al., 2009). For deep rooting traits, only 05 major QTLs were mapped as root traits (Kitomi et al., 2015; Uga et al., 2011, 2015). A cross between the deep rooting cultivar Kinandang Patong and the shallow-rooting cultivar IR64 resulted in recombinant inbred lines (IK-RILs) leading to the identification of the DRO1 gene located on chromosome 9 (Uga et al., 2011). It was reported that DRO1 considerably encodes the RGA and grain yield. The DRO2 gene found on chromosome 4 is another major QTL that encodes the RGA and was obtained by crossing Kinandang Patong and shallow-rooting cultivars (ARC5955, Pinulupot1 and Tupa729) (Uga et al., 2013b). Furthermore, the DRO3 gene found on chromosome 7 also had a great impact on the RGA due to its involvement in the DRO1 genetic pathway (Uga et al., 2015). Genome wide expression profiling also led to the identification of *OsAHL1*, a novel gene involved in drought tolerance and avoidance in rice plants. Later, genes were found to regulate root development, oxidative stress and chlorophyll content under drought stress conditions (Zhou et al., 2016). Norton and Price (2009) identified 04 QTLs for seminal root morphology and 2 QTLs for root gravitropic responses. By using 124 recombinant inbred lines, QTLs such as Soil Surface Rooting 1 (qSOR1) were found on chromosome 7 in rice (Uga et al., 2012). In addition, 2 QTLs for canopy temperature and 6 for leaf water potential in RILs were identified in crop plants (Liu et al., 2005). Lou et al. (2015) identified 6 QTLs by using 1,019,883 SNPs. In drought stress, 1 QTL for leaf drying, 1 for SPAD and 2 QTLs for canopy temperature were identified to manage stress (Prince et al., 2015). It is concluded that the introduction of traits contributes to drought tolerance and avoidance in rice to enhance yield (Fukai and Cooper, 1995; Nguyen et al., 1997). Moreover, a considerable number of QTLs have been mapped for osmotic adjustment in crop plants, even though very few loci have a major impact during stress conditions (Lilley et al., 1996). On the other hand, DRO1 was found to enhance yield under drought stress (Uga et al., 2013a).

Genomics plays a pivotal role in deciphering the new genome sites that code for critical traits and apprehending the nucleotide variations linked to specific variant phenotypes. Specific Rice SNP-seek database was updated to gain insights into nucleotide and phenotypic variants (Locedie et al., 2017). The nucleotide variants were curated by databases of rice such as, Gramene (Tello-Ruiz et al., 2016), NCBI (Sherry et al., 2001), RiceVarMap (Zhao et al., 2015), RMBreeding (Zheng et al., 2015), and IC4R (The IC4R Consortium, 2015).

## TRANSCRIPTOMICS

Transcriptomic studies involve studying total transcripts found in cells/tissues or organisms. Through transcriptome-wide studies, it has been revealed that a large number of TFs have helped to confer drought tolerance in rice. Broadly, two classes of TFs have been identified to mediate signaling and physiological responses in plants such as, ABA independent and ABA dependant activation of TFs for drought tolerance. ABA dependant TFs includes (1) basic leucine zipper (bZIP) and (2) NAM, ATAF, and CUC2 (NAC). The bZIP class of TFs plays a pivotal role in dehydration induced by ABA signaling under drought conditions (Yang et al., 2010). Moreover, ABF-3 mediated drought tolerance was more efficient than *DREB1A/CBF3* triggered drought stress tolerance (Oh et al., 2005). NAC TFs are another potential member that belongs to the ABA dependant group, which consists of a DNA binding NAC domain (Palaniswamy et al., 2006; Guo et al., 2008). The SNAC1 is typical of the NAC family and is overexpressed in transgenic rice lines showing a higher seed setting rate and spikelet fertility under severe drought stress (Hu et al., 2006).

Two families of TFs are discussed in ABA-independent signaling pathways, zinc fingers and AP2/ERFs. Former TFs consist of Zn ions coordinated to motifs that stabilize the protein folds. These TFs include *WRKY, ZFP252,* and *Zat10/STZ* genes (Xu et al., 2008; Wu et al., 2009; Xiao et al., 2009). Among these, STZ was reported to enhance the fertility of spikelets and grain yield under drought stress (Xiao et al., 2009). Under the control of the drought inducible promoter *OsHVA22P*, *AtDREB1A*/*CBF3* gene overexpression enhanced drought tolerance in rice plants to increase the yield and spikelet fertility. Large numbers of TFs are deployed to impart drought tolerance in crop plants, but their successful introduction in crop plants is still in its infancy.

Tian et al. (2015) assembled the *de-novo* transcriptome of common wild rice (*Oryza rufipogon* Griff.) and identified several drought responsive and related genes in root tissue based on transcriptomic data, thus, providing critical data pertaining to genetic and genomic studies in rice. Analyzing the transcript and metabolic responses of two rice cultivars with contrasting drought-tolerance to long-term drought, Ma et al. (2016) showed that a large number of DEGs related to photosynthesis were upregulated in the drought tolerant variety and concluded that well-maintained photosynthesis under drought is a critical aspect for improved drought-tolerance in rice. An RNA-seq based transcriptomic profile between OsMIOX-overexpressing (OE) and wild-type (WT) rice plants revealed that this unique monooxygenase plays an essential role in drought tolerance. Significantly, upregulated DEGs in

OE lines were associated with TFs, plant hormone transduction and sugar metabolism (Shi et al., 2020). Luo et al. (2020) examined the morphological differences between upland and lowland rice ecotypes. Furthermore, genetic and transcriptomic divergences between these contrasting rice ecotypes under well-watered and drought conditions were observed using RNA-seq. The results revealed that the expression divergences were higher in upland rice than in lowland rice, and it was concluded that this transcriptomic divergence contributed to their morphological differences in drought tolerance.

Xia et al. (2020) investigated the temporal transcriptomic data of 12 rice genotypes varying in naturally induced drought in field conditions. They highlighted that the drought tolerant varieties had higher proportions of upregulated DEGs related to fucose, trehalose, and raffinose metabolic processes that were specifically induced. In addition, the DEGs related to proteins and their modifications such as protein peptidyl-prolyl isomerization, histone deacetylation, transcriptional attenuation and ferric iron transport, were induced earlier in drought tolerant genotypes. An integrated phenotypic and transcriptomic landscape of 61 rice (*Oryza sativa*) varieties grown in an upland field with highly diverse below-ground traits under mild drought stress was reported (Kawakatsu et al., 2020). Rice accessions were classified into four admixture groups based on phenotypic variation and the transcriptomic analysis showed admixture group-specific enrichment of stress-related genes. Signaling network and key TFs were identified by co-expression network analysis of DNA affinity purification followed by sequencing (DAP-seq) datasets and were found to negatively regulate crown root diameter. Tarun et al. (2020) carried out co-expression network analysis of the transcriptomes obtained from emerging panicle tissues and flag-leaf in drought-tolerant yield recurrent parent and introgression line under drought stress. Results indicated that plants scavenge ROS and enhanced protein turnover as pivotal mechanisms induced in both tissues during drought stress conditions.

Liang et al. (2021) conducted comparative RNA-seq analysis of a moderately tolerant breeding line and drought susceptible elite varieties during the grain-filling stage and observed that the tolerant line had much earlier responses at the transcriptomic level. Genes and gene families related to TFs, drought tolerance genes, and reactive oxygen species (ROS) scavengers were significantly altered. More recently, Tiwari et al. (2021) carried out time-based comparative transcriptomic profiling of two varieties that showed contrasting drought tolerance. Comparatively, a significant proportion of the identified DEGs related to phytohormone signaling, stress-response, photosynthesis, anti-oxidative mechanisms and TFs were higher in the drought tolerant variety. Furthermore, QTL mapping could distinguish drought-responsive traits at the chromosomal level, because a very high number of DEGs associated with drought stress was observed in the drought tolerant variety.

Studies have revealed that to unravel the complexity of expression levels, it is important to investigate the transcriptomes as a function of development and environmental conditions (Dayan et al., 2015; Liu et al., 2015; Gould et al., 2018). Furthermore, the transcriptomic investigation of rice lines under two different drought stress conditions could help to elucidate the possible molecular switches adapted by rice crops (Gould et al., 2018). The variation induced by stress tolerant and stress sensitive cultivars of rice at the molecular level was identified by microarray analysis (Walia et al., 2005; Lenka et al., 2011; Ray et al., 2011). The studies evaluated the differential expression of genes by both tolerant and sensitive cultivars of rice under stress conditions (Walia et al., 2005; Lenka et al., 2011). Therefore, it is important to gain in-depth knowledge about the complexity of transcriptional regulation in rice during drought and salinity stresses using advanced technologies.

## PROTEOMICS

This approach involves studying the proteomes of a cell/organ/tissue and organism for experimental purposes (Rakwal and Agrawal, 2003). Proteomes are largely influenced by the environmental conditions that occur in the immediate vicinity of biological systems. Therefore, it is pertinent that genomes can produce a large number of proteomes according to the prevailing conditions. Little progress has been made in rice proteomics compared to genomic studies. Proteome maps of rice seeds, roots and leaves investigated at different developmental stages augment the development of stress tolerant rice varieties (Komatsu et al., 1999; Koller et al., 2002; Tanaka et al., 2005; Nozu et al., 2006). Approximately 31 drought responsive proteins have been identified in crop plants (Muthurajan et al., 2010). The preliminary Rice Proteome Database was available (Komatsu et al., 2004; National Institute of Agrobiological Sciences) and the data might be used to process the comparative proteomic analysis of drought tolerant and control varieties of rice.

A tandem mass tag (TMT)-based proteomic approach was applied to compare the roots, flag leaves and spikelets of a wild type and its near-isogenic line (NIL) harboring QTL qDTY 12.1, a large effect QTL for rice yield under drought (Raorane et al., 2015). The proteomic data correlated with drought-specific morpho-physiological responses and tissue-specific differences in protein abundance were observed. The DAPs were related to respiration, photosynthesis, energy generation and carbon-nitrogen acquisition/remobilization largely through the pathways of sugar/starch and amino acid metabolism. Paul et al. (2015) carried out comparative proteomic analysis to understand the metabolic networks regulated by the proteomes of roots in transgenic and wild type rice plants. Later studies led to the identification of DREB1A overexpression in rice plants by employing two-dimensional gel electrophoresis (2DE)-coupled with MALDI TOF MS/MS cultivated under drought stress. Most DAPs were linked to carbohydrate metabolism and defense, and a novel protein, R40C1, significantly accumulated only in the roots of transgenic plants. Agrawal et al. (2016) employed the 2DE-MALDI-TOF-MS/MS approach to compare the root cytoplasmic proteome of a drought tolerant rice variety for drought tolerant rice grown under normal and PEG-induced drought conditions. Major proportion of the identified differentially abundant proteins (DAPs) were involved in bioenergy, metabolism, cell defense and rescue, protein

biogenesis, protein storage and cell signaling, and the authors proposed that protein biogenesis, cell defense, and efficient homeostasis could render better drought-adaptation. Wu et al. (2016) carried out comparative label-free shotgun proteomics and TMT labeling proteomics of two rice cultivars with contrasting genetic backgrounds and levels of tolerance to drought. Proteomics results indicated alteration in multiple stress and defense response related proteins. Notably, a ClpD1 protease was upregulated several folds only in the drought tolerant variety, and porphyrin and chlorophyll biosynthesis pathways were down-regulated. A comparative proteomic analysis of a susceptible rice cultivar and its stress-resistant somaclonal mutant line showed that a large proportion of DAPs primarily related to retrotransposons were predominantly identified in the resistant line, suggesting that the gene expression associated with drought tolerance mechanisms is under strong epigenetic regulation. It was also suggested based on proteomic analysis that photosynthetic adaptation through NADP(H) homeostasis contributes to drought tolerance in rice (Chintakovid et al., 2017).

