Physiological and Proteomic Analysis Revealed the Response Mechanisms of two Different Grought-resistant Maize Varieties

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Abstract

Background: Drought stress seriously limits the seedling growth and yield of maize. Despite previous studies on drought resistance mechanisms by which maize cope with water deficient, the link between physiological and molecular variations are largely unknown. To reveal the complex regulatory mechanisms, comparative physiology and proteomic analyses were conducted to investigate the stress responses of two maize cultivars with contrasting tolerance to drought stress.

Results: Physiological results showed that SD609 (drought-tolerant) maintains higher photochemical efficiency by enhancing CEF (cyclic electron flow) protective mechanism and antioxidative enzymes activities. Proteomics analysis revealed a total of 198 and 102 proteins were differentially expressed in SD609 and SD902, respectively. Further enrichment analysis indicated that drought-tolerant 'SD609' increased the expression of proteins related to photosynthesis, antioxidants/detoxifying enzymes, molecular chaperones and metabolic enzymes. The up-regulation proteins related to PSII repair and photoprotection mechanisms resulted in more efficient photochemical capacity in tolerant variety under moderate drought. However, the drought-sensitive 'SD902' only induced molecular chaperones and sucrose synthesis pathways, and failed to protect the impaired photosystem. Further analysis indicated that proteins related to the electron transport chain, redox homeostasis and heat shock proteins (HSPs) could be important in protecting plants from drought stress.

Conclusions: Our experiments explored the mechanism of drought tolerance, and obtained detailed information about the interconnection of physiological research and protein research. In summary, our findings could provide new clues into further understanding of drought tolerance mechanisms in maize.

Background

Maize, an important cereal crop for food, feed and fuel, always subjected to different abiotic stresses during their life cycle [1]. Water resources shortage has become a major challenge for agricultural production and social development in the arid region of northwest China [2]. As the main grain crop in this region, significant portions of the maize suffer from drought-induced yield losses. Thus, greater yield stability through improved drought tolerance is commonly listed as an objective of the breeders [3]. Plant breeders have used a wide range of technologies to successfully breed varieties that perform well under drought stress conditions. Therefore, understanding the drought tolerance mechanisms of drought-tolerant maize varieties is essential for genetic manipulation and/or cross breeding in maize.

The responses of plants to drought stress are highly complex, especially chloroplast metabolism. Photosynthesis, the most fundamental process, is also severely affected by drought stress [4]. Under water stress, photosynthesis activity is disturbed due to chlorophyll degradation, stomatal closure, reduced activity of enzymes (such as Rubisco) and photochemical efficiency of Photosystem II (PSII) [5]. Down regulation of PSII activity will result in an imbalance between the light absorption and utilization [6]. Excess light energy generates active oxygen species (O_2^- , 1O_2 , H_2O_2 , OH), which are potentially

detrimental and can inhibit the repair of PSII [7]. Tolerant genotypes have a highly active system to against ROS, such as enzymatic [SOD, POD, CAT, GR] and non-enzymatic (carotenoids and anthocyanins) [6]. During the past years, major research efforts have focused on the various of physiological, biochemical, basic molecules and transcriptome analysis [8–12]. Nevertheless, researches on drought tolerance at transcriptome level cannot provide us a fully understanding of regulation mechanism. Furthermore, transcript abundance usually are not in accordance with the protein abundance and physiological performance [13]. Consequently, we still lack comprehensive information on the interacting processes of maize leaves under drought stress.

Recently, proteomics is used as a particularly powerful tool that can provide us an overview of cellular and molecular changes under drought stress. For differential proteome analysis, techniques such as two-dimensional electrophoresis (2-DE) and two-dimensional differential gel electrophoresis (2D-DIGE) have been widely applied to analyze wheat [14] and rice [15] under stress conditions. However, a number of inherent limitations of 2D-gel-based approaches including the low identification rate of proteins, low reproducibility and the difficulty in separating low-molecular-weight proteins [16, 17]. To better understand these changes, isobaric tags for relative and absolute quantification (iTRAQ) can identify more numerous proteins and provides more reliable quantitative information than 2-DE analysis [18]. To date, proteomic studies on drought stress were mainly focused on maize inbred lines [19–21]. The combination of iTRAQ-based quantitative proteomics and detailed physiological analysis to study the response mechanism under drought stress remains incipient.

In this work, we took two hybrids maize cultivars, drought-tolerant (Shaandan 609) and drought-sensitive (Shaandan 902), as materials and systematically compared their differences in physiological level under moderate drought by measuring leaf chlorophyll content, photosynthetic energy efficiency and antioxidant enzyme activities. Subsequently, the protein expression levels between the two maize varieties were systematically compared with iTRAQ tandem mass spectrometry technology and further experimentally verified by qRT-PCR. Our intentions were: (i) evaluate and compare the drought response mechanisms of tolerant and sensitive maize cultivars through physiological and proteomic approaches. (ii) made precise correlations between physiological observations and proteomic patterns and constituted a link between molecular and physiology under drought stress. These results will help us to better understand the drought tolerance mechanisms and will be useful for future crop improvement in maize.

