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# EFFECTS OF DROUGHT STRESS ON ANTIOXIDANT ENZYMES IN SEEDLINGS OF DIFFERENT WHEAT GENOTYPES

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**Abstract**

The aim of this experiment was to study the relationship between the physiological and biochemical indices and drought resistance of different wheat varieties. The response parameters evaluated were: relative water content (RWC), malondialdehyde content (MDA) and isozymes including superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in seedlings of four genotypes, including Changwu134, Xinong2208, Xinong928 and Shaan253. The wheat seedlings were subjected by -1.0MPa PEG-6000 solution for 1~6 d in hydroponic condition and the quantities of antioxidant enzymes was measured. The results showed that the expression of SOD isozymes in seedling leaves was strongest in Changwu134 and weakest in Shaan253. CAT isozymes were induced well and increased readily in Changwu134 and Xinong928 seedling leaves than in Xinong2208 and Shaan253 during drought stress. The expression pattern of POD isozymes was similar to CAT. It is well known that SOD, CAT and POD enzymes play a significant role in determining response of wheat genotypes to drought stress. The results of this study suggested that the amount of antioxidant enzymes determine drought stress tolerance in wheat.

**Key words:** Wheat, Drought resistance, Antioxidant enzymes, Isozymes.

## Introduction

Wheat (*Triticum aestivum* L.), one of the most important food crops, can be grown in a wide variety environments. The lack of adequate available water is the most common limitation for wheat production in low rainfall and poorly irrigated areas. Water stress results in a significant reduction in the yield (Bano *et al*., 2012). Water deficit is one of the most important environmental factors that restricting plant survival and crop productivity in arid regions (Kafi & Salehi, 2012). Plants usually experience water supply fluctuations during life cycle due to changing climatic factors (Tan *et al*., 2006; Izabela *et al*., 2013). Among all the abiotic stress, drought probably has the most significant effect on growth and yield which plants may be encounter in both natural and agricultural systems (Bartels & Sunkar, 2005). Therefore, it is important to study the mechanism of drought resistance of plant species in order to improve their agronomic characters to facilitate developing cultivar with increased resistance.

To diminish the adverse effect of drought stress and ensure crops under optimal growth conditions, plants have evolved some defense mechanisms such as an increase in the content of reactive oxygen species (ROS) (Dhanda *et al*., 2004; Li *et al*., 2004; Rauf *et al*., 2007; Miller *et al*., 2010; Zhang *et al*., 2012). Plants usually increase the activity of anti-oxidative peroxidases or glutathione reductase in response to the drought induced oxidative stress (Neill *et al*., 2002; Gill & Tuteja, 2010; Miller *et al*., 2010). Oxygen is crucial to the presence of most life, however toxic reactive oxygen species (ROS), including the superoxide anion radical, the hydroxyl radical and hydrogen peroxide, tend to the increase when the plants are exposed to stress. ROS are also associated with injuries, they are collectively referred to as oxidative stress, among the most profound damaging factors in plants. As a result of all this, the plants have developed a number of antioxidant defense mechanisms to protect themselves from ROS. These mechanisms draw on a series of antioxidant enzymes, including Superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), as well as the low molecular weight antioxidants, including ascorbic acid, GSH, phenolic compounds and so on (Noctor & Foyer, 1998; Asada, 1999; Young *et al*., 2007). Amjad (2011) proved the hydrogen peroxide scavenging system was more actively induced by water deficit in different wheat genotypes.