Wang et al. (2017) performed physiological measurements and conducted 2DE-based proteomic analysis of rice flag leaves at the flowering and milk stages to understand the drought responsive mechanism. Based on the proteomic analysis, it was postulated that at the flowering stage, $CO_2$ assimilation and ATP synthesis were disrupted, while at the milk stage, both $CO_2$ assimilation and photosynthesis were impaired. However, there was an increased abundance of DAPs related to defense and antioxidant machinery, which suggests redox imbalance and activation of the ROS scavenging system in drought stressed plants (Saini et al., 2021).

To understand the role of jasmonic acid under drought stress, studies were conducted to compare the morpho-physiological traits and the root proteome of a wild type (WT) rice plant with its jasmonic acid biosynthesis mutant *coleoptile photomorphogenesis 2* (*cpm2)*, disrupted in the allene oxide cyclase (AOC) gene. Studies revealed that roots of *cpm2* mutants compared to wild type had higher water use efficacy, high ABA concentration in shoots, and higher stomatal conductance under drought conditions. Moreover, the TMT technique was used to analyze the root proteome to better understand this difference at the molecular level. Identification of other DAPs proposed increased energy metabolism (i.e., increased mobilization of resources) and ROS scavenging in *cpm2* under drought. In addition, it was revealed that there was an abundance of proteins pertaining to cell growth, secondary metabolism and cell wall synthesis in *cpm2* mutants grown under drought stress. A clear understanding of adaptation and responses in roots of *cpm2* mutants under drought stress was elucidated by proteome-guided metabolites, transcripts and histological investigations (Dhakarey et al., 2017). By conducting comparative morphological and proteomic analysis of two rice genotypes, 5 DAPs, including chitinase, were found to be abundant under drought stress (Anupama et al., 2019).

Cunha et al. (2019) investigated APX found in rice thylakoids in response to mild drought stress by carrying out physiological measurements and leaf proteomic analysis of thylakoidal APX knockdown rice plants (apx8) and non-transformed control plants. A correlation was observed between the sensitivity of plants to mild drought stress and the lower accumulation of DAPs related to several metabolic processes, especially photosynthesis, photorespiration and redox metabolism, in *apx8* plants. Although *apx8* effectively induces other compensatory antioxidant mechanisms in well-watered conditions, the *apx8* plants could not maintain $H_2O_2$ homeostasis and accordingly avoid adverse conditions under mild drought conditions. To carry label-free proteomic studies, Hamzelou et al. (2020) exposed eight genotypes of upland and lowland rice plants to drought stress at the late vegetative stage. The majority of the identified DAPs under drought conditions were related to photosynthesis, oxidative stress response, proteolysis, and translation of stress-responsive proteins, such as heat shock, and LEA proteins (late embryogenesis-associated proteins) sharply increased under drought stress. Du et al. (2020) investigated the physiology of rice grown under two nitrogen management modes by proteomics and metabolomics approaches to investigate their yield formation and the mechanism of nitrogen regulation for drought tolerance. It was evident from the proteomic analysis that the most altered biological processes and pathways were related to the biosynthesis of primary and secondary metabolites important for the growth and metabolism of crop plants.

The rice proteomes are assessed by several gel and off-gel based proteomics techniques. For instance, proteome changes are analyzed by a combination of proteomic techniques such as 2-DE matrix-assisted laser desorption ionization-tandem time of flight (MALDI-TOF/TOF) and 1-DE/LC-Fourier transform-ion cyclotron resonance (FT-ICR) MS based analyses (Wan and Liu, 2008). Moreover, several proteomic databases for rice have been established and updated to curate the sequences for desirable traits. For example, the Rice Yield-related Database (RicyerDB) was created to curate the research related to yield related traits in rice by assessing the genomic and proteomic data inputs, and it provides a vast literature source related to where we can browse, investigate and analyze the desirable genes related to yield (Jiang et al., 2018). Systemic proteome analysis through a wide range of proteomic techniques is a powerful tool to investigate the complete proteomes of rice plants at different levels, such as the organelle, cell, tissue, organ and organism levels, and further provides insights into assessing proteomes once plants are exposed to stressful conditions.

## METABOLOMICS OF RICE PLANTS

Metabolomic studies involve comprehensive and systematic identification and quantification of endogenous metabolites from biological samples and have rapidly evolved and advanced in recent years (Ryan and Robards, 2006; Kumar et al., 2017).

### Some Major Breakthroughs in Metabolomics-Related Articles on Rice From 2015 to 2021

These include: Nam et al. (2016) – studies carried in transgenic rice to unravel the metabolomic changes in grains of well-watered

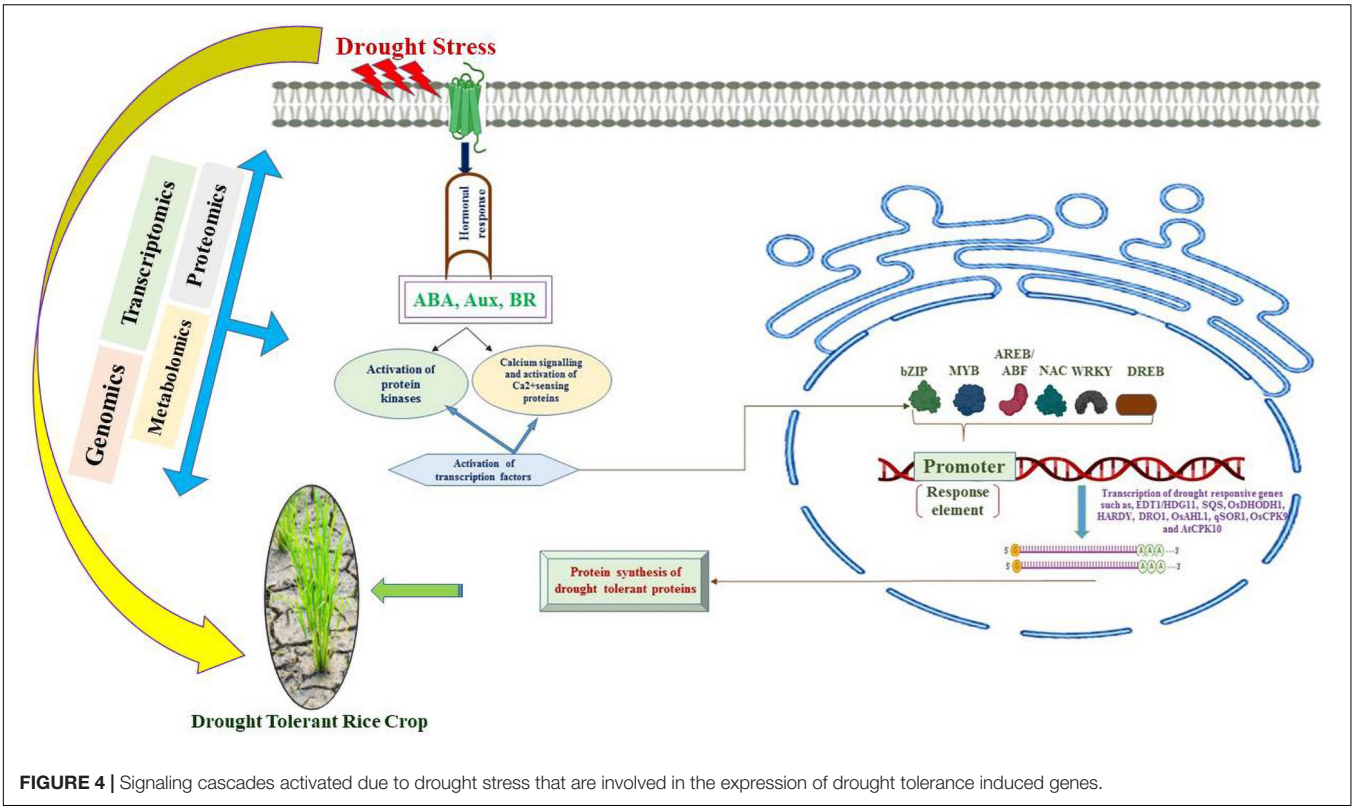


**FIGURE 4 |** Signaling cascades activated due to drought stress that are involved in the expression of drought tolerance induced genes.

and drought conditions; Ma et al. (2016) – studied the key pathways by transcriptomic and metabolomic studies to maintain photosynthesis under the drought and the consequent drought-tolerance in rice; Xiong et al. (2019) – Analysis of grain yield reduction in rice by comprehensive proteomic, metabolomic, and physiological analyses of grain under abrupt drought–flood alternation stress; and, Du et al. (2020) – Combinatorial proteomic, metabolomic and physiological studies of rice growth and grain yield with heavy nitrogen application before and after drought.

Metabolomics analysis in plant systems is rapidly advancing because it measures total, or groups of metabolites expressed in few samples in specific time-periods. Metabolomes of higher plants could comprise hundreds of thousands of metabolites out of that number, so little is known (Wang S. et al., 2019). Qualitative and quantitative measures of plant metabolomes mirror their responses to organic phenomenon and abiotic stimuli, genome, and physiological standing, serving as a connecting link between genotypes and phenotypes. They provide a considerable contribution to stress biology by deciphering numerous compounds such as by-products of stress metabolism, signaling molecules, and compounds, that square measure a part of the plant acclimatization method (Lanzinger et al., 2015; Ramalingam et al., 2015). Metabolites such as amino acids, fatty acids, soluble sugars, nucleotides, organic acids, phenolics, peptides, cofactors, and secondary metabolites act as cellular measurements that changed during drought stress (Das et al., 2017; Arora et al., 2018). Various metabolomic approaches elucidated the wide range of metabolite markers expressed in response to abiotic stressors (Ghatak et al., 2018). Several of these metabolites square measure crucial parts of the plant's weapons system. Polyamines and phenoplast compounds are major substantial plant secondary metabolites that impart tolerance, and square measures are interpreted as replacements for biostimulants underneath environmental stress, especially drought stress conditions (Aninbon et al., 2016; Chen D. et al., 2019). Underneath environmental stresses, metabolic changes could assert some necessary metabolic products, which illustrates that the metabolic pathways are regulated at different levels. Additionally, plants can also change metabolic pathways to accumulate giant amounts of energy, which is a prerequisite to resist environments (Ullah et al., 2017). Metabolic analyses have shown that changes in metabolites under drought responses in various plant species are pivotal for developing adaptations. In Arabidopsis, most amino acid intermediates (such as from proline, glutamine, tryptophan, alanine, aspartate, ornithine, isoleucine, leucine, and valine) from the TCA cycle (such as 2-oxoglutarate, cis-aconitate, and succinate), flavonoids (such as quercetin and cyanidin) and lipids accumulate under drought stress (Tarazon et al., 2015; Pires et al., 2016). Similarly, in rice the metabolomic data suggest that in response to increasing water stress, rice cultivars that were drought tolerant accumulated higher levels of carbohydrates (fructose, glucose, and myo-inositol) compared with the susceptible cultivars (Barnaby et al., 2019).

