Identification of key modules and hub genes regulating drought stress response in rice drought sensitive line PY6 by weighted gene co-expression network analysis

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Abstract

Background: Drought stress is an adverse factor with deleterious effects on several facets of rice growth. However, the mechanism underlying drought resistance in rice remains unclear. In order to genetically understand the potential molecular mechanism for drought response in rice, a drought sensitive Chromosome Segment Substitution Line (CSSL) PY6, which was constructed by the introgression of genomic segments of drought sensitive variety LAMBAYEQUE1 into drought-resistance variety PR403 via backcrossing, was used to map the QTL locus *dss-1* for its sensitive phenotype, and to reveal the impact of *dss-1* on the transcriptional profiling of PY6 via RNA-seq and WGCNA (weighted gene co-expression network analysis) analysis.

Results: The genetic linkage analysis showed that *dss-1* was located on the short arm of chromosome 1 of rice. In contrast to PR403, the over-accumulation of H₂O₂ and MDA that might result in drought sensitive phenotype was observed in PY6 under drought stress. In the analysis of RNA-seq data, the identified differentially expressed genes (DEGs) mainly enriched in photosynthesis-related GO terms and exhibited a down-regulation pattern of their expressions in both PY6 and PR403 in response to drought stress, indicating that the photosynthesis was greatly inhibited in rice. Further WGCNA analysis constructed a co-expression network with 26 gene modules in which 4 and 3 modules that were highly correlated with H₂O₂ and MDA, respectively. Likewise, the GO analysis of the differentially expressed hub genes (DEHGs) enriched in H₂O₂-correlated modules showed that the photosynthesis related GO terms were consistently over-represented. Furthermore, functional annotation of DEHGs in H₂O₂ and MDA correlated modules revealed a cross talk between abiotic and biotic stresses. This was reflected by the differential expression alterations of hub genes which were annotated as encoding MYBs, laccases, WRKYs, and PRs family proteins, and ZFP36 were notably observed between PY6 and PR403 in response drought stress.

Conclusions: Collectively, we speculated that drought-induced the inhibition of photosynthesis lead to the accumulation of H₂O₂ and MDA that can trigger the reprogramming the profiling of transcriptome in rice. This included the differential regulation of hub genes that involve in ROS eliminated pathways to prevent the damage of rice plants from oxidative stress.

Background

Abiotic stresses, such as drought, chilling, heat, and salinity, etc., are wide-spread factors with deleterious effects on several facets of plant life. However, as sessile organisms, higher plants usually adopt the 'overcome' strategy upon encountering any extreme environmental stresses, in contrast to animals, which preferentially opt for the tolerance or avoidance of unfavorable circumstances. In general, various tolerance/avoidance mechanisms (de Zelicourt et al. 2016), such as the regulation of endogenous hormone signaling (Verma et al. 2016), the increase of wax deposition in cuticle (Zhu and Xiong 2013), and the modulation of stomatal state (Huber et al. 2019), are employed in response to abiotic stresses.

Rice (*Oryza sativa* L.) is a staple food crop of the world. Drought is one of major abiotic constraints for rice production with the increasing of the global water shortage. Two important strategies, dehydration tolerance and avoidance, are adopted in plants to cope with drought stress. Dehydration avoidance depends on the development of a larger and deeper root system to uptake water from soil and reducing water loss via the modulation of stomatal opening. Dehydration tolerance mainly enhances drought resistance by the complex mechanisms in later stage of drought stress such as anti-oxidation processes, ABA signaling transduction, osmotic adjustment, and cell membrane protection (Hu and Xiong 2014; Luo 2010).

Several QTLs for dehydration tolerance and avoidance in rice were cloned via map-based cloning. Moreover, a large number of QTLs for root growth (Lou et al. 2015), osmotic pressure regulation (Lilley et al. 1996), ROS regulation, and cuticular wax accumulation which may contribute to enhance drought resistance were mapped to different locations of rice genome (Huang et al. 2019; Huang et al. 2009). *DRO1*, one of the gene that has been well-characterized, involves in the regulation of rice root development under drought stress. *DRO1* regulates cell elongation in the root tip that causes downward bending of the root to avoid water deficit by increasing deep rooting (Uga et al. 2013). *DST* was identified as negative regulator of H_2O_2 accumulation in guard cell that functions in the modulation of stomatal opening. Loss function of *DST* increases stomatal closure, resulting in enhanced drought tolerance in rice (Huang et al. 2009). In addition, *DS8* participates in the synthesis of plant cuticular wax and in the regulation of stomatal movement in response to drought stress (Huang et al. 2019). The studies of these genes indicated that intricate mechanisms are adopted in rice plants for the adaption of drought stress.

Although the over-accumulation of reactive oxygen species (ROS), including singlet oxygen, H_2O_2 , and superoxide anion, causes severe injuries to plant cells, even leading to programmed cell death (Liu et al. 2016), the generation of ROS acts as second messenger that involves in the stress signaling response, photosynthetic regulation, stomatal movement, and the development of higher plants. ROS homeostasis plays a key role in plant drought resistance regulation (Ramachandra Reddy et al. 2004; Wang et al. 2016). Plants have evolved a series of enzymatic and nonenzymatic antioxidant systems. Enzymatic system, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), participates in scavenging ROS and preventing the damage from oxidative stress (Xu et al. 2008). SODs function in the dismutation of $O_2^{\cdot -}$ to H_2O_2 and O_2 ; then the resultant H_2O_2 was catalyzed into H_2O and O_2 by the effect of CAT and APX (Mori and Schroeder 2004). Chloroplasts is an organelle for photosynthesis in plant cells. As a key process for the responsive regulation, when rice plants are subjected to abiotic stresses, photosynthetic activity is greatly inhibited (Cen et al. 2018). The inhibition of photosynthesis reduces the utilization of absorbed light energy, resulting in the generation of ROS in chloroplasts, which in turn causes the photodamage to photosystem proteins, such as D1, a core component of photosystem II (Kojima et al. 2007). Recently, the emerging evidence indicated the high correlation among antioxidant mechanism, photosynthesis regulation, and drought-tolerance (Gan et al. 2019; Ramachandra Reddy et al. 2004; Zong et al. 2016). In addition, ABA induces the accumulation of H_2O_2 via the activation of NADPH oxidase, which is activated by SnRK2s phosphorylation, and thereby, resulting in the stomatal

closure eventually. Moreover, the accumulation of ABA induces the expression of *CAT*, suggesting that a certain relationship existed between ABA signaling and H₂O₂ tuning (Bailey-Serres et al. 2019; Liu et al. 2014; Okamoto et al. 2013; Shim et al. 2018; Tang et al. 2016; Wang et al. 2018).

Recent reports have suggested that WRKY transcription factors (TFs) participate in the regulatory mechanism pathways of abiotic stresses, including drought and salinity (Xiao et al. 2013; Yokotani et al. 2013). WRKY TFs are plant specific group of zinc finger transcription factors that are characteristic of containing a conserved WRKY domain, and bind to a consensus *cis*-element W-box (TTGACT/C) (Ross et al. 2007). The majority of over 100 members of which in rice involve in the plant defense response in either a negative or positive manner (Group 2012; Ross et al. 2007). Some WRKY members have important roles in abiotic stress responses, for example *OsWRKY13* was suggested to regulate the antagonistic cross talk between drought and disease resistance pathways via the repression of *SNAC1* and *WRKY45-1* (Xiao et al. 2013; Yokotani et al. 2013). Meanwhile, pathogenesis-related (PR) proteins have been well defined as a group of proteins that are induced not only by pathogen infection such as viruses, bacteria, and fungi, but also in response to abiotic stresses, including drought and salinity (Takeuchi et al. 2011). All the PR proteins in both monocot and dicotyledon were classified into 17 families (Liu and Ekramoddoullah 2006). Among of which, PR3 and PR4 have plant chitinase activity; PR5 family of proteins includes permatins, osmotins, zeanatins, and thaumatin-like proteins (Agarwal and Agarwal 2014). Besides, the PR10 genes are belonged to a multigene family in various plants, and exert RNase activity, ligand binding, post-translational modification (phosphorylation), and phytohormone signaling in stress responses. The promoters of PR10 genes harbor *cis*-acting elements for WRKY, bZIP, ERF, MYB transcription factors, suggesting the pivotal roles of PR10 play in the stress response pathways (Agarwal and Agarwal 2014).

