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ORIGINAL ARTICLES

Soil-Water Threshold Range of Chemical Signals and Drought Tolerance Was Mediated by ROS Homeostasis in Winter Wheat During Progressive Soil Drying

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Abstract The soil-water threshold range of chemical signals and reactive oxygen species (ROS) homeostasis could have a profound impact on drought tolerance in wheat. A pot experiment was used to investigate the homeostasis between ROS and antioxidant defense at five harvest dates, and its role in the correlation between soil-water threshold range of chemical signals and drought tolerance in three wheat (*Triticum aestivum*) cultivars during progressive soil drying. The cultivars were bred at different periods, cv. BM1 (old), cv. Xiaoyan6 (recent), and cv. Shan229 (modern). They were treated with progressive soil drying. Shoot biomass was affected by drought imposed by two water treatments (90% and 55% field water capacity). The modern wheat cultivar had a lower ROS content and higher ROS-scavenging antioxidant capacity with greater soil drying (68–25% soil water content) compared with the older cultivar. The modern cultivar also had excellent adaptation to drought, with a longer survival of 22.7 days and less reduction in shoot biomass of 20.9% due to early chemical signals and better balance between ROS production and antioxidants. The older cultivar had survival of 15.3 days and 37.3% reduction of shoot biomass. A wider soil-water threshold range of chemical signals was positively correlated with improved drought tolerance and better ROS homeostasis. These results suggest that ROS homeostasis acts as a regulator in relationships between the soil-water threshold range of chemical signals and drought tolerance.

Keywords Antioxidants · Chemical signals · Drought tolerance · Reactive oxygen species · Threshold range · Winter wheat

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Introduction

Drought is one of the most important restraints on plant growth and ecosystem productivity. Crop plants have evolved appropriate mechanisms to cope with temporary or longer water limitations to survive and develop grain. One important mechanism is root-to-shoot chemical signaling, in which plants sense drought around roots and respond with root-sourced chemical signals to the shoot to elicit several adaptive responses, including decreased leaf expansion and stomatal closure (Lorena and Ernesto 2005). Increasing soil drying can induce root-sourced chemical signals, and can also cause abscisic acid (ABA) accumulation and enhancement of reactive oxygen species (ROS) generation and antioxidant defenses (Zhang and others 2006). Early triggering of root-sourced chemical signals acts as a unique nonhydraulic "early-warning" response to soil drying and could regulate the homeostasis between ROS and antioxidants. It also stabilizes metabolism in preparation for limited water availability.

In many cases, ROS generation is time-coursed, induced during perception of chemical signals, with complex upregulation of antioxidant defenses and downstream effects on both primary and secondary metabolism. ROS produced in plant cells can act as secondary messengers associated

with plant growth and yield formation (Gechev and Hille 2005). In chloroplasts, mild drought stress induces higher electron transfer from photosynthetic electron carriers toward O_2, increasing the generation of ROS such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl (OH^-). Meanwhile, root signal compounds also increase the complex activities of the antioxidant system, including nonenzymatic and enzymatic constituents. Of the nonenzymatic constituents, glutathione (GSH) and ascorbic acid (AsA) are the most important soluble antioxidants. Superoxide dismutases (SODs) catalyze the dismutation of O_2^- to H_2O_2. Catalases (CATs) are responsible for removal of H_2O_2, and the enzymes of the AsA–GSH cycle, such as ascorbate peroxidase (APX) and glutathione reductase (GR), are also involved in removing H_2O_2; all are key antioxidant enzymes in preventing oxidative damage. Furthermore, antioxidant capacity is very dependent on stress severity (Zhang and Kirkham 1994), cultivar (Zhang and Kirkham 1996), and developmental stage (Shao and others 2005). With gradual soil-water depletion, hydraulic gradients reduce the leaf relative water content (RWC) to such an extent that it decreases leaf turgor and stomatal conductance (g_s), which are defined as the appearance of hydraulic signals (Xiong and others 2006a). Chemical signaling molecules accumulate mainly in tissues with the appearance of hydraulic signals and also are associated with increased ROS concentration (Sarath and others 2007). Once the antioxidant system no longer effectively scavenges excess ROS, the balance between ROS production and antioxidant defense collapses, causing severe oxidative stress to plants coupled with senescence and finally death with reduced water availability (Lim and others 2007).

There is much evidence that chemical signals are important in the regulation of physiology, growth, and development of drought-affected plants (Davies and others 2005). However, there has been little study of changes in ROS and antioxidants with chemical and hydraulic signals during progressive soil drying for wheat (*Triticum aestivum*) cultivars bred in different recent historical periods. Our previous work has shown a correlation between a wider soil-water threshold range of chemical signals (TRc) and drought tolerance in eight old and modern wheat cultivars or six of different ploidy (Xiong and others 2006a, b, 2007). The objective of the present study was to investigate the ecophysiologic significance of chemical and hydraulic signals with ROS and antioxidant defense systems, which may elucidate some mechanisms in the correlation between TRc and drought tolerance. We quantified the soil water content (SWC) threshold at which chemical and hydraulic signals were triggered during progressive soil drying, and studied the changes in ROS and antioxidants in this process. This approach allowed the linking of diverse onsets of chemical signals with drought tolerance, a process regulated by ROS homeostasis in different winter wheat cultivars.