Both quantitative and qualitative rice metabolomics analyses help to investigate primary and secondary metabolites and

their differential expression patterns during biotic and abiotic stress conditions (Khakimov et al., 2014). Major metabolic techniques, such as nuclear magnetic resonance (NMR), GC/MS, and LC/MS in combination with Fourier transform ion cyclotron resonance (FT-ICR), have been employed to decipher the metabolite profiles of organisms in conjunction with databases, including Kyoto Encyclopedia of Genes and Genomes (KEGG) and MetaCyc (Kanehisa et al., 2012; Morreel et al., 2014; Caspi et al., 2018; Chen L. et al., 2021). The database specific to rice, including RiceCyc, is constricted based on metabolic networking and MetaCyc (Chae et al., 2007). To comprehend the basic mechanism of the response to abiotic stress, in particular drought, comparative metabolomics approaches provide a solid framework of gene expression, protein expression and other metabolites expressed temporally. Metabolomics approaches are promising technical interventions that overall serve as frameworks to obtain an in-depth biochemical and genetic portrait of organisms to pave the way for molecular breeding under stress conditions (Bino et al., 2004; Fernie and Schauer, 2009; Kusano et al., 2011).

## EPIGENOMICS FOR DROUGHT TOLERANCE

The epigenome could be outlined as the summation of all of the biochemical changes in nuclear polymer, simple proteins and tiny non-coding ribonucleic acid biogenesis of a cell. Studies on the epigenetic changes in and around polymers that regulate ordination activity are outlined as epigenetics, and the branch of genetic science that addresses epigenomic studies is named epigenomics. Plants have evolved non-heritable, extremely subtle systems to deal with varied environmental stresses. The last decade has witnessed considerable progress in understanding the signaling and metabolic pathways that are dominant in plant responses to stresses, which has been summarized in previous reviews (Ku et al., 2018; Kumar et al., 2019). Activation of signaling pathways typically results in transcriptional adjustments to initiate the expression of stress responsive proteins (Kim et al., 2019; Shahid, 2019; Wu et al., 2019). A large number of adaptations have been attributed to crop plants in response to stress factors (Thomashow, 1999). The temporal responses to stress include the expression of stress-induced proteins, RNA molecules and metabolites. These stress adaptions could be longer if phenological and morphological adjustments have been accomplished. The transcriptional reprogramming and regulation of stress-responsive genes are critical to numerous epigenetic processes and components, such as DNA methylation, protein modifications and non-coding RNAs based regulations (Deleris et al., 2016; Kim et al., 2017; Chen R. et al., 2019). Drought stress conditions primarily tend to extend demethylation. It is conjointly discovered that DNA methylation shows tissue specificity. A variation of up to 1% methylation occurs across cells, tissues, genotype and organic process stages. The DNA methylation level is reported to be lower in roots than in leaves, which indicates an important role of roots in addressing water scarcity (Suji and John, 2010). The correlation between drought stress and DNA methylation is shown in lowland and drought-tolerant rice cultivars. The drought inclined IR20 variety showed hypo-methylation under drought conditions, whereas the tolerant varieties "Paiyur" and "PMK3" showed hyper-methylation. These changes in methylation patterns were found to be responsible for the differential expression of stress responsive genes (Gayacharan, 2019). In another study conducted in rice, it was illustrated that hypomethylation has an important role within the drought tolerance attributes of rice genotypes. Epigenomic studies involve a range of techniques, such as chromatin immunoprecipitation (ChiP), ChiP-sequencing, methylated-DNA immunoprecipitation and shotgun bisulfite sequencing. For the development of resilient rice cultivars, it is pertinent to focus on the profile and functioning of epigenomic profiles, such as histone modifications, DNA methylation, diverse classes of regulatory non-coding RNAs and especially the 3D genomic structure of rice (Lu et al., 2020). The role of epigenomics studies has considerably contributed to studying the changing dynamics of expression patterns under stress conditions, thus unraveling the complexity of crop plants in response to a wide range of stress factors. In addition, a detailed account on rice epigenomics variations and their identification is critical to the characterization of phenotypes fitting to agronomic traits for providing framework for enhancing productivity and traits in rice crops (Chen and Zhou, 2013).

## CONCLUSION

The mechanism of drought tolerance is an important quantitative trait that is accompanied by several phenotypic adjustments and adaptations. A large number of stressors are supposed to be tolerated by crop plants, such as high temperatures, high irradiance, toxicities, and nutrient deficiencies due to the induction of drought stress. The past few decades have witnessed a higher prevalence of stresses due to abiotic factors imparting hindrances to growth and productivity in rice crop. Globally, scientists are extensively investigating strategies to cope with ever increasing climatic change and drought stress factors. The emergence of innovation in omics technologies has led to the deciphering of a large amount of molecular machinery to adapt to a large number of environmental factors. Besides, multiomics data integration and molecular modeling protocols can be employed to understand the complex traits to unearth the tolerance mechanism and yield of major legumes and cereal crops (Pazhamala et al., 2021; Yang et al., 2021). It is pertinent that enhancing tolerance to drought in rice requires the establishment of research programs to investigate complex networks of molecular interactions. Investigations based on transcriptomics, proteomics and metabolic studies should be initiated in unison to unravel the drought tolerance mechanisms in rice. These techniques will further widen our understanding of molecular signaling cascades and a wide range of stress responsive proteins and intracellular adjustments under stress factors (**Figure 4**).

Moreover, the cited literature validates the role of investigating QTLs and SNPs that play pivotal role of genes in drought tolerance in rice crops. This investigation at large could possibly augment our efforts to develop drought tolerant crop plants such as rice to ensure food security. The applications of markers and genomic selection are an efficient way to improve crop plants. Moreover, the development of high throughput phenomics techniques will help to augment the in-depth understanding of the mechanisms of tolerance to stressors. A low level of progress has been made with respect to proteomics, epigenomics and metabolomics in conjunction with genomic approaches. It is an important prerequisite to use the data obtained from these approaches to understand the genetic and physiological basis of drought tolerance. In addition, efforts aimed at combinatorial omics approaches and practical plant-breeding applications can accelerate progress in producing rice cultivars that are suitable for rainfed environments or to realize significant freshwater savings. Against this background, a PANOMICS platform that integrates mathematical and statistical tool boxes with omics data to facilitate improvement of crops through discovering pathways and target genes critical for resilience and creation of elite lines (Weckwerth et al., 2020). Finally, physiological and morphological adaptations accomplished by molecular mechanisms traced in resistant parent lines can be utilized to transfer the resilient genes to mainstream crop plants to become very adept to a particular environment, to establish a solid base for food security at a global level.

## AUTHOR CONTRIBUTIONS

SZ and RR conceived the idea, framed outline and contributed in writing and final editing of the manuscript. RAM contributed in writing genomics part and prepared the figures. LE prepared transcriptomics part of the manuscript. AM edited the manuscript and also contributed in preparing the proteomics and transcriptomics sections. AH, MM, and RM prepared the tables and also contributed in preparing genomics section. RS and NS contributed in writing introduction and physiological section of the manuscript. JR revised the original draft and carried out the final editing with SZ and RR. All authors contributed to the article and approved the submitted version.

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# Mitigation of Salinity-Induced Oxidative Damage, Growth, and Yield Reduction in Fine Rice by Sugarcane Press Mud Application

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Salinity stress is one of the major global problems that negatively affect crop growth and productivity. Therefore, ecofriendly and sustainable strategies for mitigating salinity stress in agricultural production and global food security are highly demandable. Sugarcane press mud (PM) is an excellent source of the organic amendment, and the role of PM in mitigating salinity stress is not well understood. Therefore, this study was aimed to investigate how the PM mitigates salinity stress through the regulation of rice growth, yield, physiological properties, and antioxidant enzyme activities in fine rice grown under different salinity stress conditions. In this study, different levels of salinity (6 and 12 dS $m^{-1}$) with or without different levels of 3, 6, and 9% of SPM, respectively were tested. Salinity stress significantly increased malondialdehyde (MDA, 38%), hydrogen peroxide ($H_2O_2$, 74.39%), $Na^+$ (61.5%), electrolyte leakage (40.32%), decreased chlorophyll content (32.64%), leaf water content (107.77%), total soluble protein (TSP, 72.28%), and free amino acids (FAA, 75.27%). However, these negative effects of salinity stress were reversed mainly in rice plants after PM application. PM application (9%) remained the most effective and significantly increased growth, yield, TSP, FAA, accumulation of soluble sugars, proline, $K^+$, and activity of antioxidant enzymes, namely, ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD). Thus, these findings suggest a PM-mediated eco-friendly strategy for salinity alleviation in agricultural soil could be useful for plant growth and productivity in saline soils.

**Keywords: anti-oxidants, ionic balance, photosynthetic pigments, press mud, rice, salt stress**

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## INTRODUCTION

Salinity stress is one of the serious limitations for plant growth, productivity, and soil health (Machado and Serralheiro, 2017; Kamran et al., 2020; Seleiman et al., 2022). It is expected that salinity contamination will severely affect 50% of the total cultivable land within 2050 (Shrivastava and Kumar, 2015). Salinity negatively affects crop production and causes huge yield and economic losses; therefore, proper strategies must be adopted to reduce salinity stress on crops and protect the soils from the devastating impacts of salinity stress (Mohanavelu et al., 2021; Monsur et al., 2022). Salinity stress is a complex process that negatively affects the plant's physiological and biochemical processes (Dustgeer et al., 2021; Sultan et al., 2021). The effects of salt ranged from seed germination to flowering and fruiting settings, which resultantly caused significant yield losses (Mbarki et al., 2020; El Sabagh et al., 2021). Moreover, salt stress also induces osmotic and ionic stress and induce the production of reactive oxygen species (ROS), which cause damages to significant molecules, namely, DNA, proteins, and cellular membranes (Ahanger et al., 2017; Hassan et al., 2019, 2020, 2021a; Mbarki et al., 2020; Hossain et al., 2021; Sultan et al., 2021; Batool et al., 2022). Salt stress also reduced the nutrient uptake and enzymes' activity, therefore significant growth and yield losses (Kamran et al., 2020; Singhal et al., 2021). Moreover, salinity stress also reduced the photosynthetic efficiency and increased the accumulation of toxic ions ($Na^+$), which cause serious growth and yield losses (Ahanger et al., 2017; Mbarki et al., 2020; Taha et al., 2020; Dustgeer et al., 2021; El Sabagh et al., 2021; Sultan et al., 2021). The adverse effects of salt stress on plants growth and development depend on different factors, namely, planting species, growth stage, environmental conditions, and salts concentration in soil solution (Aghighi et al., 2018; Monsur et al., 2020). Therefore, it is essential to find out sustainable strategies to reduce the deleterious impacts of salt stress through genetic techniques, soil conditioners, and biological products (Farid et al., 2020; Leal et al., 2020; Naz et al., 2021).