With the rapid development of next generation deep resequencing technology, RNA-seq is widely used to explorer the transcriptomic profiling of plants in response to the challenges of adverse environments. Recent studies revealed that antioxidant process, ABA pathway, and photosynthesis were the target biological processes for plants in response to environmental change at transcription level (Varoquaux et al. 2019; Zhang et al. 2016). More intriguingly, photosynthesis rate was found to be declined under drought stress, especially in drought sensitive genotype, suggesting that the enhancement of antioxidant capacity was in favor of maintaining higher photosynthetic activity, and thereby would improve drought resistance of plants (Zhang et al. 2016). Moreover, phytohormone signaling and Ca²⁺ signaling were also highlighted in transcript profiling and revealed the cross-talk among phytohormone signaling pathways (Kim et al. 2018; LY et al. 2014). In addition, a large number of transcription factor families, like WRKY, bZIP, and MYB, were identified involved in ABA-mediated signaling transduction under abiotic stresses (Borah et al. 2017; Okay et al. 2014). However, the molecular mechanism of drought tolerance/sensitive still need to be further investigation.

Weighted gene co-expression network analysis (WGCNA) is a new algorithm that is used to identify the potential gene modules with highest connectivity among genes and is correlated to certain phenotypes in the gene expression data (Langfelder and Horvath 2008; Zhang and Horvath 2005). WGCNA constructs a

co-expression network that can indicate the correlations among genes across samples. Based on the co-expressed relationships, genes with similar expression are grouped into the same module, thus suggesting that the genes in the same module may have similar function or possibly have common biological regulatory roles (Zhou et al. 2018). The method has been successfully applied in numerous study cases which were used to identify hub genes and the relationship between gene expression data and relevant phenotypes (Clarke et al. 2013; Deng et al. 2019; Du et al. 2017; Greenham et al. 2017; Shaik and Ramakrishna 2013; Zhang et al. 2018).

Here, we identified a drought sensitive rice chromosome single substitution segment line (CSSL) PY6, which carries the substitution segment derived from a drought sensitive variety LAMBAYEQUE1 by backcrossing into drought tolerance variety PR403 background. A locus *dss-1* underlying drought sensitive phenotype of PY6 was harbored in the short arm of chromosome 1. In order to investigate the impact of *dss-1* on the transcriptional profiling of PY6, RNA-seq was performed to identify differential expression genes (DEGs) under drought stress treatment. Further, WGCNA was applied to identify hub genes from the modules which were highly correlated with drought-induced physiological indicators. By this approach, we aimed to infer *dss-1* mediated potential regulatory network that are relevant to the drought stress tolerance in rice.

Results

Identification of *dss-1* locus for drought sensitive phenotype in the CSSLs

In our previous study, a set of rice CSSLs with 140 lines was constructed by the introgression of the genomic segments of drought sensitive variety LAMBAYEQUE1 into the genome of drought resistant variety PR403 through backcross (Fig. 1A). The overlapped introgressed segments defined by 167 molecular markers covered the whole genome of LAMBAYEQUE1 (unpublished data). In order to uncover the molecular regulatory mechanism underlying the drought sensitive phenotype in LAMBAYEQUE1, drought stress treatment was performed to identify the major QTL (s) for drought sensitive trait. PY6, one of the CSSLs, was highlighted for its drought sensitive phenotype. After 10-day drought treatment, the treated CSSLs were re-watered and allowed to recover for 3 days. In the screening, PY6, one of CSSLs, was identified with the significant difference of survival rate (20.0 %) by comparing with its recurrent parent PR403 (93.3 %), whereas no significant difference was observed in the survival rate of PY6 and its donor parent LAMBAYEQUE1 (Fig. 1B-C). During the drought stress treatment, the relative water content of the seedlings was declined in PY6 and PR403 with the decreasing of the soil moisture. However, starting from 120 h, the significant difference was observed between PY6 and PR403 and the former one exhibited more severe dehydration at the later stage of the treatment, suggesting the drought stress broke the balance of water uptake and evaporation in PY6 (Fig. 1D). PY6 carries a 10-Mb chromosome segment derived from LAMBAYEQUE1 on the short arm of chromosome 1. These results suggested that the segment from LAMBAYEQUE1 might harbor a major QTL locus for the drought sensitive phenotype of PY6. We designated it as *dss-1*.

To reveal the genetic basis underlying the drought sensitive phenotype conferred by *dss-1* locus, PY6 was backcrossed with recurrent parent PR403 to construct mapping population. The F1 plants exhibited drought resistance, similar to that of parental line, PR403. Then, F2 population was developed to determine the inheritance feature of *dss-1*. In a 151-individual F2 population, 111 showed drought resistance and 40 showed drought sensitive traits (resistant: sensitive $\approx$ 3:1, $\chi^2 = 0.1788 < \chi^2_{0.05} = 3.84$), indicating that drought sensitive trait is controlled by a single recessive gene. In turn, the recombinants were screened for genetic linkage analysis, and *dss-1* was finally narrowed down to 4.01-Mb interval (Fig. 1E). To further confirm the reliability of *dss-1* mapping, GWAS was performed based on 84 rice varieties that were selected from 3000 Rice Genomes Project (Wang et al. 2018). Drought stress phenotype was investigated under 20% PEG-6000 treatment. Using the general mixed linear model, two SNP located on the region of *dss-1* interval were detected associated with drought stress phenotype (Fig. 1E). Ten candidate genes for *dss-1* were identified in the vicinity around 200-kb of associated SNPs (Table S2).

Drought induced H₂O₂ and MDA accumulation the leaves of PY6

Given that the previous reports indicated that drought stress was known to inhibit photosynthetic activity plants due to an imbalance between light capture and its utilization (FOYER and NOCTOR 2000), and coupled with considerable potential for increased accumulation of superoxide and hydrogen peroxide in chloroplasts (Robinson and Bunce 2000), we examined the accumulation of ROS and the activity of its scavenging enzymes in the leaves of drought-treated PY6. Leaf samples were harvested at 4 sampling points (Fig. S1). In contrast to PR403, the increasing accumulation of H₂O₂ was detected in PY6 from points 40 to 15, whereas the enhanced content only can be observed at point 22 in PR403, thereby the significantly increased accumulation of H₂O₂ was detected in PY6 at sampling points 22 and 15 by comparing with their respective controls (Fig. 2A). The remarkable accumulation of H₂O₂ in the leaves of PY6 was confirmed by DAB staining (Fig. 2D). Consistently, the activities of POD and CAT were up-regulated in response to the accumulation of H₂O₂ (Fig. 2B, C). However, the activity of APX, a major H₂O₂-scavenging enzyme that removes H₂O₂ via the ascorbate-glutathione cycle, was not induced and no significant difference was observed between both genotypes during drought treatment (Fig. S2A). For the dynamic changes of superoxide, the content of SOA (superoxide anion) and the activity of its scavenging enzyme SOD at point 15 were exhibited significant difference between two genotypes with higher values in PY6. However, the content of SOA in whole period of drought treatment was not significantly induced in contrast to the untreated control (point 40) (Fig. 2E, F; Fig. S2B). Moreover, the contents of MDA, a major reactive aldehyde resulting from the peroxidation of polyunsaturated fatty acid (PUFA) constituents of biological membranes and acting as the indicator of the abiotic stress-induced bio-membrane damage, were shown significantly accumulated in PY6 by comparing with those in PR403 at points 22 and 15, respectively (Fig. 2G).

Taken all these together, our results suggested that the drought sensitive phenotype might be caused by the over-accumulation of ROS, especially H_2O_2 that could result in the severe damage to the plant cells and the peroxidation of cell membrane, and thereby leading to the dehydration of rice plants under drought stress.

The accumulation of ABA and stomatal modulation during drought stress treatment

As above mentioned, PY6 exhibited more severe dehydration at the later stage of the treatment, suggesting that PY6 either had a higher water transpiration or lower water uptake ability when it was subjected to drought stress. ABA is a key regulator for plant stress responses and regulates a series of physiological processes, including tolerance to abiotic stresses and stomatal closure (Sah et al. 2016). In order to determine the role of ABA-mediated stomatal movement played in the drought-induced severe dehydration in PY6, the content of ABA and the status of stomatal apertures in drought treated samples were examined. The content of ABA was accumulated during drought stress treatment, although no significant difference between PY6 and PR403 was observed at any sampling point (Fig. S2C). Scanning electron microscopy was used to examine the stomata status of leaf samples. By comparison with the untreated control, the percentage of completely closed stomata in the drought-treated samples was significantly elevated. However, PY6 and PR403 have similar patterns in modulating their stomata in response to drought stress, and also no significant difference in the stomatal density was observed in both genotypes (Fig. S2D). Therefore, these results suggested that severe dehydration in drought-treated PY6 might not result from the defect of ABA-mediated stomatal transpiration regulation, but cause by the limitation of water uptake or by other water loss pathway.