Materials and Methods

Plant Materials and Growth Conditions

The experiment was conducted at the experimental station of the Institute of Soil Erosion and Water Conservation, Yangling, China (lat 34°12′N, long 108°7′E, altitude 530 m). Based on similar developmental stages and plant heights, three winter wheat cultivars were selected in a pre-experiment from eight winter wheat cultivars bred at different times and which were widely used locally at the time. These were *T. aestivum* cv. BM1, an "old" cultivar widely planted from 1950 through the 1960 s (BM); cv. Xiaoyan6, a "recent" cultivar used from 1970 through the 1980 s (XY); and cv. Shan229, a "modern" cultivar used since the late 1990 s (S). Seeds were vernalized at 4°C for 72 h and germinated in an incubating cabinet. Plants were grown for 4 months (August–December) in pots (11 cm diameter × 40 cm height) containing 1.0 kg of peat in an environmentally controlled glasshouse; air temperature was 20 ± 2°C, 50% relative humidity, and sunlight was supplemented by metal-halide lamps to provide at least 400 μmol m^{-2} s^{-1} PAR. Before sowing, 0.36 g N, 0.16 g P, and 0.21 g K were applied per pot so that nutrition was not limited. Three seeds were sown in each pot. When the first leaf had emerged, pots were thinned to one seedling per pot. To minimize soil-water evaporation, the pots were covered with 2 cm of quartz gravel. Pots were moved and rearranged daily for randomization.

Water Treatments

For 2 weeks after emergence, plants were irrigated daily to maintain 90% field water capacity (FWC). Drought stress was imposed by withholding water from pots 90 days after sowing for 24 days until all plant-available water had been used. Controls were plants that remained watered at 90% FWC. SWCs in the pots were calculated using the formula $SWC = (W_t - W_d - W_e - W_p)/(W_d \times FWC) \times 100\%$, where W_t is the temporary whole pot weight, W_d the net weight of dried soil in pot, W_e the weight of the empty pot, and W_p the estimated fresh weight of all plants in the pot. SWC was reduced during the experiment (Figure 1a), and the soil-water characteristic curve equation was $Y = 32.8\ X^{-0.2103}$ (Figure 1b).

Harvest

Nine control and nine stressed plants were harvested at five stages of increased soil drying, H1–H5 (Table 1). Mature

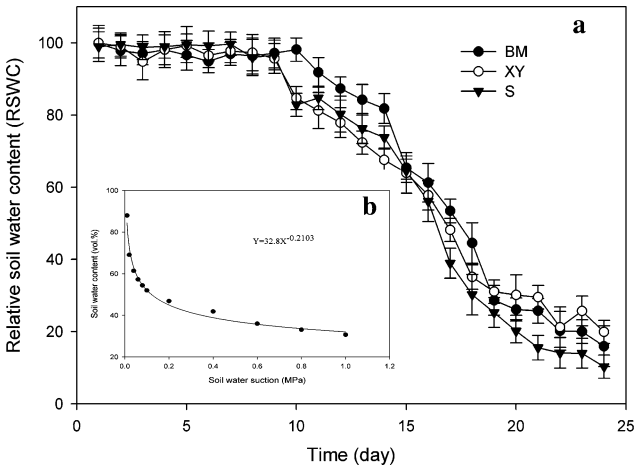

Fig. 1 Variation in relative SWC (%) as a function of time (**a**) and soil water characteristics curve (**b**) after water was withheld from well-watered plants

leaves were collected and frozen immediately in liquid N_2 for biochemical analysis after gas exchange measurement with the same leaf position in each harvest.

Gas Exchange and Relative Leaf Water Content Measurement

There were daily measurements of the gas exchange parameters stomatal conductance (g_s) and net photosynthesis rate (Pn) of the mature nonsenescent leaves (one leaf per plant, nine plants per treatment) at 9:00–11:00 a.m. with a LI-6400 portable photosynthesis system (LiCor, Lincoln, NE, USA). After measuring gas exchange, two leaf discs (5 mm in diameter) were cut with a cork borer from each measured leaf and weighed immediately for fresh weight (FW). The discs were floated in distilled water for 4 h under 10 μmol m^{-2} s^{-1} PAR, then blotted dry and weighed to obtain turgid weight (TW). Dry weight (DW) was measured after drying at 80°C in a forced-air oven for 24 h. RWC was calculated as RWC = [(FW – DW)/(TW – DW)] × 100%. RWC of each disc was measured individually.