Results

Changes of phenotype and photosynthesis parameters of two varieties under moderate drought

Under moderate drought stress, the net photosynthetic rate (P_N) and stomatal conductance (g_s) were significantly decreased compared to control plants, while the intercellular CO_2 concentration (C_i) were increased. As shown in (Fig. 1b, c, d), the changes of each parameters in SD902 were higher than SD609.

Compared with control, the intercellular CO₂ concentration in SD609 and SD902 increased by 16.3% and 20.9%, respectively.

Drought stress significantly induced different phenotypic responses between the two varieties. Under moderate drought stress, the leaf tips of SD902 curled more severely than SD609, and the seedlings of SD609 appeared greener than those of SD902 (Fig. 2a). Moreover, the chlorophyll content (SPAD) in SD609 was higher than 902 in both control and water deficit conditions (Fig. 1a), which was consistent with the visual observation.

Changes of photosynthetic characteristics and protective enzymes of two varieties under moderate drought

Drought stress also significantly changed the photosynthetic efficiency, which can be reflected by energy conversion in PSII and PSI. As shown in Fig. 3a, b, the value of Y(II) and Y(I) under drought stress were lower than under control conditions in two varieties, especially in SD902. The decline of Y(II) was accompanied by the increase of Y(NPQ) and Y(NO) in both varieties, of which Y(NO) increased significantly in SD902. Similarly, the increase in Y(I) was accompanied by the changes in Y(NA) and Y(ND). The changes in Y(NA) and Y(ND) were higher in SD902 than that in SD609 (Fig. 3a, b). The values of Y(CEF) of two varieties under moderate drought stress was examined by estimation of ETRI and ETRII (Fig. 3c, d). When exposed to moderate drought stress, values for ETRI and ETRII significantly decreased compared to control conditions. There is a clear difference in Y(CEF) between the two varieties Fig. 3e. Compared with control, Y(CEF) was increased in SD609 under drought stress whereas decreased in SD902.

The ability of the antioxidant enzymes, including SOD, POD and GR in two varieties leaves were influenced by moderate drought (Fig. 2b, c, d, e)). Nevertheless, the degree of elevation of these antioxidant enzymes was relatively higher in SD609 than in SD902. We found that the activities of SOD, POD and GR in SD609 increased by 26.4%, 20% and 40.1%, respectively. However, the activities of SOD, POD and GR in SD902 increased by 8.1%, 15.6% and 13.9%, respectively.

Comprehensive proteome analysis of SD609 and SD902

To compare the differences in protein expression levels between the control- and drought-treated maize seedlings of each genotype, we used the iTRAQ approach and performed quantitative analysis on SD609 and SD902. The present study identified 5488 proteins with a 1% FDR from 15079 distinct peptides derived from 126307 spectra. A search for proteins with fold-changes >1.5 or <0.67 ($P < 0.05$) resulted in the identification of 2559 proteins between the control and drought stress in SD609 and SD902. We further screened the specific proteins between two varieties based on their fold-changes in expression level. As shown in Fig. 4a, a total of 198 (108 increased and 90 decreased) and 102 (66 increased and 36 decreased) proteins exhibited significant changes ($P < 0.05$) in SD609 and SD902, respectively. Only 39

proteins overlapped between the two varieties, of which 26 were up-regulated and 13 down-regulated. Moreover, 159 drought responsive proteins were unique in drought-tolerant variety SD609, while 63 specific protein in drought-sensitive SD902 (Additional Table 1).

Next, the WoLFPSORT database was used to predict subcellular localization (Fig. 4b, c). Of these DEPs, the subcellular localization showed most proteins were located in chloroplasts in SD609 (Fig. 4b) and SD902 (Fig. 4c), indicating that moderate drought stress mobilized many chloroplast proteins in SD609 and SD902. We further performed gene ontology (GO) and KEGG analysis to annotate their functions (Fig. 5). Among them, Fig. 5a and Fig. 5b respectively represented the GO terms of specific DEPs in SD609 and SD902 induced by drought stress. The GO terms of shared DEPs in both two varieties were shown in Fig. 5c. The GO data revealed that the specific in SD609 (Fig. 5a) were highly enriched in photosynthesis, whereas the specific DEPs in SD902 (Fig. 5b) were mainly included response to stress. The biological process of shared DEPs in SD609 and SD902 involved photosynthesis and protein-chromophore linkage (Fig. 5c). KEGG pathway analysis was used to identify the metabolic pathways of the differentially expressed proteins in SD609 and SD902 (Fig. 5d, e). The upregulated DEPs in SD609 (Fig. 5d) under drought stress were frequently associated with photosynthesis, while downregulated DEPs were associated with photosynthesis-antenna protein and pyruvate metabolism etc. The KEGG pathways protein processing endoplasmic reticulum and photosynthesis-antenna proteins were more frequent in SD902 enriched proteins, whereas porphyrin and chlorophyll metabolism were more frequent in suppressed DEPs (Fig. 5e). Collectively, GO and KEGG enrichment results provided an overview showing that moderate drought leads to the accumulation and suppression of distinct sets of proteins in SD609 and SD902.