Comparative transcriptional profiling of PY6 under drought stress

To further unveil the impact of *dss-1* on drought stress-induced H_2O_2 accumulation that might lead to the drought sensitive of PY6, we investigated the transcriptional profiling of PY6 and PR403 in response to drought stress through RNA-Seq. Principal components analysis (PCA) was initially performed based on all the transcriptional profiling data to detect the variations among the samples with the imposition of the drought treatment (Fig. 3A). PC1 explained 37.98% of total variation. The samples of CK40 and CK22/20 of both PY6 and PR403 were clearly separated by PC1, indicating the effect of the development imposed on the variations, while PC1 also separated drought-treated samples of points 22 from 20, indicating the effect of imposition of drought treatment on the transcriptomes of the samples. PC2 explained 27.94 % of total variation and clearly separated drought-treated samples from the parallel untreated samples (Fig. 3A). Moreover, both PY6 and PR403 samples of point 20 exhibited the remarkable divergence from other sampling points, indicating the dramatic transcriptomic reprogramming occurred at this sampling point.

Drought-induced DEGs were identified by comparing with untreated controls (Table S3). It was worth noting that the development-dependent DEGs were detected in the same sampling points of well-watered samples with the same criterion and they were excluded from the DEGs set to avoid the misleading to understand drought-induced transcriptomic responses (Table S3-5~12). Finally, in the two sampling points of PR403, 3052 DEGs including 1135 up-regulated and 1917 down-regulated were detected at point 22 (Table S3-1), while 5821 DEGs were detected at point 20, including 2383 up-regulated and 3438 down-regulated DEGs (Table S3-2). In PY6, 3145 (1748 up-regulated/1397 down-regulated) and 5529 (2655 up-regulated/2874 down-regulated) were detected at point 22 and 20, respectively (Table 1; Table S3-3, -4). Among the resultant DEGs, venn diagram analysis showed that 1142 DEGs were common between PR403 and PY6 at point 22, while 3184 were common between PR403 and PY6 at point 20, which accounted for 38.4% and 48.7% of total DEGs in both sampling points, respectively (Fig. 3B-C, Table 1), demonstrating that PY6 has a similar genetic background and a similar drought stress response to those of PR403 from another side.

To verify the data quality of RNA-seq, five genes was selected for qRT-PCR. Similar trends of expression changes were observed between qRT-PCR and RNA-seq, indicating that transcriptomic data are reliable for further analysis (Fig. S3A-E, Table S1).

Gene ontology enrichment analysis

GO enrichment analysis was performed to enrich the DEGs into the functional categories of biological processes (BP), cellular components (CC), and molecular function (MF) subgroup. The results showed that all the DEGs were enriched into the GO terms that were related to photosynthesis in chloroplasts (Fig. 4A-D). For instance, in terms of BP group, the GO terms including photosynthesis (GO: GO:0015979), photosynthesis, light harvesting (GO:0009765), photosynthesis, light harvesting in photosystem I (GO:0009768), photosynthesis, light reaction (GO:0019684), chlorophyll biosynthetic process (GO:0015995), and porphyrin-containing compound biosynthetic process (GO:0006779), were highly significantly over-represented. The over-represented GO terms, such as chloroplast part (GO:0044434), chloroplast envelope (GO:0009941), chloroplast thylakoid (GO:0009534), chloroplast thylakoid membrane (GO:0009535), chloroplast thylakoid lumen (GO:0009543) in CC (Fig. S4A-D), and the over-represented terms of MF, such as pigment binding (GO:0031409), chlorophyll binding (GO:0016168) (Fig. S5A-D), supported the identified DEGs were related to the photosynthetic modulation in response to drought stress. In addition, superoxide metabolism related GO terms superoxide metabolic process (GO:0006801) and regulation of superoxide metabolic process (GO:0090322) were highly enriched by which of the DEGs identified in point 20 of PR403, suggesting the involvement of the ROS scavenging processes in drought stress response (Fig. 4C). GO enrichment suggested that photosynthetic modulation played important role in the regulatory network of rice plants in response to drought stress, thereby chloroplast is the target organelle for drought stress response.

Construction of the co-expression network and identification of drought-induced hub genes

To further investigate the impact of *dss-1* on the regulatory network in response to drought stress and to identify the specific genes that are highly correlated with drought-induced physiological alterations in rice, we performed WGCNA analysis. After removed the genes with low FPKM ($\text{FPKM} < 1$), a total of 23178 genes was used to construct a scale-free co-expression network based on the soft-thresholding power of $\beta = 12$ (Fig. S6). In the results of WGCNA, the clusters with highly inter-connected genes were defined as modules; and the genes in the same modules have high correlation coefficients among each other. A total of 26 modules were identified via the Dynamic Tree Cut method (core parameter: $\text{MEDissThres} = 0.25$) (Fig. 5A). In the correlation of physiological traits with over-represented modules, the intriguing preferences were observed in the results of the correlation. In general, the modules including Black, Blue, and Tan were exhibited negatively correlated with the majority of physiological traits, whereas Royalblue, Brown, Red, Grey60, Orange, and Green modules were exhibited positively correlated with the traits, indicating that the genes clustered in a module have similar expression alternation pattern in response to drought stress (Fig. 5B). Considering the significant difference of the contents of H_2O_2 and MDA were observed during drought stress, we focused on the correlations of H_2O_2 and MDA with gene modules and identified hub genes that were highly correlated with the two drought-related physiological indicators (Fig. 2A, D, G; Fig. 5B, C, D). With the cut-off threshold of $\text{GS} > 0.4$, in the total of 26 modules, Black (741 genes; $R^2 = -0.69$, $P = 3.0 \times 10^{-5}$), Blue (4810 genes; $R^2 = -0.57$, $P = 9.0 \times 10^{-4}$), Grey60 (148; $R^2 = 0.67$, $P = 4.0 \times 10^{-5}$), and Green (1099 genes; $R^2 = 0.61$, $P = 3.0 \times 10^{-4}$), were considered as the modules that were highly correlated with H_2O_2 , and Red (913 genes; $R^2 = 0.63$, $P = 2.0 \times 10^{-4}$), Brown (3132 genes; $R^2 = 0.56$, $P = 1.0 \times 10^{-3}$), and Royalblue (45 genes; $R^2 = 0.68$, $P = 4.0 \times 10^{-5}$) modules were considered highly correlated with MDA (Fig. 5B, D). The genes in which the modules were clustered showed highly correlated with their respective modules (Fig. S7A, B). Furthermore, hub genes were identified based on the following cutting-off thresholds, $\text{GS} > 0.60$ and $\text{MM} > 0.80$ for Black, Blue, Green, and Royal blue module, and $\text{GS} > 0.5$ and $\text{MM} > 0.8$ were used for Grey 60, Red, and Brown module according to the strengthen of threshold. After removed the genes with $|\text{Log}_2\text{FC}| < 1$, all the obtained hub genes were significantly differentially altered their expressions in PY6 and PR403 under drought stress and were highlighted in the further functional analysis (Fig. S10A-F; Table S4-1~-7). Hereafter, we designated these hub genes as DEHGs.

Functional enrichment analysis of hub genes correlated with H_2O_2 accumulation

GO enrichment analysis was performed to functional clustering of the hub genes in the modules that were highly correlated with H_2O_2 . A total of 115 and 204 DEHGs were identified in Black and Blue modules, respectively (Table S4-1, -2). In line with the above-mentioned results, these hub genes were highly enriched in the GO terms, including chloroplast (GO:0009507), chloroplast envelope (GO:0009941), thylakoid (GO:0009579), chloroplast stroma (GO:0009570), chlorophyll binding (GO:0016168), photosynthesis (GO: GO:0015979), photosystem I (GO:0009522), photosynthesis, light harvesting

(GO:0009765), and photosynthesis, light reaction (GO:0019684), that are related to photosynthesis ($Qvalue \leq 0.05$) (Fig. S8A-B; Table S6-1, -2). Interestingly, in agreement with previous reports (Ma et al. 2016), the expression of the majority of the DEHGs in modules Black and Blue was down-regulated without remarkable Log_2FC differences ($|\text{Log}_2\text{FC}_{\text{PY6}} - \text{Log}_2\text{FC}_{\text{PR403}}| \geq 1, P < 0.05$) observed between PY6 and PR403 during drought treatment (Fig. 6A, B; Fig. S10A,B), implying that PY6 and PR403 have a common drought responsive regulation and their photosynthetic activities were inhibited in response to the drought stress.

