

MEMORY RESPONSE OF PEPPER ANTIOXIDANT SYSTEM TO CYCLES OF DROUGHT AND RE-WATERING STRESS

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Abstract

Stress causes a stress memory in some plants, which appears to play a vital role in responding to repeated stress conditions. However, it's uncertain whether pepper has drought stress memory and, if so, what the physiological process is. We investigated the creation of pepper drought memory following the half-wilting and re-watering treatment cycles and the induction of the reaction oxygen species (ROS) scavenging system by evaluating antioxidant content, activity, and gene expression of antioxidant enzymes. The results indicated this treatment can significantly increase the ASA and GSH content in pepper seedlings under 5 cycles of drought and re-watering stress, and significantly increase the POD, SOD, CAT, APX, GPX, and GR activities and gene expression; This physiological and biochemical change improves the ability of pepper seedlings to remove ROS, so its ROS content is lower than that of untreated pepper seedlings under drought stress. In the end, the MDA content was remarkably lower than the untreated, indicating that the damage to the plants was less than that of the untreated. These results indicate that the antioxidant defence system is associated with memory response to circular drought and re-watering stress in pepper.

Key words: Pepper (*Capsicum annuum* L.); Drought and re-watering stress; Antioxidant defense system; Stress memory.

Introduction

Plants are subjected to a variety of environmental challenges throughout their lives (Bruce *et al.*, 2007). Their stress responses after that are frequently different from the first time (Ding *et al.*, 2013; Avramova, 2015). Repeated exposure to identical stress conditions can improve a plant's stress resistance, implying that plants have a "memory" for similar environmental stress. (Wang *et al.*, 2014; Avramova, 2015; Li *et al.*, 2015). Multiple droughts with re-watering treatments at the seedling stage of plants are effective methods for plants to increase their stress resistance, but the possible physiological mechanisms are still unclear. Water is an important substance for plant life activities and has a vital limiting role for plant growth and development, photosynthesis and yield. Water stress can cause the water potential of the leaves to drop and cause the stomata to close, thereby limiting the absorption of CO₂ and photosynthesis, seriously affecting the plant's growth, and ultimately leading to plant death (Wang *et al.*, 2003; Mahajan & Tuteja, 2005; Beck *et al.*, 2007; Farooq *et al.*, 2009). Many cultivation strategies have been used in agricultural production to improve the drought tolerance of crops. Drought and re-watering therapy during the seedling stage is one of them, and it's a good way to boost the plants' drought tolerance. However, there hasn't been any mention of it in pepper (*Capsicum annuum*).

In the process of drought stress, reactive oxygen species (ROS) such as H₂O₂ and O₂⁻ was accumulated in the plants, leading to cell damage (Gechev *et al.*, 2002; Luna *et al.*, 2004). At the long-term evolution process of plants, a more complete antioxidant system has been formed to scavenge ROS, thereby maintaining the balance of ROS (Langebartels *et al.*, 2002; Bai *et al.*, 2006). In the initial stage of adversity stress, to resist the ROS

poisoning of cells, plant cells often actively initiate these ROS scavenging mechanisms. Studies have shown that in the process of drought conditions, the content of antioxidant and activity of an antioxidant in plants increases (Foyer & Noctor, 2005; Gill & Tuteja, 2010; Bian & Jiang, 2009; Osipova *et al.*, 2011). These studies indicate that the antioxidant defence system have a vital function in plants respond to drought stress.

Despite the fact that plants have a variety of techniques to cope with dryness, drought remains a major limiting factor in plant dispersion and growth (Guo *et al.*, 2016). Drought and re-watering stress memory (DRW) has been studied in willow switch (Zhang *et al.*, 2018), corn (Virilouvet *et al.*, 2018), and rice (Zhang *et al.*, 2018) (Li *et al.*, 2019). Pepper, on the other hand, does not appear to have the same physiological responses, and the mechanism of pepper DRW processing "memory" is unclear. Pepper is a very important vegetable. Pepper often suffers from drought, which has a serious impact on its growth and yield. We have used 5 circulars DRW in the late stage of seedling to improve drought tolerance after transplanting in pepper output, although the theoretical basis for this is absent. . In this study, by measuring the changes of ROS and MDA and antioxidant content, gene transcription level and protein activity of an antioxidant enzyme in pepper seedling leaves under 5 circulars DRW, to explore the effect of antioxidant system in improving drought resistance of pepper plants provides new insights.

