African Journal of Biotechnology Vol. 9(49), pp. 8378-8391, 6 December, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Over expression of CuZn superoxide dismutase (CuZn SOD) and ascorbate peroxidase (APX) in transgenic sweet potato enhances tolerance and recovery from drought stress

Yan-Yuan Lu[1,2], Xi-Ping Deng[1*] and Sang-Soo Kwak[3]

[1]State key laboratory of soil erosion and dry land farming on loess plateau, northwest A and F University, xinong road No.26, yangling, Shaanxi 712100,P.R. China.
[2]College of life sciences, northwest A and F University，yangling, Shaanxi 712100, P.R. China.
[3]Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Oun-dong 52, Yuseong-gu, Daejeon, Republic of Korea.

Accepted 1 November, 2010

To understand the antioxidant stress of transgenic sweet potato containing elevated Cu/Zn superoxide dismutase (Cu/Zn SOD) and ascorbate peroxidase (APX) in chloroplasts, we analyzed plant growth, yield, water status, photosynthetic activity and several antioxidant enzyme activities under drought and post-drought re-watering conditions. Three water stress regimes - no, moderate and severe water stress - were applied. Our results indicated that the number of transgenic plants (TS) was a function of water deficit and recovery period. Compared with non-transgenic plants (NS), the expression of antioxidant enzymes (SOD, APX and CAT) in transgenic plants was profoundly increased under drought stress and rewatering periods. Transgenic plants exhibited better growth, photosynthetic activity (Fv/Fm) and water status under drought stress, but tuberization was poor. Compared with NS plants, TS plants exhibited low levels of malondialdehyde (MDA) and electrolyte leakage (EL). In addition, TS plants recovered faster upon release from drought stress. These results showed that expression of Cu/Zn SOD and APX in chloroplasts of sweet potato enhanced drought resistance and capacity for recovery from drought stress. However, tuber formation in TS plants was constrained by drought stress.

Key words: Transgenic sweet potato, antioxidant enzyme, drought, recovery.

INTRODUCTION

Plants are often exposed to abiotic stresses such as salinity, extremes of temperature, ozone and drought, which reduces yields by up to 70% worldwide. Drought is one of the major environmental stresses limiting plant growth, and consequently, crop yield (Reddy et al., 2004).

Acclimation of plants to drought is often associated with increased levels of reactive oxygen species (ROS), such as superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-) and singlet oxygen ($_1O^2$). Excessive levels of ROS can damage lipids, cellular structures and macromolecules, DNA and carbohydrates, while causing photoinhibition of photosynthetic apparatus; it can even lead to cell death if not scavenged well in the proper time (Sofo et al., 2005). Therefore, how to remove ROS becomes critical for plant growth and survival during stressful conditions (Sandermann, 2004).

*Corresponding author. Email: dengxp@ms.iswc.ac.cn. Tel: +86 29 87012437. Fax: +86 29 87012210.

Abbreviations: ROS, Reactive oxygen species; **SOD,** superoxide dismutase; **APX,** ascorbate peroxidase; **CAT,** catalase; **POD,** peroxidase; **MV,** methyl viologen; **NS,** non-transgenic plants; **PEG,** polyethylene glycol; **RWC,** relative water content; **MDA,** malondialdehyde; **TBARS,** 2-thiobarbituric acid-reactive substances; **NBT,** nitro blue tetrazolium; **TS,** transgenic plants; **EL ,** electrolyte leakage.

Plants have developed various mechanisms to cope with the oxidative stresses caused by unfavorable environments, such as modulating the expression of stress tolerance genes and synthesizing compatible solutes (Ahmad et al., 2010). Antioxidant defense systems are well know for scavenging ROS produced in different stressful conditions, such as activation of the antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and peroxidase (POD) (Allen et al., 1997; Kwon et al., 2002). The SOD converts superoxide radicals ($O_2^{-\cdot}$) into hydrogen peroxide (H_2O_2). POD reduces H_2O_2 to water using various substrates as electron donors. APX uses ascorbate as an electron donor to reduce H_2O_2 to water. Also, CAT dismutates H_2O_2 into water and oxygen. Numerous studies demonstrate that antioxidant defense system improves the relationship between enhanced or constitutive antioxidant enzyme activities and increased resistance to drought stress (Türkan et al., 2005). Previous studies showed that the over-expression of antioxidant genes resulted to enhanced tolerance of drought in transgenic plants (TS). However, drought tolerance is a complex trait that is influenced by coordinated expression of a network of genes (Xiao et al., 2007), which cannot be increased substantially by introducing any single gene, suggesting that over-expression of one enzyme may not alter the function of the entire antioxidant pathway. Previous studies also showed that simultaneous expression of antioxidant genes enhanced protection compared with single gene expression in transgenic plants (Zhou et al., 2008). Kwon et al. (2002) demonstrated that simultaneous expression of Cu/Zn SOD and APX genes in tobacco chloroplasts enhanced tolerance to methyl viologen (MV) stress compared to expression of either of these genes alone. Subsequently, Tang et al., (2006) and Lee et al., (2007a) reached similar conclusions in transgenic potato and tall fescue. Simultaneous expression of choline oxidase (codA), superoxide dismutase (SOD) and ascorbate peroxidase (APX) in potato resulted to enhanced protection of these SSAC plants and lower levels of H_2O_2 compared with expression of both SOD and APX genes in SSA and non-transgenic plants (NS) after methyl viologen, drought and salt-mediated oxidative stress (Ahmad et al., 2010).

Sweet potato (*Ipomoea batatas* L.) is characterized by a simple sink and source relationship in the biomass production system, suggesting that the crop has two beneficial features: a quick yield during a short growth period and a stable productivity (Kubota, 2003). Therefore, sweet potato is cultivated worldwide as a valuable source of food, animal feed and industrial raw material. Up to date, it is one of the most important food crops in many regions of the world, particularly in China, Indonesia, South Korea and Papua New Guinea, ranking fifth after wheat, maize, rice and potato (Fan et al., 2005). Although sweet potato is considered to be drought-tolerance compared with other crops, long and severe drought can significantly reduce productivity, especially tuber yield. The recent global warming tendency could accelerate, worldwide, the frequent occurrence of water deficit. Thus, it is crucial to develop crops with increased drought tolerance by gene transfer approaches. A number of attempts have shown the possibility for enhanced tolerance to extreme environments through introduction of genes encoding antioxidant enzymes (Kasukabe et al., 2006).

Transgenic sweet potato plants expressing both Cu/Zn SOD and APX genes in chloroplasts under the control of *SWPA2* promoter enhanced tolerance of oxidative stress induced by MV and chilling (Lim et al., 2007). Li et al. (2006; 2007) used polyethylene glycol (PEG) in hydroponics to test response of plants under a given osmotic potential and found that transgenic sweet potato plants showed increased tolerance of oxidative stress caused by PEG stress. However, we noticed that most of the previous reports focused on transgenic plants responses to oxidative stress during stressful conditions, and less has been done about the capacity of recovery from drought (Flexas et al., 2004). Furthermore, there is little understanding of the regulatory mechanisms of plant recovery from different water stress intensities (Kirschbaum, 1987). Therefore, further investigations are needed to elucidate the mechanisms of recovery from drought in the field, especially when an increased frequency of drought events is predicted for the coming decades. This could also improve predictions about ecosystem productivity or better irrigation systems (Cai et al., 2005; Galle et al., 2007; Miyashita et al., 2005; Souza et al., 2004).

In the present investigation, the potential role of antioxidant enzymes in enhancing tolerance to drought stress and recovery capacity of transgenic sweet potato was examined by analyzing enzymes (SOD, APX and CAT) activities. We also investigated the effects of drought on the basic parameters, including relative water content (RWC), chlorophyll fluorescence (F_v/F_m), malondialdehyde (MDA) and leakage of electro (EL) in leaves of sweet potato.

MATERIAL AND METHODS

Non- transgenic sweet potato (*Ipomoea batatas L. cv. Yulmi*) and transgenic lines, over-expressing both of CuZnSOD and APX in chloroplasts (Kim et al., 2003; Li et al., 2004) were selected for the study. Construction of the expression vector is shown in Figure 1. Transgenic gene identification (isoenzyme analysis) and plant regeneration were done at Environmental Biotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (Lin et al., 2007); these were transplanted to water and soil.

Experimental setup

A pot experiment to evaluate drought tolerance and recovery capacity of transgenic and non- transgenic sweet potato lines was carried out in the experimental field of the Institute of Soil and

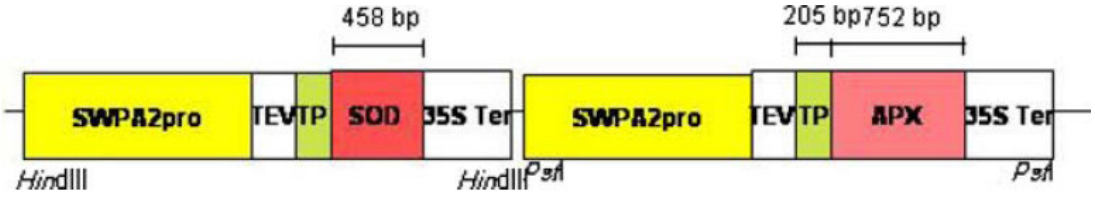

Figure 1. Construction of expression vector, TS *SWPA2*pro Sweet potato peroxidase (*SWPA2*) promoter. SOD =cassava CuZnSOD (mSOD1), APX = pea ascorbate peroxidase, TEV = tobacco etch virus 5'-untranslated region, 35S = Ter CaMV 35s terminator, = TP chloroplast-targeted transit Peptide (Lim et al., 2007).

