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Review Article
Roles of Glycinebetaine on Antioxidants and Gene Function in Rice Plants Under Water Stress

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Abstract
Antioxidants activities and gene expression in rice up-regulated during water stress condition. The activation of antioxidants (enzymatic and non-enzymatic) such as glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) in plants is related to the Reactive Oxygen Species (ROS) accumulation in plant under stress conditions. Glycine betaine, however, plays a pivotal role as an osmoprotectant in response to water stress. Exogenous application of glycine betaine improves the activities of antioxidants and expression of gene, which might lead to the improvement and sustainability of rice production during climate change conditions. Understanding the association of antioxidant enzyme activities and its expression in response to water stress is essential for further understanding the molecular mechanisms by which controlling antioxidant defense for drought tolerance. This also will be useful information on theory basis for drought resistance breeding and cultivation of rice as an effort to sustain rice production. In this review, literatures for the potential of glycinebetaine to improve antioxidants defense activity and gene expression during the water stress condition in related to sustainable rice production have been discussed.

Key words: Reactive oxygen species, antioxidants, glycinebetaine, gene expression, water stress

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Water is a major component that greatly important to most of the land plants by which functioned as tissue constituent, metabolite and minerals translocation, chemical reaction and cell enlargement[1]. Rice is one of the monocot plants, which is predominantly at risk to water stress, which affected about more than 50% of the world rice production[2], which reduced rice production[3,4]. Various physiological processes have been affected by water stress in rice such as net photosynthesis[5], transpiration rate[6], stomatal conductance, intercellular CO_2, photosystem II (PSII) activity[4], relative water content[7,8] and membrane stability index[9]. Borrmann *et al.*[10] stated that the oxidative state have been detected in water stressed-plants including rice. Different water levels affect phytoavailability of macronutrients[11-14] and micronutrients with time as well as plant physiological parameters[11,15-17]. In contrast, flooding condition reduced redox condition in the soil[18-20]. Reactive Oxygen Species (ROS) is known as anaerobic metabolism by-products, which partially reduced molecular oxygen forms and enhanced by the unavoidable outflow of electrons into the molecular oxygen while the transportation of electron activities. This event has taken place during the drought stress through the metabolic oxidizing and electron transport system disruption arising in the mitochondria, chloroplasts, microbodies and plasma membranes[21]. The excessive accumulation of ROS can instantly cause lipids peroxidation, denaturation of protein, the mutation of DNA and oxidative damages of cells, inactivate metabolic enzymes and attack membrane lipids by which eventually leading to cell death[22].

During biotic and abiotic stress conditions, plant has activated ROS scavenger defense system to compensate the effect of ROS[23,24], physiological functions and rice yield[3,6] through glutathione biosynthesis[16,25,26]. Islam *et al.*[27] stated that antioxidants could be initiated in organelles, which include different enzymes related to antioxidants. The respond of antioxidants are vital as works of ROS scavenging scheme whereas, expressions of these antioxidants could advance rice tolerance abilities to counteract drought stress[28]. In addition to that, glutathione increased yield and plant parameters[29-31] which likely enhances the production of crop by the application of GB through reducing stress effect.

In addition to antioxidant defense system, glycinebetaine (GB) plays a major function in enhancing plant tolerance to drought[27] whereas, GB has shown a great potential as one of the scavenger mechanisms and work effectively more than the other compatible solutes in cytoplasmic osmotic adjustment of plant responses to osmotic stresses[32]. The GB would also play role as protein-compatible hydrotrope as well as a scavenger of hydroxyl radical which could stabilize the structure of many macromolecules including oxygen-evolving photosystem II protein pigment complex[33]. Therefore GB might have potent function to activate antioxidant defense to the rice plants for sustainable rice production under low water condition.