Antioxidant enzyme is one of the most important components in the active oxygen removal system. Superoxide dismutase (SOD: EC 1.15.1.1) is a major scavenger of $O2^-$ and it can catalytic the disproportionation reaction of superoxide radical anions into oxygen and hydrogen peroxide (Scandalios, 1993). According to the different ions bounded in the reactive reaction centre of the enzyme, there exist three forms of SOD isoenzymes: SOD containing copper/zinc (Cu/ZnSOD), or manganese (MnSOD) or iron (FeSOD), which are all localized in different cellular compartments (Bowler *et al*., 1994). It is essential for the cell to remove the highly toxic $H_2O_2$ produced during dismutation to avoid inhibition of enzymes such as those controlling the Calvin cycle in the chloroplast (Creissen *et al*., 1994). Meanwhile the $H_2O_2$ can be scavenged by catalase (CAT: EC 1.11.1.6) and a variety of peroxidases (POD: EC 1.11.1.7). CAT breaks down $H_2O_2$ into water and molecular oxygen, whereas POD decomposes $H_2O_2$ by oxidation of co-substrates, such as phenolic compounds and/or antioxidants (Mittler, 2002). There is growing evidence that together with osmotic adjustment and ion compartmentalization is also vital in mitigating the adverse effects of the stress (Ashraf, 2009; Wang *et al.*, 2013).

There are extensive studies on the relationship between the activity of antioxidant enzymes and drought resistance in wheat. However, the conclusions are often contradictory. In this study, four different wheat genotypes were subjected by PEG6000 with -1.0MPa osmotic potential for 6 days. Changes in antioxidant enzymes were monitored in different wheat genotypes to develop relationship between antioxidant enzymes and mechanisms of drought tolerance.

## Materials and Methods

**Plant material and treatments:** Two drought resistant wheat genotypes (Xinong928 and Changwu134) and two drought sensitive genotypes (Shaan253 and Xinong2208) were used in this study. Wheat seeds of uniform size were surface sterilized for 8 min in 0.1% $HgCl_2$ and subsequently washed three times in distilled water after fully flushing with tap water, then germinated in the dark for 48 h.

The seeds with uniform radicle length were placed in petri-dishes containing layers of filter paper. When the wheat seedlings grew to single-leaf, they were watered with Hoagland solution every three days and cultured in a growth chamber under a constant day/night temperature regime of 25℃/20 ,16h/8h (light/dark) with a light intensity of 350u $mol.m^{-2}.s^{-1}$. When the wheat seedlings grew to the period of two true leaves, they were subjected by -1.0MPa PEG6000 for 6 days and replaced the solution of PEG6000 once per day. The second fully expanded leaf was taken after 0, 1, 2, 3, 4, 5 and 6 days of treatment as test materials.

**The relative water content (RWC):** Relative water content (RWC) was measured following the method of Gao (2006): the young and fresh leaves were cut and weighed as fresh weight (Wf). Saturated weight (Wt) was measured after allowing leaves to soak in water for 24h, then the leaves were oven-dried at 8℃, and reweighed as dry weight (Wd); The RWC of the leaf was calculated by the formula: RWC=[ (Wf – Wd ) / (Wt - Wd)] ×100%. All treatments were replicated 3 times.

**The malondialdehyde content (MDA):** The content of malondialdehyde (MDA) was measured using the method of Sun & Hu (2005). Approximately 0.15 g of wheat seedling leaf was homogenized in 3ml of 5% trichloroacetic acid (TCA) on the ice bath. The homogenate was centrifuged at 4000 rpm for 5 min at 25℃. 5 ml of 0.5% thiobarbituric acid (TBA) was added to the supernatant. The mixture was kept in a boiling water bath (100 ) for 10 min, and then quickly cooled on ice. The content was re-centrifuged at 1000 rpm for 5 min. The supernatants was collected and used for the measurement of the absorbance at 450 nm, 532 nm and 600 nm. All treatments were replicated 3 times.

**Preparation of extracts of antioxidant enzymes:** 0.5 g of wheat seedling leaves were weighed and homogenized in 50mM sodium phosphate buffer (pH 7.8) containing 5mM EDTA, 2mM ASA and 1% (W/V) soluble polyvinylpyrrolldone (PVP) on the ice bath. The homogenate was centrifuged at 12000 rpm for 15 min at 4$^{o}$C and the supernatant was used for assaying the activities of isozymes.

**Poly-acrylamide gel electrophoresis (PAGE) and activity analysis:** Equal amounts of concentrated protein from wheat seedling leaves were subjected to polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). SOD Isozymes were separated on a 10% native-PAGE following the method described by Beauchamp & Fridovich (1971), SOD isozymes were stained by incubating in a solution containing 2.5mM NBT for 25 min, followed by incubation for 20 min in the dark in 50mM potassium phosphate buffer (pH 7.8) containing 28 uM riboflavin and 28mM tetramethylethylenediamine. The gel was placed in distilled water and exposed on a light box for 15 min at room temperature.