Water Conservation, Yangling Shaanxi, China, from 30th May to 15th September, 2007. Treatments were a factorial combination of three watering regimes – severe drought-stress (35% of the field water capacity), moderate drought-stress (60% of the field water capacity) and adequate water supply (control - 80% of the field water capacity) with transgenic and non- transgenic sweet potato lines; re-watering was done at the end of the growth phase. Treatments were arranged into a completely randomized design with three replications. Tip cuttings (4 to 5 cm long) of each cultivar were planted in 30 cm plastic pots containing 1/2 Hoagland solution, which was changed every 5 days. After rooting (about 15 days), the plants were replanted in plastic pots (40/ 60/ 20 cm, 4 pots per treatment, 2 plants per pot) with 12 kg soil mixture of 25% sand and 75% commercial soil. A total of 8.63 g fertilizer (2.8 g N: 1.43 g P: 4.4 g K = 1:0.51:1.57) was added to each pot during the experiment.

Containers of plant were covered with 50 g perlite in order to decrease evaporation from the soil surface. Plants were grown in a naturally lit greenhouse under semi-controlled environment (sheltered only from rainfall). Plants were allowed to grow for about a month before the drought stress was imposed (Lin et al., 2007). All pots were weighed and watered to maintain desired soil water conditions (80% ± 5, 60% ± 5, 35% ± 5 of field capacity, respectively) every day.

Mature, healthy, fully expanded third, fourth and fifth leaves from 96-day-old plants were sampled at three intensities of water stress and on the1st, 2nd, 3rd, 5th, 7th and 9th day of recovery. Sampling of control, stressed and recovered plants was done simultaneously. The plants were divided into leaf, stem, root and tuber and then oven-dried at 80°C to obtain dry weight. For the biochemical assays, the leaves were frozen in liquid nitrogen after measuring their fresh weight and stored at -80°C.

Water relations

Leaf water potential (Ψ_L) was measured using the pressure chamber (Model 3000, Santa Barbara, USA). All measurements of water potential were done between 6:30 and 7:30. Relative water content was measured according to the method of Smart and Bingham (1974) with some modifications. After fresh weight (W_f) determination, leaves were floated on distilled water for 24 h, obtaining the turgid weight (W_t). Dry weight (W_d) of leaves were determined after oven-drying at 80°C for 72 h; the RWC was determined using the formula:

$$RWC\ (\%) = (W_f - W_d) / (W_t - W_d) \times 100$$

Relative electrolyte leakage and lipid peroxidation

Twelve leaf discs (1 cm in length) obtained by stiletto, were place in 10 ml falcon tubes and then vacuumed for 30 min. Electrical conductivity of the bathing solution (L1) was measured after incubation at 25°C under light for 30 min using a conductivity meter (Model DDS-307, USA). The tubes were then placed in a preheated 95°C water bath and incubated for 10 min. The solution was cooled to room temperature and the electrical conductivity (L2) was measured. Relative electrolyte leakage was calculated according to the following formula:

$$EL = L1 / L2*100\%$$

Lipid peroxidation was estimated by measuring the content of 2-thiobarbituric acid-reactive substances (TBARS) in 0.5 g leaf fresh weight according to Ozkur et al., (2009). Malondialdehyde (MDA) content was determined spectrophotometrically at A532 and corrected for nonspecific turbidity at A600.

Antioxidant enzymatic activity assays

Sweet potato leaves were homogenized on ice with a mortar in a 0.1 M potassium phosphate buffer (pH 7). The homogenate was centrifuged at 10000 g for 15 min at 4°C.The supernatant was used immediately for enzyme assays. SOD activity was determined by monitoring the photochemical reduction of nitro blue tetrazolium (NBT) spectrophotometrically at 560 nm (Giannopolitis and Ries, 1977). Activity was expressed as unit min^{-1} g^{-1} fresh weight (FW). APX activity was also determined spectrophotometrically at 290 nm after the oxidation of ascorbate to dehydroascorbate (extinction coefficient of 2.8 Mm^{-1} cm^{-1}), as described in Nakano and Asada (1981). The activity of APX was presented as mmol ascorbate oxidized unit min^{-1} g^{-1} FW. CAT activity was assayed according to the method described in Aebi (1984). The activity was determined by the decrease at 240 nm for 1 min due to H_2O_2 consumption.

Photosynthetic activity

The photosynthetic activity was estimated by chlorophyll fluorescence determination of photochemical yield (*Fv*/*F*m), which represented the maximum quantum yield of photosystem II, using a portable PAM-2000 fluorometer (Walz-Effeltrich, Germany) after 30 min of dark adaptation. Measurements were performed on the third leaves of three plants per treatment using saturating light flashes at 0, 1, 2, 3, 5, 7 and 9 days of re-watering after drought treatment. Chlorophyll content in leaves was measured with SPAD-502.

Data analysis

The data were subjected to analysis of variance using the Statistical Analysis System (SAS) version 8.1; treatment means were compared using Duncan's multiple range tests at the 5% level of significance.

Table 1. Effect of moderate and severe drought on the growth of transgenic (TS) and non-transgenic (NS) sweet potato.

Parameter	Control		Moderate stress (MS)		Sever stress (SS)	
	TS	NS	TS	NS	TS	NS
Branch (unit)	3.5 ± 0.50	3.5 ± 0.70	3.7 ± 0.47	3.0 ± 1.15	2.6 ± 0.57	2.5 ± 0.57
Caulis (cm)	144.33 ± 5.85	137.25 ± 7.13	80.33 ± 3.39	60.0 ± 5.59	73.0 ± 4.24	53.0 ± 6.05
Green leaf (unit)	43.330 ± 4.04	40.5 ± 3.87	29.66 ± 4.11	22.25 ± 5.05	24.5 ± 3.7	20.25 ± 2.62

The data were collected from 106-day–old of plants. Plants growing in well-watered conditions served as control. Values are means ± SD of three replicates.

RESULTS

Plant growth response to drought stress

The growth of TS and NS plants was not different under well-watered conditions, showing that the introduction of both Cu/Zn SOD and APX had little effect on plant growth under normal conditions (Table 1). Moderate drought slightly increased branching in TS plants (3.7) and decreased same in NS plants (3.0) as compared to well watered plants (3.5). Both TS and NS plants were badly affected by severe drought; both moderate and severe drought reduced plant Caulis and green leaves. Under moderate drought, the average caulis length and number of green leaves in TS plants were reduced by 64 and 13.7 cm, respectively. The corresponding decrease was 77.3 and 18.3 cm in NS plants as compared to controls. Increase in drought intensity caused further reduction; there was no statistical difference between TS and NS. These results suggested that some level of water deficit (moderate drought), elevated both Cu/Zn SOD and APX expression in chloroplasts of sweet potato, and could alleviate the limit on growth induced by water deficit. However, under severe stress, TS plants did not show obvious advantage in protecting plant growth.

Membrane damage under drought stress and re-watering conditions

Disruption of the membrane integrity caused by stress can be estimated by measuring the leakage of cyto-plasmic solutes from leaf slices. Under well watered conditions, there were no differences in electrolyte leakage between TS and NS plants (Figure 2). Moderate drought increased relative electrolyte leakage by 1.9 and 2.4 fold in TS and NS plants (Figure 2A) and then increased further to 2.3 and 2.7 fold under severe drought (Figure 2B). The relative electrolyte leakage declined gradually with the days after re-watering. A significant difference between TS and NS occurred on the first 3 days after re-watering from severe drought.

Malondialdehyde, as the final product of cellular mem-branaceous lipid peroxidation, is a key parameter for evaluating the extent of damage in plants. The level of MDA was increased dramatically by severe drought condition (Figure 3), but TS plants showed better tolerance of water stress than NS plants. The level of MDA was 1.9 and 2.6 fold of control in TS plants under moderate and severe water stress whereas in the same condition, NS plants were 2.3 and 3.6 fold of control, respectively. In response to rewatering, both plants showed similar change trends relative to electrolyte leakage. The MDA of TS plants recovered to near normal levels when the plants were re-watered from both stress cycles for 4 days, whereas NS plants could not recover to the same levels even after 9 days recovery from severe drought conditions.

Changes in leaf relative water content (RWC) under drought stress and re-watering conditions

The relative water contents (RWC) of drought-stressed plants were measured over a specified time-period during drought stress and recovery. After almost 96 days of withholding water, we noted significant reductions in the water contents of plants, and the reduction became more severe under severe water stress. Compared to control, the reduction in RWC in the TS plants was only 18 and 12% in severe (Figure 4B) and moderate drought stress (Figure 4A), respectively. In the NS plants, 25 and 16% reductions were observed. The leaf relative water content of both lines was strongly affected by re-watering, especially when they were released from severe drought. The TS plants showed better recovery than NS plants, in the sense that they recovered faster. TS plants main-tained significantly higher water contents than the NS plants in water stress period and on the 1st, 2nd and 3rd day after re-watering, neither of them recovered fully from severe stress even after 9 days re-watering.