Photosynthesis-related DEPs observed in tolerant variety SD609

Photosynthesis proteins in tolerant variety SD609

Photosynthesis is the main physiological process of plants and responds quickly to stress. A total of 36 DEPs in SD609 (10 up-regulated and 24 down-regulated) were related to electron transport (Additional Table 1). Among these proteins, five proteins associated with PSII were increased under drought stress, including PSII repair protein PSB27-H1, Oxygen-evolving enhancer (OEE) protein 1-1, OEE1, OEE2-1 and PSII 11kD protein. The protein level of 14 light-harvesting Chl a/b binding protein complexes (LHCs) (such as LHCII, LHCP) were decreased by 0.22- to 0.55-fold. The protein Plastoquinol-plastocyanin reductase and plastocyanin in Cytochrome b6/f complex were also up-accumulated by 1.7-fold relative to control. Nine proteins (4 up-regulated and 5 down-regulated) annotated in PSI were also changed under drought stress, of which the Ferredoxin 2 (FDX2), FDX5, two PSI reaction center subunit IV A proteins were increased, other proteins such as PSI subunit O, PSI-G, PSI-K as well as PSI-B were decreased. Moreover, four chlorophyll biosynthesis proteins were down-regulated induced by drought. In the dark (Calvin cycle)

reactions of photosynthesis, nine phosphoenolpyruvate carboxylase (PEPC) proteins were decreased by 0.5- to 0.66-fold. The expression profiles of the photosynthesis proteins suggest that the system structure and the ability of electron transport is enhanced in the drought-tolerant seedling leaves but reduced in the drought-sensitive seedling leaves under the same drought stress.

Reactive Oxygen Species (ROS) scavenging proteins in tolerant variety SD609

Plants have evolved the antioxidant defense system including enzymatic and non-enzymatic mechanisms to scavenge the increase of ROS under water deficit conditions. In our study, 16 proteins in ROS scavenging were observed in SD609 (Additional Table 1). The expression of SOD in SD609 was up-regulated by 2.4-fold, and five peroxidase proteins was enhanced under drought stress. Moderate drought stress also activated proteins associated with the ascorbate-glutathione (AsA-GSH) cycle and thioredoxin-peroxiredoxin (Trx-Prx) pathway, such as glutathione reductase (GR), together with 2-Cys peroxiredoxin BAS1, thioredoxin M (TrxM).

Energy metabolism-related proteins in tolerant variety SD609

Consistent with enriched GO biological process terms, mitochondrial electron chain and ATP synthesis-related proteins linked with energy metabolism were changed in SD609 under drought stress (Additional Table 1). Here, electron transport proteins such as cytochrome c oxidase subunit and NADH-ubiquinone oxidoreductase B18 subunit were increased. Furthermore, two ATP synthase subunit proteins were also induced by drought. Their changes imply that SD609 enhanced energy production to cope with moderate drought stress.

Photosynthesis-related DEPs observed in sensitive variety SD902

The photosynthesis-antenna proteins pathway was significantly enriched in SD902 under water deficit conditions (Fig. 5e). However, the number of photosynthesis proteins detected in SD609 is higher than those in SD902 (Additional Table 1). Eight DEPs (LHCs) involved in photoreaction were up-regulated by approximately 1.7-fold. Besides, 17 DEPs participating in photosynthesis were down-regulated. These 17 DEPs were grouped into three types: (i) five proteins related to Chl biosynthesis (for example, uroporphyrinogen decarboxylase and NADPH-protochlorophyllide oxidoreductase) were detected under drought stress. (ii) four Ferredoxin-NADP(H) oxidoreductase (FNR) protein, which catalyzes the electron transfer between NADP(H) and ferredoxin (Fd), were decreased in abundance. (iii) eight pyruvate, phosphate dikinase (PPDK) protein involved in carbon fixation were significantly down-regulated in

response to moderate drought stress. The down-regulation of most photosynthetic proteins may explain the changes in photosynthetic parameters of SD902 under drought stress. Besides, six sucrose synthase proteins involved in starch and sucrose metabolism were up-accumulated in SD902 under drought stress.

Common DEPs between two maize varieties

In our results, 39 proteins shared in SD609 and SD902 were evident (Additional Table 1), including GO enrichment (Fig. 5c) of both datasets for: photosynthesis and generation of precursor metabolites and energy. The most abundant photosynthesis-related proteins were LHCs and NADPH-protochlorophyllide oxidoreductase, in which the membrane proteins of LHCs bind chlorophyll and transfer energy to the reaction centers for photosynthesis. Among the 35 identified DEPs, most protein were increased in two varieties. As shown in Additional Table 1, the heat shock protein (HSP26) and other small heat proteins (sHSP), glutathione reductase, were up-regulated in both varieties under drought stress. By contrast, the LHCs was increased in SD902, but decreased in SD609.