CAT isozymes were separated on 7% native-PAGE gels and were measured as described by Sun (2003) with some modification. After electrophoretic separation, gels were incubated in 100 ml of 1.0% ice-cold starch solution. The gels were washed and stained for 10 min with 100 ml of 0.5% KI and 0.5 ml glacial acetic acid. The isoenzymes appeared as colorless bands on Prussian-blue background.

POD isozymes were separated on 7% native-PAGE gels as per the method described by Rahnamal and Ebrahmzdeh (2006) with some modification. The gel was covered with a solution containing 50 ml of 50mM sodium acetate buffer (pH 6.0), 3.4 ml of 0.5 % $H_2O_2$ and 3.4 ml of 0.25% (w/v) 0-dianisidine. Finally, the gel was agitated until a brown color appeared (30~60 min). The gels were scanned using a gel imaging system.

## Results

**Effects of drought stress on the relative water content (RWC) in wheat seedlings:** RWC is regarded as a measure of plant water status, reflecting the metabolic activity of the plant organization. It is usually used as an index of dehydration in most plants (Anjum *et al*., 2011; Huang *et al*., 2013). RWC is closely related to physiological function of plants, and it is often used to show the relationship between plant leaves and water content. RWC also indicates the ability to sustain their water content and wilting degree of leaves. As shown in Fig. 1, with increased time of drought stress, the RWC of leaves of four wheat cultivars seedling decreased significantly. According to the standard of Hsiao (1973), RWC values of Xinong928 and Shaan253 seedling leaves decreased by more than 10% on the first day, and the loss was set as a moderate stress state. The RWC of Changwu134 and Xinong2208 seedling leaves declined slowly during the first two days of drought stress. The RWC of Shaan253 seedling leaves dropped by more than 20% severe stress state the fourth days after stress. Xinong2208 and Xinong928 exhibited severe stress on the fifth day. Only Changwu134 was still in a moderate stress state on the fifth day. The results showed that in sustaining water content: Changwu134 was strongest, Xinong928 and Xinong2208 were second, and Shaan253 was weakest.

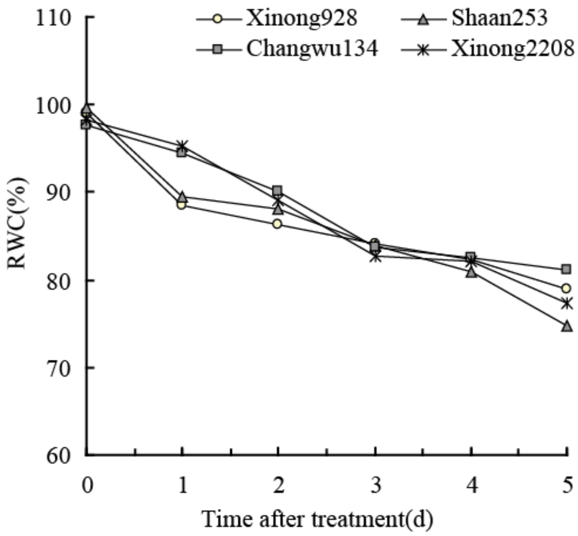


Fig. 1. RWC of wheat seedling leaves subjected to drought stress.

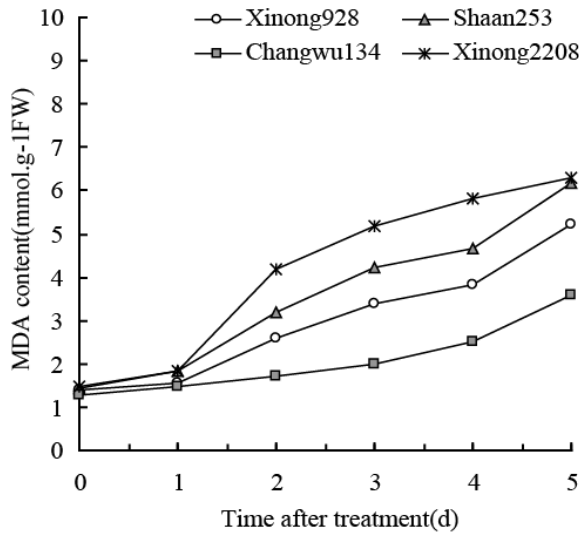


Fig. 2. MDA content in wheat seedling leaves subjected to drought stress.