FUNCTION OF GLYCINEBETAINE AND ITS NATURAL ACCUMULATION

Glycinebetaine or (N, N, N-trimethyl glycine) derivative of glycine has been classified among of the many quaternary ammonium compounds found abundantly in plants[34]. It is also extensively well-suited solutes that induced naturally and synthesized in chloroplast responses to drought[35]. The GB plays a pivotal character in defensive cellular constructions and vital metabolite character in plants for instances, safety of proteins, enzymes and membranes against damage due to stresses by stabilizing macromolecules (e.g. photosynthetic machinery) and quaternary structure of proteins as well as maintaining ROS scavenging capacity[36]. The GB has also been continuing the cellular osmotic equilibrium as well as the turgor pressure to protect cell from dehydration and regulated signaling transduction by regulating water potential flow into cells[36] in response to water stress conditions.

EFFECTIVE LEVEL OF EXOGENOUS GB IN REPLY TO WATER STRESS

Glycine betaine (GB) has been reported to accumulate naturally in many plants, such as sugar beet, spinach, barley, wheat and sorghum in reaction to numerous stresses[36]. However, rice is the only cereal plant that has been recognized neither synthesizes nor accumulates GB regardless of stress condition[37]. In rice plant, choline monooxygenase and betaine aldehyde dehydrogenase encoding genes has been found, however, there is no accumulation of GB reported under stress condition[37].

Interestingly, positive properties of GB application on growth of plants under drought stress have been well documented on several plants such as tobacco, wheat, corn, rice and cotton see review by Ashraf and Foolad[36]. In rice, the application of exogenous GB during abiotic stresses especially drought has stimulated and then increased the growth of rice[38]. Exogenous use of GB also was reported towards improving photosynthetic abilities by improving chlorophyll content in leaf as well as improved height of plant and yield traits such as panicle, fertility and weight of grains during the harvesting stage[38]. Apart from that, GB improved membrane stability through minimizing membrane ion leakage, reduced H_2O_2 and MDA content in leaf, lowering water potential whereas allowed additional water taken up to compensate water shortages in plant tissues during water stress condition[39].

Many studies have shown different results from the use of GB in different plant species due to different method and concentrations applied[36-39]. The most effective method and concentration of exogenous GB in rice for instances, has been reported as foliar application at 100 mM and 100 mg L^{-1} during 85 days after sowing and five leaf stage respectively[39]. In tobacco, foliar application of 0.1 M GB during early drought has increased fresh weight, dry weight and area of leaf compared to 0.3 M of GB[37]. Therefore, the most effective level of exogenous GB in tobacco has been proved at level of 0.1 M. In addition to that, the positive effect of foliar exogenous GB application also has been reported in wheat, at 50 mM which enhance various growth attributes, yield and increased the level of some key metabolites, while in corn, it increased yield at 15-34% and improved plant height and chlorophyll when applied at 150 ppm before flowering[40]. In similar study capacity, GB has been also reported to act as osmoregulating substance and enhanced water stress of corn at level of 15 mM and enhances growth at level of 100 mM[40]. Increasing of seed cotton yield and number of boll produced in cotton via foliar application of GB during drought stress also has been documented by Miri and Armin[40].

In spite of positive effects of exogenous GB application have been intensively studied, there are few reports showing contradictories and even negative properties of GB in drought condition. There is a report shown that foliar of exogenous GB application did not touch yield and physiology of cotton[41]. Besides that, over-accumulation of GB has been reported increased photosynthesis rate during drought stress in wheat[42]. Surprisingly, it is contrast to with reported by Shahbaz *et al.*[38], which all wheat cultivars shown a substantial decrease in photosynthetic in reaction to water stress. Furthermore, the application of exogenous GB has also shown to decreased leaf area of wheat [42], however, Shahbaz *et al.*[38] on other hand shown that exogenous GB increased the leaf area of wheat. In addition to that, negative effects of exogenous GB has been also reported for example, in tobacco, foliar application of GB at 0.3 M has caused leaf scorching by which decreased 20% of number of green leaf. Similarly, in corn, GB application at 20 mM significantly decreased the growth and yield. Moreover, there is still lack info obtainable on the exogenous GB application effects particularly on rice during water stress occurrence and its action as mechanism(s) at both molecular and cellular levels are yet fully understood. Thus, deeper understanding on these mechanism(s) of its effect is crucially needed to advance drought tolerance ability in rice production.