Materials and Methods

Materials: In all the trials in this investigation pepper (*Capsicum annuum* L. CV. Qiubei) was used. Pepper seeds that were full-grained and of good quality were chosen and disinfected with a 75 percent alcohol solution for 8S. Pepper

seeds were germinated at 28°C in a petri dish with a layer of disinfectant filter paper on the bottom. The germinated seeds were planted in a 32-hole plug with the substrate (perlite: mulch soil = 1:2 volume ratio) and grown in a light incubator maintaining temperature 28±1°C, relative humidity 65-75 percent, light intensity 200 mol s⁻¹ m⁻², and the photoperiod 16L/8D. To continue cultivation, pepper seedlings with 5-6 true leaves were moved to the cultivation chamber. The seedlings were watered as usual and used in future trials. (1) Seedlings were watered normally as a control (CK); (2) Seedlings were subjected to natural drought as a drought treatment (T); (3) Seedlings were subjected to natural drought and re-watered when half-wilted until the leaves returned to normal. To improve stress memory, the DRW was repeated for 5 rounds. The seedlings were watered normally after 5 cycles of DRW and then naturally drought treated as the DRW (R5). Every morning at 10:00, the leaves of CK, T, and R5 were gathered on the 0th, 1, 2, 3, 4, and 5th days following treatment (Generally, the leaves of seedlings after 5 days of natural drought are half wilted). MDA, ROS, and antioxidant content determination, antioxidant enzyme activity, and gene expression analysis were all performed on leaves that had been treated with liquid nitrogen and maintained at -80°C.

Physiological and biochemical measurements: The O₂⁻ production rate was investigated according to the method of Elstner & Heupel (1976). The content of O₂⁻, H₂O₂, MDA, AsA, GSH were measured using classic methods (Elstner & Heupel, 1976; Patterson *et al.*, 1984; Hodges *et al.*, 1999; De Pinto *et al.*, 1999; Griffith, 1980). The activities of POD, SOD, CAT, APX, GPX and GR were analyzed following the procedure of Kochba *et al.*, (1977),

Giannopotics and Ries (1977), Jiang *et al.*, (1982), Chen and Asada (1989), Flohe & Gunzler (1984) and Foyer and Halliwell (1976).

Relative gene expression analysis: Gene expression was performed according to the method of Bian & Jiang (2009). Genespecific RT-PCR primers are designed by Primer 5.0 for RT-PCR analysis. Prime pairs were shown in Table 1. The 2^{-ΔΔCT} method was used to measure the relative gene transcription level (Livak & Schmittgen, 2001).

Data processing: All experiments performed for three technical and three biological replicates. Analysis of variance was performed using SPSS13.0 and difference significance detection was carried out using Duncan's new multiple range method. Values are the mean ± SD.

Results

Effect of DRW on the production rate and content of O₂⁻: According to studies, the accumulation of O₂⁻ in pepper seedlings that were irrigated routinely remained essentially constant. In the first two days under drought stress, the rate and amount of O₂⁻ in all materials were not significantly different from the CK. The production rate and content of O₂⁻ in R5 pepper seedlings were increased, but at a slower rate; however, the production rate and content of O₂⁻ in T pepper seedlings were increased rapidly, and was significantly higher than that of T and R5 plant seedlings (Fig. 1), indicating that 5 circulars DRW could reduce the production rate and content of O₂⁻ in pepper seedlings.

Table 1. Specific primers used for qRT-PCR.

Gene	Primer sequence	T _a °C	GenBank ID
<i>POD</i>	F: ATG CCC TGG TGT TGT TTC R: CCT TGG CTT GCT GTC CTG	55°C	FJ596178
<i>Mn-SOD</i>	F: TGG AGC CTT AGA ACC TGC R: CCG CCA TTG AAC TTG ATA	55°C	AF036936
<i>Cu/Zn-SOD</i>	F: TGT CGC CGT CCT TAG CAG C R: TTC CGC CAG CGT TTC CAG T	55°C	AF009734
<i>Iron-SOD</i>	F: GGG GAA GCA TCA CAG GAC C R: AGC CCA CCC AGA ACC AAA T	55°C	AY173123
<i>CAT1</i>	F: AGG AGG TGG ATT ATT TGC C R: CCA GCC TGC TTG AAG TTG T	60°C	AF227952
<i>CAT2</i>	F: TAC CAC AAG ATT ACA GGC ACA R: TTG GAG CAT TAG CAG GAA G	55°C	AY128694
<i>CAT3</i>	F: CCC ACA AGA TTA CAG GCA CA R: CAT TCA AGA CAC GAG GAG G	55°C	AY128695
<i>APX</i>	F: TCC TAT TAT GCT CCG TCT CG R: AAC AGG TGG TTC TGG CTT G	57°C	DQ002888, AY078080
<i>GPX</i>	F: CTG ACT AAT TCA AAC TAC ACC GAC AT R: ACC ATC ACC AAA GAA CCC ACC T	60°C	AJ973135
<i>GR</i>	F: ATG GAT GGG AAG TGA ATG AGA R: TTA GAA TGT GCT TTG CCG AAT	57°C	AY547351
<i>β-ACTIN</i>	F: TGC AGG AAT CCA CGA GAC TAC R: TAC CAC CAC TGA GCA CAA TGT T	57°C	DQ252512

Effect of DRW on H_2O_2 content: Under drought stress, DRW has a significant impact on H_2O_2 levels in pepper seedlings. Under drought stress, the H_2O_2 level of R5 pepper seedlings was increased on the third day and was higher than the CK. On the third day, the H_2O_2 level in T pepper seedlings began to rise fast, and it was much higher than the H_2O_2 content in R5 pepper plants (Fig. 2). This revealed that pepper seedlings may lower the emergence of H_2O_2 and the damage caused by drought stress after 5 DRW circulars.