For salt-affected reclamation, applying organic amendments like farmyard manures, press mud (PM), and green manuring is considered a simple and practical economic approach (Hassan et al., 2021b). Press mud is a by-product of the sugarcane industry produced in large quantities. The agricultural use of PM has shown significant improvement in nutrient uptake and soil health (Chattha et al., 2019). Sugarcane PM is considered to positively affect the soil structure, soil organic matter, nutrients uptake, and soil microbial activities (Nawaz et al., 2017; Chattha et al., 2019). Therefore, PM could serve as an important amendment to improve salt-affected soils' productivity in this context. PM application induced salinity tolerance by enhancing the leaf water status, membrane stability, photosynthetic efficiency, accumulation of osmolytes, and $K^+$ (Sheoran et al., 2021a). Press mud application improves nutrient uptake and significantly increases growth and biomass productivity under salinity stress (Imran et al., 2021). Moreover, PM also mobilizes the $CaCO_3$ to maintain the optimum $Ca^{2+}$ availability, improve soil structure stability, and increase the leaching of $Na^+$ in salt-affected soils (Muhammad et al., 2019). In addition, PM also serves as a potential nutrient source and soil conditioner for reclamation of salt-affected soils (Kumar S. et al., 2017).

Rice (*Oryza sativa* L.) is an imperious staple food crop around the globe; however, salinity stress is a significant threat to rice productivity. Rice plants alleviate the salinity-induced damages by maintaining ionic balance, osmotic adjustments, scavenging ROS, maintaining nutrient uptake and cell signaling, and increasing hormonal accumulation (Liu et al., 2021). However, limited studies are conducted to determine the impact of PM on crops grown under salt stress conditions. Therefore, more investigations are required to explore the mechanism behind PM-induced salinity tolerance in field crops. To our best knowledge, no study is available related to the effect of sugarcane PM on photosynthetic pigments, physiological attributes, element concentration, antioxidant defense, and growth and yield of rice crops growing in salt-stressed conditions. Thus, we hypothesized that the application of PM would improve salinity tolerance in rice crops by favoring photosynthetic pigments, physiological attributes, and antioxidant defense. Therefore, this study was aimed to explore the impact of PM photosynthetic pigments, physiological features, $H_2O_2$ and malondialdehyde (MDA) production, soluble protein, free amino acid and elements accumulation, antioxidant defense, and growth and yield of rice crop.

## MATERIALS AND METHODS

### Experimental Details

This experiment was carried out at the Agronomy Farm in a complete randomized design (CRD) with a factorial combination having three replications. The investigation was composed of several salinity treatments; control, 6 and 12 dS $m^{-1}$, and diverse levels of PM control, 3, 6, and 9%. For achieving the desired rates of PM (3, 6, and 9%), we used PM at the rate of 300, 600, and 900 g/pot. The PM was thoroughly mixed in soil and left for 7 days. The rice variety Kisan-basmati was used in this study, which is sensitive against salinity stress. Rice plants were grown at Agronomy Farm for 4 weeks to reach the transplanting stage; after that, they were transplanted to experimental pots with a volume of 8 kg and diameter of 32 cm. In each pot, 8 kg of soil was filled and eight seedlings were transplanted in each pot. The soil used in the study had a sandy loam texture with pH (7.82), organic matter (0.82%), and available nitrogen (N), phosphorus (P), and potassium (K): 0.04%, 6.60 and 156 ppm, respectively. Ammonium phosphate (5.58 g) and sulfate of potash (1.87 g) were added to each plot to fulfill nutrient requirements. The fertilizers were applied for soil analysis, and these fertilizers were readily available in the market; therefore, they were used to meet nutrient needs. According to treatments, the imposition of various salt stress levels was applied following the procedure used previously by Khaliq et al. (2015). A soil sample from the collected soil was taken, soil paste was made, and then left for 2 h to attain equilibrium. The soil extract was then collected using filter paper. After that, the soil mixture was ovendried,

and the percentage of soil saturation was calculated using the following equation.

$$Saturation\ (\%) = \frac{Loss\ in\ soil\ weight\ on\ drying}{Weight\ of\ soil\ after\ drying} \times 100$$

The concentration of salts (6 and 12 dS $m^{-1}$) to achieve the desired treatment levels treatments was calculated using the following equation (Khaliq et al., 2015).

$$Nacl\ required\left(\frac{g}{kg}\right) = \frac{TSS \times 58.5 \times saturation(\%)}{100 \times 1000}$$

Here TSS refers to total soluble salt, which was determined as ($EC_2$ – $EC_1$) × 10. $EC_1$ refers to the initial EC of soil, whereas $EC_2$ refers to the desired EC as per treatments.

## Growth Parameters

Three plants were uprooted carefully from each pot, and roots and shoots were separated and washed to remove any debris. After that, the length of three roots and shoots was measured, and the average was taken. Later on, they were weighed to determine the fresh weight and ovendried (70°C) for 8 h to determine the dry weights. Moreover, three plants in each pot were selected, leaves were counted, and the average was worked out. The growth traits were collected at the flag leaf stage of the plant. The pots were well irrigated after 24 h. Plants were carefully uprooted from the pots to avoid any damage to roots.

## Determination of Photosynthetic Pigments

Leaves were selected at the flag leaf stage to determine the concentrations of chlorophyll and carotenoid by the methods of Lichtenthaler (1987). The leaves rice (1 g) was taken and homogenized in 85% of acetone solution, and the extract was centrifuged for 15 min at 1,000 rpm. Absorbance was recorded using a spectrophotometer, and the concentration of photosynthetic pigments was determined using the below equations.

$$chlorphylla\left(\frac{mg}{g}FW\right) = \frac{12.7\,(OD663) - 2.69(OD645) \times V \times W}{1000}.$$

$$chlorphyll\ b\left(\frac{mg}{g}FW\right) = \frac{12.9\,(OD645) - 4.68(OD663) \times V \times W}{1000}$$

$$Total\ chlorphyll\left(\frac{mg}{g}FW\right) = \frac{2.02\,(OD645) - 8.02(OD663) \times V \times W}{1000}$$

In the above equations, $V$ and $W$ refer to the volume of acetone and the weight of the plant sample. Moreover, carotenoids were calculated using the following equation.

$$Carotenoids\left(\frac{mg}{g}FW\right) = OD480 + (0.114 \times OD663) - (0.638 \times OD645)$$

## Measurement of Relative Water Content and Electrolyte Leakage

For the determination of RCW, leaf samples were collected at the flag leaf stage and weighed for the determination of fresh weight (FW). After that, leaf samples were dipped in water and weighed to determine the turgid weight (TW). Later on, turgid leaves were ovendried, and dry weight was taken. Finally, RWC was determined by the following equation given by Mostofa and Fujita (2013).

$$RWC\ (\%) = \frac{FW - DR}{TW - DR} \times 100$$

Fresh leaves (0.25 g) were added in 25 ml of distal water after 24 h $EC_1$ was recorded using an EC meter. Then the test tubes were heated in the water bath for 50 min at 90°C, and $EC_2$ was recorded. The final value of electrolyte leakage was calculated by the equation:

$$EL\% = (EC1 \div EC2) \times 100.$$

## Determination of $H_2O_2$ and Malondialdehyde Levels

Hydrogen peroxide concentration in rice samples was measured at the flag leaf stage by Velikova et al. (2000). A total of 0.5 g rice plant sample was grounded in 5 ml of TCA solution and centrifuged. After that, samples were placed in test tubes, 1M potassium iodide (KI) and 100 µl potassium phosphate buffers were added and left at room temperature for 30 min, and absorbance was noted at 390 nm to determine $H_2O_2$ contents. MDA contents in rice plant samples were estimated by Rao and Sresty's (2000) procedures. For this, 0.5 g frozen sample of rice plants was homogenized in 5 ml of TCA solution and centrifuged at 12,000 rpm for 15 min. After that, the mixture was added with 5 ml of thiobarbituric acid (TBA), heated for 30 min, later on, cooled quickly, and absorbance was noted at 600 nm.

## Determination of Total Soluble Proteins and Free Amino Acids

The concentration of TSP in the rice plant sample was measured at the flag leaf stage using the methods described by Bradford (1976). A total of 0.5 g of plant samples were grounded in phosphate buffer (5 ml) and centrifuged at 15,000 rpm for 15 min. After that, 1 ml plant extract was taken in test tubes containing 3 ml of Bradford reagent and set aside for 15 min at room temperature. Later on, TSP concentration in collected samples was recorded at 595 nm using a spectrophotometer. The total free amino acids (FAA) in the rice sample at the flag leaf stage were determined with Hamilto and Van-Slyke (1943). We took 1 ml of plant extract and placed it in test tubes containing 1 ml of ninhydrin and pyridine solution, and samples were

placed in the water bath at 90°C for 30 min. After that, volume was brought to 25 ml, and FAA concentration was recorded at 570 mM using a spectrophotometer. To determine soluble sugars, plant supernatant was prepared, and 1–2 drops were placed on the prism of a digital refractometer to measure soluble sugar value. Bates et al. (1973) methods were used to determine proline contents in rice samples. A total of 0.5 g of rice samples were extracted with 3% sulfosalicylic acid solution (10 ml) and centrifuged for 10 min at 10,000 rpm. Afterward, acid-ninhydrin was added to the supernatant and placed at 90°C in the water bath for 30 min, and absorbance was recorded at 520 nm to determine proline concentration.