Changes in leaf water potential during drought stress and re-watering periods

The leaf water potential (ψ_L) of both lines was decreased progressively by drought stress (Figure 5). Long term moderate water deficit caused 3.5 and 4.4 bar reduction in TS and NS plants, respectively, as compared to well

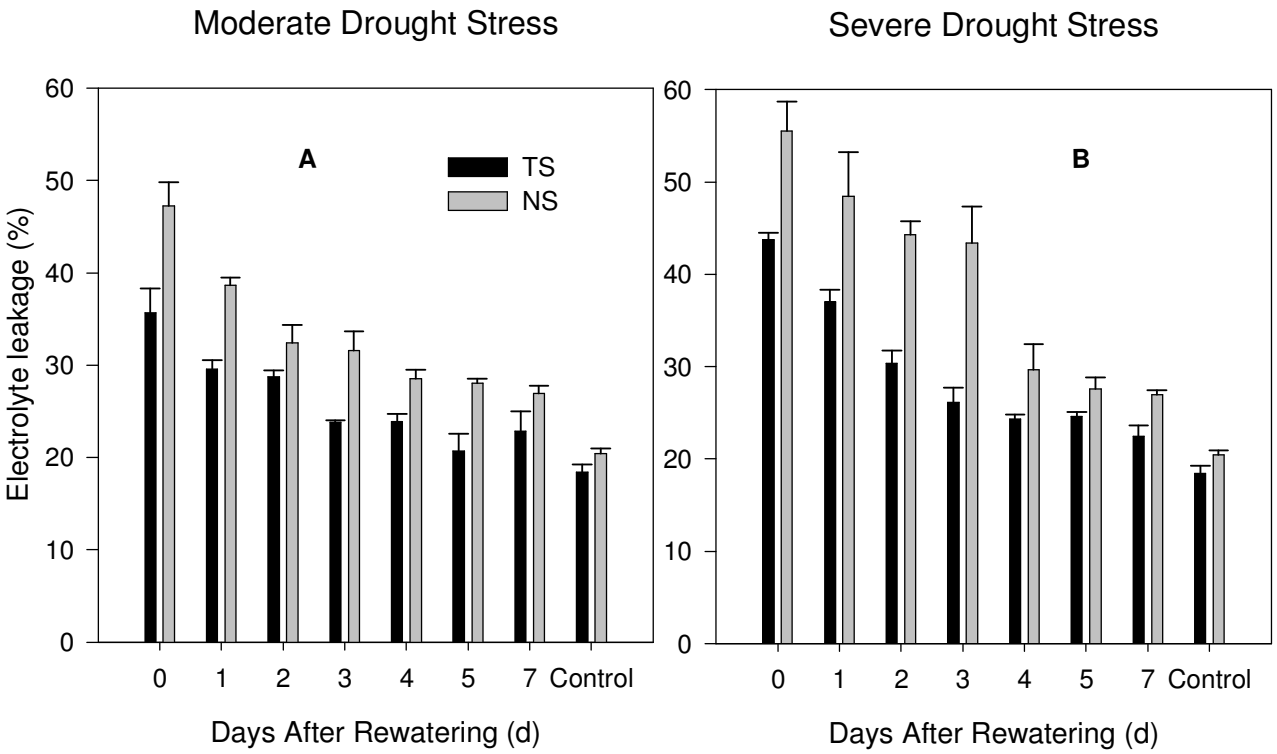

Figure 2. Effect of drought and re-watering on electrolyte leakage (EL) in transgenic (TS) and non-transgenic sweet potato (NS). After 96 days of drought treatment, the electrolyte leakage of leaf discs was measured after 0, 1, 2, 3, 4 5, and 7 days of re-watering. Plants growing in well watered conditions served as control. Values are means ± SD of three replicates.

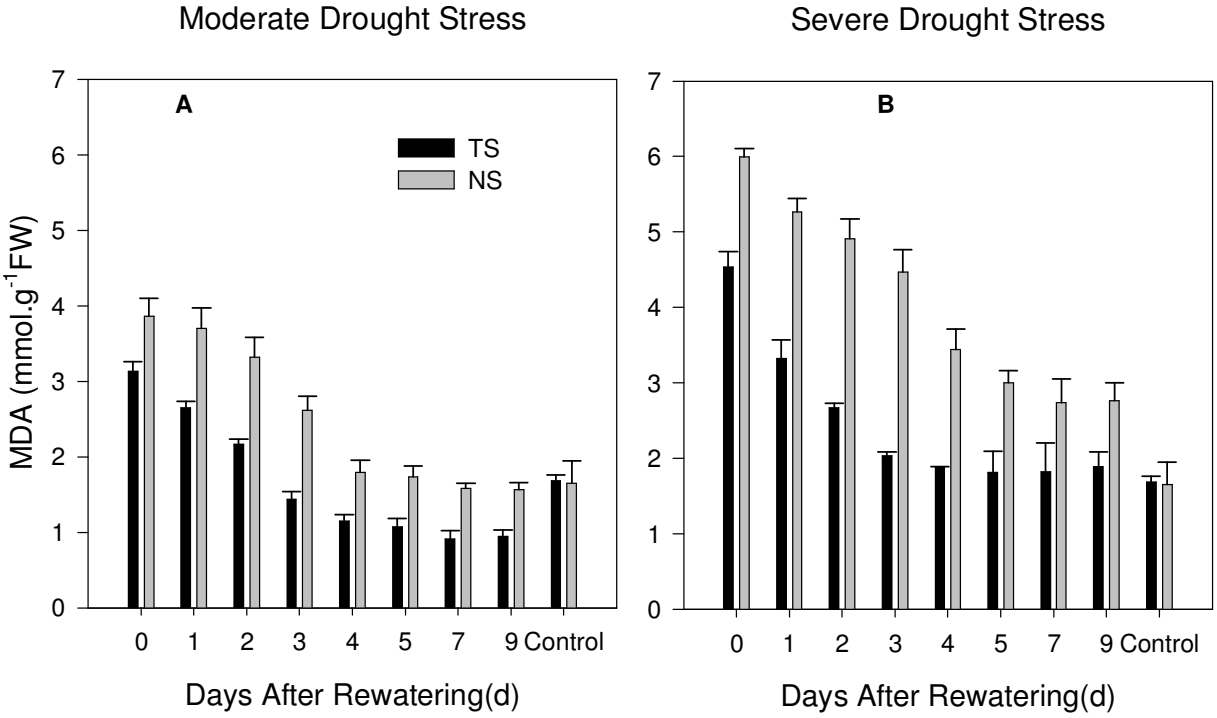

Figure 3. Effect of drought and re-watering on MDA and EL in transgenic (TS) and non-transgenic (NS) sweet potato. After 96 days of drought treatment, MDA in leaf discs was measured at 0, 1, 2, 3, 4, 5, 7 and 9 days after re-watering. Plants growing in well watered conditions served as control. Values are mean ± SD of three replicates.

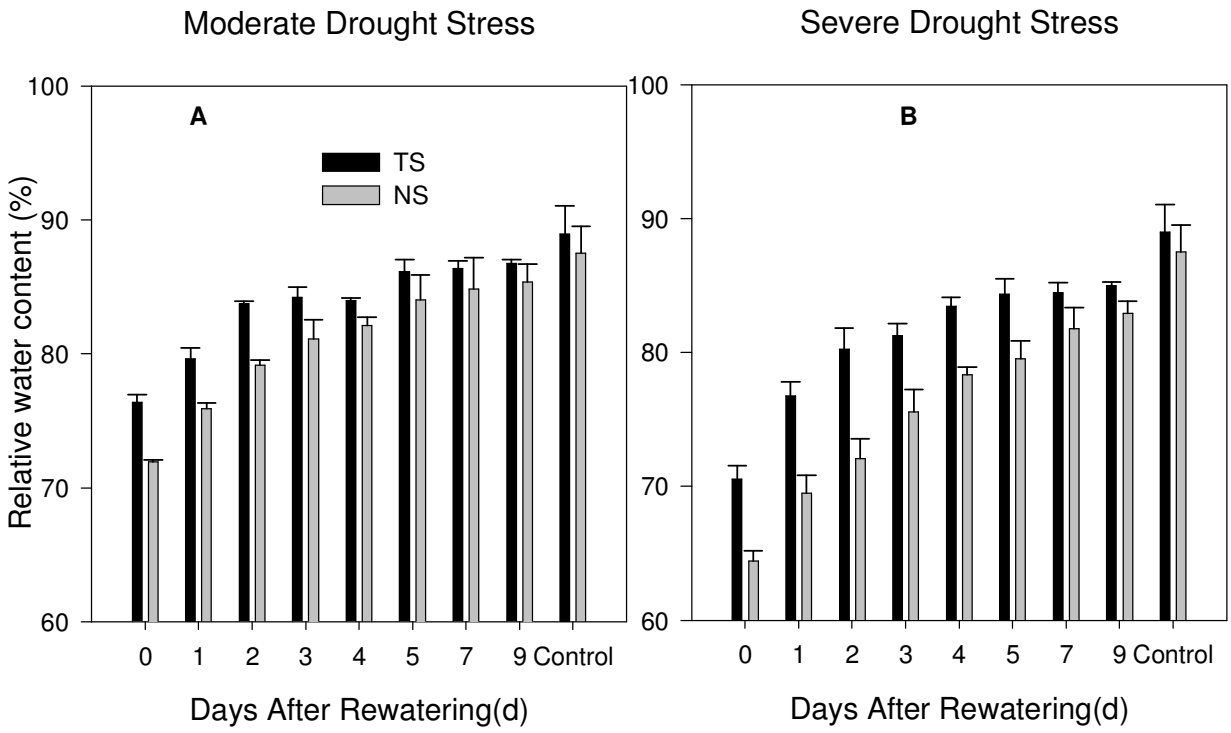

Figure 4. Effect of drought and re-watering on leaf relative water content (RWC) in transgenic (TS) and non-transgenic (NS) sweet potato. After 96 days of drought treatment, the RWC of leaf discs was measured at 0, 1, 2, 3, 4, 5, 7 and 9 days after re-watering. Plants growing in well watered conditions served as control. Values are mean ± SD of three replicates.