Effect of DRW on MDA content: The results showed that 5 circulars drought and re-watering treatments could significantly reduce the MDA content in pepper seedlings under drought stress, and reduce the damage of drought stress to cells. Under drought stress, the MDA content of R5 pepper seedlings was slightly lower than that of the CK at the initial stage. As the drought stress continued, the MDA content increased slightly, but it reached a significant level compared with the CK over the same period, but it was significantly lower than that of T pepper seedlings (except day 0) (Fig. 3). It showed that 5 circulars drought and re-watering treatments can significantly reduce the damage of drought stress to pepper seedling.

Effect of DRW on protein activity and gene transcription level of antioxidant enzyme: DRW has the potential to boost the activity of the POD enzyme, improve its ability to eliminate ROS, and reduce ROS generation in pepper seedlings. POD protein activity was increased in T pepper seedlings under drought stress, but it was much lower than that of R5 pepper seedlings, implying that its ability to breakdown active oxygen was also significantly lower than that of R5 pepper seedlings (Fig. 4). The gene transcription level data revealed that the *POD* gene transcription level and POD protein activity trended in the same direction (Fig. 4).

SOD protein activity in pepper seedlings was increased rapidly under drought stress. But SOD protein activity in R5 pepper seedlings was increased sharply, and also the ability to remove $O_2^{\cdot-}$ was sharply enhanced which effectively prevented $O_2^{\cdot-}$ excessive accumulation. Under drought stress, the SOD protein activity in T pepper seedlings also showed a rising trend, but their SOD protein activity was extremely significantly lower than that of R5 pepper seedlings (Fig. 4). The gene transcription level results indicated that the *SOD* gene transcription level was consistent with the trend of SOD protein activity (Fig. 4).

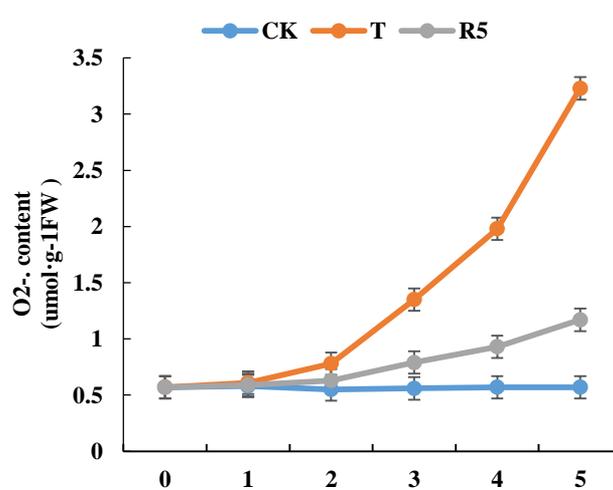
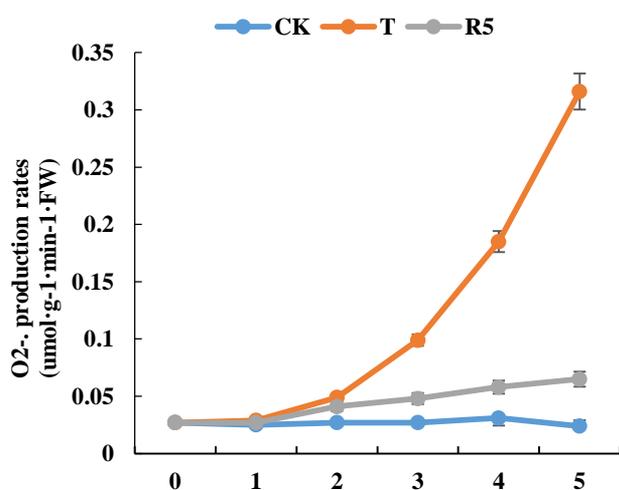


Fig. 1. Effect of DRW on the $O_2^{\cdot-}$ production rate and content.

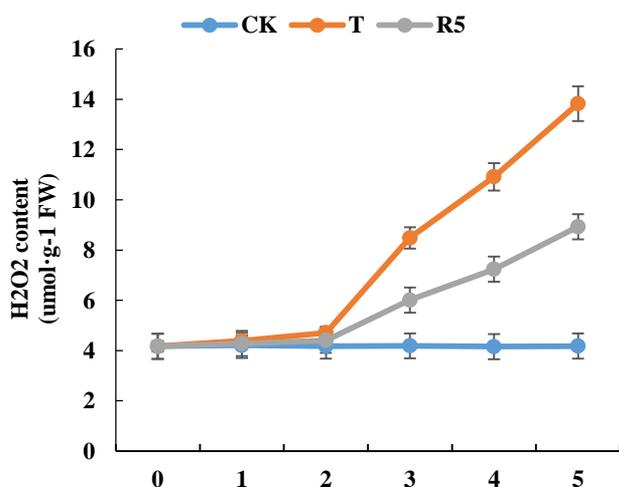


Fig. 2. Effect of DRW on H_2O_2 content.