## Antioxidant Enzyme Activity

The activities of all the antioxidants were measured at the flag leaf stage. To determine the ascorbate peroxide (APX), we took plant extract and mixture contained 100 μl of enzyme extracts, 100 μl of ascorbate (7.5 mM), 100 μl of $H_2O_2$ (300 mM), and 2.7 ml of potassium buffer (25 mM); 2 mM CA having 7.0 pH was added in the plant extract. Absorbance was noted at 290 nm with a spectrophotometer to determine APX activity (Nakano and Asada, 1981). After that, APX was determined at 290 nm using the spectrophotometer. The activity of ascorbic acid (AsA) was measured using the procedure described by Mukherjee and Choudhuri (1983). A total of 0.5 g rice samples were standardized with 10% tri-chloro-acetic acid solution (5 ml) and centrifuged at 8,000 rpm for 10 min. Afterward, 2 ml of supernatant was taken, and 0.5 ml DTC reagent was added and incubated for 3 h and then cooled. The procedure given by Aebi (1984) was used to determine the catalase (CAT) contents in rice samples. Test tubes containing 100 μl of $H_2O_2$ (5.9 mM) and 1,000 μl of buffer along with the 100 μl of plant extract were taken and centrifuged at 15,000 rpm for 30 min, and absorbance was noted at 240 nm using a spectrophotometer. The peroxidase (POD) content was measured by the procedure of Zhang (1982). We took reactants having 100 μl of extract enzyme + 2,700 μl of 50 mM potassium buffers + 100 μL guaiacol and 100 μl of $H_2O_2$, which was added into plant sample. Afterward, rice samples were homogenized with 5 ml of potassium phosphate buffer (50 mM having 7.0 pH) under ice-cold conditions and centrifuged for 15 min at 15,000 rpm. Absorbance was recorded using a spectrophotometer at 470 nm.

## Determination of Elemental Concentration

Rice plant samples were taken and washed to remove any contamination. After that, sample were ovendried and grinded to make the powder. Finally, grinded samples were digested at a hot plate after adding the mixture of HCL and $HNO_3$, and the filtrate was obtained and diluted by adding water. The concentration of ions ($Na^+$ and $K^+$) in rice was measured using a flame photometer (Jones and Case, 1990).

## Determination of Yield Attributes

The tillers of all the plants in every pot were counted and averaged to determine the tillers/pot. Similarly, panicles on each plant in every pot were calculated, and the average was taken. Five panicles from each pot were selected, panicle lengths were measured, and grains/panicles were counted. The full pots were harvested, and grains were separated from the panicle and weighed to determine the grain yield/pot.

## Statistical Analysis

The observed data were analyzed with computer-based software STATISTIX 8.1 using analysis of variance, and the least significant differences (LSD) test at 0.05 probability level was used to determine the significant difference among means (Steel et al., 1997). Moreover, Sigma-plot (8.0) software was used to prepare the graphs.

# RESULTS

## Effect of Press-Mud Amendment on Growth Attributes of Rice Plants Grown Under Salinity Stress

The results indicated that salinity markedly reduced the growth-related parameters of rice (**Table 1**). Shoot and root length decreased significantly (0.05 P) by 37.5 and 24.1% at 6 and 12 dS $m^{-1}$, respectively (**Table 1**). Likewise, salt stress also diminished biomass production. The maximum reduction in roots fresh weight (16.7%), shoots fresh weight (35.6%), root dry mass (22.2%), and shoot dry mass (37.4%) at 12 dS $m^{-1}$ was compared with control (**Table 1**). However, PM application protecting the rice plants from harmful impacts of salinity stress significantly improved the growth traits. Application of PM (9%) increased shoot and root length by 44.7 and 27.9%, shoot and root fresh biomass by 40.7 and 7%, and shoot and root dry biomass by 41.5 and 23.9%, respectively, under 12 dS $m^{-1}$ salinity stress (**Table 1**).

## Effect of Press-Mud Amendment on Photosynthetic Pigments of Rice Plants Grown Under Salinity Stress

Salt stress significantly ($p = 0.05$) reduced chlorophyll and carotenoid contents in rice seedlings (**Figure 1**). The chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids showed a reduction of 15.1, 39.4, 25, and 37.2% under a salinity stress level of 12 dS $m^{-1}$ (**Figure 1**). The PM application significantly increased photosynthetic pigments under salinity stress (**Figure 1**). An escalation in chlorophyll a (28%), chlorophyll b (41.6%), total chlorophyll (33%), and carotenoid (42.4%) was recorded with PM (9%) under salt stress as compared with control (**Figure 1**).

## Effect of Press-Mud Amendment on Electrolyte Leakage and Relative Water Contents of Rice Plants Grown Under Salinity Stress

The PM application markedly reduced the EL and considerably increased the RWC (**Figure 2**). Maximum EL (37.2%) and minimum RCW (50.6%) were recorded at 12 dS $m^{-1}$ salt

**TABLE 1 |** Effect of different levels of press mud application on growth attributes of rice plants grown under different levels of salinity stress.

| SL | PM L | SL (cm) | RL (cm) | SFW (g) | RFW (g) | SDW (g) | RDW (g) | LPP |
|---|---|---|---|---|---|---|---|---|
| 0 dS $m^{-1}$ | Control | 49.7e ± 0.9 | 11.8bc ± 0.7 | 55.0g ± 0.8 | 3.7cd ± 0.04 | 10.4ef ± 0.7 | 1.4e ± 0.02 | 23f ± 0.80 |
| | 3% | 66.0d ± 0.8 | 14.7a ± 0.9 | 71.0d ± 0.6 | 3.8c ± 0.03 | 13.9c ± 0.8 | 1.5d ± 0.01 | 28cd ± 0.8 |
| | 6% | 75.7b ± 10 | 14.9a ± 0.8 | 80b ± 1.30 | 3.9b ± 0.05 | 15b ± 1.30 | 1.7b ± 0.02 | 31b ± 0.70 |
| | 9% | 80.0a ± 1.3 | 15.6a ± 1.0 | 85a ± 1.40 | 3.9a ± 0.15 | 16.1a ± 0.8 | 1.8a ± 0.04 | 33a ± 0.80 |
| 3 dS $m^{-1}$ | Control | 35.1j ± 0.8 | 9.7e ± 0.70 | 41.0j ± 0.8 | 3.4fg ± 0.01 | 6.7i ± 0.9 | 1.1h ± 0.03 | 18.5h ± 1.2 |
| | 3% | 41.2i ± 1.2 | 10.8cd ± 0.4 | 44.0i ± 0.7 | 3.5f ± 0.02 | 9.1g ± 0.3 | 1.3f ± 0.03 | 20.2g ± 1.2 |
| | 6% | 61.8e ± 0.9 | 12.8b ± 0.6 | 66.0e ± 0.8 | 3.6e ± 0.02 | 12.5d ± 0.5 | 1.5d ± 0.01 | 27d ± 0.90 |
| | 9% | 71.0c ± 0.8 | 14.8a ± 0.8 | 74.7c ± 1.0 | 3.7d ± 0.03 | 14c ± 0.40 | 1.6c ± 0.01 | 29c ± 0.60 |
| 6 dS $m^{-1}$ | Control | 29.0k ± 0.6 | 9.2e ± 0.80 | 35.1k ± 0.6 | 3.0k ± 0.08 | 5.5j ± 1.0 | 1.1i ± 0.03 | 17.5h ± 0.8 |
| | 3% | 41.0i ± 0.9 | 10.1de ± 0.8 | 41.2j ± 1.1 | 3.2j ± 0.03 | 8.5h ± 0.9 | 1.2g ± 0.02 | 20g ± 0.40 |
| | 6% | 44.0h ± 0.7 | 11.7bc ± 0.7 | 49.7h ± 0.9 | 3.3i ± 0.03 | 9.5g ± 1.1 | 1.3f ± 0.04 | 21g ± 0.60 |
| | 9% | 55.0f ± 0.4 | 12.2b ± 0.9 | 61.5f ± 1.1 | 3.4h ± 0.02 | 11e ± 0.80 | 1.4e ± 0.02 | 25.5e ± 0.7 |
| LSD at 0.05 P | | 0.746 | 0.533 | 0.744 | 0.042 | 0.739 | 0.020 | 0.659 |

*SL, salinity levels; PML, press mud levels; SL, shoot length; RL, root length; SFW, shoot fresh weight; RFW, root fresh weight; SDW, shoot dry weight; RDW, root dry weight; LPP, leaves per plant. The values given in the table are the mean of three replicates with ± S.E. and different letters with each meaning showing the significant difference at $p \leq 0.05$.*

stress compared with control. Moreover, PM application (9%) reduced electrolyte leakage by 42% and increased RWC by 34% under salt stress (12 dS $m^{-1}$) conditions as compared with control (**Figure 2**).

## Effect of Press-Mud Amendment on Malondialdehyde and $H_2O_2$ Contents of Rice Plants Grown Under Salinity Stress

Salt stress significantly increased the MDA accumulation, and maximum MDA contents (6.55 μmol $g^{-1}$ FW) were recorded in 12 dS $m^{-1}$ salt stress without PM application while the lowest MDA contents (4.2 μmol $g^{-1}$ FW) was noted in control with 9% PM application (**Figure 2**). The application of salinity stress and PM also significantly affected $H_2O_2$ contents (**Figure 2**). The maximum concentration of $H_2O_2$ (15.82 μmol) was observed under 12 dS $m^{-1}$ salinity stress without PM application, and the minimum concentration of $H_2O_2$ (7.73 μmol) was noticed under control conditions. The application of PM significantly reduced $H_2O_2$ accumulation; however, application of PM (9%) remained the top-performing, and it reduced the $H_2O_2$ accumulation by 14 and 17% and at 6 and 12 dS $m^{-1}$, respectively (**Figure 2**).

## Effect of Press-Mud Amendment on Total Soluble Protein, Free Amino Acids, Soluble Sugars, and Proline Content of Rice Plants Grown Under Salinity Stress

Salinity stress considerably reduced TSP and FAA concentrations (**Figure 3**), and a reduction of 38 and 39% in TSP and FAA, respectively, was recorded at 12 ds $m^{-1}$ salt stress (**Figure 3**). However, PM appreciably increased the accumulation of both TSP and FAA. The application of PM (9%) increased the TSP and FAA by 18 and 19%, respectively, at 12 dS $m^{-1}$ salt stress (**Figure 3**). The results indicated that soluble sugars (SS) and proline contents were significantly increased under salt stress (**Figure 3**). Further application of PM also increased the accumulation of SS and proline (**Figure 3**). The PM application (9%) increased SS by 27 and 28% at 6 and 12 dS $m^{-1}$ salt stress, respectively, while it increased the proline contents by 33 and 41% at 6 and 12 dS $m^{-1}$ salt stress, respectively, as compared with control (**Figure 3**).

## Effect of Press-Mud Amendment on the Activity of Antioxidant Enzymes of Rice Plants Grown Under Salinity Stress

Results revealed that salt stress and PM application considerably increased the antioxidant enzyme activities (**Figure 4**). The PM (9%) increased activities of CAT (12 and 15.8%) and APX (10 and 13%) under both levels of salt stress (**Figure 4**). Likewise, POD and AsA activities also increased by 52 and 38% with PM (9%) under a salt stress level of 12 dS $m^{-1}$ compared with control treatment (**Figure 4**).