Comparison of Drought Tolerance Among Cultivars

Measurement of the tolerance of the three wheat cultivars to continuous natural soil drying was conducted in a movable transparent rain shelter to avoid any dry-season rain (Xiong and others 2006b). Nine seedlings in total were planted 3 cm apart in a 7.5-L pot. Each pot was divided into three equal zones and three seedlings of the same cultivar were planted in each zone. Soil moisture in the pots was maintained at field capacity until the beginning of drought treatment, when water supplies were stopped simultaneously and all pots dried naturally. When water content of leaves went below the permanent wilting point,

Table 1 Stress level (% of FWC), gas exchange parameters, and leaf relative water content (%) were studied at five harvest times during the progressive soil drying experiment

Harvest	Time (days)			Stress level (% of FWC)			Stomatal conductance (g_s) (mol m^{-2} s^{-1})			Net photosynthesis rate (Pn) (μmol CO_2 m^{-2} s^{-1})			Relative water content (leaf RWC) (%)		
	BM	XY	S	BM	XY	S	BM	XY	S	BM	XY	S	BM	XY	S
Start	1	1	1	90.0 ± 2.0	90.0 ± 2.6	90.0 ± 1.8	0.38 ± 0.002	0.41 ± 0.004	0.42 ± 0.002	15.9 ± 0.06	16.3 ± 0.17	16.5 ± 0.01	82.0 ± 3.5	90.8 ± 6.1	95.8 ± 7.8
H1	3	3	3	85.6 ± 7.0	85.0 ± 6.5	86.9 ± 6.0	0.37 ± 0.007	0.39 ± 0.006	0.41 ± 0.003	15.8 ± 0.59	16.4 ± 0.29	16.4 ± 0.40	83.3 ± 12.3	92.8 ± 10.9	93.8 ± 6.1
H2	11	10	10	59.5 ± 8.1	59.7 ± 7.3	62.9 ± 7.7	0.34 ± 0.019	0.35 ± 0.006	0.37 ± 0.012	15.6 ± 0.11	16.1 ± 0.39	16.2 ± 0.29	82.1 ± 3.5	91.7 ± 14.9	92.7 ± 11.2
H3	13	13	13	53.1 ± 8.4	54.2 ± 7.6	54.1 ± 8.4	0.26 ± 0.010	0.27 ± 0.014	0.31 ± 0.028	14.2 ± 0.22	14.9 ± 0.57	15.1 ± 0.36	81.9 ± 4.7	91.6 ± 8.3	90.6 ± 9.1
H4	15	16	18	46.0 ± 7.6	43.2 ± 6.8	35.1 ± 8.1	0.15 ± 0.015	0.21 ± 0.031	0.25 ± 0.010	9.8 ± 0.37	10.1 ± 0.36	11.6 ± 0.22	77.5 ± 10.2	87.1 ± 9.6	87.1 ± 8.3
H5	21	21	21	25.4 ± 9.0	26.0 ± 8.2	25.7 ± 9.0	0.07 ± 0.013	0.11 ± 0.015	0.10 ± 0.009	5.80 ± 0.22	7.20 ± 1.03	7. 1 ± 0.17	62.1 ± 10.9	79.2 ± 11.0	80.2 ± 5.3

Values are means ± standard error (SE) ($n = 3$)

at which the leaves could not recover, the days to reach the corresponding leaf RWC were determined by repeated measures. The mean of survival days (SD) was calculated from six replications for each variety.

Two groups of plants were compared to determine the effect of drought on shoot biomass from the third leaf stage: One group was maintained at 90% soil FWC, the second at about 55% FWC, in the range of chemical signals operation, so that the effect of chemical signals on shoot biomass could be tested (Xiong and others 2007), all with five replications. When matured, the plants were harvested and shoot biomass determined after oven-drying for 48 h at 80°C.

ROS Concentration

H_2O_2 was measured by absorbance of the titanium-peroxide complex at 415 nm (Brennan and Frekel 1977), calibrated against a standard curve of known H_2O_2 concentrations. O_2^- production was measured using nitrite formation from hydroxylamine in the presence of O_2^- (Ke and others 2002). One gram of leaf segments was homogenized with 3 ml of 65 mmol L^{-1} potassium phosphate (pH 7.8) and centrifuged at 5000 *g* for 10 min. The incubation mixture contained 0.9 ml of 65 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride, and 1 ml of supernatant. After incubation at 25°C for 20 min, 17 mM sulfanilamide and 7 mM α-naphthylamine were added. After reaction at 25°C for 20 min, the absorbance was measured in aqueous solution at 530 nm.

Enzyme Assays

Frozen leaf segments were crushed into fine powder with a mortar and pestle under liquid N_2. Soluble proteins were extracted by homogenizing the powder in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP), with addition of 1 mM AsA for the APX assay. The homogenate was centrifuged at 15,000 *g* for 20 min at 4°C and the supernatant used for the following enzyme assays. Protein content was determined with BSA as standard (Bradford 1976).