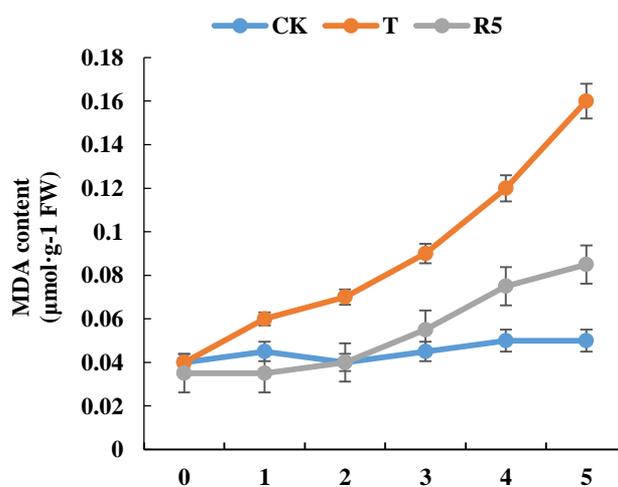


Fig. 3. Effect of DRW on MDA content.

The findings of the experiments revealed that during drought stress, the protein activity of CAT in pepper seedlings was increased. R5 pepper seedlings, on the other hand, had a faster rate of growth in CAT protein activity than T pepper seedlings, and their protein activity was noticeably higher. This increased CAT protein activity improved cells' ability to breakdown H_2O_2 and lowers H_2O_2 's damaging effects on cells (Fig. 4). The gene transcription level data revealed that the *CAT* gene transcription level and CAT protein activity trended in the same direction (Fig. 4).

Effect of DRW on antioxidant content: The findings revealed that the DRW might boost AsA levels, improve ROS removal, and improve resistance to oxidative stress. The AsA content of R5 pepper seedlings was significantly higher than that of the CK under drought stress, and their ability to clear ROS was also significantly higher than that of the CK. However, the AsA content of T pepper seedlings continued to decline in the late drought period, significantly lower than that of the CK and extremely significantly lower than that of R5 pepper seedlings (Fig. 5). In the early stages of dryness, as shown in Figure 5, the GSH content in pepper seedlings gradually grew and was higher than the CK; As the drought continued, the amount of GSH in R5 pepper seedlings increased, as did their ability to destroy ROS. T pepper seedlings' GSH content began to drop dramatically on the fourth day of dehydration, and was lower than CK, and much lower than R5 pepper seedlings' GSH content.

Effect of DRW on the protein activity and gene transcription level of antioxidant substances-related enzymes: In the early stages of drought, the protein activity of APX in pepper seedlings was nearly identical to that of CK (Fig. 6). The activity of the APX protein in pepper seedlings was increased as the drought proceeded. However, the APX protein activity in R5 pepper seedlings increased dramatically, significantly higher than in CK and T pepper seedlings, indicating that its ability to remove H_2O_2 was also significantly higher than in CK and T pepper seedlings, preventing excessive H_2O_2 accumulation and affecting plant growth. The relative transcription level of the *APX* gene was largely compatible with the trend of APX protein activity, according to the gene transcription level data (Fig. 6).

Under drought stress, GPX protein activity in pepper seedlings was stimulated and showed an increasing trend. However, since the first day, the GPX protein activity in R5 pepper seedlings was increased dramatically and was significantly higher than that of T pepper seedlings. Higher GPX protein activity indicates a greater ability to remove H_2O_2 , which can prevent excessive H_2O_2 accumulation in the plant and affect cell activity. T pepper seedlings' GPX protein activity was slightly higher than that of the CK in the early stages of drought stress (the first three days), and it was not significantly higher than that of the CK until the fourth day, but it was extremely significantly lower than that of R5 pepper seedlings, indicating that their ability to remove H_2O_2 was lower than that of R5 pepper seedlings (Fig. 6). The relative transcription level of the *GPX* gene was largely compatible with the trend of GPX protein activity, according to the gene transcription level data (Fig. 6).

Under drought stress, GR protein in pepper seedlings was increased. R5 pepper seedlings had a faster rate of GR protein activity enhancement than T pepper seedlings, and their GR protein activity was also significantly higher than T pepper seedlings. The ability to produce GSH was also far superior to that of T pepper seedlings (Fig. 6).