Protein interaction network in tolerant variety under moderate drought

To predict the protein interactions and functional relations among DEPs, protein-protein interaction network analysis was performed with confidence scores > 0.5 to identify the interactions of specific DEPs in SD609 (Fig. 6). Four main groups of interacting protein species were identified in the network. The functions of the proteins in these four clusters are generally focused on photosynthesis, ROS scavenging, protein folding and energy metabolism. Most proteins in these clusters were increased, which displays the pivotal response of these proteins under drought conditions. It is found that plastocyanin (103629356) interacts with 24 other proteins, and OEE1 (100272890) is also linked with 24 other proteins, such as PSI-K, Cytochrome b6/f complex proteins and TRM1. Furthermore, ATP synthase subunit (100281924) interacts with 21 other proteins. These results indicate proteins involved in different metabolic pathways responded to drought stress by interacting.

Expression levels of genes encoding DAPs in response to moderate drought

Next, the transcriptional expression in thirteen selected proteins were measured by real quantitative real-time PCR (qRT-PCR). qRT-PCR results showed that the expression patterns of half of DEPs were coincided well with their corresponding coding genes. Of these 13 proteins, seven genes expression (Fig. 7a, d, e, h, i, j, k) were consistent with proteomic results. The expression patterns of other six genes showed opposite trends with their homologous proteins. These results may be due to a time delay between mRNA and

proteins or posttranscriptional and transcriptional regulatory mechanisms. Thus, most of the qRT-PCR results confirmed our proteomic results.

Discussion

Physiological responses of two varieties to moderate drought

Generally, the photosynthetic rate is usually affected by stomatal limitations and non-stomatal limitations under drought stress [22]. we found that the P_N and g_s of SD609 were higher than those of in SD902. The C_i increased in the two varieties, indicating that the decline in the net photosynthetic rate of the two varieties may be caused by non-stomatal factors.

Plants have evolved complex mechanisms to deal with drought stress. By comparing the energy conversion, we found Y(II), Y(I), ETRII and ETRI in the leaves of SD902 were significantly lower than those of in SD609, indicating that moderate drought reduced the rate of LEF of the two varieties [12]. In addition, compared with SD609, Y(NO) and Y(NA) in SD902 were significantly increased. We speculate that the PSI and PSII photoinhibition were observed in SD902. Meanwhile, the Y(CEF) in SD609 is significantly higher than that in SD902. These results showed that SD609 can coordinate the changes in electron transfer between PSII and PSI better than SD902. In tolerant varieties (SD609), the plant was able to maintain high PSII and PSI activity, reduces excitation pressure on PSII and PSI and protects the photosystems.

Improving the scavenging activity of ROS is one of the adaptive approaches in response to drought stress [23]. The activities of SOD, POD and GR were significantly enhanced in SD609, indicating that SD609 synthesized different antioxidant enzymes to reduce the production of ROS. This results generally agreed with a previous study in other plants [10]. Thus, SD609 showed more well physiological performance under drought stress. All these protective effects help SD609 to survive under stressful conditions.

Fine control of photosynthetic electron transport chain in tolerant variety

Photosynthesis is one of the key processes to be easily affected by water deficits. In plants, photosynthesis involves storage the energy from light, captured by photosynthetic electron transport reactions, and reduced carbon in Calvin-Benson cycle [24]. The electrons released from water are transferred from PSII to PSI via the plastoquinone (PQ) pool, the Cytb₆f and the plastocyanin (PC), to ultimately reduce ferredoxin and NADP⁺. This electron transport pathway is termed linear electron flow (LEF). In addition, the cycling electron flow (CEF) around PSI, which also passes through the Cytb₆f complex, can generate ΔpH and drives ATP synthesis but without concomitant generation of NADPH [25].