Total SOD (EC 1.51.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) (Giannopolitis and Ries1977). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the NBT reduction monitored at 560 nm.

CAT activity (EC 1.16.1.6) was measured by the disappearance of H_2O_2 (Aebi 1984). The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 200 µl enzyme extract. The reaction was initiated by adding the enzyme extract and monitoring the change in absorbance at 240 nm (extinction coefficient 39.4 M^{-1} cm^{-1}) and 25°C for 3 min.

APX activity (EC 1.11.1.11) was measured in a 1 ml reaction volume containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM H_2O_2, and 0.5 mM AsA. Adding H_2O_2 started the reaction and the decrease in absorbance at 290 nm (extinction coefficient 2.8 mM^{-1} cm^{-1}) was recorded for 1 min to determine the oxidation rate of AsA (Amako and others 1994).

GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) for 3 min in 1 ml of assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na_2EDTA, 0.15 mM NADPH, 0.5 mM oxidized GSH (GSSG), and 100 µl enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH (Schaedle and Bassham 1977).

AsA and GSH Content

AsA was determined as described by Wang and others (1991). Approximately 0.2 g of leaves was homogenized in 3 ml of cold 5% (w/v) trichloroacetic acid containing 4% PVP and centrifuged at 16,000 *g* for 10 min. The supernatant mixtures were used for AsA assay. GSH content was determined spectrophotometrically at 412 nm in acid-soluble extracts (Griffith 1982), with modifications. The samples were neutralized with 500 mM potassium phosphate buffer (pH 8.0) and incubated with 10 mM 5,5′-dithio-bis(2-nitrobenzoic acid) for 15 min. Total GSH content was determined in neutralized samples after reduction of GSSG with 1 U of wheat GR (Sigma Chemical Co., St. Louis, MO, USA), 1 mM EDTA, 3 mM $MgCl_2$, and 150 µM NADPH.

Statistical Analysis

The values of leaf g_s, leaf RWC, SOD, CAT, APX, GR, AsA, GSH, H_2O_2 and O_2^- were expressed relative to the control plants. The results presented are the means of three samples and two technical replicate measurements. Results were analyzed by two-way analysis of variance (LSD) and means were compared by Duncan's multiple range tests at $P < 0.05$. The relationships of g_s and RWC to SWC were evaluated using a linear-plateau model.

The relative values of g_s and RWC equal

$$1 \text{ if } C_i \leq \text{SWC} \leq 1 \quad (1a)$$

$$1 - [A \times (\text{SWC} - C_i)] \quad \text{if SWC} \leq C_i \quad (1b)$$

where A is the slope of the linear Eq. 1b and C_i is the threshold of SWC at which the measured traits started to diverge, that is, increase or decline from 1. Data were

subjected to ANOVA procedures (SAS Inc., Cary, NC, USA) to estimate A and C_i in the linear-plateau model [Eq. 1]; PROC NLIN of PC SAS was employed. Statistical separations between different plant physiologic processes were from comparisons of coefficients in Eq. 1b at $P < 0.05$ (Liu and others 2003).

Results

Soil Water Relations, Stomatal Conductance, and Leaf Relative Water Content

The SWC was monitored and maintained stable by adding controlled amounts of water before sampling. SWC declined during the drying cycle (Figure 1a) at a rate of about 0.26, 0.25, and 0.27% h^{-1} in light periods for cultivars BM, XY, and S, respectively, from 90–15% FWC. There was no significant difference in the rate of soil drying between cultivars (Table 2).

The different times of the harvests H1–H5 meant that SWC varied (Table 1). At harvest H1 there were no significant changes in leaf g_s, Pn, and RWC, with values similar to controls. At H2, the plants had reduced g_s and slightly reduced Pn and leaf RWC, contributing to the maintenance of plant water status through improved/stabilized water uptake capacity at constant or decreasing transpiration. This time marked the appearance of root-sourced chemical signals. At H3, with progressive soil drying, g_s was significantly lower, leaf RWC was slightly but not significantly decreased, and Pn was significantly decreased. This time was the operating period of chemical signals. At H4, plants were stressed, leaf RWC was significantly lower, and this marked the appearance of hydraulic signals. At H5, plants were suffering very severe stress, leaf RWC was markedly lower, and there were senescence symptoms of yellowing and shedding of older leaves (Table 1, Figure 2c).

The g_s and RWC in well-watered controls (90% FWC) and drought-affected plants were plotted as time courses (Figure 2a, c). Immediately after imposing water stress, g_s remained stable at higher soil moisture, similar to controls. As soil water was depleted, g_s fell significantly lower than controls at 11, 10, and 10 days for BM, XY, and S, respectively. By the end of the drying cycle, the g_s was near zero (Figure 2a). Drought stress significantly decreased leaf RWC at 15, 16, and 18 days for BM, XY, and S, respectively (Figure 2c).