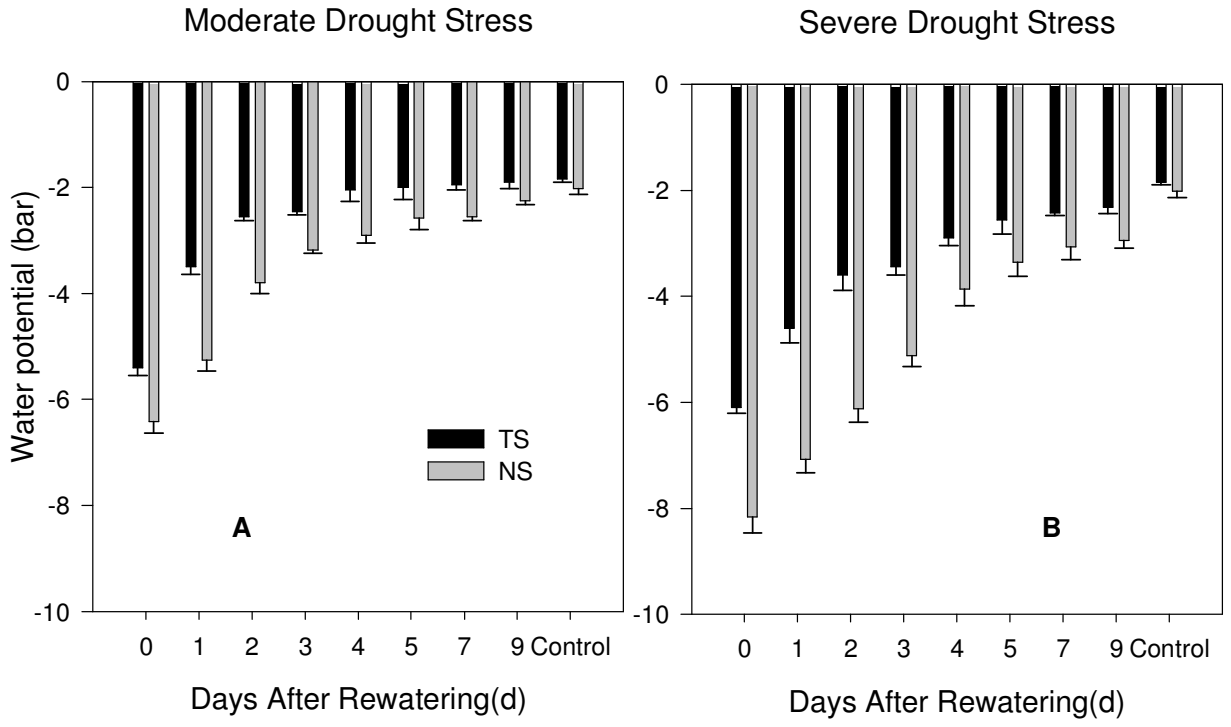

Figure 5. Effect of drought and re-watering on leaf water potential (ψ) in transgenic (TS) and non-transgenic (NS) sweet potato. After 96 days of drought treatment, the leaf water potential was measured at 0, 1, 2, 3, 4, 5, 7 and 9 days after re-watering. Plants growing in well watered conditions served as control. Values are means ± SD of three replicates.

watered values (Figure 5A), and then was increased to 4.2 and 6.2 bar, respectively because of increase in stress intensity (Figure 5B). The result indicated that TS plants had enhanced ability to maintain water balance under stress environment. Re-watering improved leaf water status of plants, however, the extent of recovery depended on the stress level. On release from moderate drought, the water potential of TS plants recovered rapidly to an almost stable level on the 2nd day of re-watering, whereas NS plants took more time to get to the same level. Both plants recovered completely after 9 days of re-watering treatment. On termination of severe drought, TS plants showed much higher recovering capacity than NS plants, especially on the first 3 days of re-watering. However, both lines could not recover to the well watered levels even at the end of treatment, indicating that severe drought induced permanent damage of the photosynthetic apparatus in existing leaf tissues. These results suggested that simultaneous over-expression of both chloroplast-targeted Cu/Zn SOD and APX in transgenic sweet potato improved their water status under drought, especially during recovery period.

Changes in antioxidant enzyme activities during drought stress and re-watering periods

Drought stress induced some antioxidant enzymatic activities (APX, CAT and SOD) based on the different responses to recovery (Figure 6). Moderate drought stress increased the activities of SOD by 2.3 and 1.9 fold in TS and NS plants, respectively (Figure 6A). With increases in drought intensity, they were increased to 2.7 and 2.2 fold, respectively (Figure 6B). Re-watering restrained SOD activity in both lines. Upon watering from moderate stress, the SOD activity in TS plants was maintained at a higher level during the first 2 of re-watering, and then declined rapidly, whereas NS plants decreased directly after re-watering. On the 3rd day, both lines almost returned to the control level. In the recovery period of severe drought, TS plants still showed higher SOD activity than NS plants, although this activity decreased rapidly after re-watering days.

APX activity of plants showed a similar change as SOD in response to moderate drought and the following recovery: increasing in stressful condition and decreasing further in re-watering period (Figure 6C, D). Obviously, APX activity was more affected by moderate drought than SOD; it increased 4.4 and 3.2 fold in TS and NS plants, respectively (Figure 6C). Unlike SOD, the APX activity decreased with further increases in stress intensity, but had a greater increase after 1 day re-watering, and then dropped gradually (Figure 6D). Though TS and NS plants showed a similar trend, the APX activity in TS plants was much higher than NS plants, especially during the first 3 days of re-watering.

Obviously, the CAT activity in plants was less affected by drought and re-watering than SOD and APX activity. Compared to control, the CAT activity of TS and NS plants increased by 122 and 85% under moderate drought (Figure 6E) and decreased to 103 and 57% under severe drought (Figure 6F). During the recovery period, there were similar changes in APX activity.

The results suggested that previous water stress intensity is a crucial factor affecting both the velocity and extent of recovery after re-watering. However, TS plants always showed higher level of all scavenging enzyme activities both in response to stress and recovery.

Changes in leaf chlorophyll content and photosynthetic activity (Fv/Fm) during drought stress and re-watering periods

Damage of the photosynthetic apparatus by drought stress was determined via measurement of the fluorescence parameters (Fv/Fm) (Figure 7). The Fv/Fm of TS and NS plants was similar under control conditions (0.87) but decreased to 0.82 and 0.71 under moderate and severe drought in TS plants; the NS plants experienced 0.79 and 0.67 decrease under the same condition. Upon watering from moderate drought stress, the Fv/Fm of both TS and NS plants increased drastically after 2 days of recovery and then continued to rise gradually; both lines recovered to the control levels after 9 days of rewatering (Figure 7C). Following rewatering from severe drought, the Fv/Fm of TS plants was far superior to that of NS plants. TS plants increased slowly on the first 2 days and then rapidly, whereas the NS plants had negligible recovery on the first 5 days of re-watering. However, both TS and NS plants could not attain full recovery even after 9 days re-watering (Figure 7D).