**Effects of drought stress on malondialdehyde (MDA) content in wheat seedlings:** Malondialdehyde (MDA) is one of the key products of lipid peroxidation by thiobarbituric acid reaction. The MDA content is an indicator of membrane lipid peroxidation which could reflect the degree of damage at adverse conditions. As shown in Fig. 2, the MDA content of Xinong2208 and Shaan253 seedling leaves significantly increased on the second day of drought stress. Following five days of continuous drought stress, MDA content increased at a greater rate in Xinong2208 and Shaan253 seedling leaves than that in Changwu134 and Xinong928. The results showed that: under continuous drought stress, the oxidative damage of the leave in Xinong2208 and Shaan253 cultivars were greater than that in the Changwu134 and Xinong928 cultivars.

**Effects of drought stress on SOD isozymes in wheat seedlings:** Under drought stress conditions, two major SOD isozyme bands were clearly observed in all four wheat varieties (Fig. 3). The SOD isozymes of seedling leaves were increasingly expressed in Xinong928 in one to two days of drought stress, then became slightly weaker in the final days. During continuous drought stress, the expression of SOD isozymes in seedling leaves was strongest in Changwu134 and the Xinong2208 was stronger than Xinong928. The expression of SOD isozymes was weakest in Shaan253 seedling leaves.

**Effects of drought stress on CAT isozymes in wheat seedlings:** During continuous drought stress, seven main CAT isozyme bands were clearly observed. The expression of two main isozymes, CAT and CAT , were strong in four different genotypes wheat. The expression of these two CAT isozymes induced by drought stress was strong and stable in Xinong928 seedling leaves (Fig. 4), but it was gradually decreased in Shaan253. Under drought stress, the expression difference of the four different genotypes wheat can be clearly seen from the four isozyme bands, CAT , CAT , CAT and CAT . Fig. 4 showed that the four CAT isozymes, CAT , CAT , CAT and CAT , were expressed increased in the Xinong928 cultivar leaves. However, the four CAT isozymes was very poor in the seedling leaves of Shaan253.

In the Fig. 5 shown that the CAT and CAT isozymes were induced and increased rapidly in Changwu134 seedling leaves. But it increased slowly in Xinong2208 seedling leaves. Throughout the drought stress period, CAT , CAT and CAT had increasingly expression in Changwu134 seedling leaves, but the expression was weak and turned weaker rapidly in Xinong2208 seedling leaves.

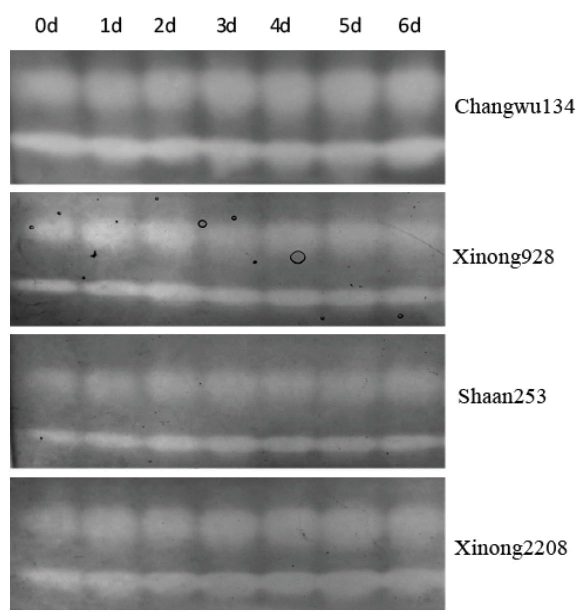


Fig. 3. SOD isozymes in the leaves of wheat seedlings (Changwu134, Xinong928, Shaan253 and Xinong2208) subject to drought stress.