The g_s decreased when SWC fell below 59.5, 59.7, and 62.9% for BM, XY, and S, respectively, before leaf RWC began to decrease (Figure 2b). With soil water depletion, leaf RWC decreased; when SWC was reduced to 46, 43.2, and 35.1% for BM, XY, and S, respectively, leaf RWC was significantly lower (Figure 2d). The SWC thresholds of leaf g_s and RWC, relative to well-watered controls, were determined by linear-plateau functions (Eq. 1; Figure 3). When SWC decreased below the thresholds, g_s and RWC declined linearly. The SWC thresholds for g_s were 64, 69.9, and 70.8% (Figure 3a) and for leaf RWC they were 50.5, 50.4, and 46.2% (Figure 3b), in BM, XY, and S, respectively. The threshold range of chemical signals (TRc) was the difference between the SWC thresholds of leaf g_s and RWC (Figure 3). The modern cultivar had the widest soil moisture threshold range (46.2–70.8% FWC, that is, 24.6%), the old cultivar had the narrowest range (50.6–64% FWC, that is, 13.4%), and the recent cultivar's range was between 50.4 and 69.9% FWC (that is, 19.5%).

ROS, Enzyme, and Nonenzyme Antioxidants During Progressive Soil Water Drying

The relative values of ROS production (Figure 4), enzymes such as SOD (Figure 5a), CAT (Figure 5b), APX (Figure 5c), and GR (Figure 5d), and nonenzymes, including AsA (Figure 5e) and GSH (Figure 5f), were recorded during soil water depletion for the three wheat cultivars. As soil water was depleted, water stress increased O_2^- and H_2O_2 production. Production of O_2^- and H_2O_2 had the same trend in the five sampling times (H1–H5; Figure 4). There was significantly increased H_2O_2 and O_2^- production at H2. At H4, O_2^- and H_2O_2 production reached maximum values. At H5, O_2^- and H_2O_2 production significantly decreased for BM, and

Table 2 Survival time (days) after drying, reduction in shoot biomass (%), threshold range of SWC, and rate of soil drying (% h^{-1}) of wheat grown in a climate-controlled greenhouse

Cultivars	Survival time (days)	Reduction in shoot biomass (%)	Threshold range of SWC	Rate of soil drying (% h^{-1})
BM	15.3 ± 1.1^{b}	37.3 ± 8.6^{b}	0.13 ± 0.01^{b}	0.26 ± 0.06^{a}
XY	17.3 ± 2.3^{ab}	30.6 ± 11.9^{ab}	0.20 ± 0.03^{ab}	0.25 ± 0.07^{a}
S	22.7 ± 1.5^{a}	20.9 ± 4.9^{a}	0.25 ± 0.04^{a}	0.27 ± 0.08^{a}

Soil-water thresholds are expressed as the soil water content (SWC) at which relative values of biophysical parameters began to diverge from controls. The rate of soil drying was the reduction percentage of SWC per hour within the light period H1–H5. Means within columns with the same letter are statistically similar (Duncan's multiple range test, $P < 0.05$). For details see Eq. 1 and Figure 3. Values are means ± SE ($n = 3$)