Plants growth and harvest index under drought stress

Plants were harvested and data collected are shown in Figure 9. Water stress decreased the aboveground biomass (Figure 9A) but improved root development (Figure 9B). Transgenic lines had greater shoot and root weights compared to NS lines. Moderate and severe drought stress reduced the entire dry weights of TS plants by 41.5% while NS plants were reduced by 61.7% (Figure 9C). However, drought stress resulted in a significant increase in the root-shoot ratio, especially in TS plants which increased by 62.2 and 35.3% under moderate and severe drought while NS plants were reduced by 32.7 and 30.9%, respectively (Figure 9D). The root-shoot ratio was significantly higher in TS plants than in NS plants, and it seems that the root was more sensitive to drought stress. However, the tuber yields of TS plants were badly affected by drought stress (Figure 9E), and consequentially, the harvest index decreased significantly

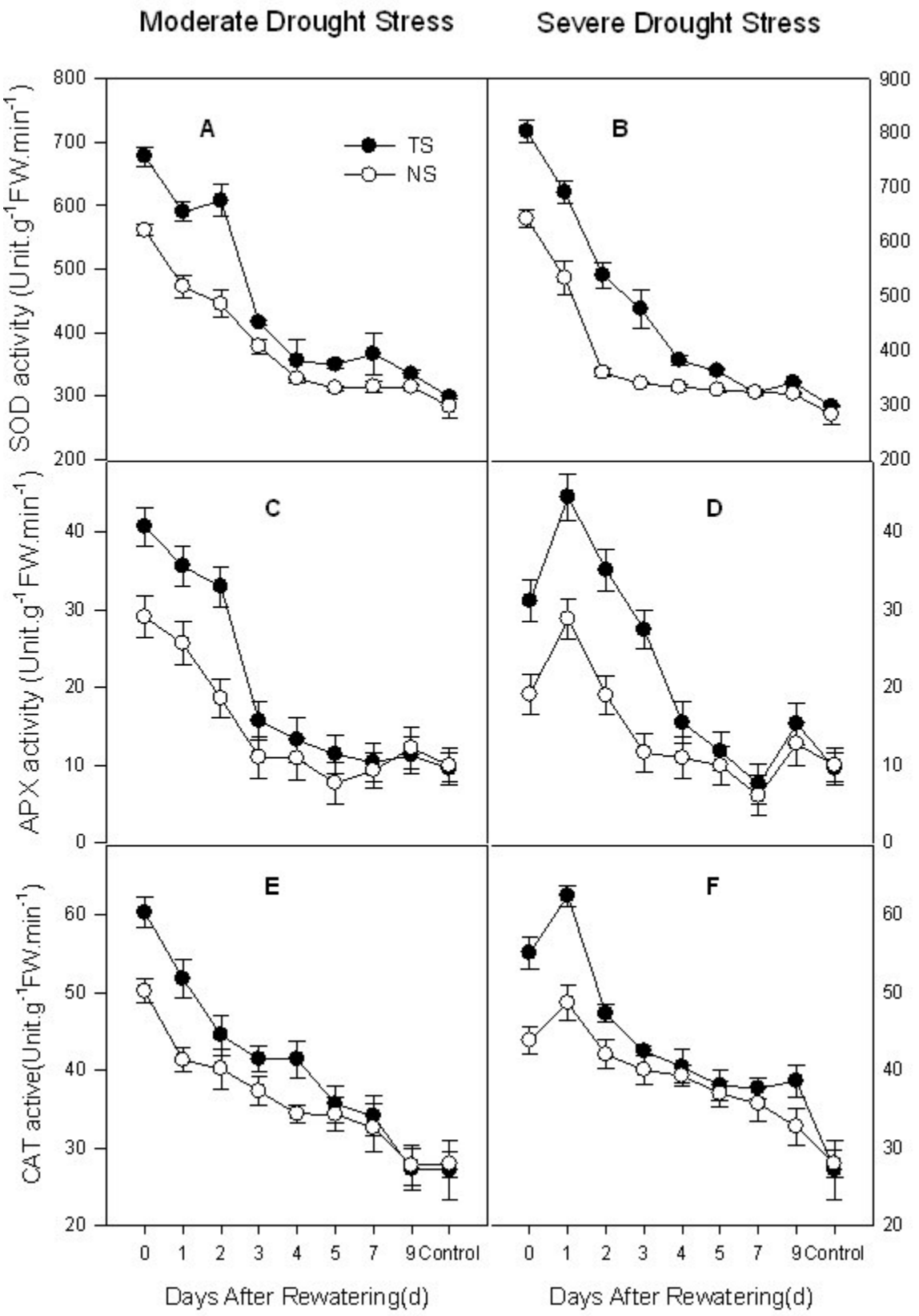

Figure 6. Effect of drought and re-watering on antioxidant enzymatic activity SOD (A, B), APX (C, D), CAT (E, F) in transgenic (TS) and non-transgenic (NS) sweet potato. Antioxidant enzymes in leaves were measured at 0, 1, 2, 3, 4, 5, 7 and 9 days after re-watering. Plants growing in well watered conditions served as control. Values are means ± SD of three replicates.

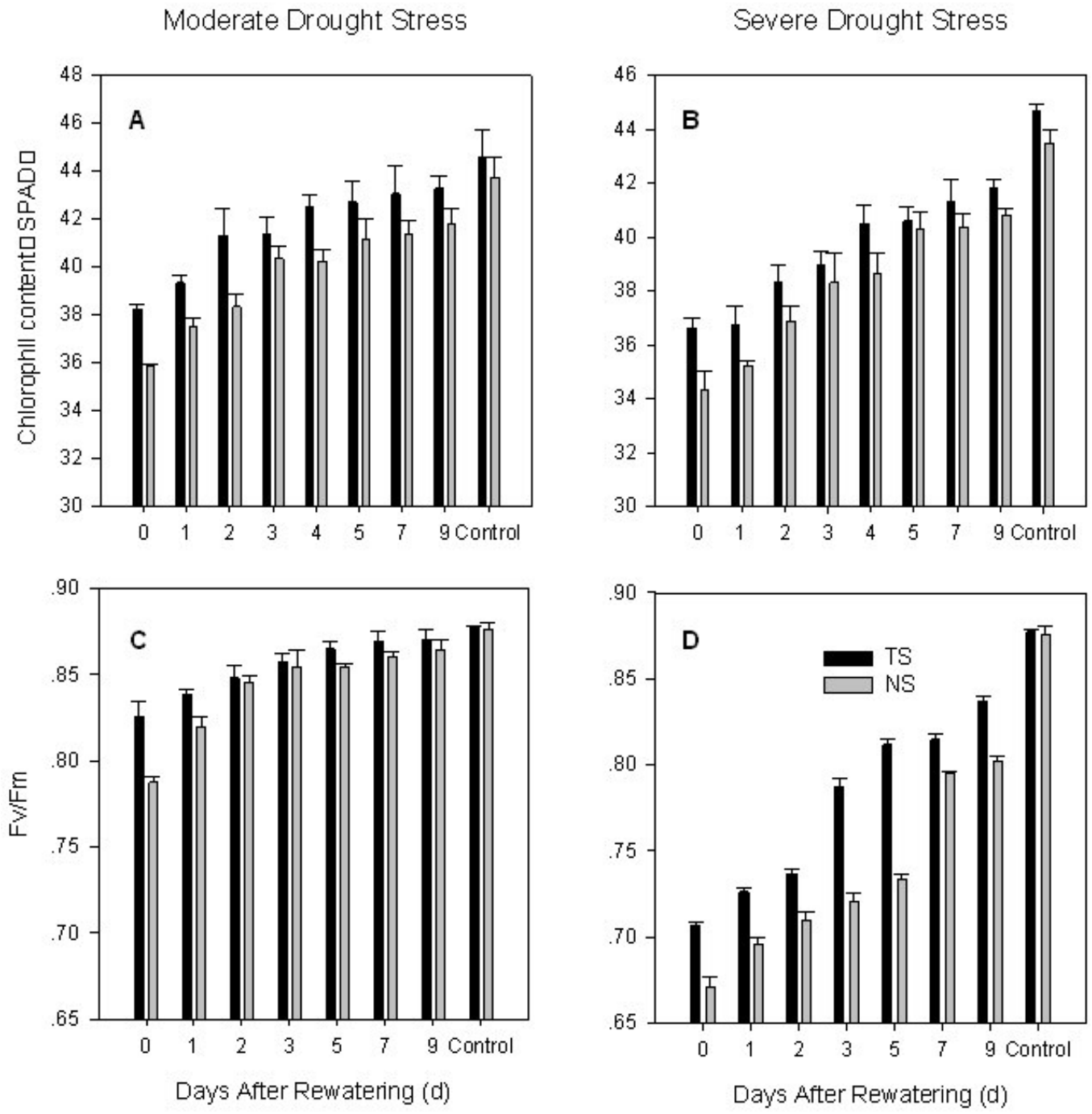

Figure 7. Effect of drought and re-watering on chlorophyll contents (A, B) and photosynthetic activity (Fv/Fm) (C, D) in transgenic (TS) and non-transgenic (NS) sweet potato. After 96 days of drought treatment, Chl content and Fv/Fm of leaves were measured at 0, 1, 2, 3, 5, 7 and 9 days after re-watering. Plants growing in well watered conditions served as control. Values are means ± SD of three replicates.