Discussion

Plants that have been exposed to abiotic stress in the past may respond differently to subsequent stress (Auler *et al.*, 2017; Ding *et al.*, 2012, 2014; Avramova *et al.*, 2015). Drought preconditioning can have an impact on memory (Avramova *et al.*, 2015). Drought stress causes an increase in plant ROS, which act as a signal in the plant's response to stress. However, excessive ROS in cells can cause oxidative stress, disrupt the biofilm, and alter the DNA structure (Møller & Sweetlove 2010; Suzuki *et al.*, 2011). When plants are subjected to drought stress, their antioxidant system plays a critical role in their ability to withstand the stress. Plants have antioxidant enzymes and tiny compounds that can scavenge ROS and help them adapt to drought stress (Thapa *et al.*, 2011).

POD, SOD, CAT, APX, GPX, GR, and other antioxidant protection reactions in plants are connected with POD, SOD, CAT, APX, GPX, GR, and others (Reddy *et al.*, 2004; Navrot *et al.*, 2007; Beck *et al.*, 2007; Jaleel *et al.*, 2009). These enzymes collaborate to transform O_2^- and H_2O_2 into H_2O and O_2 , thereby easing oxidative stress and lowering plant damage. When plants are exposed to adversity, they often actively adjust the concentration of antioxidant molecules (AsA, GSH, etc.) in order to eliminate ROS, resist, and slow down their cell damage (Reddy, 2004; Fazely, 2007; Yang *et al.*, 2008; Faize *et al.*, 2011; Vaseva *et al.*, 2012).

We investigated the MDA produced as the drought progressed to represent peppers' recall abilities. R5 pepper seedlings had a reduced MDA level, which could signal that they were less damaged and had superior drought tolerance. The first drought stress generated a significant increase in the formation of ROS, which worked as a cell signal to trigger a series of physiological and biochemical processes in response to the drought.

Under drought stress, the concentration of ROS in pepper seedlings increases. These ROS operate as second messengers in the plant, activating the antioxidant system and allowing the plant to adjust to drought stress. The transcription levels of *POD*, *SOD*, and *CAT* are up-regulated as the drought develops, and the protein activities of POD, SOD, and CAT increase, boosting the ability to eliminate ROS. However, *POD*, *SOD*, and *CAT* transcription levels and protein activities were significantly higher in R5 pepper seedlings than in T pepper seedlings, implying that R5 pepper seedlings' ability to remove ROS is significantly stronger than that of T pepper seedlings, resulting in lower ROS content in R5 pepper seedlings. After 5 rounds of DRW, the protein activities of POD, SOD, and CAT, as well as the transcription level of coding genes in pepper seedlings (R5), were significantly higher than in pepper seedlings (T) that had never been exposed to drought stress. It implies that pepper seedlings have drought memory, and that the antioxidant enzyme system is involved in the establishment of drought memory.

To adapt to drought stress, these ROS operate as second signals, regulating antioxidant content (AsA, GSH, etc.) and related enzyme protein activities. The content of antioxidants (AsA, GSH, etc.) was increased steadily during the early stages of the drought. However, the performance was inconsistent later on: the antioxidant substance content in R5 pepper seedlings continued to rise, and the ability to remove ROS was increased. However, the antioxidant content of T pepper seedlings continued to decline, and the ability to remove ROS was decreased. As the drought progressed, the transcription levels of genes encoding *APX*, *GPX*, and *GR* were increased, as did the related protein activities. *APX*, *GPX*, and *GR* gene transcription and protein activity were considerably higher in pepper R5 seedlings

than in T pepper seedlings. As a result, R5 pepper seedlings had a much higher ability to eliminate ROS than T pepper seedlings, as well as a significantly higher ability to create GSH than T pepper seedlings. Finally, the R5 pepper seedlings are better at eliminating ROS than the T pepper seedlings, decreasing and reducing the oxidative stress and damage induced by dryness, and boosting drought resistance. After 5 rounds of DRW, the content of antioxidant substances (AsA, GSH), related enzyme protein activities, and gene transcription level of pepper seedlings (R5) were significantly higher than those of pepper seedlings (T) subjected to drought stress for the first time, implying that antioxidants and related enzymes were involved in the formation of drought memory in pepper seedlings.