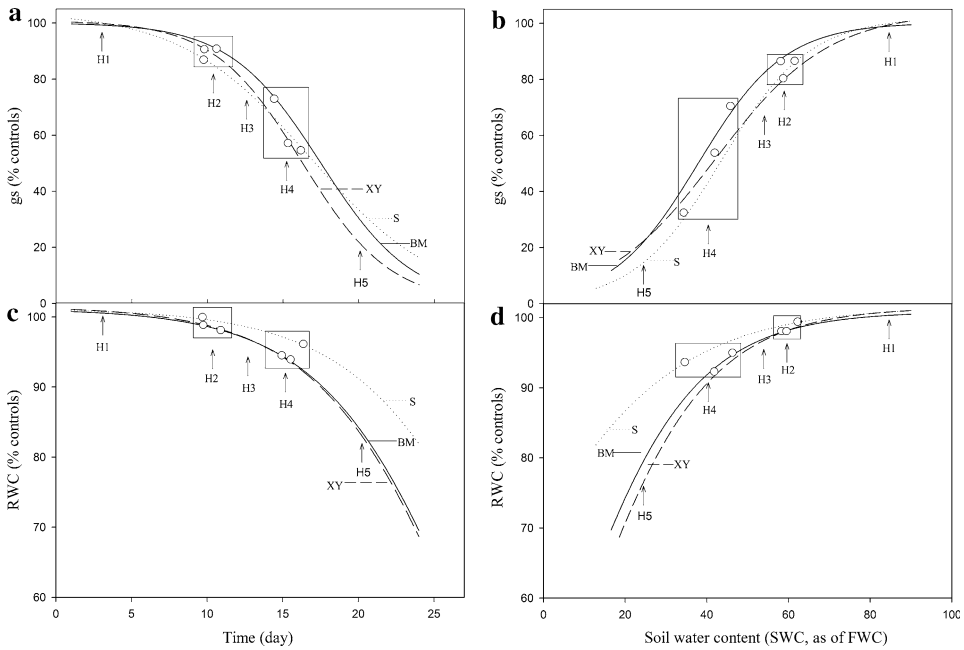

Fig. 2 Level of physiologic functions in drought-stressed wheat plants (% of controls) as a function of time. Stomatal conductance (**a**) and relative water content (**c**). Sigmoidal curves ($Y = 100/[1 + \exp(-X - X_0)]/b$) were fitted by 18 measurements with three replicates (stomatal conductance) and 9 measurements with three replicates (relative water content). Arrows indicate the five harvest times (H1–H5) and the two insets represent the points of chemical and hydraulic signals. Level of physiological functions in drought-stressed wheat plants (% of controls) as a function of soil water content (SWC). Stomatal conductance (**b**) and relative water content (**d**).

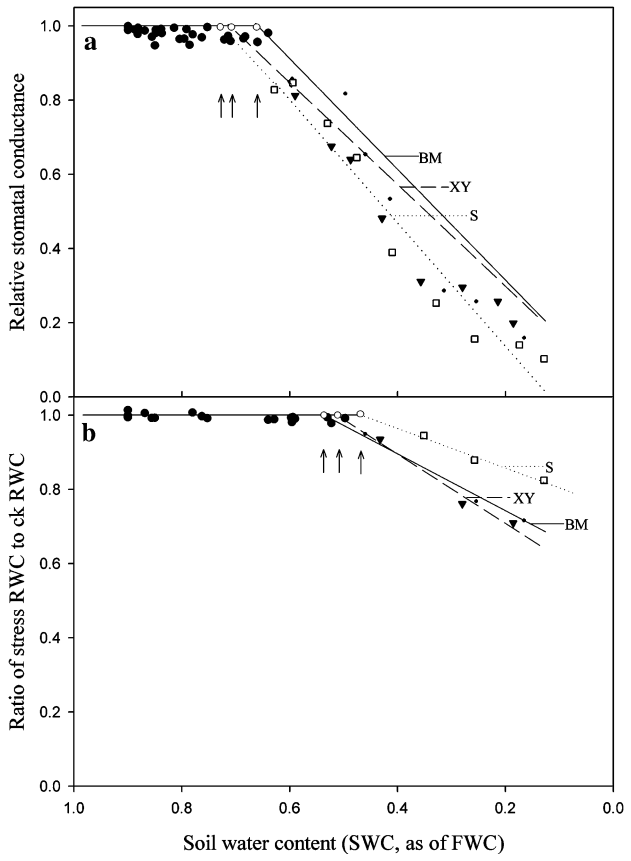

Fig. 3 Relationships between relative stomatal conductance (**a**) and ratio of stress RWC to control RWC (**b**) and the SWC for wheat (% of controls) grown in a climate-controlled greenhouse. Curves in a and b were fitted by linear-plateau functions [Eq. 1]. Arrows indicate the threshold values (see Table 1)

H_2O_2 production by XY and S also significantly declined; however, O_2^- production in XY and S did not significantly decrease. In all three cultivars, the relative values of O_2^- and H_2O_2 remained significantly higher than controls at H5 (data not shown). There were no significant differences in the O_2^- and H_2O_2 production of all three cultivars over H1–H2, but ROS production in S was slightly below that of the other cultivars. At H3, H_2O_2 production was the same in all three cultivars, with BM slightly higher than other cultivars. However, O_2^- production was significantly different among all cultivars; with XY the highest and S the lowest. At H4 and H5, H_2O_2 production in BM was significantly higher than the other cultivars, with XY and S similar. At H4, O_2^- production in BM was significantly higher than other cultivars; and at H5, O_2^- production in BM and XY were similar and greater than S (Figure 4b). Based on these results, BM (old) with a high basal level of ROS, generated much more ROS than S (modern) with progressively greater drought stress, particularly in period H4–H5.

At H1–H2 (89–60% FWC), there were no significant differences in accumulation rates of ROS among cultivars (see the peak curve slope of Figure 4c and d). They began to diverge, especially when SWC fell below 59% and BM had a significantly higher accumulation rate of ROS than S (Figure 4c, d). Furthermore, ROS production decreased when SWC fell below 38%, but the decreased rates were not the same, BM had the least and S the highest reduction rate.