ACTIVATION OF ANTIOXIDANTS DEFENSE SYSTEM IN RESPONSE TO WATER STRESS IN RICE

Water stress leads various effects such as reduction of cell division and vegetative growth of plants. Once plants challenge drought, their stomata is closed, which resulting in decrease of CO_2 adjustment in leaves and form ROS, such as O_2. The ROS-scavenging system is broken due to extreme buildup of ROS, which causes cell damage followed by cell death[43]. In a similar study, it was suggested that the precise measurement of enzyme activities and expression during drought stress as an approach to studying the scavenging system connection during the water stress condition. Moreover, Li *et al.*[44] proposed that minor drought effectively changes enzymes response of antioxidant in rice to get used with intermediate drought stress condition. Hence, the enhancement of antioxidant component including enzymatic, non-enzymatic and enzyme of AsA-GSH cycle might be a best strategy to reduce the ROS damage and trigger drought resistance of plants[45]. Additionally, higher level of antioxidant activity might pay to as drought tolerance by which growing defense capacity to counteract oxidative damage[45]. Therefore, as an effort to protect cells and tissue from oxidative stress, rice has evolved sophisticated and complex antioxidant defense system to scavenge excessive accumulation of ROS[46] in response to various oxidative stresses. These antioxidant enzymes have been found in diverse organelles including chloroplast, mitochondria, peroxisome and interestingly, their pathway have been well-coordinated[47].

ENZYMATIC ANTIOXIDANT SYSTEM (SOD, GPX, CAT)

Superoxide dismutase (SOD) is the first barrier to withstand ROS in most subcellular compartments. The SOD is a group of metalloenzyme which remove superoxide anion ($O_{2.-}$) by disproportioning it into O_2 and H_2O_2. The CAT is the first enzyme that was discovered and categorized in peroxisomes and considered as unique because it is not requiring a reducing equivalent. This enzyme degrades H_2O_2 into H_2O and O_2. The GPX with similar function eliminates excess H_2O_2 by reducing it to H_2O_2 into H_2O via electron donor. Through metabolite analyse and enzyme assay, the activities of antioxidant in related to water deficit. They observed decreasing in SOD, CAT and GPX activities when exposed to water stress treatment. By regard to the results, the authors claimed that the degree of activity changes of these enzymes however were not very significant. The effect of drought stress on these enzymatic antioxidant systems in two rice cultivars, which were Malviya-36 and Pant-12 in response to mild and high drought stress level by antioxidant enzymes assay[46]. They first observed total SOD significantly increased in shoots and roots of both cultivars during both drought conditions compared to unstressed seedlings. Thus, the results were contrast with the previous report[46,47]. However, the activity of GPX drastically declined when exposed at higher level of drought. In the other hand, CAT activities decline under many stress conditions observed by Radotic *et al.*[48]. In this occurrence, the authors concluded that, CAT might not an effective scavenger of H_2O_2. Nevertheless, few current researches have proved that, SOD and CAT can effectively scavenging ROS formation at the same time reduced the negative impact of drought stress[49]. The different in results from both studies might be due to increase of antioxidants activity is normally connected to the mark of drought that subjected by plants[49].

ENZYMES OF THE CYCLE OF ASCORBATE-GLUTATHIONE (APX, DHAR, MDHAR, GR)

In order to sense and respond to oxidative stresses, the balance proportion of AsA to DHA and GSH to GSSG is essential. The recycling path of AsA and GSH is known as AsA-GSH cycle. The AsA-GSH sequence is existed in subcellular compartments including chloroplast and cytosol[27]. In chloroplast, H_2O_2 is mainly eliminated by the successive redox reactions by APX, monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) and GR. It was reported that, APX reduce the excess of H_2O_2 in cells to water by utilized two molecules of AsA (MDHA and DHA) in ascorbate-glutathione pathway[47]. Sharma *et al.*[50] suggested the important character of APX to detoxify H_2O_2 that increase of c-APX motion in rice under drought stressed seedling parallel to decrease of H_2O_2 as observed. In addition to that, increase of AsA restoration system capacity by synthesis of DHAR, MDHAR and GR had proved as the primary response to mitigate water stress in plant[46,48]. The MDHAR catalyse the restoration of AsA from MDHA radical via electron donor by NADPH. The DHAR in another event reduced DHA to AsA to prevent DHA from decomposed into oxalate and tartrate due to highly unstable pH by using reduced equivalents from GSH as electron donor. In order to maintain high cellular ratio of GSH/GSSG, GR catalyse the reduction of GSSG to GSH by NADPH[47]. During higher level of stress however, ascorbate-glutathione enzymes activities were observed to decline. This might be due to enhance of ROS production that interacts with the enzymes, which further lead to the probable oxidation and inactivation[45].