## Effect of Press-Mud Amendment on the Elemental Concentration of Rice Plants Grown Under Salinity Stress

Salt stress significantly increased the $Na^+$ contents while it reduced the $K^+$ accumulation. Conversely, PM appreciably reduced the $Na^+$ while increasing $K^+$ accumulation (**Figure 5**). The application of a PM (9%) reduced the $Na^+$ contents by 26 and 48% under moderate (6 dS $m^{-1}$) and stronger (12 dS $m^{-1}$) salt stress levels (**Figure 5**). Moreover, the application of PM (9%) increased the $K^+$ contents by 18 and 20% at moderate (6 dS $m^{-1}$) and stronger (12 dS $m^{-1}$) salt stress (**Figure 5**).

## Effect of Press-Mud Amendment on Yield and Yield Parameters of Rice Plants Grown Under Salinity Stress

Salinity caused a significant decrease in yield attributes of rice crops (**Table 2**). However, PM appreciably improved the yield traits of rice (**Table 2**). The maximum tillers (8.3), panicle length

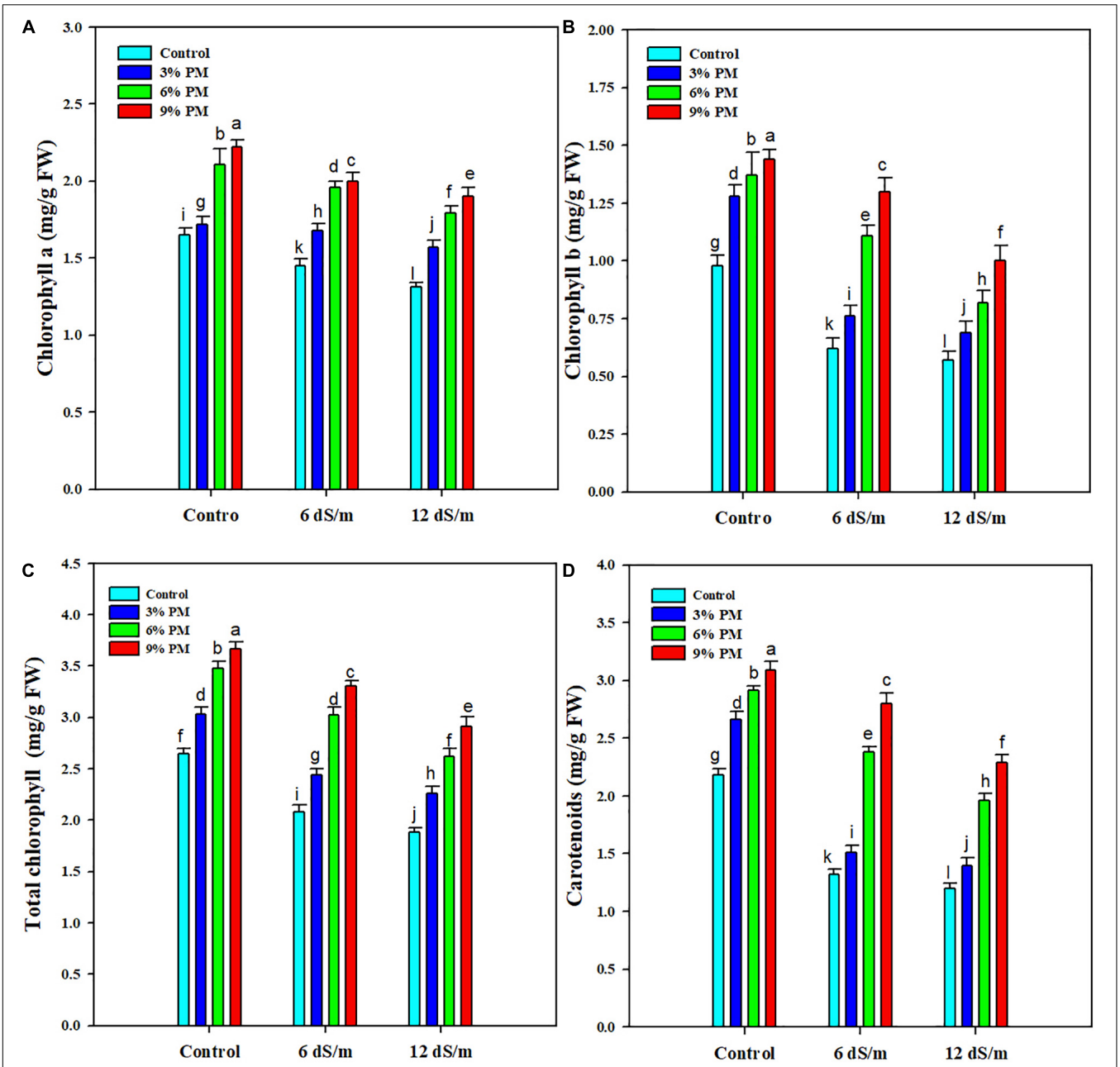


**FIGURE 1 |** Effect of different levels of press mud application on chlorophyll **(A)**, chlorophyll **(B)**, total chlorophyll **(C)**, and carotenoid **(D)** contents of rice crop grown under different levels of salinity stress. The bars indicate the means of three replications with ± S.E. and a different letter indicating significant differences at $p < 0.05$.

(20.6 cm), and panicle/plant (12.5) were noted in control (no salt stress) with the application of 9% PM, whereas the lowest tillers (3.8), panicle length (8 cm), and panicle/plant (6.5) were recorded at 12 dS $m^{-1}$ without PM application (**Table 2**). Likewise, PM application also markedly increased the grains/panicle, thousand-grain weight (TGW), and grain yield/pot under normal and salt stress conditions. The application of 9% PM remained at the top position, and it significantly improved the grains/panicle (195 and 198%), TGW (21.03 and 23.61%), and grain yield/pot (105 and 86%) at 6 and 12 dS $m^{-1}$ salt stress (**Table 2**).

## DISCUSSION

Mitigation of salinity stress through eco-friendly approaches is highly demanding in plant production for sustainable agriculture and global food security. In this study, salinity stress decreased rice plants' growth, biomass production, and photosynthetic efficiency and induced oxidative stress. However, it was evident that the SPM significantly alleviated salinity stress and improved rice growth and yield by improving physiological and biochemical attributes. Salinity stress induced a substantial

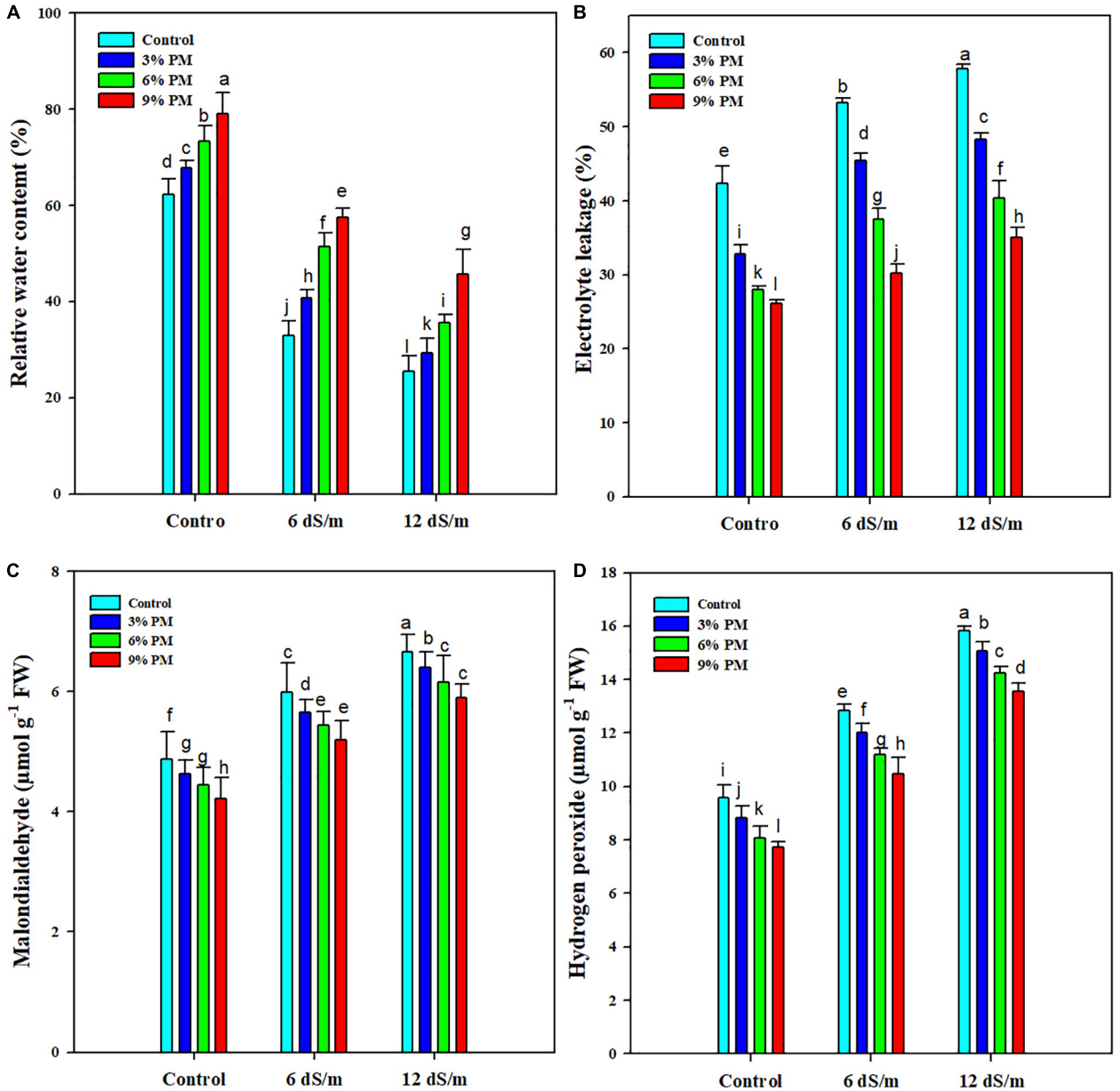


**FIGURE 2 |** Effect of different levels of press mud application on RWC **(A)**, EL **(B)**, MDA **(C)**, and $H_2O_2$ **(D)** contents of rice crop grown under different levels of salinity stress. The bars indicate the means of three replications with ± S.E. and a different letter indicating significant differences at $p < 0.05$.