Here, GO and KEGG enrichments showed that photosynthesis was the most significantly process in SD609 under drought stress. Our results showed that drought induced the expression of Psb27, OEE1-1 and OEE2-1. Psb27, a component of the PSII complex, has also been shown to play a role in PSII assembly or during the repair cycle [26], and also increased in pearl millet under drought stress by RNA-Seq approach [27]. The expression of OEE1 is also considered to be the rate-limiting step in the assembly of PSII subunit [28]. Therefore, our data suggests that maintaining the capacity of PSII through facilitating assembly and recovery is one of the mechanisms responding to drought. Cytochrome b6-f complex protein mediates electron transfer between photosystem I and II, controls state transitions in the thylakoid membrane, and governs cyclic electron flow around PSI [29]. Subsequently, plastocyanin accepts electrons from cytb6f and transferred to Fd via PSI, at which point they can take two pathways: the LEF from water to NADP^+ via FNR to produce NADPH, then enters the Calvin cycle, and the CEF pathways, whereby electrons are transferred back to the PQ, have protective effects against environment cues [30]. Corresponding with previous studies [31-33], here, drought increased the expression of Cytb6f complex protein, plastocyanin and Fd proteins which participate in cyclic electron flow, consistent with the measurement result of Y(CEF) in SD609 (Fig. 3e). Nevertheless, the LHC in concert with chlorophyll biosynthesis proteins, which major in capture and transfer light energy to the reaction center of PSII, were down-regulated in SD609 but up-regulated in SD902. This may demonstrate that these thylakoid membrane complexes of PSII were vulnerable to drought stress, and in line with the decrease in the efficiency of photochemical energy conversion by PSII and PSI (Y(II) and Y(I)) under stress conditions (Fig. 3a, b). Down-regulation of these proteins are suggestive of a regulatory mechanism in SD609 to resist drought. Minimization of light absorption by reduced amounts of LHCP and Chl contents would down-regulate the electron transport to reduce the ROS production under stress conditions. These are in agreements with previous reports [34, 35], although contrasting findings have also been reported [36]. The actual mechanisms are yet to be understand. As the final step of linear electron flow transferring electrons from Fd and then reduce NADP^+ to support carbon fixing [37], the activity of FNR will clearly effect the partitioning of electrons between LEF and CEF, irrespectively of whether it is directly involved in CEF [38] or not [39]. The reduced levels of FNR in SD902 demonstrating that drought stress diminished photosynthetic electron transfer efficiency, and ultimately resulted in a lower level of various fluorescence kinetics parameters and photosynthesis rate in SD902 compared to SD609 during drought stress. Overall, the regulation of the partitioning of electrons between the cyclic and linear pathways thus play a key role in the adaption of the plant [38]. Our study implies that appropriately repairment of PSII and activation CEF may help minimize the energy loss caused by a reduction of light absorption efficiency in SD609 response to moderate drought stress.

Drought stress also influences the capacity of carbon assimilation in photosynthesis [40]. PEPC is involved in photosynthetic carbon fixation that catalyzes the conversion of phosphoenolpyruvate to oxaloacetic acid in the presence of HCO_3^- of C_4 plants as part of a CO_2 pump and provides oxaloacetic acid to the tricarboxylic acid cycle (TCA) [41]. Previous study has also concluded that PEPC can inhibited by malate under drought stress [42]. Correspondingly, PPDK catalyzes the phosphoenolpyruvate regeneration phase of the C_4 carbon fixation pathway [43]. Our findings revealed that PEPC and PPDK

proteins were considerably decreased to cope with water stress in SD609 and SD902, respectively, as was the case in maize and wheat [43, 44]. It is noteworthy that the reduced levels of PPDK in SD902 could perhaps also associated with a greater plant susceptibility to drought due to this enzyme involving in the production of NADPH, which is an important component of various cell antioxidative and osmoprotective mechanisms. Thus, the reduction of different carbon fixation enzymes may also be one of the reasons for the divergence in photosynthesis observed in SD609 and SD902 under moderate drought stress.

ROS scavenging pathway is activated in tolerant variety under moderate drought

Plants exposed to drought significantly generate ROS, which, on one hand can function as signaling molecules to regulate many physiological processes, and on the other hand, can disturb the intracellular redox balance and cause oxidative damage to cells [45, 46]. Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system comprising of the nonenzymic (ASA, GSH) as well as enzymic antioxidants (SOD, CAT, APX, MDHAR) [47]. Among the 16 antioxidant-related proteins in SD609, CuZn-SOD acts as the first line of defense by converting O_2^- into H_2O_2 , and its overexpression produces enhanced tolerance to differential environmental stresses [48]. The elimination of H_2O_2 in plant cells depends mainly on POD, CAT, and Prx, as well as other main enzyme in AsA-GSH cycle [49, 50]. The expression of POD-related proteins in SD609 were significantly increased under drought stress, which is in accordance with a previous report [51]. The Trx-Prx pathway is one of the key antioxidant systems in plants. Prx can convert H_2O_2 into water and alcohols through Cys [52, 53]. TrxM is a member of Trx family, mainly participates in the removal of ROS in the chloroplasts and regulation of the Prx activity. Our results demonstrated that Trx-Prx pathway plays an important role in alleviating oxidative damage caused by drought stress in SD609. Besides, GR can reduce GSSG to GSH and maintain the GSH pool in AsA-GSH cycle, and GST may play an important role in scavenging lipid hydroperoxide (LOOH) [46]. Consequently, in our paper, moderate drought also could initiate the AsA-GSH and GST pathway to reduce oxidative cell damage in SD609.