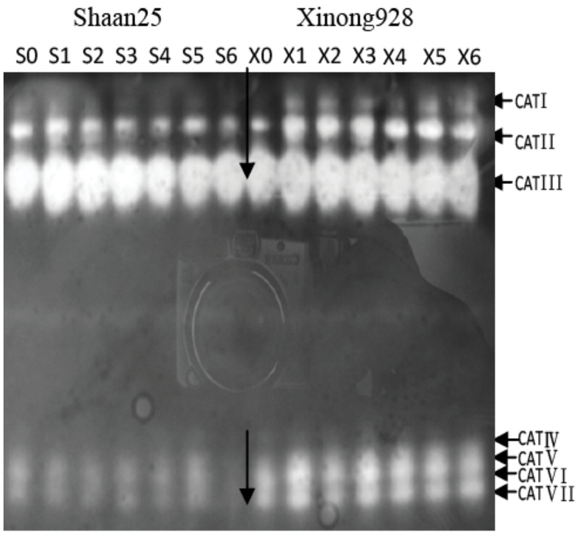


Fig. 4. CAT isozymes in the leaves of wheat seedlings (Shaan253 and Xinong928) subjected to drought stress.

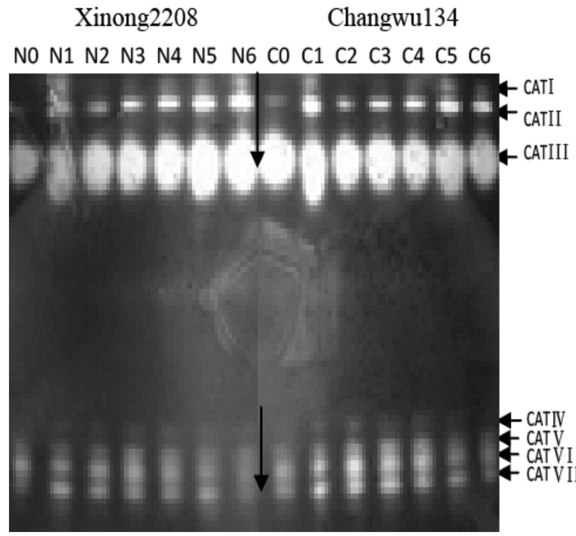


Fig. 5. CAT isozymes in the leaves of wheat seedlings (Xinong2208 and Changwu134) subjected to drought stress.

S: CAT isozymes bands of Shaan253 after 0d,1d,2d,3d,4d,5d and 6d's treatment ; X: CAT isozymes bands of Xinong928 after 0d,1d,2d,3d,4,5d and 6d's treatment; N: CAT isozymes bands of Xinong2208 after 0d,1d,2d,3d,4d,5d and 6d's treatment ; C: CAT isozymes bands of Changwu134 after 0d,1d,2d,3d,4d,5d and 6d's treatment.

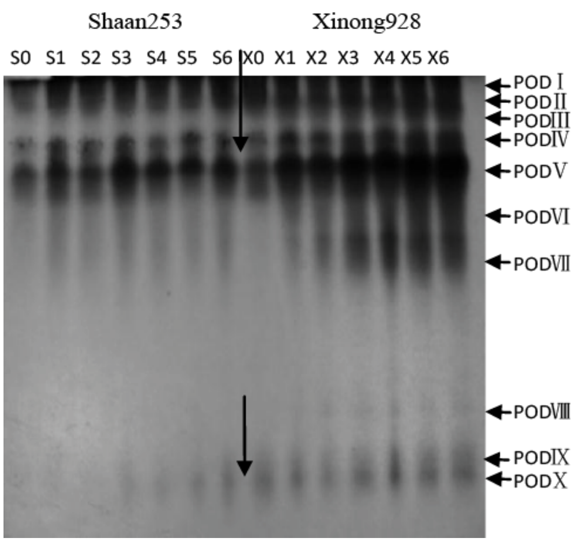


Fig. 6. POD isozymes in the leaves of wheat seedlings (Shaan253 and Xinong928) subject to drought stress.