Grey60 module was enriched with 41 DEHGs (Table S4-3). The expression of most of them was elevated under drought stress (Fig. S10C). Among of which, eight genes were showed differential expression alterations with the remarkably different magnitudes at least in one sampling point between two genotypes, exhibiting a strong dynamic induction in response to drought treatment ($|\text{Log}_2\text{FC}_{\text{PY6}} - \text{Log}_2\text{FC}_{\text{PR403}}| \geq 1, P < 0.05$) (Fig. 6C; Table 2; Table S6-3). The expression of Os01G0842500 and Os03G0273200, both encoding the proteins that are similar to laccase and were enriched in GO terms apoplast (GO:0048046)/lignin catabolic process (GO:0046274)/cytoplasmic membrane-bounded vesicle (GO:0016023)/oxidoreductase activity (GO:0016491), was consistently enhanced in two points of both PY6 and PR403, and showed higher magnitudes in PY6 (particularly at point 20) (Fig. 6C; Table 2). Five genes (Os01G0702700, Os06G0131700, Os05G0543600, Os03G0433200, and Os04G0508500) which are annotated as transcription factors and mainly enriched in the GO terms including DNA binding (GO:0003677), sequence-specific DNA binding transcription factor activity (GO:0003700), and response to freezing (GO:0050826), were significantly consistently induced to a certain extent at point 22 and were down-regulated at point 20 in PR403, whereas they were significantly induced only at point 20 in PY6 with the exception of Os03G0433200 (Fig. 6C; Table 2). Os01G0702700, Os05G0543600, and Os04G0508500 are belonged to MYB family TFs, and Os06G0131700 is NAC TF. Recent study suggested that Os06G0131700 (*OsSWN1*) were related to secondary cell wall formation (Chai et al. 2015), implying that the secondary cell wall formation may play a certain role in drought stress resistance. In addition, Os08g0189200 is annotated as a gene encoding Germin-like protein 8-3 and was suggested involved in disease resistance (Davidson et al. 2010). A different up-regulated pattern of this gene was observed in PY6 and PR403 in response to drought stress (Fig. 6C; Table 2).

A total of 96 hub genes were identified in Green module, including 36 down-regulated and 60 up-regulated genes in response to drought (Fig. S10D; Table S4-4). GO enrichment analysis showed that the majority of the over-represented GO terms are related to oxidation-reduction process (GO:0055114), response to salt stress (GO:0009651), carbohydrate metabolic process (GO:0005975), biosynthetic process (GO:0009058), and metabolic process (GO:0008152), etc. These hub genes were sub-localized to distinct cellular components and have various molecular functions (Fig. S8D; Table S6-4). However, no GO term was significantly over-represented with the exception of iron-sulfur cluster binding (GO:0051536) in our analysis (Table S6-4). It's worth noting that only 1 hub gene OS04G0685300, encoding a protein with harpin-induced 1 domain, was showed significant differential expression alteration with a higher up-regulated magnitude at two points of PY6 (2.03/2.67) by comparing with those in PR403 (Table S4-4).

All these together, the functional annotation of the hub genes identified in the H₂O₂-related modules suggested both PY6 and PR403 experienced drought-triggered photosynthetic activity inhibition and led to the accumulation of H₂O₂ in rice plants. While the hub genes with highly differential expression alternations identified in Grey60 might be responsible for the accumulation of H₂O₂ in rice plants.

Functional enrichment analysis of hub genes correlated with MDA accumulation

MDA is an important indicator of membrane lipid peroxidation. Red, Brown, and Royal blue modules were highly correlated with MDA under drought treatment. There were 303, 304, and 13 hub genes were identified in these modules, respectively (Table S4-5~-7). For Red module, GO analysis showed that the hub genes involve in biotic stress and defense response (Fig. S9A; Table S6-5). Among these hub genes, 29 were showed differential expression alterations at least in one sampling point between two genotypes ($|\text{Log2FC}_{\text{PY6}} - \text{Log2FC}_{\text{PR403}}| \geq 1$, $P < 0.05$) (Fig. 7A; Table 3). Strikingly, most of them consistently showed a similar expression pattern in both genotypes with the elevated transcripts at point 22 and then declined to comparable or even lower level in contrast to their respective untreated control (Fig. S10E; Table 3). GO analysis revealed that these genes involved in biotic stress responses, for example, the GO terms defense response, response to biotic stimulus, response to stress, and response to endogenous stimulus were over-represented in the results (Fig. 7A). Of which, 8 genes were belonged to WRKY family TFs (Fig. 7A; Table 3). Specifically, *OsWRKY70* (Os05g0474800) and *OsWRKY76* (Os09g0417600) were suggested to participate in ABA signaling and biotic stress response (Yokotani et al. 2013; Zhang et al. 2015). OS01g0185900 and OS05g0322900 were exhibited relative higher up-regulated expression at point 22, whereas another two genes OS05g0571200 and OS11G0117600 have relative higher expression at point 20 of PY6 by comparison with those in PR403 (Fig. 7A; Table 3); Considering the accumulation of MDA in PY6 and PR403, these implied that four genes might positively correlate with the over-accumulation of MDA and lead to the death of PY6 under drought stress. Moreover, *PR-10a* (OS12g0555300), which functions in the downstream of jasmonic acid pathway and is positively regulated by WRKY TFs in response to drought and high salt stresses (Takeuchi et al. 2016; Takeuchi et al. 2011), and three other PR-10 protein family hub genes (Os12g0555200, Os12g0555500, and Os12g0555000), which annotated as the genes encoding for PR10 family proteins probenazole-inducible protein PBZ1 that were involved in disease resistance, exhibited higher magnitude of the up-regulated expressions at two sampling points of PY6 in contrast to PR403 (Fig. 7A; Table 3). *BSR-d1/ZFP36* (Os03g0437200), encoding C₂H₂-type zinc finger protein, participates in ABA-OsMPK transduction and results in the accumulation of H₂O₂ (Li et al. 2017; Zhang et al. 2014). Similarly, it also has a higher up-regulated expression in PY6 than those in PR403 (Table 3). In addition, the expression of OS02g0121700 and OS02g0570400 was dramatically induced in PY6 in the whole period of drought treatment, whereas they were only significantly induced in point 22 of PR403 and in turn lowered their level to untreated control (Fig. 7A; Table 3). They encode terpenoid synthase domain containing protein and ent-kaurene synthase 1A that involve in gibberellins (GA) biosynthesis, respectively, suggesting the roles of GA played in drought stress response in rice.

OS04g0109100, a gene was annotated as concanavalin A-like lectin/glucanase, has a similar expression pattern in both genotypes.

Totals of 18 and 13 DEHGs were identified in Brown and Royalblue modules, respectively (Fig. 7B, C; Table 4; Table S5). GO analysis revealed that the DEHGs in Brown module play different roles in diverse biological processes, including response to stress, response to endogenous stimulus, protein phosphorylation, protein metabolic process, lipid metabolic process, carbohydrate metabolic process, protein folding, regulation of transcription, and signal transduction (Fig. 7B; Table S6-6). Almost all of which were induced at point 22 and repressed at point 20 by drought in both genotypes. Although they have a similar alteration pattern, the different extent of the down-regulation was observed in several hub genes, for example OS11G0569300, OS01G0971800, OS04G0675400, and OS09G0243200 showed more lower extent of down-regulation at point 20 of PY6, while OS07G0201500 and OS11G0209600 showed more lower extent of down-regulation at point 20 of PR403 (Fig. 7B; Table S5). In module Royalblue, only one GO term was significantly over-represented due to the lesser number of DEHGs. The striking feature of the expression pattern of the hub genes was that their transcripts were dramatically induced by drought at point 22 of PY6 and they were also slightly reduced their levels in next sampling point. Interestingly, Os04g0494100 and Os11g0592200 were annotated as the genes encoding PR3 and PR4 family proteins that have plant chitinase activity. Os03g0663600 and Os12g0630200 are two genes for encoding pathogenesis-related thaumatin-like proteins which is belonged to PR5 family, suggesting that the roles of these genes played in the cross talk between drought and biotic stresses in this study (Fig. 7C; Table 4; Table S6-7).

Discussion

In this study, a CSSL PY6 was adopted to characterize a QTL locus *dss-1* for drought sensitive phenotype and to investigate the impact of *dss-1* on the reprogramming of transcriptional profiles of PY6 in response to drought stress by RNA-seq and WGCNA analysis.