Shared pathways between two maize varieties in response to drought

Dehydration stress also affects the quantity and quality of normal plant proteins and, as a result, stress related proteins including HSPs are induced [54]. Here we found that heat shock proteins in SD609 and SD902 respond to drought stress. It should be noted that different classes of HSPs were induced in two varieties, which indicated the expression pattern of HSPs were genotype-specific [54]. Some members of HSP90, HSP70 and sHSP induced in SD902 to prevent cellular damage, whereas most sHSPs were upregulated in SD609 under drought stress. Individual members of each class of HSPs have particular functions, but the cooperation between different HSP networks appears to be a central principle of the

integrated HSP machinery [55]. In addition to prevent protein aggregation and use ROS as a signal molecule under stress conditions in our study, HSPs might, as a major class of stress-responsive proteins, also play a role via cross-talk with other mechanisms and function synergistically with other components to decrease cellular damage [56]. Additionally, further research should be devoted to investigating the crosstalk between HSPs and other stress response mechanisms in maize to provide a further understanding of acquired stress tolerance.

Proposed molecular model of drought-tolerant maize varieties

Considering both the photosynthetic parameters and the identified drought-responsive proteins in SD609, our finding indicates that when maize leaves are subjected to water deficit conditions, the different sensitivity genotypes to drought is associated with changes in a limited fraction of proteins. In SD609, most photosynthesis and ROS scavenging proteins were up-regulated in the leaves, concomitant with the occurrence of complex changes of energy metabolism and the establishment of a new homeostasis while few proteins altered in SD902 following drought treatment. A model of the response of photosynthetic electron transfer proteins to drought in SD609 was shown in Fig. 8. Drought stress decreased chlorophyll biosynthesis and LHC proteins, which reduced photochemistry and photosynthetic electron transport. However, the PSII assembly, cytb6f and PSI proteins were accumulated under moderate drought, which functioned in the assembly and stabilization of PSII as well as increased the CEF around PSI and thereby enhanced the tolerance of SD609 to drought. Drought also activated ROS scavenging system, improved the production of NADPH and ATP and facilitated protein folding. Similar results were reported in *Brachypodium distachyon* under H₂O₂ stress [57] and ginger under drought and shading conditions [58].

Conclusion

In the present study, our physiological data suggest that tolerant variety exhibited better under moderate drought owing to its enhanced photoprotection mechanisms and improved ROS scavenging ability. Proteomic data confirmed the physiological results and analyzed that a high tolerance of SD609 associated with: (i) high photochemical efficiency via stimulating photoprotective mechanism. (ii) Efficient antioxidant system by up-regulating ROS scavenging enzyme proteins. (iii) Modulating protein metabolism to prevent proteins aggregation. While drought-sensitive SD902 only activated sucrose metabolism and protein metabolism is not enough to cope with moderate drought. Further, qRT-PCR analysis results confirmed the iTRAQ sequencing data. Overall, our results provide a clear relationship between the physiological mechanisms and molecular events of maize under drought stress and nominate a list of targeted proteins for further investigation.

Methods

Plant materials and treatments

No permissions were necessary to collect plant materials. Two maize (*Zea mays*. L) cultivars, drought-tolerant maize Shaandan609 (91227 x Chang7-2) and drought-sensitive Shaandan902 (K22 x K12), commercial hybrids, were obtained from Shaanxi Dadi (Seed Company, Shaanxi, China). Maize seeds were grown in plastic pots (26 cm diameter × 38 cm high) filled with 18 kg air-dried soil, composed of 1.62% organic matter and 0.064% total nitrogen [59]. All experiments were conducted in a greenhouse at the Northwest A&F University in Shaanxi, China. The average temperature for day/night was 35/30°C, respectively, relative humidity was 50-60%, the photoperiod for the day/night cycle was 16/8h, and the maximum illumination intensity was about $2,000 \pm 50 \text{ mmol m}^{-2} \text{ s}^{-1}$. At the sixth leaf stage, plants were divided in two different watering treatments: (i) well-watered control treatment (CK): plants were watered every day to maintain soil water content (SWC) at between 90% and 100%; (ii) drought-stress treatment (DS): plants were irrigated to the extent of 60-70% of soil water content. The second or third top fully expanded leaves were selected to determine the photosynthetic parameters with six replications. The third top fully expanded leaves were sampled and stored at -80°C for proteome analysis. Three biological replicates were set for each treatment.

Fluorescence parameter determination

The energy conversion efficiencies in PSII, PSI and CEF activity were measured using a saturation-pulse Dual-PAM-100 (Heinz Walz, Effeltrich, Germany) on the upper second or third fully expanded leaves according to Zhou et al [12]. The following parameters were assessed: maximum quantum yield of primary PSII photochemistry [$F_v/F_m = (F_m - F_0)/F_m$], the effective quantum yield of PSII photochemistry [$Y(II) = (F_m' - F_s')/F_m'$], The quantum yield of PSI [$Y(I) = (P_m' - P)/P_m'$], the quantum yield of non-regulated energy dissipation of PSII [$Y(NO) = F_s'/F_m'$], the quantum yield of non-photochemical energy dissipation due to the acceptor side limitation [$Y(NA) = (P_m - P_m')/P_m'$], the quantum yield of non-photochemical energy dissipation due to the donor side limitation [$Y(ND) = 1 - P700_{red}$], non-photochemical quenching [$Y(NPQ) = (F_m/F_m') - 1$], the ratio between the electron transport rate around PSII (ETR_{II}) and PSI (ETR_I): $ETR_I = Y(I) \times PPFD \times 0.85 \times 0.5$, $ETR_{II} = Y(II) \times PPFD \times 0.85 \times 0.5$, where 0.85 represents the leaf absorbance and 0.5 is the proportion of absorbed light energy allocated to PSI or PSII. The cyclic electron flow value (CEF) was estimated as $ETR_I - ETR_{II}$ [32].