DISCUSSION

The frequency of drought is likely to increase in the future and plant growth and productivity will be greatly limited by water deficit, especially in arid/semi-arid regions. Drought tolerance in plants involves modulating multiple genes and coordinating the action of various genes from different pathways. To date, many studies have demonstrated that multiple genes transfer can act additively or synergistically to enhance tolerance of abiotic stress such as drought. For instance, our work, together with the earlier reports on tall fescue (Lee et al., 2007a), tobacco

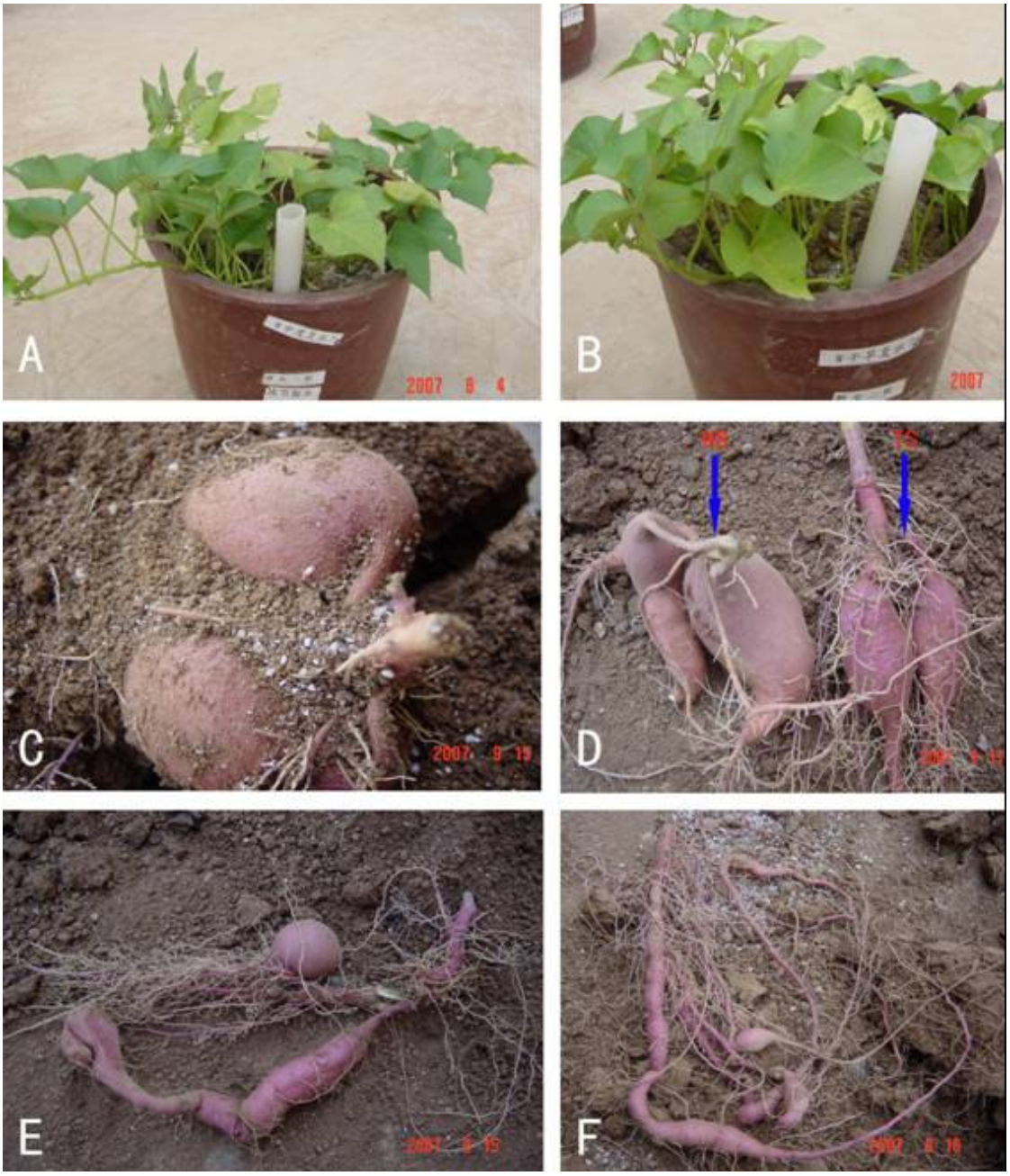

Figure 8. Effect of drought tress on visual rating of plants growth and storage root formation in transgenic (TS) and non-transgenic (NS) sweet potato. Observations were made on 106-day–old plants. (A) Growth performance of TS under severe drought; (B) growth performance of NS under severe drought; (C) storage root formation in control condition; (D) differential tuber development in TS and NS plants under moderate drought; (E) tuber development in NS plants under severe drought; (F) tuber development in TS plant under severe drought.

(Lee et al., 2007b), sweet potato (Lim et al., 2007), rice (Wang et al., 2005) and potato (Van der Mescht et al., 2007) demonstrated that development of transgenic plants, such as direct modification of the expression of antioxidant enzymes, SOD, GR and APX in chloroplasts, was an effective way of enhancing the protection of plants against various oxidative stresses (Badawi et al., 2004; Van Camp et al., 1997). However, very few studies have focused on the recovery capacity of transgenic plants. The rate and extent of plant recovery from stressful conditions may be used as an important index in evaluating the stress tolerance of transgenic plants.

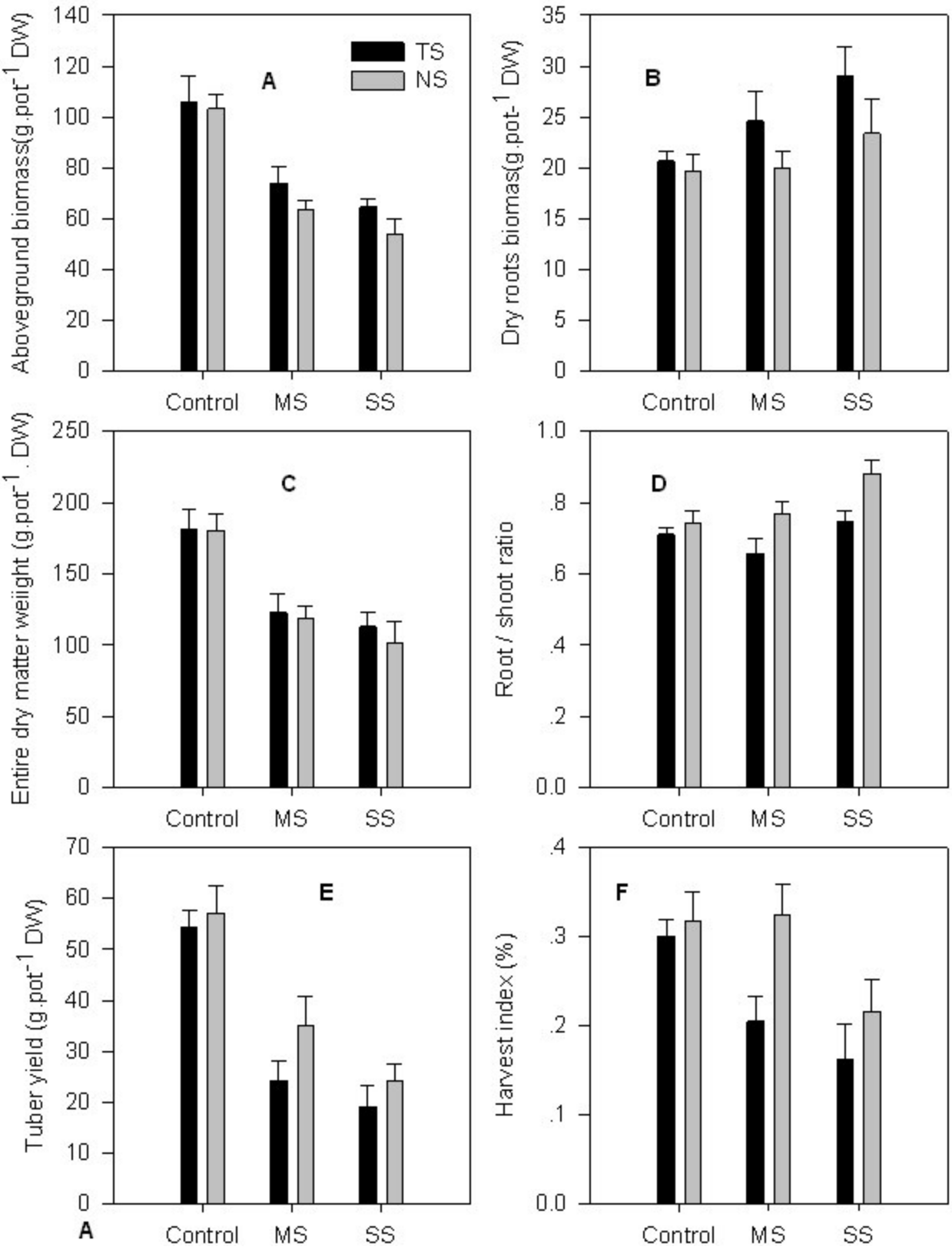

Figure 9. Effect of moderate and severe drought on dry matter production (A, B, C), tuberous root yield (E) and harvest index (F) in transgenic (TS) and non-transgenic (NS) sweet potato. The data were collected from 106-day–old plants. Plants growing in well watered conditions served as control. Values are means ± SD of three replicates. MS: Moderated drought stress; SS severe drought stress.

In this work, we observed that the introduction of foreign genes did not affect the growth of plants under normal conditions (Table 1). The caulis and leaf area of non- transgenic and transgenic plants were similar. However, drought stress injured plants both at tissue and cellular levels. The development of caulis was impaired and leaf was lost or turned yellow. Transgenic plants delayed leaf wilting and maintained growth, thereby experiencing lower damage than NS plants.