The activities of several antioxidant enzymes (Figure 5a–d) increased and the content of AsA and GSH decreased (Figure 5e, f) after increased ROS production

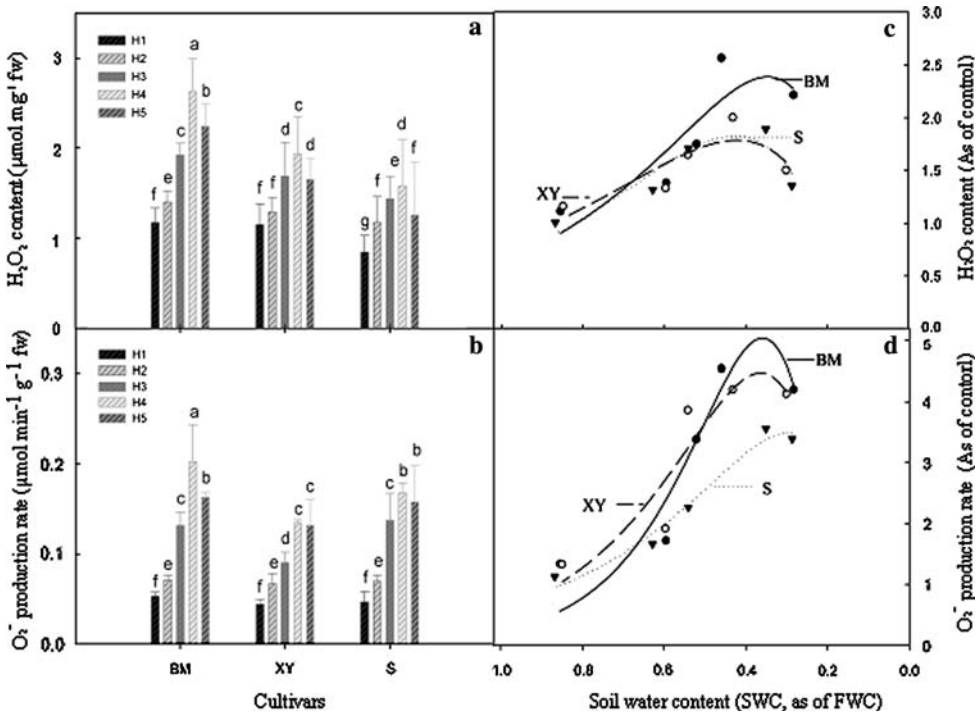

Fig. 4 H_2O_2 (**a**) and O_2^- (**b**) were recorded in leaves of winter wheat during progressive soil drying at five harvest times (H1–H5). Relationships of H_2O_2 (**c**) and O_2^- (**d**) to SWC for three wheat cultivars. Single peak curves [$f = a \times \exp(-0.5 \times (\ln(x/x_0)/b)^2]$ were fitted by five measurements with three replicates

induced by drought. Activities of the four enzymes increased with soil water depletion until a maximum level at the appearance of hydraulic signals; enzyme activities decreased thereafter. For SOD, there were no significant differences in the three cultivars at the same harvest times, but S had a slightly higher SOD activity than BM. For CAT, there was a significant difference in the three cultivars at the appearance of hydraulic signals (H4). CAT activity of S was significantly higher than that of other cultivars at H4. For APX and GR, activity in S was significantly higher than in BM at H3–H5. In general, over H2–H5 the trend in the four enzyme activities was S > XY > BM. However, cultivars had different rates of decrease of AsA and GSH. There was a significant decline in AsA in the BM leaves during mild water stress (that is, up to H2). In contrast, AsA decreased in S and XY leaves until H3 (Figure 5e). Furthermore, AsA content in S was much higher than that in BM at the same harvest time, and BM had the highest rate of decrease of AsA. There was no significant change in leaf GSH contents in the three cultivars at H1. However, at H2, BM began to decrease faster than other cultivars, and the GSH content in XY and S was significantly higher than in BM. Similarly, GSH content in S was significantly higher than that in BM at H2–H5. Therefore, AsA and GSH contents decreased with soil water depletion, most markedly in BM and least for S during H2–H5 (Figure 5e, f). Two-way ANOVA showed that stress significantly affected ROS content, nonenzyme, and enzyme antioxidants at H2–H5 (data not shown). However, there were differences in cultivar contents of ROS, nonenzyme, and enzyme antioxidants. There were significant effects on the contents of O_2^- ($P < 0.01$), APX ($P < 0.01$), GR ($P < 0.01$), AsA ($P < 0.0001$), and GSH ($P < 0.01$); other parameters were not significantly affected. The interactions of stress and cultivars affected the contents of ROS, nonenzyme, and enzyme antioxidants differently. There was a significant interaction in the content of CAT ($P < 0.01$), APX ($P < 0.001$), GR ($P < 0.01$), and GSH ($P < 0.01$). There were no significant changes in soluble protein content among the cultivars at the same harvest time (data not shown).

Drought Tolerance

Drought tolerance was determined by SD and the ability to minimize growth reductions. The reduction percentage (RP) of shoot biomass was the difference between controls and stress treatment relative to controls (Table 2). SD differed among cultivars. The older cultivar BM died first at 15.3 days after watering ceased, and the RP of 37.3% was the largest of the cultivars under drought conditions. Hence, drought tolerance of BM was the poorest. In contrast, the modern cultivar S had the longest SD at 22.7 days and RP the lowest at 20.9%. XY was intermediate with SD of 17.3 days and RP of 30.6%. Therefore, the cultivars' sequence of drought tolerance was S > XY > BM.