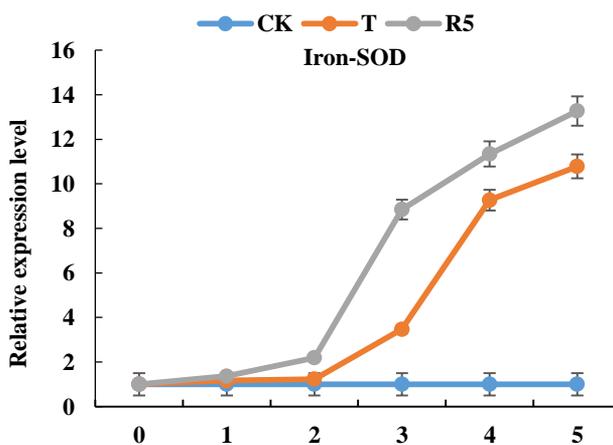
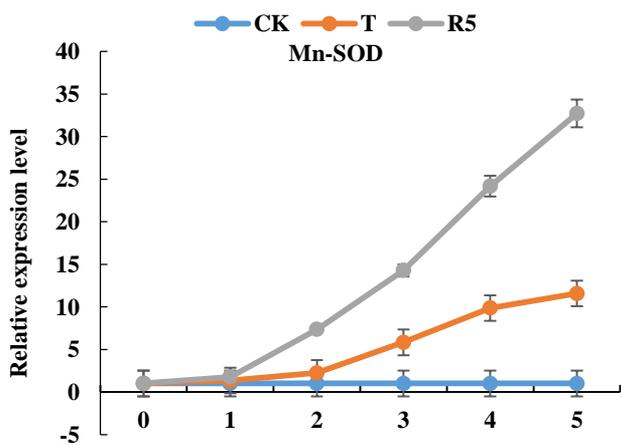
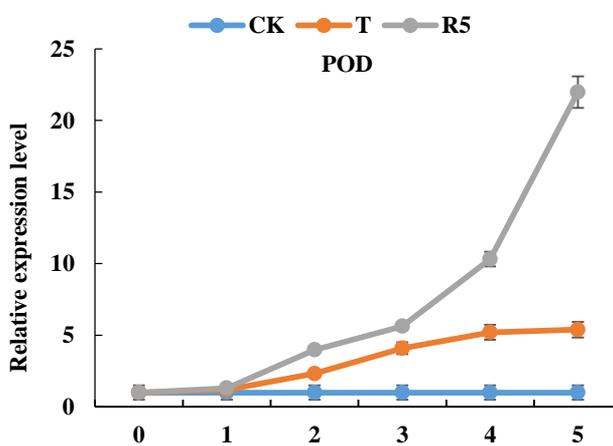
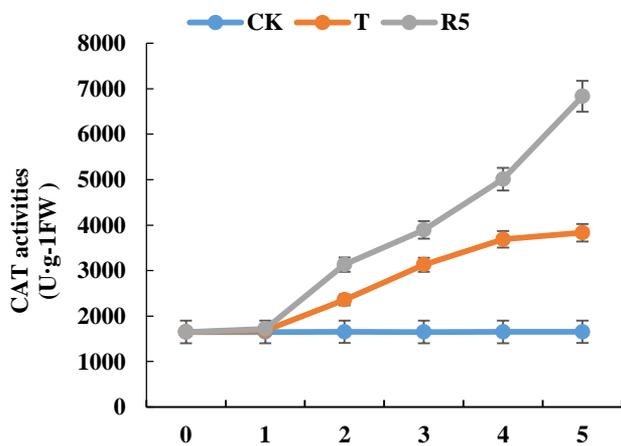
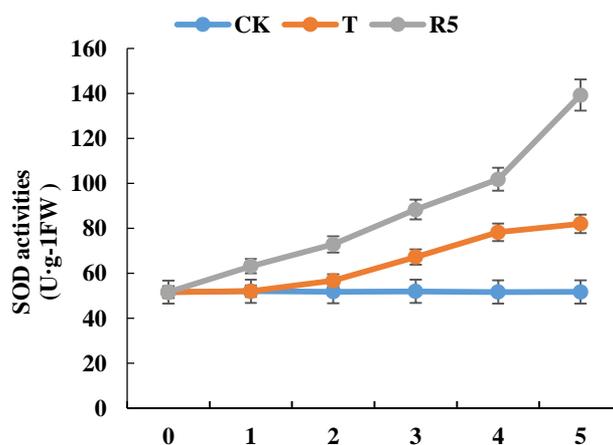
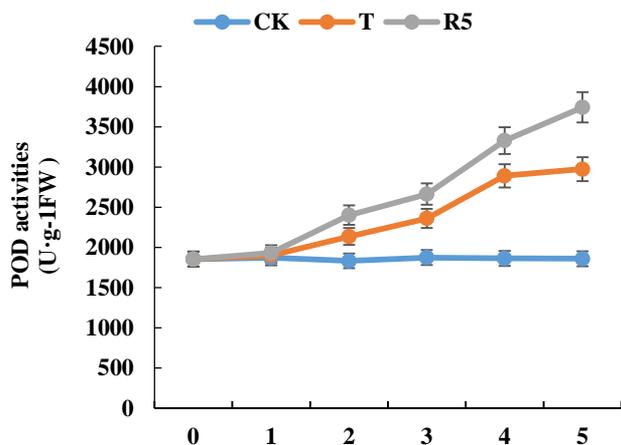


Fig. 4 (Cont'd.)