Drought-induced the over-accumulation of H₂O₂ might lead to the sensitive phenotype of *dss-1*

Reactive oxygen species (ROS) (e.g. O₂²⁻, H₂O₂, OH[·], ¹O₂) is unavoidable toxic byproducts of plants in response to abiotic/biotic stresses (Choudhury et al. 2017). The over-accumulation of ROS in cells causes severe oxidative damage to membranes (lipid peroxidation), proteins, RNA, and DNA molecules (Mittler 2002). Higher plants have thus evolved dedicated scavenging pathways to protect themselves from ROS toxicity, including detoxifying enzymes, CAT, SOD, POD, and APX, as well as antioxidant ascorbate-glutathione (GSH) cycle (Mittler 2006; Mori and Schroeder 2004). In this study, the significant over-accumulation of H₂O₂ was detected in PY6 in contrasting to PR403 during drought treatment (Fig. 2A, D). Regarding to the drought sensitive phenotype of PY6, which conferred by the introgression of *dss-1* into the background of PR403, our results suggested that the important relationships were evident

among *dss-1*, H₂O₂ accumulation, and sensitive phenotype. That is, we speculated that the introgression of *dss-1* might directly or indirectly lead to the over-accumulation of H₂O₂ in the rice cells. The over-accumulation of H₂O₂ causes oxidative damages to cell membrane (lipid peroxidation), which was reflected by the measurements of MDA, and others, and further leads to the death of rice plants under severe drought stress condition.

Photosynthesis regulation, a common regulatory process for rice in response to drought stress

Chloroplast is important organelle for the fixation of light energy for the life of higher plants, and any other life in our biosphere (Cai et al. 2019). Thus, photosynthesis is the major biological process occurred in chloroplast. Beyond its normal role in photosynthesis, the increasing evidence suggested that chloroplast is a targeted organelle that involves in environmental stress responses via positively or passively tuning the photosynthetic activity to adapt to the environmental changes (Gan et al. 2019). In GO enrichment analysis, the drought-induced DEGs identified either in PR403 or PY6 were mainly enriched in photosynthesis-related GO terms (Fig. 4; Fig. S4, S5); As well, the analysis of differentially expressed hub genes clustered in the modules Black and Blue showed that the similar set of GO terms that are related to photosynthesis process in chloroplasts were over-represented (Fig. S8A, B). In terms of the expression alteration during drought treatment, in line with previous studies (Ma et al. 2016), the majority of hub genes clustered in modules Black and Blue were consistently declined to a similar extent in both PY6 and PR403 (Fig. S10A, B), implying that the photosynthetic activity of both genotypes was dramatically inhibited in response to drought stress. The inhibition of photosynthesis reduces the utilization of absorbed light energy. The excess light energy results in the generation of toxic ROS (Kojima et al. 2007). Therefore, photosynthetic activity regulation could be a common regulatory process that are shared in diverse varieties for the adaptation of rice in response to environmental stresses. The inhibition of photosynthesis in both genotypes of this study might be correlated to the accumulation of H₂O₂ in rice cells. Regarding to the differential accumulation of H₂O₂ in the leaves of PY6 and PR403, it might result from the effect of the introgression of *dss-1* into the PR403 on the differential expression changes of the hub genes identified in other modules. Furthermore, previous studies considered that the improvement of the photosynthetic capacity was important for drought resistance in plants (Zhang et al. 2016). However, in this study, no significant expression difference was observed in the photosynthesis-related DEGs identified in both PR403 and PY6, suggesting that *dss-1*-underlied the drought sensitive phenotype was not caused by the severe inhibition of photosynthesis, but by the over-accumulation of H₂O₂.

H₂O₂-correlated hub genes differentially regulated in response to drought stress

Transcription factors are key players in the regulatory networks for plant responding to unfavorable stresses. The MYB family is one of the largest transcription factor families in plants with a conserved

MYB binding domain and it has been showed to be essential for plants in response to abiotic stresses (Li et al. 2015), as well as, to be induced by H₂O₂ in soybean (Wang et al. 2017). In this study, five differentially expressed genes that are annotated as transcription factors, including 3 MYB, 1 NAD, and 1 SHR families, were highly clustered in Grey60, a module that was statistically correlated with the accumulation of H₂O₂ during drought stress. Among them, Os01G0702700, Os05G0543600, and Os04G0508500 are MYB family transcription factors. They were exhibited an elevated expression in both genotypes in response to drought stress (Table 2). However, three genes were showed a higher magnitude of up-regulations in the point 22 of PR403. Considering the lower accumulation of H₂O₂ and MDA in PR403, therefore, the MYB family TFs might be induced by drought stress or other signaling messengers and triggered the activation of downstream components of drought responsive signaling pathways, including the up-regulation of stress responsive genes which involve in increasing the content of osmo-protectants and the activities of the POD, CAT, and SOD, and maintain a sub-lethal content of H₂O₂ in plant cells during drought stress, suggesting that the MYB TFs identified here were positively correlated with drought resistance (Fig. 2; Fig. 6C; Table 2). In addition, strikingly, Os01G0842500 and Os03G0273200, both of which were annotated as putative laccase-encoded genes, were consistently exhibited elevated expression in both genotypes in response to drought. However, PY6 had a relative higher upregulated expression in the point 20, this implying the over-accumulation of the transcripts of the genes for laccase might be correlated to the drought sensitive phenotype of PY6. Paradoxically, the overexpression of another laccase-encoded gene *OsChi1* (Os01g0827300) in *Arabidopsis* resulted in an increased tolerance to drought and salinity stresses (Cho et al. 2014). Thus, further study needs to be performed to explorer the function of the laccases identified in the study and reveals the discrepancy appeared here.

Moreover, it has been demonstrated that laccases were regulated by MYB TFs and involved in lignin biosynthesis in secondary wall formation of *Arabidopsis* and *Miscanthus* (He et al. 2019; Zhou et al. 2009); as well, a rice laccase encoding gene *OsChi1* was reported that played an important role in the ROS signaling pathway (Cho et al. 2014), suggesting that, in present study, MYB TFs-regulated laccases with the differential expression alteration participated in the improvement of drought stress tolerance (Fig. 8).

MDA-correlated hub genes differentially regulated in response to drought stress

The majority of DEHGs clustered in the modules that were highly correlated with MDA have been showed to be involved in plant defence signaling pathways. These included WRKY family TFs and PR family proteins (Table 3-4; Table S5). In general, 8 differentially expressed WRKY TFs identified in Red module, in contrast to PR403, were showed a higher degree of induction at point 22 and a lowered level at point 20 in PY6 (Table 3). Similarly, four members of PR10 family encoding genes identified in Red module, and three other families of PR proteins (PR3, PR4, and PR5) identified in Royalblue module exhibited similar alteration pattern in response to drought stress. These suggested that a cross talk existed between

abiotic and biotic stress responsive pathways during drought stress. Recent reports revealed that some common components were shared by the signaling pathways for abiotic and biotic stress responses, including the phytohormones abscisic acid, salicylic acid, and jasmonic acid, the *cis*-acting regulatory elements, and protein kinase cascades (Verma et al. 2016). Plants can rapidly respond to environmental changes by the synergistical or antagonistic regulations of the cross talks among the pathways via their convergent nodes (Verma et al. 2016). *OsWRKY76* (OS09G0417600), one of a hub gene of Red module, encodes a group IIa WRKY TFs in rice. It has been showed that overexpression of *OsWRKY76* in rice plants suppressed a specific set of PR genes and elevated the expression of abiotic stress-associated genes, and thereby, resulted in the increased susceptibility to *M. oryzae*, whereas improved tolerance to chilling stress (Yokotani et al. 2013). Moreover, *OsWRKY13* was suggested to regulate the antagonistic cross talk between drought and disease resistance pathways via the repressions of *SNAC1* and *WRKY45-1* (Xiao et al. 2013). For the group of identified PR encoding genes, OS12G0555000 (*RSOsPR10*), a gene encoding rice root-specific pathogenesis-related protein PBZ1, was induced by drought and salinity stresses, as well as jasmonic acid; however, it was strongly inhibited by salicylic acid (Takeuchi et al. 2016; Takeuchi et al. 2011). Moreover, the expression of *RSOsPR10* was postulated to repress by SA-induced *OSTGAs* or *OsWRKYs* (Takeuchi et al. 2011). Thus, WRKY TFs and PR family proteins encoded genes identified here might act as the convergent nodes and be responsible for the cross talks between abiotic and biotic stresses. In addition, *ZFP36* (OS03G0437200), a gene encoding C2H2 Zinc finger protein (Brs-d1), is a key factor for regulating rice cell redox status and involves in ABA-induced up-regulation of the expressions and the activities of SOD and APX. Overexpression of *ZFP36* enhanced the tolerance to drought and oxidative stresses, but reduced the resistance to rice blast (Li et al. 2017; Zhang et al. 2014). However, a dramatic elevated expression of *ZFP36* was exhibited in drought sensitive genotype PY6, suggesting it might play a synergistical role in the regulating network with WRKY TFs and PRs for rice plants in response to drought stress.