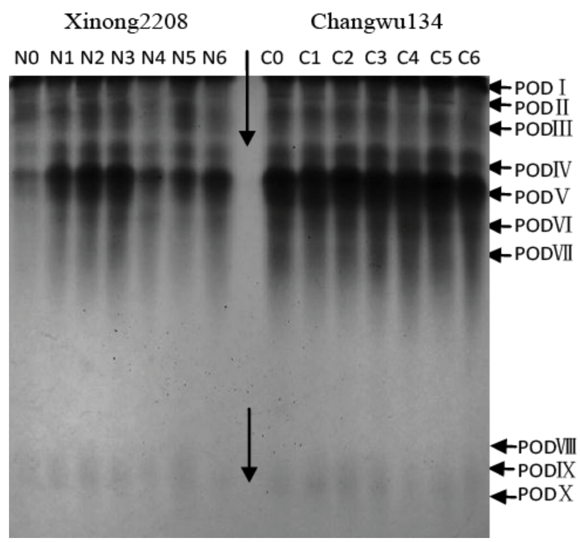


Fig. 7. POD isozymes in the leaves of wheat seedlings (Xinong2208 and Changwu134) subject to drought stress.

S: POD isozymes bands of Shaan253 after 0d,1d,2d,3d,4d,5d and 6d's treatment ; X: POD isozymes bands of Xinong928 after 0d,1d,2d,3d,4d,5d and 6d's treatment; N: POD isozymes bands of Xinong2208 after 0d,1d,2d,3d,4d,5d and 6d's treatment ; C: POD isozymes bands of Changwu134 after 0d,1d,2d,3d,4d,5d and 6d's treatment.

**Table 1. Expression of antioxidant enzymes response to durative drought stress in different wheat genotypes.**

| Wheat genotypes | Antioxidant isozymes | | | |
|---|---|---|---|---|
| | **MDA** | **SOD** | **CAT** | **POD** |
| Changwu134 | Smallest | Strongest | Stronger; sensitive | Strong; insensitive |
| Xinong928 | Small | Weak | Strongest; sensitive | Strong; sensitive |
| Xinong2208 | Great | Strong | Weak; sensitive | Weak; sensitive |
| Shaan253 | Great | weakest | Weakest; insensitive | Weak; insensitive |

**Effects of drought stress on POD isozymes in wheat seedlings:** As shown in Fig. 6 and Fig. 7, only in Xinong928 ten POD isozymes were clearly seen as compared to nine POD isozyme bands in the others cultivars. On the first day of drought stress, the expression of four isozymes, containing POD , POD , POD and POD , clearly increased in the seedling leaves of Shaan253 and Xinong928. But the width of expression enhancement was bigger in Xinong928. On the second day the expression became weak. The expression became strong on the third day, and increased again on the sixth day (Fig. 6). Under continuous drought stress, the expression of PODIX and PODX enzymes was stable with very large quantity. The expression of the PODIX and PODX started to increase slightly after three days of drought stress, but the PODVIII didn't appear obvious expression all the time.

Seven isozyme bands, POD , POD , POD , POD , POD , POD and POD , were observed in the seedling leaves of Xinong2208 and Changwu134, but with obvious differences as seen in Fig. 7. On the first three days of drought stress, the expression of these POD isozymes increased in the Xinong2208 seedling leaves. However, the expression became rapidly weaker on the fourth day of drought stress and increased slightly on the fifth and sixth day. During the sustained drought stress, the POD isozymes had the instability of expression in the Xinong2208 seedling leaves. Though the expression of the seven POD isozymes wasn't clearly induced by drought stress in the seedling leaves of Changwu134, the expression was stable all the time during the continuous drought stress period. Meanwhile, the amount of expression in Changwu134 seedling was larger than that in Xinong2208. The expression of POD and POD in Changwu134 was stronger than in Xinong928.

The difference expression of antioxidant enzyme isozymes and the content of MDA in four different genotypes of wheat were analyzed and compared during the continuous drought stress. The results are shown in Table 1.