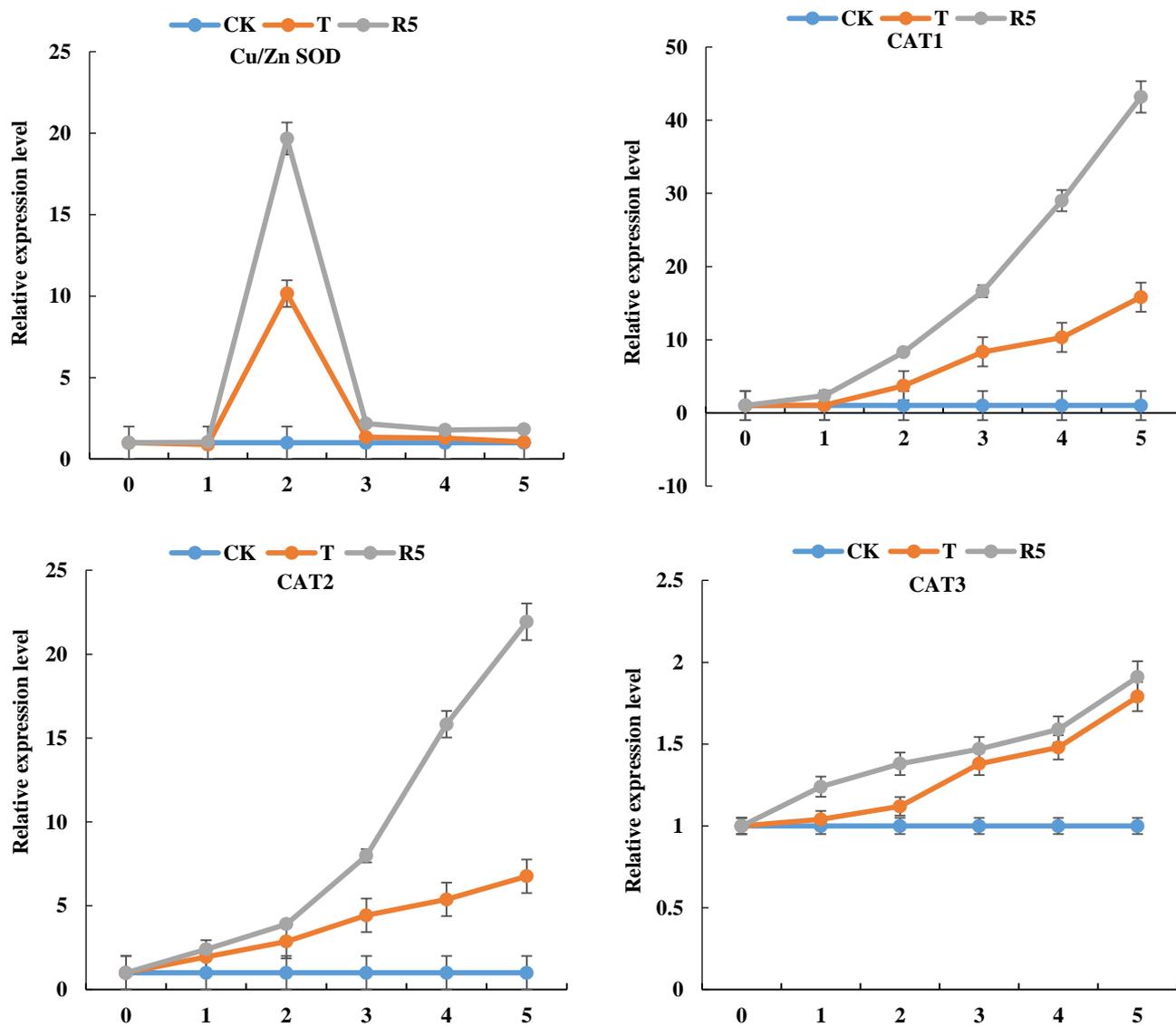


Fig. 4. Effect of DRW on protein activity and gene transcription level of antioxidant enzyme.