increase in $Na^+$ accumulation, decreasing photosynthetic pigments and leaf water contents disturbed nutrient and water uptake, thereby reducing growth and biomass production (Mahmood et al., 2021; Nahar et al., 2022). However, the PM amendment appreciably alleviated the salinity stress and improved the growth and biomass production. PM application enhances soil organic matter content, which improves the nutrient and water uptake and maintains better synthesis of photosynthetic pigments, resulting in a significant improvement in growth and biomass production (Budiyanto, 2021; Imran et al., 2021). The favorable conditions created by SPM also improved the antioxidant activities and osmolytes activities, which protected the cellular structure, proteins, and lipids from the toxic effect of salinity, and enhanced plant growth and biomass production (Imran et al., 2021; Sheoran et al., 2021a). In this study, salinity stress significantly reduces the photosynthetic pigments. Excessive $Na^+$ ions participate in ROS production by working as signaling molecules in transduction pathways (Fatima et al., 2021). The excessive accumulation of $Na^+$ denatures the enzyme needed to synthesize chlorophyll and therefore reduces the synthesis of chlorophyll (Alzahib et al., 2021). However, PM alleviated this reduction, which indicates

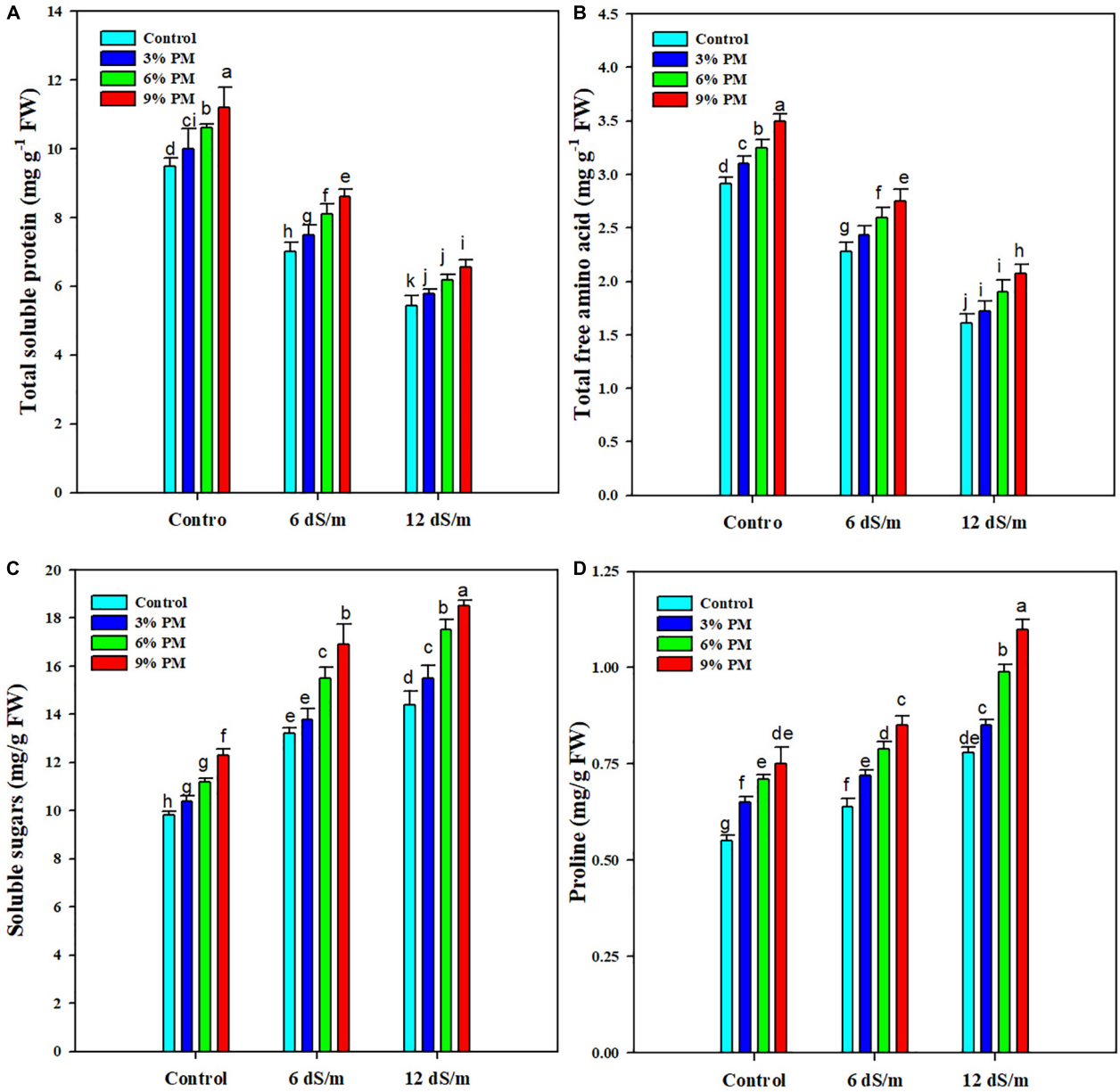


**FIGURE 3 |** Effect of different levels of press mud application on TSP **(A)**, FAA **(B)**, soluble sugars **(C)**, and proline **(D)** contents of rice crop grown under different levels of salinity stress. The bars indicate the means of three replications with ± S.E. and a different letter indicating significant differences at $p < 0.05$.

PM application reduced the excessive $Na^+$ accumulation, which protects photosynthetic apparatus from damaging effects of $Na^+$ and reduces the activities of chlorophyll degrading enzymes, improving chlorophyll synthesis under salinity stress (Sheoran et al., 2021a). In rice plants, salinity stress significantly reduced RWC (**Figure 1**). Plants exposed to salinity stress face the osmotic challenge that reduces the water uptake. Besides this, ABA-mediated stomatal closure affects the transpiration pull and leads to low/no water uptake by plant roots and entails low RWC in plants (Meguekam et al., 2021). In contrast, PM improved RWC, which could be due attributed to an increase in cell turgor pressure, water uptake, and reduced transpiration rate, which resulted in a significant increase in RWC (Kumar and Chopra, 2016; Soni et al., 2016).

Salinity significantly increased the accumulation of MDA and $H_2O_2$. The increased $H_2O_2$ accumulation interrupts normal cell functioning by causing oxidative damage and substantially reducing growth and productivity (Hassan et al., 2020; Sultan et al., 2021). Membrane damage is the primary effect of salinity stress, and increased MDA accumulation under salt stress that can be attributed to salt-induced membrane damage (Fatima et al., 2021). Salt stress significantly increased EL; however,

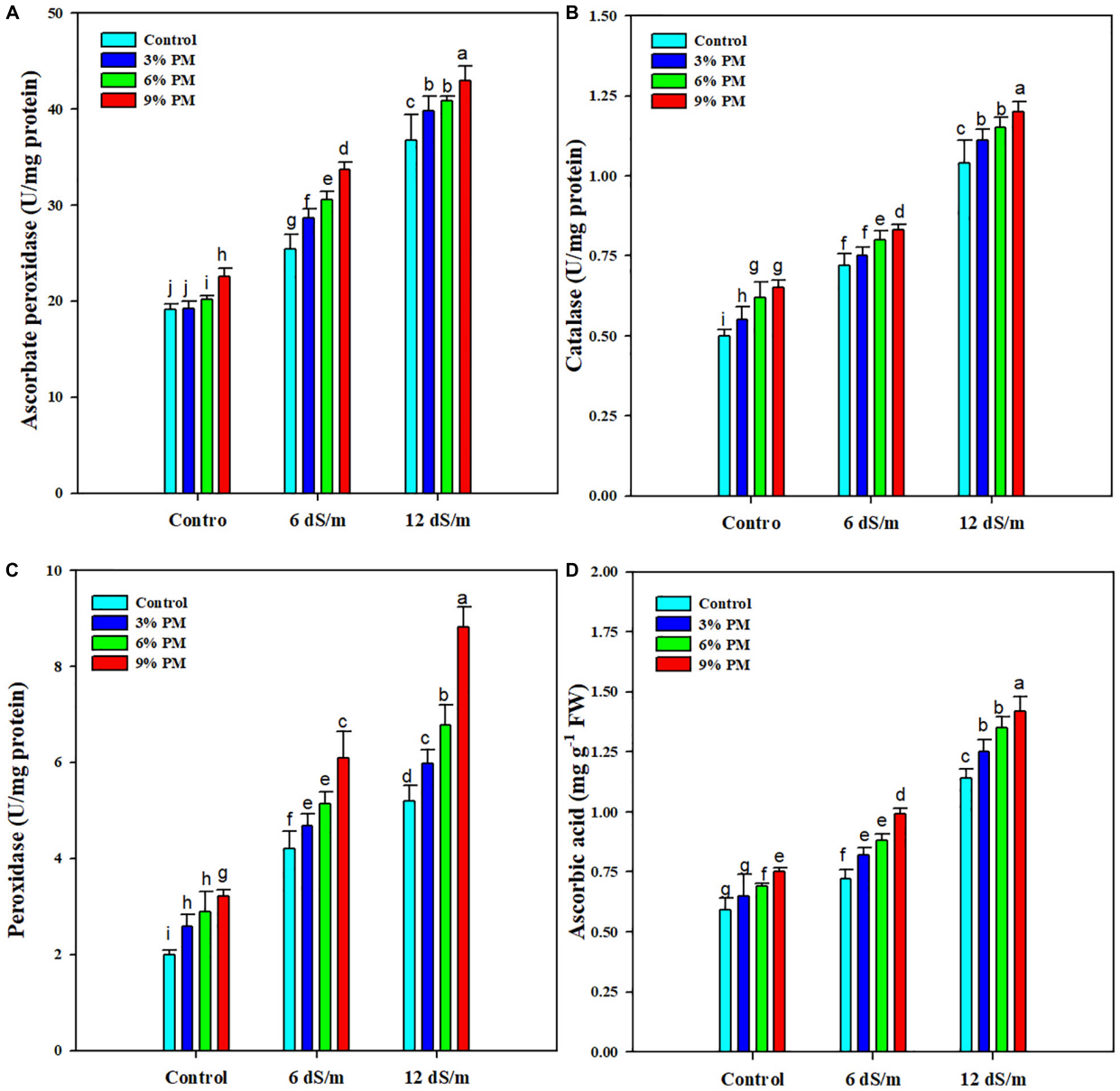


**FIGURE 4 |** Effect of different levels of press mud application on APX **(A)**, CTA **(B)**, POD **(C)**, and AsA **(D)** contents of rice crop grown under different levels of salinity stress. The bars indicate the means of three replications with ± S.E. and a different letter indicating significant differences at $p < 0.05$.

the PM amendment markedly reduced the EL. Salt stress increases ROS production, which damages cell membranes and consequently increases the EL (Sultan et al., 2021). Membrane integrity plays an imperious role in salt tolerance, and reduction in EL with PM application was linked with lower MDA and $H_2O_2$ accumulation owing to improved antioxidant activities (APX, CAT, POD, and AsA) and accumulation of proline and soluble sugars (Sheoran et al., 2021a).