## Discussion

Plants often face a series of biotic and abiotic stress under the severe environmental conditions and drought is one of the major abiotic factors that adversely affect crop growth and productivity worldwide (Muhammad *et al*., 2014). Drought is expected to cause serious plant growth problems for more than 50% of the arable lands by 2050 (Vinocur & Altman, 2005). The osmotic effect caused by drought stress can induce a wide range of responses such as growth inhibition, synthesis of some nontoxic compounds, and increase in osmotic potential of the cell in natural and agricultural habitats. When exposed to drought stress conditions, the plants were more susceptible to damage brought by overproduction of ROS. Increased drought tolerance of plants was associated with changes in antioxidant enzymes and maintenance of $H_2O_2$ level. Increased peroxidase activities and superoxide dismutase in response to water stress were reported by Kukreja *et al*. (2005). The SOD activity is responsible for scavenging $O^{2-}$radical to produce $H_2O_2$, increased within three days of drought at tillering stage in both drought susceptible and drought tolerant cultivars (Smirnoff, 1998; Bano *et al*., 2012). It is accepted that $H_2O_2$ is important in complex signal transmission networks. There are many stress reactions involving $H_2O_2$, the homeostasis of $H_2O_2$ is mostly due to catalase scavenging and regulating functions. Thus, $H_2O_2$ accumulation was possibly the trigger of SOD activation. So, the balance between ROS and the antioxidative system is crucial for survival and adaptation to stress.

Stress-induced variation of antioxidant is dependent on the severity and duration of the treatment and also the species and age of the plant. For example, in wheat, SOD activity increased or remained unchanged in the early phase of drought but decreased with prolonged water stress. A large number of studies on various species indicated that drought stress alters the amount and the activities of the enzymes involved in scavenging ROS. In this study, two SOD isozyme bands, seven CAT isozyme bands and nine POD isozyme bands were clearly detected after native polyacrylamide gel electrophoresis (PAGE) analysis in the seedling leaves of wheat under continuous drought stress. We expected that these isozymes were the key isozymes involved in removing ROS in the seedling leaves of wheat. The difference expression of these antioxidant enzymes was very obvious in different genotypes of wheat under continuous drought stress. MDA is a common and important index for evaluating the redox status, lower MDA content means higher antioxidative ability and reflecting higher drought resistance (Izabela *et al.*, 2013). In our experiment, it was shown that the content of MDA was lower and it can delay the progress of water loss, thus they can improve the drought resistance of plants.

Superoxide dismutase (SOD) is a potent antioxidant because it can catalyzes superoxide free radical dismutation into $H_2O_2$ and $O_2$ and be recognized as early defense against free radicals of oxygen (Liu & Huang, 2000). It is the main antioxidant enzyme defender against oxidative stress caused by ROS, which is a major scavenger of $O^{2-}$ free radicals converting into $H_2O$ and $O_2$ by catalase and a variety of peroxidases (Smirnoff, 1998). So, the concentration of $H_2O_2$ should correlate with SOD activity and the ability of species to adapt to the environment. In this study, under the continuous drought stress conditions, SOD isozymes were abundantly expressed in Changwu134 seedling leaves. At the same time, CAT isozymes and POD isozymes were also abundantly and stably expressed. These three key antioxidant enzymes acted synergistically and scavenged the ROS efficiently in vivo. However, under continuous drought stress, the expression of SOD isozymes didn't enhance synergistically. This led to more serious oxidative damage to the cells (Fig. 2). It was mainly because SOD isozymes had stronger expression, but the expression of CAT and POD isozyme didn't increase accordingly. He *et al*. (2012) also found that the bands and activities of protective enzymes were differently under different PEG stress in *dendranthema indicum*. This may lead to the accumulation of $H_2O_2$ in cells. Although $H_2O_2$ inhibited the activity of SOD isozymes, serious oxydic damage can occur with increased active ·OH. Soon Lim *et al*. (2007) found that the CuZnSOD gene of chloroplasts transferred into tobacco can increase the activity of CuZnSOD enzyme in the transgenic plants. The expression of CuZnSOD and APX simultaneously increased and it also improved the ability of anti-oxidation under conditions of stress such as chilling. Young *et al*. (2007) also found that the simultaneous expression of CuZnSOD enzyme and other kinds of antioxidant enzymes can more efficiently improve the ability of multiple environmental stress resistance. We obtained the similar results by the researches in the other transgenic plants. In other words, these transferred antioxidant enzyme genes didn't provide individual protective action. In this study, the expression quantity of CAT isozymes and POD isozymes was very large in Xinong928 seedling leaves under the drought stress, but the expression of SOD isozymes was weaker than that in Changwu134 seedling leaves. Result of our study support the following conclusion: the expression of massive SOD isozymes improves the seedling tolerance to drought stress. The expression of SOD isozymes in different cultivars followed the order: Changwu134> Xinong2208> Xinong928> Shaan253.