NON- ENZYMATIC ANTIOXIDANT SYSTEM (ASA, GSH)

Glutathione and AsA in the other hand are non-enzymatic antioxidants within the cell which play important roles in diverse biological processes such as signal transduction, synthesis of protein and nucleic acids and cell growth/expansion by controlling procedures from cell elongation and mitosis the senescence to cell death[51]. The ROS are detoxified via non-enzymatic reactions involving small antioxidants molecules including GSH and AsA (also known as AA) in hydrophilic environment[50]. In rice, AsA has been widely reported to alter in response to various oxidative stresses[46]. However, at higher degree of drought stress, these non- enzymatic has been reported to decline with the increase of superoxide anion and lipid peroxidation compared to mild stress condition[45,46]. In conclusion, the authors suggested that the antioxidants protection system superficially flops to counteract the oxidative damage in rice to compensate higher level of water deficit.

ROLES OF EXOGENOUS GB IN ENHANCING ANTIOXIDANT ACTIVITY UNDER WATER STRESS IN RICE

Abundant studies have been reported on the failure of antioxidant defense system to confer high degree level of drought. Alternatively, GB has proved to enhance antioxidant defense mechanisms to counteract the damages cause by various stresses[36]. The GB could protect plants to fight oxidative stress by eliminating ROS both directly or by enhancing AsA-GSH cycle[36]. Similarly, the application of GB exogenously has been found to enhance the antioxidant enzymes activities in wheat seedlings under water stress treatment. This was mainly due to the improved of photosystem II (PSII) activity, improved starch metabolisms and care of necessary leaf water balance of cell osmotic.

To further elucidate the roles of GB, Farooq *et al.*[39] studied the physiological roles of exogenous GB application in aromatic rice cultivar. The GB was used as both foliar and seed application at different concentration levels during the four-leaf stage subjected to drought stress. Interestingly, results suggested that GB application induced enzymatic activities in rice seedlings particularly SOD, APX and CAT activities. The GB has proved to enhance the cellular integrity and allowed rice to maintain its water status resulting in improving of photosynthesis and general metabolism activities. The authors also suggest that foliar application of GB shown better results as compared to seed treatments at 100 mg L^{-1} which also can be applied at critical stages of rice under drought stress treatment. In another piece of work, Farooq *et al.*[52] evaluated the possible roles of compounds including GB, salicylic acid (SA), brassinosteroids (BR), nitrous oxide (NO) and spermine in improving drought tolerance of rice. The GB was used as foliar application and applied at leaf stage of super-basmati cultivar exposed to drought condition, by maintaining 50% of field capacity.

SOD, GPX AND CAT GENES EXPRESSION IN WATER STRESS IN RICE

Drought has been known to induce the appearance of genes by which, more than 5,000 to 6,000 genes were reported to up-regulated and down-regulated respectively in reply to drought condition[53]. Previously, multiple isoforms of antioxidant had been recognized in plants including rice, which may response differentially to the developmental of environment stresses including SOD, CAT and APX isoforms[38-40].