Salinity stress significantly reduced TS and FAA while it increased the accumulation of soluble sugars and proline. The higher FAA accumulation creates a potential osmotic gradient which facilitates inward water movement and prevents the plants from toxic effects of salinity stress (Sultan et al., 2021). PM increases nitrogen uptake, which increases protein synthesis because nitrogen is an integral component of proteins. Another possible reason for this increase in protein concentration might be increased antioxidant activities due to PM, which protected the proteins from damaging effects of salinity stress and improved their accumulation under salinity stress. Increased protein accumulation regulates metabolic processes and antioxidant activities, which enhances salt tolerance (Fahad and Bano, 2012; Farouk and Al-Huqail, 2020). The increase in sugars accumulation improves salt tolerance (Fahad and Bano, 2012), and in this study, the PM amendment significantly

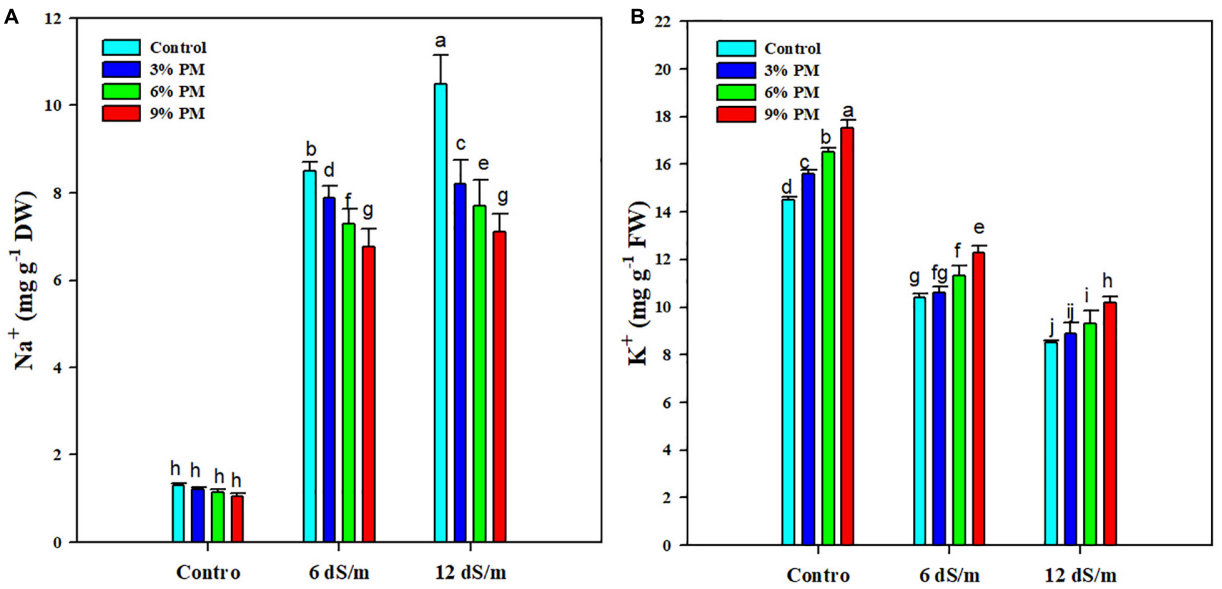


**FIGURE 5 |** Effect of different levels of press mud application on $Na^+$ **(A)**, and $K^+$ **(B)** contents of rice crop grown under varying levels of salinity stress. The bars indicate the means of three replications with ± S.E. and a different letter indicating significant differences at $p < 0.05$.

**TABLE 2 |** Effect of different levels of press mud application on yield and yield attributes of rice plants grown under different levels of salinity stress.

| SL | PML | NT | PL (cm) | PPP | GPP | TGW (g) | GY/pot (g) |
|---|---|---|---|---|---|---|---|
| 0 dS $m^{-1}$ | Control | 5.9e ± 0.6 | 13.5g ± 0.8 | 9.4cd ± 1.2 | 12.0g ± 0.8 | 18.82de ± 0.59 | 26.6g ± 0.9 |
| | 3% | 7.1bc ± 0.8 | 17.1c ± 0.6 | 10.5bc ± 0.5 | 16.5d ± 0.5 | 19.60cd ± 0.46 | 34.3d ± 0.8 |
| | 6% | 8.0a ± 0.8 | 19.7ab ± 0.4 | 11.5ab ± 1.2 | 19.5b ± 0.5 | 21.30b ± 0.78 | 39.5b ± 0.6 |
| | 9% | 8.3a ± 0.6 | 20.6a ± 1.1 | 12.5a ± 1.1 | 21.0a ± 0.7 | 23.33a ± 0.85 | 42.5a ± 0.9 |
| 3 dS $m^{-1}$ | Control | 4.2i ± 0.7 | 8.9j ± 0.01 | 7.3ef ± 1.1 | 6.1k ± 0.9 | 16.83g ± 0.58 | 18.0j ± 0.6 |
| | 3% | 5.0fg ± 0.8 | 11.1i ± 0.2 | 8.6de ± 1.2 | 9.0i ± 0.8 | 18.00ef ± 0.46 | 22.5i ± 2.4 |
| | 6% | 6.6c ± 0.4 | 16.0cd ± 0.7 | 10.6bc ± 0.4 | 15.1e ± 0.6 | 19.80cd ± 0.53 | 32.4e ± 1.7 |
| | 9% | 7.6b ± 0.4 | 18.2c ± 0.8 | 11.5ab ± 0.9 | 18bc ± 0.9 | 20.37bc ± 0.96 | 37.4c ± 1.6 |
| 6 dS $m^{-1}$ | Control | 3.8j ± 0.1 | 8.0k ± 0.8 | 6.5f ± 1.0 | 4.5l ± 0.5 | 15.67g ± 0.74 | 15.9k ± 0.7 |
| | 3% | 4.6h ± 0.9 | 9.8ij ± 0.5 | 7.5ef ± 0.8 | 7.4j ± 0.6 | 17.23fg ± 0.46 | 19.3j ± 0.3 |
| | 6% | 5.4f ± 0.5 | 12.2gh ± 0.6 | 8.4de ± 0.6 | 10.5h ± 0.3 | 19.43cd ± 0.21 | 24.4h ± 1.1 |
| | 9% | 6.3cd ± 0.6 | 14.9f ± 0.4 | 9.5cd ± 0.8 | 13.4f ± 0.7 | 19.37cd ± 1.00 | 29.5f ± 1.5 |
| LSD at 0.05 P | | 0.490 | 1.014 | 0.707 | 0.351 | 1.14 | 0.834 |

*SL, salinity levels; PML, press mud levels; NT, number of tillers; PL, panicle length; PPP, panicle per plant; GPP, grains per panicle; TGW, thousand-grain weight; GY, grain yield. The values given in the table are the mean of three replications with ± S.E. and different letters with each meaning showing the significant difference at $p \leq 0.05$.*

increased the accumulation of soluble sugars in rice plants. Proline accumulates in plants in response to salinity stress which confers salt tolerance (Sultan et al., 2021). Proline accumulation was significantly increased under salt stress, and further SPM also increased the proline accumulation. Press mud application increases the activity of the proline synthesis enzyme (pyrroline-5-carboxylate reductase), which increases proline synthesis and accumulation and improves salt tolerance (Kumar A. et al., 2017; Makarana et al., 2019; Sheoran et al., 2021a).

The activities of antioxidant enzymes were increased under salinity stress, which was further increased by PM application. The increase in activities of antioxidants substantially scavenges the ROS and protects the plants from the damaging effects of salt-induced oxidative stress (Parveen et al., 2019; Mahmood et al., 2021). However, the mechanism behind PM-induced increase in antioxidant activities is still unexplored. Therefore, further studies must be conducted to explore the mechanism behind the increased antioxidant activity with PM supplementation. An inadequate $K^+$ supply under salt stress reduces the photosynthetic rate and causes oxidative damage, which are primary reasons for a reduction in growth and yield (Hasanuzzaman et al., 2018; Dustgeer et al., 2021). $Na^+$, is a toxic ion, interferes with $K^+$ uptake, which disturbs the stomatal conductance, water uptake and induces necrosis and water loss, therefore, causing significant loss in growth and yield (Larbi et al., 2020).

Additionally, at the early stages of salinity stress, higher $Na^+$ concentration also disturb the $Ca^{2+}$ level and subsequently impair the $Ca^{2+}$ availability to young leaves (Talaat and Shawky, 2022). A particular amount of $Ca^{2+}$ is needed to maintain membrane integrity leaves (Talaat and Shawky, 2022); therefore, a reduction in rice growth in the current study can be linked with a decrease in membrane permeability due to poor $Ca^{2+}$ uptake. However, PM appreciably reduced the $Na^+$ accumulation in rice plants by increasing the $K^+$ accumulation. Press mud application ensures the desirable $Ca^{2+}$ availability in soil by mobilizing $CaCO_3$, improving soil structural stability, and increasing leaching of $Na^+$ (Prapagar et al., 2012), thereby reducing the salinity-induced oxidative damages (Sheoran et al., 2021b). Additionally, PM being an excellent nutrient source ensures a better supply of $K^+$ in salt-affected soils, and $Na^+$ uptake minimizes the salt-induced toxic effects (Kumar S. et al., 2017).

Salinity stress significantly reduced the yield and yield contributing traits of rice crops. Increased $Na^+$ accretion in rice leaves due to salt stress-induced early leaf senescence, reduced the panicle formation and assimilated production, reducing the growth and yield traits (Mannan et al., 2013). Salt stress also reduces photosynthetic pigments and disrupts osmolytes accumulation, plant water relationships, membrane integrity, and $K^+$ uptake and therefore causes a reduction in yield and yield traits (Al-Ashkar et al., 2019; Otie et al., 2021). Press mud alleviated the adverse impacts of salinity stress and improved rice yield and yield traits. Improved soil properties, nutrient uptake, photosynthetic pigments, antioxidant activities, osmolytes accumulation, $K^+$ uptake, and reduced MDA and $H_2O_2$ following PM application substantially improved the yield and yield traits.

## CONCLUSION

Rice development and yield were substantially hampered by salinity stress due to a considerable rise in MDA, $H_2O_2$, $Na^+$, and electrolyte leakage. Surprisingly, salinity-induced negative effects were restored mainly due to the application of sugarcane press mud. The application of press mud (9%) significantly improved rice growth and yield due to improved photosynthetic pigment, relative water contents, osmoregulating compounds, and $K^+$ accumulation. It reduced MDA, $H_2O_2$, $Na^+$, and electrolyte leakage through triggered antioxidant activities. Therefore, the use of press acerbated deleterious impacts of salinity stress provides strong evidence for the role of press mud in improving the salinity tolerance in rice plants. However, more genomics, transcriptomic, proteomics, and metabolomics studies are direly needed to underpin the mechanism associated with press mud-induced salinity tolerance in rice plants.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

## AUTHOR CONTRIBUTIONS

IK and MC conceived the idea. AM performed the experiment. IK, MC, and MH prepared the draft of manuscript. MBi, MA, MRi, WS, MS, MAR, MBr, MZ, and AE reviewed and edited the final version. All authors contributed to the article and approved the submitted version.

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