CAT can efficiently break down high concentrations of $H_2O_2$ in the seedling leaves of wheat and reduce the damage of ·OH produced by $H_2O_2$. The level of hydrogen peroxide is controlled by CAT in plant cells. The expression difference of CAT isozymes can be reduced by different degrees of drought stress. In this study, no new bands of CAT appeared in the seedling leaves, and only the expression quantity of partial CAT changed along with drought stress. Similar conclusion was reported by Bian and Jiang (2009) for Kentucky bluegrass. The expression of CAT isozymes increased immediately in Xinong928 and Changwu134 seedling leaves which may explain in part high drought tolerance of these cultivars. Furthermore, prolonged expression of CAT isozymes under continuous drought stress efficiently reduce the damage of $H_2O_2$ and ·OH for cells. However, the expression of CAT isozymes was very weak in drought sensitive Shaan253. The expression of CAT isozymes increased slightly in the seedling leaves of Xinong2208, the enhanced amplitude was small, but the expression turned weak quickly under the continuous drought stress conditions which led to the inefficient removal of $H_2O_2$ in Xinong928 and Changwu134. Under prolonged drought stress, the damage to Xinong2208 seedling leaves was greater. The quantity of CAT isozymes expression in different cultivars followed the order: Xinong928> Changwu134> Xinong2208> Shaan253.

POD exists widely in different plants tissues. It is another enzyme involved in the removal of $H_2O_2$. It should be noted that this enzyme participates in controlling slight changes in hydrogen peroxide concentration. Tayebeh and Hassan (2010) found that the POD isoforms were highly capable of increasing the number and intensity in oilseed rape plants after water stress. PODs also influence plant growth, development, lignification, suberization, and cross-linking of cell wall compounds (Passardi *et al.*, 2005). Studies have shown that drought-tolerant plants often have higher POD activity than sensitive plants under stress conditions (Wang *et al*., 2009). In this study, the expression of POD isozymes induced by drought stress increased rapidly in Xinong2208 and Shaan253 seedling leaves. It can't effectively slow down the oxidative damage of cells, even if it may lead to more serious damage. We surmised this was one of the reasons that Xinong2208 and Shaan253 seedling performed poorly when exposed to continuous drought stress.

## Conclusion

Activities of ROS increased in response to water stress (Kukreja *et al*., 2005). Through the trends of the expression of SOD, CAT and POD can be seen that Changwu134 wheat cultivar has the strongest capacity of drought resistance among the four wheat cultivars. The study revealed that greater tolerance to drought stress of Xinong928 cultivar was linked to abundance of CAT and POD activities. However, under continuous drought stress, both Xinong928 and Changwu134 cultivars appeared to lose their drought tolerance. The expression of SOD isozymes were useful quantity in Xinong2208, but the CAT and POD had not stable expression and no synergetic effects.

Through comprehensive analysis the expression of the three antioxidases and relative water content of the four cultivars, we conclude that the seedling leaves which have strong ability of drought tolerance will expression a plenty of SOD isozymes as well as the CAT and POD isozymes to make the plants have stronger drought resistance when under the condition drought stress. This may reveal the appropriate relationship between the physiological and biochemical indexes and the mechanism of drought resistance in wheat.

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