Protein extraction, trypsin digestion and iTRAQ labeling

Protein extraction was performed according to the previous report with a slight modification [59]. Briefly, the samples grinded in liquid nitrogen were harvested to a 5-mL centrifuge tube and sonicated three times on ice using a high intensity ultrasonic processor (Scientz), then lysed with lysis buffer (8 M urea, 2 mM EDTA, 10 mM DTT, and 1% protease PMSF (Beyotime)). The lysate was centrifuged at 12,000 g at 4°C for

30 min. The protein level in the supernatant was quantified with BCA kit according to the manufacturer's instructions.

For trypsin and iTRAQ labeling, the protein solution from each sample was reduced with 10 mM DTT at 37°C for 30 min, alkylated by 25 mM iodoacetamide for 15 min at room temperature in the dark, and then digested with 1:50 trypsin-to-protein mass ratio overnight and 1:100 for a second 4 h-digestion. The digested samples were incubated for 2h at room temperature and pooled, desalted and dried by vacuum centrifugation, then labeled with iTRAQ reagent according to the manufacturer's instructions.

HPLC Fractionation and LC-MS/MS Analysis

iTRAQ labeled peptides were fractionated by high pH reverse-phase HPLC using Waters Bridge Peptide BEH C18 (130 Å, 3.5 µm, 4.6*250 mm). Peptides were first distributed into 60 fractions using a gradient of 2% to 98% acetonitrile over 88 min. Then, the peptides were reconstituted into 12 fractions, concentrated by vacuum centrifugation, and the tryptic peptides were dissolved in 0.1% formic acid and directly loaded onto a reversed-phase analytical column. The gradient contained 0.1% formic acid with an increase from 5% to 45% over 58 min, climbing to 80% in 2 min at a constant flow rate of 300 nL/min on an ultimate system.

The peptides were subjected to NSI source for LC-MS/MS analysis, which was performed on a Q Exactive HF coupled to UPLC. The m/z scan range was 400 to 2000 for full scan, 60,000 resolution for intact peptides. Ion fragments were detected at a resolution of 15,000 and the 15 most intense precursors were selected for subsequent decision tree-based ion trap higher energy collision induced dissociation (HCD) fragmentation at the collision energy of 27% above a threshold ion count of 1e5 in the MS survey scan with 20.0s dynamic exclusion. Full width at half maximum (FHMW) at 400 m/z is used coupled with an automatic gain control (AGC) setting at 1e6 ions and fixed first mass at 100 m/z.

Protein identification and quantification

The resulting MS/MS raw data were converted to mgf format profile with the software mascot 2.3.02 (matrix science). Trypsin was chosen as enzyme and two missed cleavages were allowed. Carbamidomethylation (C) was set as a fixed modification and oxidation (M), acetylation in N-Term were set as variable modification. The searches were performed using a peptide mass tolerance of 20 ppm and a product ion tolerance of 0.05 Da, resulting in 0.05 false discovery rate (FDR).

Identified proteins that differed between stressed and non-stressed plants with a fold change >1.50 or <0.67 ($p < 0.05$) were defined as significant differentially expressed proteins (DEPs). The gene ontology enrichment analysis and KEGG pathway enrichment were performed using the agriGO (<http://systemsbiology.cau.edu.cn/agriGOv2/>) and KEGG database (<https://www.genome.jp/kegg/>), respectively. Only the GO terms or KEGG pathways with P-value less than 0.05 can be defined as

statistically significant. Protein interaction network was constructed using the STRING database (<https://string-db.org/>) and Cytoscape 3.7.2 software. The interaction proteins were shown with a combined score higher than 0.5. The protein subcellular localization prediction was used WoLFPSORT (https://www.genscript.com/psort/wolf_psort.html).

Quantitative real-time PCR

Thirteen candidate different expression genes in samples were selected to verify iTRAQ results by quantitative real-time PCR. The gene-specific primers used in this assay are on Table 2S and the gene (gene ID: GRMZM2G046804) was used as internal control. The results were calculated by the $2^{-\Delta\Delta CT}$ method [60].

Statistical analysis

The physiological assay and qRT-PCR results were analyzed by SigmaPlot 11.0 software. Significant differences between the controls and treatments were determined by Tukey's tests at significance level $P < 0.05$. Bioinformatic analysis and graphics were performed with the R package.