The MDA and electrolyte leakage of leaf were measured to determine whether over-expression of Cu/Zn SOD and APX are helpful in scavenging excess ROS. The results showed that MDA accumulation and EL dramatically increased in NS plant but less in TS plants (Figure 2 and 3), suggesting that ROS were more efficiently scavenged by simultaneous over-expression of both Cu/Zn SOD and APX in the chloroplasts of transgenic plants. In addition, the MDA accumulation and EL of transgenic plants were restored to relatively low levels earlier than NS plants after re-watering, suggesting the advantage of transgenic plants in recovery period.

Rao et al. (2006) showed that re-establishment of vegetative plant growth after a period of water stress depended on the recovery of leaf water potential after re-watering and the faster the recovery, the better the overall plant production. The RWC and water potential of tested plants decreased slightly in moderate water stress (Figure 4 and 5A), and dropped further in severe water stress (Figure 4 and 5B). The reduction in water potential and RWC in TS plants was less than NS plants. Also, the water potential and RWC of TS plants recovered earlier than NS plants. The results suggested that this improvement might be due to leaves of transgenic lines over-expressing both Cu/Zn SOD and APX.

Water stress induces oxidative stress through generation of ROS, and the ability of the plant to mobilize enzymatic defense against uncontrolled production of ROS may be an important facet of their drought tolerance (Dhindsa and Matowe, 1981). Many previous works proved that transgenic plants with elevated levels of chloroplast - targeted antioxidant genes such as SOD, APX, or GR exhibited enhanced protection against oxidative stress (Allen et al., 1997; Gupta et al., 1993; Payton et al., 2001). In this work, water deficit stress increased the activities of all the scavenging enzymes (SOD, APX and CAT) according to water stress intensity (Figure 6). The SOD and APX appeared to be important enzymes for overcoming drought-induced oxidative stress as these enzymes could be the first line of defense during drought acclimation process. During the period of recovery, APX and CAT increased on the first day of re-wetting and then declined (Figure 6 D and F), suggesting that these enzymes play more important roles in recovery process. The differential response of the antioxidant enzymes with increasing stress intensity followed by recovery, highlight the different role of each in the drought acclimation process of sweet potato. The coordinated defense helped the plants to recover in terms of growth on re-watering after stress imposition. Also, transgenic plants maintained higher activities of SOD, APX and CAT during drought stress and the entire period of re-watering than NS plants. There was some evidence that comparatively, higher activities of antioxidant enzymes and metabolites enhanced plants' tolerance of various abiotic stresses (Kornyeyev et al., 2003; Perl et al., 1993; Tang et al., 2006). Thus, our experimental result indicated that over-expression of both Cu/Zn SOD and APX in transgenic sweet potato enhanced antioxidant enzyme activities and conferred better tolerance and recovery from drought stress than control plants.

Fluorescence can provide insights into the ability of a plant to tolerate environmental stress and reflect how much the photosynthetic apparatus are damaged by these stresses (Fracheboud et al., 1999; Maxwell and Johnson, 2000). Thus, in this work, we use photosynthetic activity to evaluate the possibility that the gene transfer technology could be useful for improving tolerance of sweet potato against drought stresses. We found that moderate drought stress had less effect on the Fv/Fm of both lines, especially in transgenic lines, which had a high value of about 0.8 in leaves (Figure 7C). This observation is consistent with previous studies: the value of Fv/Fm in sweet potato was nearly completely unaffected in the presence of very large reductions in photosynthetic rates under field-imposed drought stress (van Heerden and Laurie, 2008). After re-watering, the Fv/Fm increased in both examined plants to near normal levels with no significant difference between transgenic and non-transgenic plants. This may mean that functional damage in the photo-system did not occur in the leaves. On the other hand, long term severe drought imposed, caused a decrease in Fv/Fm in TS and NS plants (Figure 7D), but TS plants could maintain a higher level of Fv/Fm than NS plants. This suggests that, although the photosynthetic activity decreased because of injury of electron transfer in photochemical system II, TS plants could effectively protect photosynthetic apparatus from drought by over-expression Cu/Zn SOD and APX. Upon re-watering, the Fv/Fm of TS plants could be restored to control level rapidly from moderate drought, and full recovery after 2 days of re-wetting, whereas NS plants showed a delayed recovery during the period of re-watering. The results also showed that under moderate soil water deficit, photosynthetic depression was possible because of stomatal closure or limitation, but not because of biochemical reaction. Therefore, plants could recover to control level once released from stressful conditions. However, TS and NS plants could not get full recovery even after 9 days re-watering from severe drought conditions. It is possible that they need more time to recover or the PSII thermal energy dissipation was strongly limited, due to damage to PSII structure and functionality. A previous report indicated that severe stress damaged both photosystems (Genty et al., 1987) but mild water

stress had no effect on PSII photochemistry (Meyer and Kouchkovsky, 1993). The alterations in PSII activity under moderate drought stress were related to photoinhibition rather than to a direct damage to PSII (Miyashita et al., 2005). Previous research demonstrated that the carbon balance of a plant during periods of water stress and recovery may depend as much on the velocity and degree of photosynthetic recovery as on the degree and velocity of photosynthesis decline during water depletion (Kirschbaum, 1987). Transgenic plants showed higher photosynthetic activity than NS plants under stress conditions and took shorter time to recover following re-watering, reflecting that transgenic sweet potato effectively improved carbon balance and enhanced the capacity for recovery.

Under control water conditions, TS and NS plants had similar dry matter yield (Figure 9C) suggesting that their growth was not affected by introducing foreign genes. Dry matter yield decreased significantly in both lines under drought stress. Although the reductions in dry biomass in transgenic lines were less than that in non-transgenic plants, the difference was not significant. Aboveground and root dry biomass was different in response to drought stress; the aboveground growth declined (Figure 9A) whereas root development improved (Figure 9B). Moreover, tuber development was significantly impaired by drought stress (Figure 9E). When soil moisture content was low, sweet potato had poor tuber development and vegetative growth. The interesting thing is that, compared with NS plants, the total dry matter as well as the aboveground and root biomass of transgenic plants were higher in all treatment conditions, except tuber yield. In a previous report (Watanabe et al., 1968), it was indicated that tuber formation in sweet potato was absent or delayed by low levels of soil moisture. In the present work, tuber formation was affected not only by water deficit, but also by introduction of foreign antioxidant genes. It seems that introduction of Cu/Zn SOD and APX improved the drought tolerance of transgenic plants by enhanced development of the root system and corresponding decrease or delay in tuber formation. That our results were obtained under pot condition provides another possible explanation of the loss in tuber yield; carbon was allocated towards futile root development (based on the root: shoot ratios), but plants could not have achieved real benefit from this in a system where roots were pot-bound and incapable of scavenging more water from deeper soil layers. In the field, these roots could have translated into a major benefit in terms of water supply during the very sensitive stage of tuber initiation/development. Therefore, comparative analysis of transgenic and non-transgenic plants in term of yield and growth must be further confirmed under field conditions.

Sweet potato is a special crop food that both sink and source organs could be utilized. Storage root (tuberous roots), which is rich in starch and dietary fiber is used as human food while vegetable tissue (leaf, shoot) is a good source of vitamin A. Thus, even with the reduction in tuberous root yield of transgenic lines, we anticipate that our experimental results will provide some evidence of diverse use of transgenic plants such as utilization of vegetative tissues or growth in marginal soil.

Acknowledgments:

This work was financially supported by National Basic Program of China (2009CB118604), Chinese Natural Science Foundation (50779063) and International Science & Technology Cooperation Program of China (2010DFA91930).

Conclusion

In conclusion, transgenic sweet potato over-expressing both Cu/Zn SOD and APX under the control of the oxidative stress-inducible promoter, *SWAP2*, had normal growth and appearance when grown under normal conditions, but exhibited enhanced tolerance and recovery from drought stress via improved synergistic and protective effects of SOD, APX and CAT activity. Thus, it maintained higher leaf water status and photosynthetic activity. The improvement in drought tolerance and total dry matter yield of transgenic sweet potato suggests that this crop has prospect for wide application as an industrial material and may help to overcome the existing global controversy over the value of transgenic food.

REFERENCES

Aebi H (1984). Catalase *in vitro*. Methods Enzymol. 105: p. 121.

Ahmad R, Kim YH, Kim MD, Kwon SY, Chung WI, Lee HS, Kwak SS (2010). Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. Physiologia Plantarum, 138: 520-533.

Allen RD, Webb RP, Schake SA (1997). Use of transgenic plants to study antioxidant defenses. Free Radic. Biol. Med. 23: 473-479.

Badawi GH, Kawano N, Yamauchi Y, Shimada E, Sasaki R, Kubo A, Tanaka K (2004). Over-expression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. Physiologia Plantarum, 121: 231-238.

Cai ZQ, Chen YJ, Guo YH, Cao KF (2005). Responses of two field-grown coffee species to drought and re-hydration. Photosynthetica, 43: 187-193.