The SOD isoforms has been classified in different groups based on their metal cofactor such as manganese (Mn), ferum (Fe) and copper/zinc (Cu/Zn). There are three isoforms have been found in chloroplasts namely (FeSOD, MnSOD, CuZnSOD), while one in mitochondria (MnSOD), two in peroxisomes (MnSOD and CuZnSOD) and one in cytosol and extracellular space (CuZnSOD) respectively as documented by Pang and Wang[47]. In order to improve describe the SOD in responses to drought, several studies have been performed at gene expression level as well as enzyme activity[29]. Previously, evaluated SOD enzymes activity and gene expression level of eight SOD isoforms by using spectrophotometry and real-time quantitative PCR (qPCR), respectively[43-47]. This study was conducted under 100% irrigation (control) and 50% of water restriction (drought treatment) for two rice cultivars namely, Primavera (drought sensitive) and Douradao (drought tolerance) at two developmental stages (vegetative and reproductive). In leaf tissues, these genes were up-regulated for both cultivars but more genes were expressed at vegetative stage as compared to reproductive stage. During the vegetative stage, all of the eight genes (CuZnSOD1, CuZnSOD2, CuZnSOD3, CuZnSOD4, CuZnSOD5, MnSOD, FeSOD1 and FeSOD2) were expressed in Douradao cultivar instead of only seven genes (CuZnSOD1, CuZnSOD3, CuZnSOD4, CuZnSOD5, MnSOD, FeSOD1 and FeSOD2) were expressed in Palmivera. At reproductive stage on the other hand, only four genes expression increased for the tolerant cultivar (CuZnSOD3, CuZnSOD4, FeSOD2 and MnSOD) while six genes were observed for sensitive cultivar (CuZnSOD1, CuZnSOD2, CuZnSOD5, MnSOD, FeSOD1 and FeSOD2)[54,55]. In root tissue, four genes are overexpressed in the tolerant cultivar, whereas five genes were up-regulated at the reproductive stage. This perhaps due to different upland rice varieties marked different drought tolerance mechanisms and the expression levels of drought stress-related genes varied significantly with rice varieties[54]. In either case, the authors suggested that, significant increase of the expression in both cultivars were directly connected to amplified levels of SOD activity at the reproductive phase. However, decreased of SOD activity for sensitive cultivar and increased for tolerance cultivar was observed during the vegetative phase similarly as reported by Basu *et al.*[55]. Other detoxification of ROS mechanisms, including APX, CAT and GPX enzymes must be performing this protection function as well[56]. In spite of increased of antioxidant enzymes activity is variable between plant species and cultivars under drought stress[57], undoubtedly, the authors reported the different induction patterns in SOD activity level and/or gene expression in rice plants ought to be powerfully consideration to elucidating the drought tolerance cellular mechanisms.

Previously, Teixeira *et al.*[58] have identified the presence of eight ascorbate peroxidases isoforms in rice nuclear genome via *in sillico* analysis including; OsAPx1 and OsAPx2 (cytosolic), OsAPx3 and OsAPx4 (putative peroxisomal) and OsAPx5, OsAPx6, OsAPx7 (putative chloroplastic) and OSAPX8 (putative thylakoid-bound). The characterization of these isoforms by Teixeira *et al.*[59], revealed that, OsAPx1, OsAPx6 and OsAPx7 transcripts were accumulated in roots while OsAPx2, OsAPx3, OsAPx4, OsAPx5 and OSAPX8 transcripts preferentially accumulated in shoots. There are several reports on modulated of APX expression encoding genes by diverse environmental including water, salt, high temperatures and freezing stresses, attack of pathogen, treatment of H_2O_2 and finally abscisic acid[58,59]. For further elucidation, the responses of two *cAPX* genes to three abiotic stresses treatments in order to study on the cAPXs transcript level by using northern blot analysis, RNA blot analysis together with APX enzyme assays[43-46]. Surprisingly, the expressions of OsAPx1 and OsAPx2 were not up-regulated during drought, salt and chilling stress which is contrast with previously reported by Teixeira *et al.*[59], where salt stress induced OsAPx2 gene regulation. According to these results, the authors conclude that it may due to different intensities of stress treatments or differences in the environmental conditions as also reported by Zhang *et al.*[54].