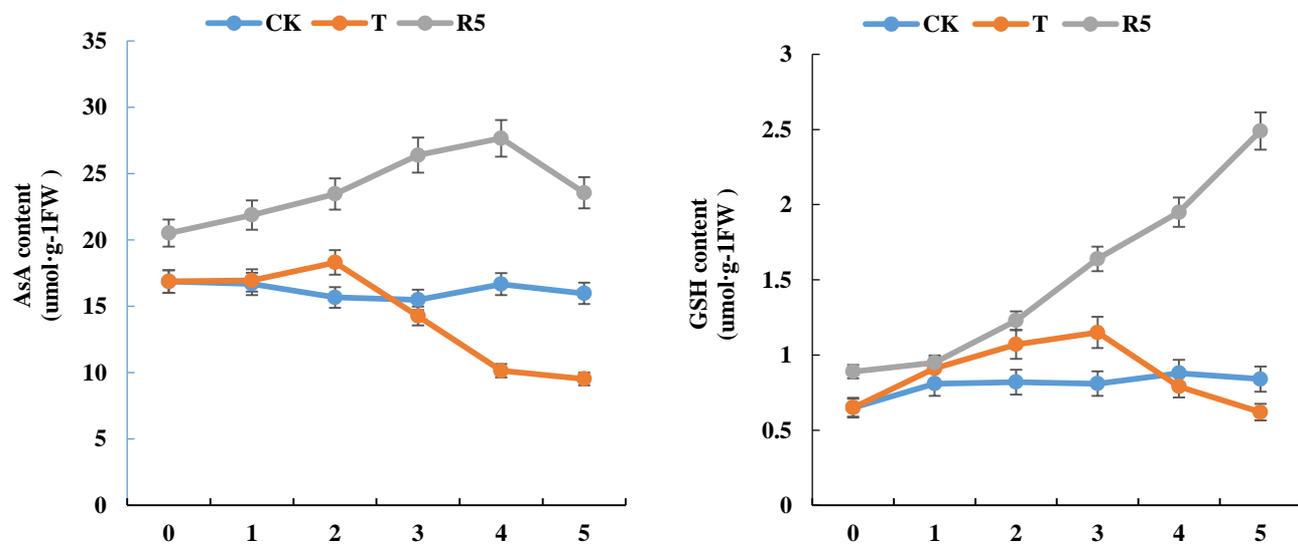


Fig. 5. Effect of DRW on Antioxidant content.

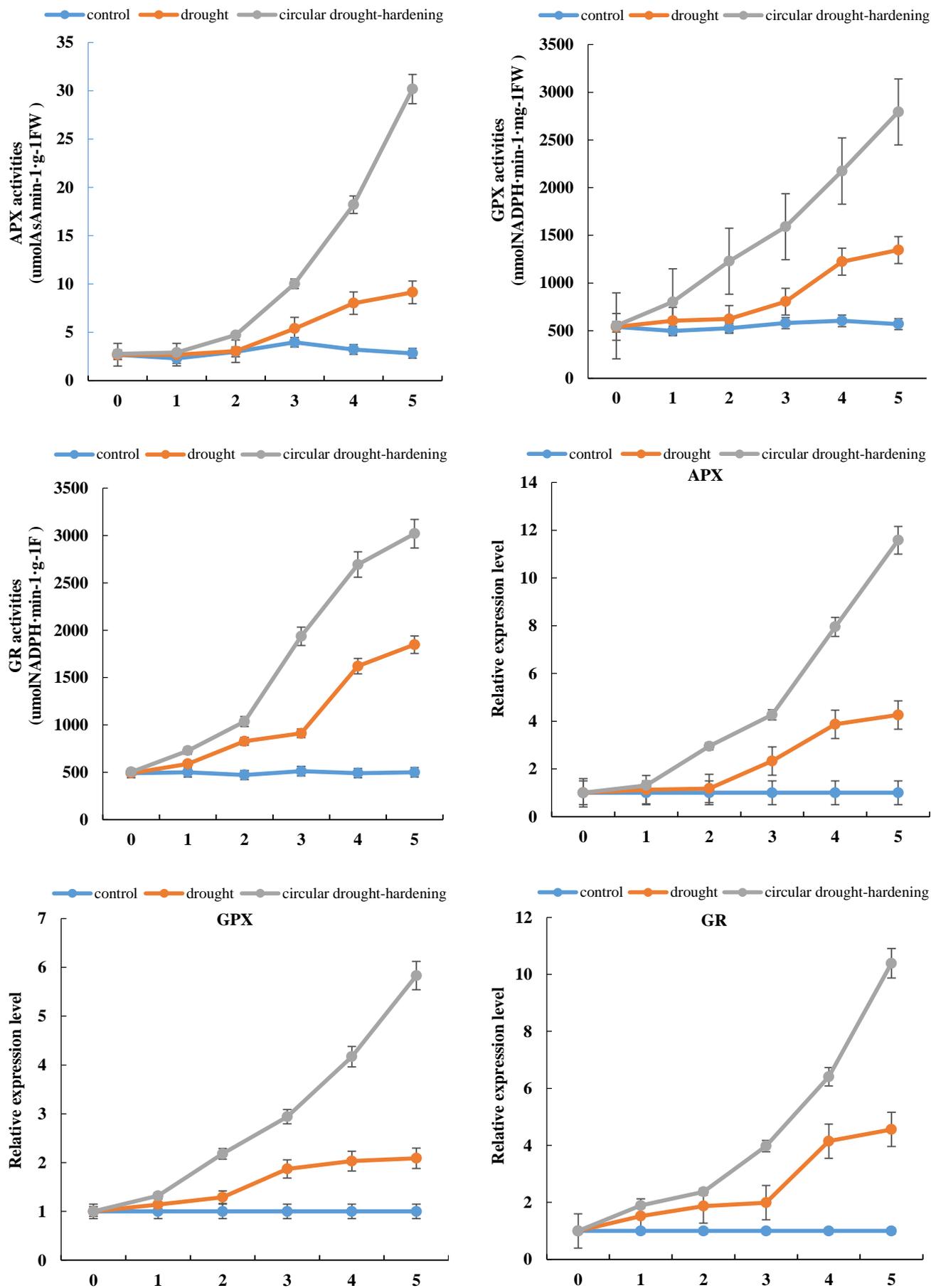


Fig. 6. Effect of DRW on the protein activity and gene transcription level of antioxidant substances-related enzymes.

Acknowledgements

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