Abbreviations

POR, NADPH-protochlorophyllide oxidoreductase; MgPME, Magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase; Mg-chelatase, Mg-protoporphyrin IX chelatase; LHCP, Chlorophyll a-b binding protein; Psb27-H1, Photosystem II repair protein PSB27-H1 chloroplastic; OEE1-1, Oxygen-evolving enhancer protein 1-1 chloroplastic; OEE2-1, Oxygen-evolving enhancer protein 2-1 chloroplastic; PQ, Plastoquinol; Cyt b_6f , Cytochrome b_6/f complex; PC, Plastocyanin; Fd, Ferredoxin; FNR, Ferredoxin-NADP oxidoreductase; CEF, Cyclic electron flow; HrBP1, Harpin binding protein 1; HSP17, 17.0 kDa class II heat shock protein; HSP26, Heat shock protein 26; HSP70, Heat shock 70 kDa protein 14; SOD [Cu-Zn], Superoxide dismutase [Cu-Zn] 4A; GST, Glutathione transferase; GR, Glutathione reductase; Prx, Thioredoxin-dependent peroxiredoxin; Trx, Thioredoxin family protein.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Proteome information and other data produced during this study are included within the article and its additional files.

Competing interests

The authors declare there are no competing interests.

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Author contributions

RHZ and HJL designed the experiments; HJL, MY and CFZ performed experiments together; HJL and YFW analyzed the data, prepared figures and wrote the paper; RHZ revised the manuscript; All authors read and approved the final manuscript.

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Figures

Figure 1

Changes in chlorophyll content and gas change of two maize varieties under moderate drought. (A) total chlorophyll content (SPAD), (B) Net photosynthetic rate (PN), (C) stomatal conductance (g_s), (D) substomatal CO_2 concentration (C_i). All data represent means + standard errors of three replicates. Values with different letters indicate significant difference at $P < 0.05$ level between treatments based on one-way ANOVA.

Figure 2

Morphological and antioxidant activities of SD609 and SD902 in response to drought stress. (A) Growth of SD609 and SD902 under well-watered (CK) and moderate-drought (DS) stress conditions. Upper left: seedlings of SD609 under well-watered (CK) conditions. upper right: seedlings of SD609 under moderate-drought (DS) stress. Lower left: seedlings of SD902 under well-watered (CK) conditions. Lower right: seedlings of SD902 under moderate drought. (B) Superoxide dismutase (SOD) enzyme activity. (C) Peroxidase (POD) enzyme activity. (D) (GR) enzyme activity. All data represent means + standard errors of three replicates. Values with different letters indicate significant difference at $P < 0.05$ level between treatments based on one-way ANOVA.

Figure 3

Changes of photochemical efficiency of two maize varieties under drought stress. (A) Drought induced changes in PSII and PSI parameters of SD609. Y(II), the effective quantum yield of PSII photochemistry; Y(NPQ), the quantum yield of PSII regulated nonphotochemical energy dissipation; Y(NO), the quantum yield of nonregulated nonphotochemical energy dissipation; Y(I), the effective quantum yield of PSI photochemistry; Y(NA), the nonphotochemical quantum yield of the PSI acceptor side. Y(ND), the nonphotochemical quantum yield of the PSI donor side. (B) Drought induced changes in PSII and PSI parameters of SD902. (C) CEF activity of SD609 and SD902 under moderate drought stress. (D) The chlorophyll contents in two maize varieties. Each parameter represents means + standard errors of three replicates. The different letters above the columns indicate significant difference at $P < 0.05$ level between treatments.

Figure 4

(A) UpSet plot between sets of proteins (Log2 FC > 1.5) in two maize varieties under drought stress. (B) Localizations of identified proteins in SD609. (C) Localizations of identified proteins in SD902.

Figure 5

(A) Functional classification of unique differentially expressed proteins in SD609 under drought stress. (B) Functional classification of unique differentially expressed proteins in SD902 under drought stress. (C) Functional classification of shared differentially expressed proteins in two maize varieties under drought stress. (D) Bubble plot for KEGG pathway enrichment of DEPs in SD609. X-axis represents the number of mapping proteins and y-axis describes the enrichment scores ($-\log_{10}[\text{enrichment } P\text{-value}]$). The bubble size indicates the proteins expression in the KEGG pathways ($(\text{abs}(\log_2\text{fold change})) * 2$). (E) Bubble plot for KEGG pathway enrichment of DEPs in SD902.

Figure 6

Analysis of the protein-protein interactions network in SD609 in response to drought stress.

Figure 7

Confirmation of proteomic results by quantitative real-time PCR (qRT-PCR). (A-D) qRT-PCR analysis of four differentially expressed proteins shared between SD609 and SD902. (E-K) qRT-PCR analysis of seven differentially expressed proteins in SD609. (L-M) qRT-PCR analysis of seven differentially expressed proteins in SD902. Values were presented as mean $\pm$ SD (n = 3).

Figure 8

Model of proteins mechanisms in SD609 under moderate drought stress. The expression pattern of each protein is shown in box with colors.

Supplementary Files

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