The impact of *dss-1* on the reprogramming of hub genes in response to drought stress

Taken all these together, hence, a working model was proposed here to depict the *dss-1* mediated possible mechanism for rice plants in response to drought stress at transcriptional level (Fig. 8). When rice exposed to severe drought stress, stress-induced the dehydration of rice plants and the stomatal closure of leaf guard cells result in the inhibition of photosynthesis activity and enhancing the ABA biosynthesis. The inhibition of photosynthesis reduces the utilization of absorbed light energy and results in the generation of toxic ROS, including H₂O₂. Overaccumulation of ROS can oxidative stress damage to the plant cells and lead to the drought sensitive phenotype of rice. Drought stress signal, the accumulated ABA, and H₂O₂ signal are integrated and perceived by rice cells and triggered the activation of responsive signaling pathways. First, the expression of series of TFs, including MYBs, WRKYs, ZFP36, NAC, etc., were differentially reprogrammed and induced the cross talk between drought and biotic stresses. Then, the induction of TFs regulates the expression of downstream responsive genes, including a number of PR family proteins, laccases, and among others, to mobilize multi facet of the resources in vivo to respond to

drought stress, and eventually lowers the content of ROS in plant cells and ameliorates the drought-induced sensitive phenotype of rice. The introgressed *dss-1* might exert its effect on the hub genes which were identified in our study to differentially reprogram of their expressions, and therefore leading to the differential accumulation of ROS in drought resistance and sensitive varieties. The differential activation of ROS-related responsive pathways that would diverge the drought resistance/sensitive phenotypes of rice varieties.

Conclusion

Taken together, our results suggested that chloroplast is the targeted organelle for PR403 and PY6 in response to drought stress regulation. Functional annotation analysis of the hub genes in the modules that were highly correlated with H₂O₂ and MDA accumulations during drought treatment provided us a global picture for the impact of *dss-1* on the reprogramming of regulatory mechanism (s) at transcriptional level. Specifically, the differential expression patterns of *MYBs*, *WRKYs*, PR protein, and *ZFP36*, etc., showed in both genotypes suggested their roles in the variation of ROS and MDA accumulations, and thereby resulting the sensitive phenotype of rice in response to drought stress.

Materials And Methods

Plant materials, growth, and treatment conditions

The growth of rice seedlings were performed according to previously described methods (Cen et al. 2018) with minor modification. In brief, rice plants were grown in the equal amount of paddy soil. As well, equal amount of water was watered routinely. The drought treatment was performed according to the methods as described (Zong et al. 2016). Soil moisture was monitored regularly to determine the stages of drought stress using EM-WSYP soil moisture detector (Hengmei Company, Shandong, China). The leaves of 3-week old soil-cultured rice seedlings (three biological replicates) in the greenhouse (day35 °C/night30 °C) were collected at four soil moisture points, 40 (soil moisture percentage 40%, represent well-watered condition), 22 (soil moisture percentage 22%, drought treatment 96h from 40% soil moisture), 20 (soil moisture percentage 20%, drought treatment 120h), and 15 (soil moisture percentage 15%, drought treatment 168h) (Fig. S1) and snap frozen in liquid-nitrogen for RNA-seq and physiological index measurement, respectively. The samples harvested at point 15 were only for physiological measurement analysis. Drought stress treatment for hydroponically cultured seedlings in 96-well plates was performed in rice nutrient solution supplemented with 20% (w/v) PEG 6000. Survival rate was determined after 5-day treatment and re-watered for 3 days, respectively.

RNA-seq library construction and transcriptomic data processing

The library of transcriptome was generated by NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's instruction. After digestion, purification, and PCR amplification,

cDNA fragment (250~300bp) was purified by AMPure XP beads. The cDNA sequencing was performed on an Illumina 2500 platform, and paired-end reads were generated. After removed low quality reads from data, the clean reads were mapping to the reference genome (the rice annotation project database, <https://rapdb.dna.affrc.go.jp/index.html>). DEGs between each treated point and untreated control were detected using DEseq with $|\log_2FC| \geq 1$ (FC, the abbreviation of fold change) and $padj \leq 0.05$ as thresholds to identify drought- induced DEGs, respectively. As well, the development-dependent DEGs were detected in the same sampling points of well-watered samples with the same criterion. As the development-dependent DEGs could be caused by the development of rice plants, they were excluded from the final dataset.

The Go enrichment analysis were performed on PlantRegMap (<http://plantregmap.cbi.pku.edu.cn/>). The over-represented GOs were visualized using ggplot2 and GOplot R package (Walter et al. 2015). Heatmap was generated by MEV v4.9 (Saeed et al. 2003).

Co-expression network analysis

The WGCNA R-package was used to construct co-expression network and analyze the correlation of modules and physiological data according to the method as described (Langfelder and Horvath 2008). Raw transcriptomic datasets of all samples were filtered to remove any genes with an FPKM <1 even in one replicate of either sampling point of the samples. Clean data was used to generate co-expression network. First, the soft threshold power was estimated by the pickSoftThreshold function, which provides the appropriate soft-thresholding power for network construction. Second, a correlation matrix was constructed based on obtained soft threshold power, and in turn calculated topological overlap matrix (TOM) from the transformed correlation matrix. Finally, the genes were grouped based on the topological overlap dissimilarity (1-TOM) by average hierarchical clustering using hclust function. Gene modules were then identified using a dynamic tree cut algorithm (minimum cluster size 30, merging threshold function 0.25). Module membership (MM) was calculated based on Pearson correlation between the expression level and the module eigengenes to identify hub genes within the modules. Genes with higher MM indicate the genes with higher connectivity within the module.

To correlate the physiological data with the network, module eigengenes were correlated with each physiological data, respectively. Gene significance (GS) was used to correlate physiological data with the expression data of individual genes.

Genome-wide association study analysis

A total of 84 varieties are selected from 3024 diverse accessions of 3K project (Wang et al. 2018). The SNPs information was obtained from public database (IRRI, <http://irri.org/>). GWAS analysis for survival rate were using Efficient Mixed-Model Association eXpedited (EMMAX) software package.

Analysis of the movement of the stomatal aperture

Four-week seedlings were selected for scanning electron microscopy (SEM) analysis. The middle part of penultimate leaves was cut into 0.5-cm pieces in length and snap frozen with liquid nitrogen to maintain the stomatal morphology. FEI QRATRO S scanning electron microscope (Thermo Fisher Scientific, USA) with a freezing stage was used to examine the leaf stomatal apertures. The opening status and density of stomata were analyzed with Image J software. Four random fields were selected to count the number of stomata in each replicate.

Quantitative real time reverse transcription PCR analysis

Total RNA was isolated from leaves samples (three biological replicates per sample). cDNA synthesis was performed by reverse transcription (RT) with the Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Cat# K1622) according to the manufacturer's protocol. The sequences of genes were downloaded from RAP-DB (<https://rapdb.dna.affrc.go.jp/index.html>). The primers for related genes were obtained from qPrimerDB (<https://biodb.swu.edu.cn/qprimerdb/>) (Table S1) (Lu et al. 2018). qPCR was performed on a Roche Lightcycler 480 Real-Time PCR System in 10 µL reactions with the SYBR Green PCR Master Mix kit (BIORAD, USA), following the manufacture's protocol. The relative expression of each gene was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The *actin* gene (Os11g0163100) was used as internal reference for analysis.

Determination of relative water content of leaves

The leaf samples were collected at each sampling point and weighted to determine the fresh weight (FW). Then oven-dried to a constant dry weight (DW). The relative water content was calculated according to $RWC (\%) = [(FW - DW) / FW] \times 100\%$.

Examination of ABA, ROS and its scavenging enzyme activity

A total of 0.15-g fresh leaf samples of PR403 and PY6 were collected and ground in liquid nitrogen into fine powder, and then resuspended in 1.35 ml PBS buffer (10 mM, pH 7.2). After centrifugation at 5000 rpm for 5 min, the supernatant was collected for subsequent assay. The contents and activity of H₂O₂, SOA, ABA, SOD, CAT, POD, and APX were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Mskbio Company, Wuhan, China) following the manufacturer's instruction.

3,3'-Diaminobenzidine (DAB) staining was performed to detect the accumulation of H₂O₂ in leaves. The leaves of 3-week old seedlings were collected for staining. Samples were vacuum-infiltrated for 10 min, then incubated in DAB solution (1 mg/mL in water, pH 5.8) for 12h in the dark. For the detection of superoxide, leaf pieces were vacuum-infiltrated as described above in 0.1% nitroblue tetrazolium (NBT) with 0.1% Triton X-100 in 10 mM of phosphate buffer (pH 7.2) for 10 min. Then the samples were boiled in absolute ethanol to remove the chlorophyll (Cen et al. 2018).

Declarations

Compliance and ethics The author(s) declare that they have no conflict of interest.