The CAT isoforms have been classified as three groups based on gene structure and expression[60]. Interestingly, each group of catalase have display protective tissues, gene expression of cellular and diurnal fluctuation outlines of transcript stages for instances; CatA genes corresponded to CatB to CatC. The class I CAT also mainly reported to be involved in photorespiration. Nevertheless, the function of CatA (class II) is remaining unclear but it is assumed to be mainly involved in protection to counteract environmental stress[60,61] because this class is normally expressed in vascular tissues and leaves. In order to gain better understanding on CAT isoforms function, Joo *et al*.[62] analysed the expression pattern of catalase genes (CatA, CatB, CatC) using RNA gel blot analysis and quantitative real-time PCR (qRT-PCR). The rice plants were treated with ABA, drought, high salinity and hydrogen peroxide. Interestingly, the authors found that catalase genes of rice were organ dependent. The expression levels of rice catalase gene are differentially regulated by the environmental conditions. The authors also confirmed the involvement of rice catalase genes in related to environmental stress response and photorespiration which agreed to the report by Iwamoto *et al*.[63], who found strong expression of CatA, CatB and CatC in the leaf sheath, roots and rice blade leaf and both CatA and CatC might be involved in H_2O_2 removal whereas produced in mitochondria or during photorespiration.

ROLES OF EXOGENOUS GB IN UP-REGULATING GENE EXPRESSION UNDER WATER STRESS

In spite of some transgenic plants have been successfully made to accumulate GB competently but the level of GB synthesis shown only at low level as compared to GB accumulator's plants[32,33]. In different study, Kathuria *et al*.[64] had also successfully raised transgenic *indica* rice from soil bacterium *A. globiformis* which expressing *codA* gene by mRNA and protein level analysis. Therefore, exogenous application of GB to non-accumulator plants has been abundantly studied as an alternative in the mean to improve the stress tolerance. However, knowledge on how GB would affect the level of gene expression and regulation, which related to plant water stress tolerance, is limited. Considering this situation, the elucidation of the GB roles in up-regulating the expression of signalling pathways castoff by plants in response to the stresses may lead to formulating new approaches to improve plant water stress tolerance.

Another piece of work have been reported by Lou *et al*.[65] on the effects of three level of GB which were 0 mM, 20 mM and 50 mM on antioxidant enzymes gene expression of perennial ryegrass under 0.5 mM cadmium (Cd) stress treatment. The expression level was measured by Real-time quantitative PCR (RT-qPCR). Without GB treatments, the expressions level of MnSOD, FeSOD, CytCu/ZnSOD, ChlCu/ZnSOD and POD genes were reported to increase at 2 h, 48 h and 7 days after treated under cd stressed condition. Previously, Sharma *et al*.[66] and Li *et al*.[67] also reported that gene expression alterations induced by heavy metals in plants. The authors also indicate that, up-regulation of all gene expressions induced by GB would create additional antioxidant to scavenge an extreme accumulation of ROS.

CONCLUSION AND FUTURE RECOMMENDATIONS

Water stress is known widely as a main limiting factor in rice production worldwide for a extended time. Abundant evidence showed the activation of antioxidant defense organization in rice to confer water stress such as SOD, APX, CAT, MDHAR, DHAR, GPX and GR. This is because, the ultimate functions and mechanisms of each antioxidants are yet fully understood. The antioxidant enzymes isoforms in rice plant on other hand perhaps trigger the adaptive mechanisms to reduce the effect of water stress through the variations in gene appearance and physiological parameters. There is evidence which shows that both antioxidant activity and gene expression are correlated. However, the information on the up-regulation of these genes in water stress related to rice is still nebulous and still unclear. The introduction of osmolytes compounds such as GB plays adaptive characters in arbitrating osmotic adjustment that protects cells from water stress through alternative approach to desiccate the accumulation of water stress in rice by enhancing antioxidants activity and up-regulate the expression level of genes.

Despite application of exogenous GB have been widely studied, only certain antioxidants are mostly reported in subjected to water stress in rice. Moreover, the studies have not discussed about the enhancement of these antioxidants in more details.

However, there is still lack of evidences or information on the roles of GB in enhancing these antioxidants activity at the full of set in related to drought condition in rice. Therefore, in the immediate future, further research is required clarifying the functions of GB on the antioxidant activities and their corresponding genes expression in rice plant related to water stress.

SIGNIFICANCE STATEMENT

This study discovers the role of betaine on antioxidant and gene expression in rice plants under stress condition that can be beneficial for plant physiologist and breeders to understand for further improvement of rice plants to cope climate change condition.

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