Dhindsa RS, Matowe W (1981). Drought tolerance in two mosses: correlated with enzymatic defence against lipid peroxidation. J. Exp. Bot. 32: p. 79.

Fan T, Stewart BA, Payne WA, Wang Y, Song S, Luo J, Robinson CA (2005). Supplemental irrigation and water-yield relationships for plasticulture crops in the Loess Plateau of China. Agron. J. 97: 177-188.

Flexas J, Bota J, Cifre J, Escalona JM, Galmés J, Gulias J, Lefi EK, Martinez-Canellas SF, Moreno MT, Ribas-Carbó M (2004). Understanding down-regulation of photosynthesis under water stress: future prospects and searching for physiological tools for irrigation management. Ann. Appl. Biol. 144: 273-283.

Fracheboud Y, Haldimann P, Leipner J, Stamp P (1999). Chlorophyll

fluorescence as a selection tool for cold tolerance of photosynthesis in maize (*Zea mays* L.). J. Exp. Bot. 50: p. 1533.
Galle A, Haldimann P, Feller U (2007). Photosynthetic performance and water relations in young pubescent oak (*Quercus pubescens*) trees during drought stress and recovery. New Phytologist. 174: 799-810.
Genty B, Briantais JM, Da Silva JBV (1987). Effects of drought on primary photosynthetic processes of cotton leaves. Plant Physiol. 83: p. 360.
Giannopolitis CN, Ries SK (1977). Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59: p. 309.
Gupta AS, Heinen JL, Holaday AS, Burke JJ, Allen RD (1993). Increased resistance to oxidative stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. Proc. Natl. Acad. Sci. 90: 1629-1633.
Kasukabe Y, He L, Watakabe Y, Otani M, Shimada T, Tachibana S (2006). Improvement of environmental stress tolerance of sweet potato by introduction of genes for spermidine synthase. Plant Biotechnol. 23: 75-83.
Kim KY, Kwon SY, Lee HS, Hur Y, Bang JW, Kwak SS (2003). A novel oxidative stress-inducible peroxidase promoter from sweetpotato: molecular cloning and characterization in transgenic tobacco plants and cultured cells. Plant Mol. Biol. 51: 831-838.
Kirschbaum MUF (1987). Water stress in *Eucalyptus pauciflora*: comparison of effects on stomatal conductance with effects on the mesophyll capacity for photosynthesis, and investigation of a possible involvement of photoinhibition. Planta, 171: 466-473.
Kornyeyev D, Logan BA, Allen RD, Holaday AS (2003). Effect of chloroplastic overproduction of ascorbate peroxidase on photosynthesis and photoprotection in cotton leaves subjected to low temperature photoinhibition. Plant Sci. 165: 1033-1041.
Kubota F (2003). The effects of drought stress and leaf ageing on leaf photosynthesis and electron transport in photosystem 2 in sweet potato (*Ipomoea batatas* Lam.) cultivars. Photosynthetica, 41: 253-258.
Kwon SY, Jeong YJ, Lee HS, Kim JS, Cho KY, Allen RD, Kwak SS (2002). Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen-mediated oxidative stress. Plant Cell Environ. 25: 873-882.
Lee SH, Ahsan N, Lee KW, Kim DH, Lee DG, Kwak SS, Kwon SY, Kim TH, Lee BH (2007a). Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J. Plant Physiol. 164: 1626-1638.
Lee YP, Kim SH, Bang JW, Lee HS, Kwak SS, Kwon SY (2007b). Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. Plant Cell Rep. 26: 591-598.
Li JM, Deng XP (2007). Antioxidative System Characteristics of Sweetpotato Transferred both Cu/Zn Superoxide Dismutase and Ascorbate Peroxidase Gene under W ater Stress and Rewatering. Chinese Agricultural Science Bulleti. 23, 616-621.
Li T, Kwon SY, Kwak SS, Sung CK, Lee HS (2004). Selection of transgenic potato plants expressing both Cu/Zn SOD and APX in chloroplasts with enhanced tolerance to oxidative stress. Korean J. Plant Bioltechnol. 31: 1-5.
Li Y, Deng XP, Kwak SS (2006). Drought Tolerance of Transgenic Sweetpotato Expressing Both Cu/Zn Superoxide Dismutase and Ascorbate Peroxidase. J. Plant Physiol. Mol. Biolog. 32: 451-457.
Lim S, Kim YH, Kim SH, Kwon SY, Lee HS, Kim JS, Cho KY, Paek KY, Kwak SS (2007). Enhanced tolerance of transgenic sweetpotato plants that express both CuZnSOD and APX in chloroplasts to methyl viologen-mediated oxidative stress and chilling. Mol. Breed. 19: 227-239.
Lin KH, Hwang WC, Lo HF (2007). Chilling stress and chilling tolerance of sweet potato as sensed by chlorophyll fluorescence. Photosynthetica, 45: 628-632.
Maxwell K, Johnson GN (2000). Chlorophyll fluorescence-a practical guide. J. Exp. Bot. 51: p. 659.
Meyer S, Kouchkovsky Y (1993). Electron transport, Photosystem-2 reaction centers and chlorophyll-protein complexes of thylakoids of drought resistant and sensitive Lupin piants. Photosynthesis Res. 37: 49-60.
Miyashita K, Tanakamaru S, Maitani T, Kimura K (2005). Recovery responses of photosynthesis, transpiration and stomatal conductance in kidney bean following drought stress. Environ. Exp. Bot. 53: 205-214.
Nakano Y, Asada K (1981). Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22: p. 867.
Ozkur O, Ozdemir F, Bor M, Turkan I (2009). Physiochemical and antioxidant responses of the perennial xerophyte Capparis ovata Desf. to drought. Environmental and Experimental Botany
Payton P, Webb R, Kornyeyev D, Allen R, Holaday AS (2001). Protecting cotton photosynthesis during moderate chilling at high light intensity by increasing chloroplastic antioxidant enzyme activity. J. Exp. Bot. 52: p. 2345.
Perl A, Perl-Treves R, Galili S, Aviv D, Shalgi E, Malkin S, Galun E (1993). Enhanced oxidative-stress defense in transgenic potato expressing tomato Cu, Zn superoxide dismutases. TAG Theor. Appl. Genet. 85: 568-576.
Rao KVM, Raghavendra AS, Reddy KJ (2006). Physiology and molecular biology of stress tolerance in plants. (Kluwer Academic Pub).
Reddy AR, Chaitanya KV, Vivekanandan M (2004). Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J. Plant Physiol. 161: 1189-1202.
Sandermann H (2004). Molecular ecotoxicology of plants. Trends Plant Sci. 9: 406-413.
Smart RE, Bingham GE (1974). Rapid estimates of relative water content. Plant Physiol. 53: 258.
Sofo A, Tuzio AC, Dichio B, Xiloyannis C (2005). Influence of water deficit and rewatering on the components of the ascorbate-glutathione cycle in four interspecific Prunus hybrids. Plant Sci. 169: 403-412.
Souza RP, Machado EC, Silva JAB, Lag a A, Silveira JAG (2004). Photosynthetic gas exchange, chlorophyll fluorescence and some associated metabolic changes in cowpea (*Vigna unguiculata*) during water stress and recovery. Environ. Exp. Bot. 51: 45-56.
Türkan Bor M, zdemir F, Koca H (2005). Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant P. acutifolius Gray and drought-sensitive *P. vulgaris* L. subjected to polyethylene glycol mediated water stress. Plant Sci. 168: 223-231.
Tang L, Kwon SY, Kim SH, Kim JS, Choi JS, Cho KY, Sung CK, Kwak SS, Lee HS (2006). Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. Plant Cell Rep. 25, 1380-1386.
Van Camp W, Inzé D, Van Montagu M (1997). The regulation and function of tobacco superoxide dismutases. Free Radic. Biol. Med. 23: 515-520.
Van der Mescht A, De Ronde JA, Slabbert MM, Oelofse D (2007). Enhanced drought tolerance in transgenic potato expressing the Arabidopsis thaliana Cu/Zn superoxide dismutase gene: research letter. South Afr. J. Sci. 103: 169-173.
Van Heerden PDR, Laurie R (2008). Effects of prolonged restriction in water supply on photosynthesis, shoot development and storage root yield in sweet potato. Physiologia Plantarum, 134: 99-109.
Wang LT, Wang AY, Hsieh CW, Chen CY, Sung HY (2005). Vacuolar Invertases in Sweet Potato: Molecular Cloning, Characterization, and Analysis of Gene Expression. J. Agric. Food Chem. 53: 3672-3678.
Watanabe K, Ozaki K, Yashiki T (1968) Studies on the effect of soil physical conditions on the growth and yield of crop plants. VII. Effect of soil air composition and soil bulk density and their interaction on the growth of sweet potato. pp. 65-69.
Xiao B, Huang Y, Tang N, Xiong L (2007). Over-expression of a LEA gene in rice improves drought resistance under the field conditions. TAG Theoretical and Applied Genetics 115, 35-46.
Zhou S, Chen X, Zhang X, Li Y (2008). Improved salt tolerance in tobacco plants by co-transformation of a betaine synthesis gene BADH and a vacuolar Na+/H+ antiporter gene SeNHX1. Biotechnol. Lett. 30: 369-376.