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Author contributions

JL and RL conceived and designed the experiments. BY, JBL, DW, YL, and WC performed all the lab experiments. BY, JBL, and DW conducted the field trials and collected samples for RNA-Seq. JL, BY, and WC performed the data processing. JL drafted the manuscript. JL, RL, BY, and SW revised the manuscript. All authors approved the final version of the manuscript.

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Availability of data and material

All data supporting the conclusion of this paper can be obtained from the corresponding authors under reasonable requirements.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Supporting Information

Fig. S1 Diagram showing the sampling points and the corresponding phenotypes of the plant materials.

Fig. S2 Determination of APX activity, endogenous ABA level, and stomatal status of PR403 and PY6 under drought treatment. **a** APX activity. **b** NBT staining of leaf samples. **c** Endogenous ABA content. **d** Leaf stomatal opening status during drought treatment. Top panel shows three levels: completely open, partially open and completely closed the aperture of stomata. Bottom panel shows the percentages of three levels of stomatal opening in PY6 and PR403 ($n = 100$ stomata for PR403 and PY6, respectively). Data in **a** and **b** are presented as mean $\pm$ SD ($n = 3$, $*P < 0.05$, $**P < 0.01$, Student's *t* test. Scale bars = 0.5 cm in **b** and = 5 μ m in **d**.

Fig. S3 Validation of the RNA-seq data by qRT-PCR. The *ACTIN* gene (Os11g0163100) was used as an endogenous reference for qPCR. Os01g0164600 (**a**), Os01g0289600 (**b**), Os02g0115700 (**c**), Os03g0319400 (**d**), and Os04g0610400(**e**) were selected for qPCR. The primer sequences were shown in **Table S1**.

Fig. S4 Cellular component GO terms significantly over-represented in DEGs GO enrichment analysis. **a** The DEGs-enriched GO terms at sampling point 22 of PR403. **b** The DEGs-enriched GO terms at sampling point 22 of PY6. **c** The DEGs-enriched GO terms at sampling point 20 of PR403. **d** The DEGs-enriched GO terms at sampling point 20 of PY6.

Fig. S5 Molecular function GO terms significantly over-represented in DEGs GO enrichment analysis. **a** The DEGs-enriched GO terms at sampling point 22 of PR403. **b** The DEGs-enriched GO terms at sampling point 22 of PY6. **c** The DEGs-enriched GO terms at sampling point 20 of PR403. **d** The DEGs-enriched GO terms at sampling point 20 of PY6.

Fig. S6 Soft threshold power estimation in WGCNA analysis

Fig. S7 Scatter plot of module eigengenes in the modules significantly correlated with H₂O₂ and MDA. **a** H₂O₂ accumulation correlated Black, Blue, Green, and Grey60 modules. **b** MDA accumulation correlated Red, Brown, and Royalblue modules.

Fig. S8 GO terms over-represented in the GO enrichment analysis of hub genes in the modules that were correlated with H₂O₂ accumulation. The GO terms of biological processes, cellular components, and molecular functions were over-represented in **a** Black, **b** Blue, **c** Grey60, and **d** Green modules, respectively.

Fig. S9 GO terms over-represented in the GO enrichment analysis of hub genes in the modules that were correlated with MDA accumulation. The GO terms of biological processes, cellular components, and molecular functions were over-represented in **a** Red, **b** Brown, **c** Royalblue modules, respectively.

Fig. S10 Heatmap showing the expression patterns of the hub genes in the modules were correlated with H₂O₂ and MDA accumulation. **a** Black module. **b** Blue module. **c** Grey60 module. **d** Green module. **e** Red. **f** Brown module. All the data used in the analysis was subjected to Log2 transformation.

Table S1 Primer sequences of genes using for qRT-PCR

Table S2 Candidate genes for *dss-1* in GWAS analysis

Table S3 Differentially expressed genes (DEGs) identified in RNA-seq

Table S4 Hub gene lists of the modules correlated with H₂O₂ and MDA accumulation

Table S5 Differentially expressed hub genes identified in Brown module

Table S6 GO enrichment analysis of DEGs and DEHGs identified in the study.

Tables

Table 1 The DEGs detected in PR403 and PY6 under drought stress treatment

Genotypes	Category of DEG	Sampling point	
		22	20
PR403	Up-regulated	1135	2383
	Down-regulated	1917	3438
	Total	3052	5821
PY6	Up-regulated	1748	2655
	Down-regulated	1397	2874
	Total	3145	5529
Common	Up-regulated	459	1202
	Down-regulated	683	1982
	Opposite-regulated	477	532
	Total	1619	3716

Table 2 Differentially expressed hub genes identified in Grey 60 module

Gene_id	PR403_22 ^a	PR403_20 ^b	PY6_22	PY6_20	Description	GO enrichment
Os01G0842500	4.69 ^c	4.40	5.85	8.49	Similar to Laccase (EC 1.10.3.2).	GO:0005507, GO:0009809, GO:0016722, GO:0046274, GO:0048046, GO:0052716, GO:0055114
Os03G0273200	4.65	3.70	6.79	9.00	Similar to Laccase (EC 1.10.3.2).	GO:0005507, GO:0009809, GO:0016722, GO:0046274, GO:0048046, GO:0052716, GO:0055114
Os01G0702700	2.20	0.81	0.41	1.16	Similar to Transcription factor MYB86 (Myb-related protein 86) (AtMYB86) (Myb homolog 4) (AtMyb4).	GO:0003677
Os05G0543600	2.56	1.51	0.79	2.28	Similar to Myb-related protein.	GO:0003677
Os04G0508500	3.65	3.34	0.50	1.18	MYB family transcription factor, putative, expressed.	GO:0003677
Os06G0131700	2.38	1.80	0.98	2.16	Similar to NAM protein.	GO:0003677, GO:0005634, GO:0006355
Os08G0189200	7.90	7.40	3.48	6.64	Germin-like protein 8-3, Disease resistance	GO:0005618, GO:0030145, GO:0033609, GO:0045735, GO:0046564, GO:0048046
Os03G0433200	3.04	2.62	1.94	2.63	SHR transcription factor, Regulation of the number of cortex cell layers in the root, Coordination of stomatal patterning	GO:0003700, GO:0005634, GO:0006355, GO:0008356, GO:0009956, GO:0043565, GO:0045930, GO:0048366, GO:0055072

Notes: ^a represents the samples collected at the 22% of soil moisture content; ^b represents the samples collected at the 20% of soil moisture content; ^c the value represents the Log2 transformation of the fold change of the expression.

Table 3 Differentially expressed hub genes identified in Red module

Gene_id	PR403	PR403	PY6	PY6	Description	GO enrichment
	<u>22^a</u>	<u>20^b</u>	<u>22</u>	<u>20</u>		
OS01G0730700	1.71 ^c	-0.16	3.00	0.15	WRKY transcription factor 14 (WRKY14).	GO:0003700, GO:0006355, GO:0043565
OS05G0474800	3.36	0.38	4.46	0.36	WRKY transcription factor 70.	GO:0003700, GO:0006355, GO:0043565
OS09G0417600	2.76	-1.65	3.82	0.17	WRKY transcription factor 76.	GO:0003700, GO:0006355, GO:0009617, GO:0009620, GO:0009751, GO:0010200, GO:0043565
OS08G0386200	1.95	-0.28	3.46	0.99	WRKY transcription factor 69.	GO:0003700, GO:0006355, GO:0043565
OS01G0185900	0.19	-3.15	2.43	-2.67	Similar to WRKY 1 (Fragment).	GO:0003700, GO:0006355, GO:0043565
OS05G0322900	1.25	-1.98	3.04	-0.17	Similar to WRKY transcription factor 45.	GO:0003700, GO:0009862, GO:0009864, GO:0010200, GO:0043565, GO:0045892, GO:0050832, GO:1900056
OS05G0571200	3.60	-0.37	5.70	2.53	Similar to WRKY transcription factor 19.	GO:0003700, GO:0006355, GO:0043565
OS11G0117600	3.59	-0.18	5.29	3.60	Similar to WRKY transcription factor 50 (Fragment).	GO:0003700, GO:0006355, GO:0043565
OS03G0437200	1.00	-1.14	5.03	0.67	C2H2-type zinc finger protein, Absciscic acid-induced antioxidant defence, Water stress and oxidative stress tolerance	GO:0003700, GO:0005634, GO:0006355, GO:0043565, GO:0044212, GO:0046872
OS10G0377300	5.42	0.66	3.21	-4.34	Similar to Homeobox-leucine zipper protein HOX8.	GO:0003700, GO:0005634, GO:0006355, GO:0043565
OS12G0555200	3.14	1.10	6.30	3.50	Similar to Probenazole-inducible protein PBZ1.	GO:0004864, GO:0004872, GO:0005634, GO:0005737, GO:0006952, GO:0009607, GO:0009738, GO:0010427, GO:0043086, GO:0080163
OS12G0555500	2.03	0.42	4.62	2.85	Pathogen resistance protein PBZ1, C 17 kDa RNase, Disease resistance	GO:0004864, GO:0004872, GO:0005634, GO:0005737, GO:0006952, GO:0009607, GO:0009738, GO:0010427, GO:0043086, GO:0080163
OS12G0555000	3.75	0.52	6.81	4.27	Similar to Probenazole-inducible protein PBZ1.	GO:0004864, GO:0004872, GO:0005634, GO:0005737, GO:0009738, GO:0010427, GO:0043086, GO:0080163
OS12G0555300	3.35	0.35	5.23	2.70	Similar to Pathogenesis-related protein PR-10a.	GO:0006952, GO:0009607
OS02G0282000	3.52	1.30	4.64	2.65	NB-ARC domain containing protein.	GO:0043531
OS01G0134700	2.02	-0.96	3.78	0.29	Calmodulin binding protein-like family protein.	GO:0002229, GO:0003700, GO:0005516, GO:0005634, GO:0006355, GO:0010112, GO:0010224, GO:0043565, GO:0071219, GO:0080142, GO:1900426
OS01G0508100	2.48	-1.35	4.13	0.89	Ferritin/ribonucleotide reductase-like family protein.	GO:0010112

OS02G0121700	3.68	-0.23	4.63	2.82	Terpenoid synthase domain containing protein.	GO:0000287, GO:0009507, GO:0010333, GO:0016114, GO:0034007, GO:0042742, GO:0043693
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Table 3 Differentially expressed hub genes identified in Red module (continued)

Gene_id	PR403	PR403	PY6	PY6	Description	GO enrichment
	<u>22^a</u>	<u>20^b</u>	<u>22</u>	<u>20</u>		
OS02G0570400	3.55 ^c	-0.11	5.06	2.08	Similar to Ent-kaurene synthase 1A.	GO:0000287, GO:0006952, GO:0008152, GO:0010333, GO:0034277
OS03G0103300	3.42	-1.76	4.33	1.68	Hybrid proline- or glycine-rich protein, Control of low-temperature germinability, Pre-harvest sprouting resistance	GO:0005783, GO:0009506, GO:0009707, GO:0050832
OS04G0226600	1.63	-1.76	3.56	-0.63	Similar to OSIGBa0135L04.3 protein.	GO:0004674, GO:0005524, GO:0005886, GO:0006468, GO:0006952, GO:0009506
OS05G0368000	4.03	-1.95	5.31	0.50	Non-protein coding transcript.	GO:0010112
OS07G0537900	2.97	-0.27	3.49	0.77	Similar to SRK3 gene.	GO:0004674, GO:0005524, GO:0005886, GO:0006468, GO:0006952, GO:0009506, GO:0016021
OS06G0210400	1.63	-2.81	1.79	-1.61	Concanavalin A-like lectin/glucanase, subgroup domain containing protein.	GO:0004674, GO:0005524, GO:0006468, GO:0016021, GO:0030246
OS04G0109100	5.74	-1.52	8.78	2.92	Concanavalin A-like lectin/glucanase domain containing protein.	GO:0004674, GO:0005524, GO:0006468, GO:0030246
OS04G0370900	3.91	-1.01	5.03	0.77	Similar to H0607F01.5 protein.	GO:0004672, GO:0005509, GO:0005515, GO:0005524, GO:0006468, GO:0016021, GO:0030247
OS04G0371000	4.22	-1.80	4.98	0.05	Four-helical cytokine family protein.	GO:0030247
OS08G0514000	4.02	0.95	6.61	1.49	Protein kinase, catalytic domain containing protein.	GO:0004672, GO:0005524, GO:0006468, GO:0016021, GO:0030246
OS03G0802500	1.90	-1.45	3.13	0.42	ATPase, AAA-type, C core domain containing protein.	GO:0005524, GO:0016021

Notes: ^a represents the samples collected at the 22% of soil moisture content; ^b represents the samples collected at the 20% of soil moisture content; ^c the value represents the Log2 transformation of the fold change of the expression.

Table 4 Differentially expressed hub genes identified in Royalblue module

Gene_id	PR403	PR403	PY6	PY6	Description	GO enrichment
	<u>22^a</u>	<u>20^b</u>	<u>22</u>	<u>20</u>		
Os01g0298400	1.91 ^c	-0.31	3.15	0.57	Myb transcription factor domain containing protein.	GO:0003677
Os01g0392600	-0.81	-2.14	2.14	-2.02	Conserved hypothetical protein.	
Os03g0663600	1.87	0.68	4.31	2.03	Similar to Pathogenesis-related thaumatin-like protein.	
Os12g0630200	0.44	-2.47	2.76	-0.64	Thaumatococcus pathogenesis-related family protein.	
Os11g0592200	0.33	-2.22	2.14	-1.30	Similar to Chitin-binding allergen Bra r 2 (Fragments).	GO:0042742, GO:0050832
Os04g0494100	1.63	0.65	4.83	1.19	Similar to Chitinase.	GO:0000272, GO:0004568, GO:0005618, GO:0006032, GO:0006952, GO:0008061, GO:0009611, GO:0009617, GO:0010262, GO:0016998
Os06g0487620	1.97	1.70	2.31	0.98	Conserved hypothetical protein.	
Os06g0493100	5.99	0.26	5.93	1.68	Conserved hypothetical protein.	GO:0016021
Os06g0586300	-0.01	1.13	5.08	3.48	Hypothetical protein.	
Os06g0588450	-1.00	-1.82	4.64	2.74	Hypothetical conserved gene.	
Os06g0591200	4.28	-2.17	8.51	4.08	Conserved hypothetical protein.	

Notes: ^a represents the samples collected at the 22% of soil moisture content; ^b represents the samples collected at the 20% of soil moisture content; ^c the value represents the Log2 transformation of the fold change of the expression.

Figures

Figure 1

Characterization of a drought sensitive CSSL. a Flowchart of the CSSL construction and drought sensitive CSSL identification. b Drought stress phenotype of PR403, LAMBAYEQUE1, and PY6. c Survival rate of PR403, LAMBAYEQUE1, and PY6 after the drought stress treatment. Data are presented as mean $\pm$ SD (n = 3, *P<0.05, **P<0.01, Student's t test). d Relative water content of leaves of PR403 and PY6 under drought stress treatment. (n = 3, *P<0.05, **P<0.01, Student's t test). e Fine mapping and GWAS analysis of QTL dss-1 underlying drought sensitive phenotype of PY6. Scale bar = 5 cm in b.

Figure 2

Comparison of ROS production and elimination in PY6 and PR403 under drought stress treatment. a H_2O_2 content. b CAT activity. c POD activity. d DAB staining of leaf samples of PY6 and PR403. e SOA content. f SOD activity. g The content of MDA. Data are presented as mean $\pm$ SD ($n = 3$, * $P < 0.05$, ** $P < 0.01$, Student's t test). Scale bar = 0.5 cm in d.

Figure 3

Global view of gene expression profiling under drought treatment. a Principal component analysis (PCA) of the RNA-Seq data. b-c Venn diagrams showing the number of DEGs identified at two sampling points of PR403 and PY6. $|\log_2FC| \geq 1$, $\text{padj} < 0.05$ was used as threshold for DEGs identification.

Figure 4

Top 20 of biological process GO terms significantly over-represented in DEGs GO enrichment analysis. a The DEGs-enriched GO terms at sampling point 22 of PR403. b The DEGs-enriched GO terms at sampling point 22 of PY6. c The DEGs-enriched GO terms at sampling point 20 of PR403. d The DEGs-enriched GO terms at sampling point 20 of PY6.

Figure 5

WGCNA co-expression network and module-trait correlation analysis. a A hierarchical cluster tree showing co-expression modules identified via the Dynamic Tree Cut method. Each leaf in the tree represents one gene. The major tree branches constitute 26 modules and are labeled with different colors. b The correlation of physiological indicators with WGCNA modules. Each row corresponds to a module and labeled with a color as in a; The columns correspond to physiological indicators. The color of each cell indicates the correlation coefficient between the module and physiological indicator (the top number in the cell represent correlation coefficient and bottom one in the parentheses represent P value). c Correlation of H2O2 with WGCNA modules. Color bars with the numbers in X-axis correspond to the module colors and the numbers showed in b. d Correlation of H2O2 with WGCNA modules. Color bars with the numbers in X-axis correspond to the module colors and the numbers showed in b.

Figure 6

H₂O₂

MDA

Figure 8

The possible role of dss-1 in reprogramming the transcriptional profiles of rice in response to drought stress.

Supplementary Files

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