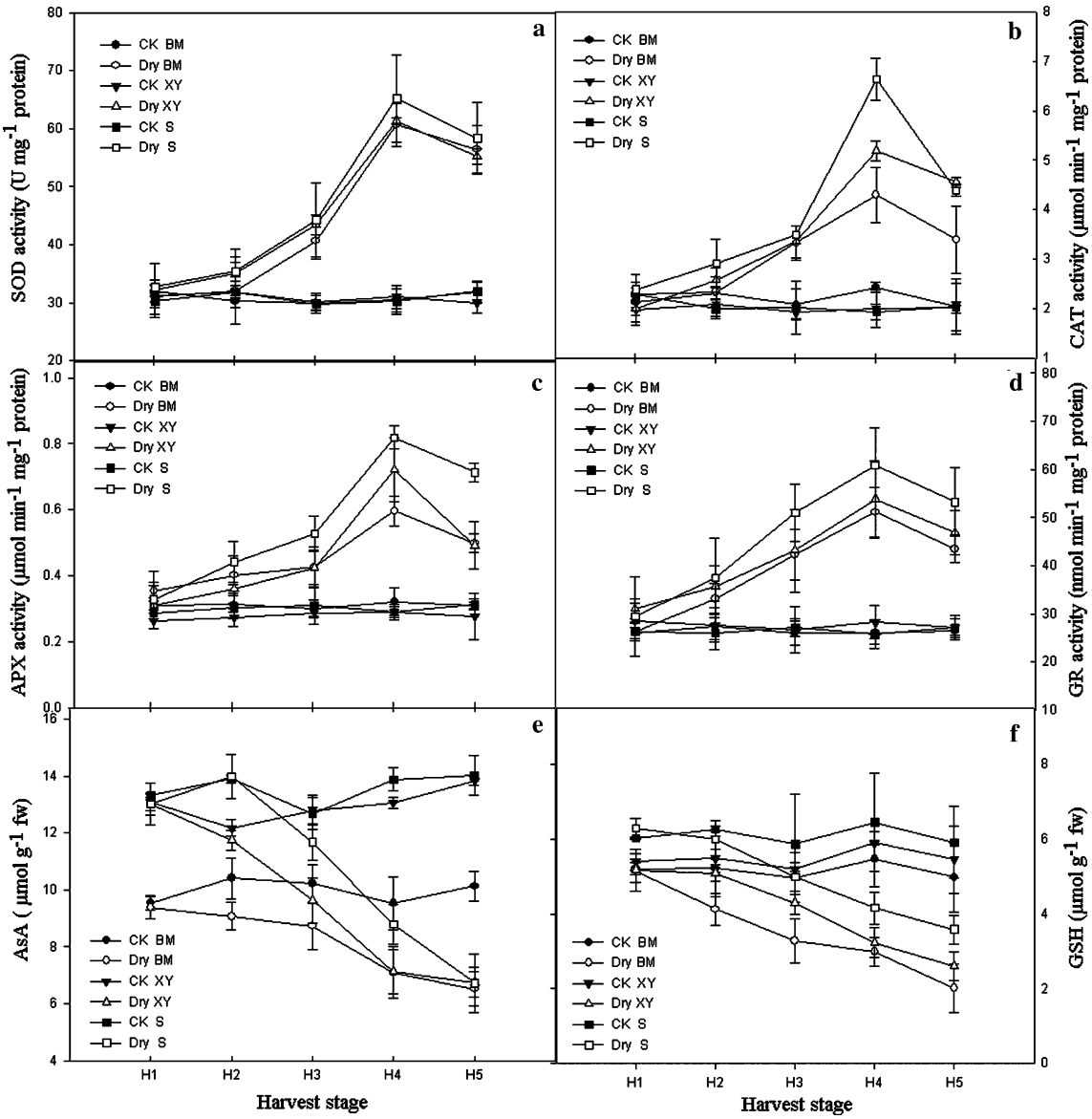

Fig. 5 Activities of enzymes SOD (**a**), CAT (**b**), APX (**c**), and GR (**d**) and nonenzymes AsA (**e**) and GSH (**f**) antioxidants at five harvest times with progressive soil drying. Values are means ± SE ($n = 6$). Means denoted by the same letter were not significantly different ($P < 0.05$) using Duncan's multiple range test

Discussion

Plant drought tolerance is a very complex mechanism. Plants may have as many regulatory patterns to respond to signals as the number of attributes embedded in drought stress, such as closure of stomata and production of antioxidants. Recent increases in understanding the mechanisms of drought adaptation have developed through studies of root-to-shoot signaling in the control of stomatal conductance and growth in water-stressed plants (Davies and Zhang 1991; Dodd 2005). Soil moisture can represent an available resource pool, the status of which should logically control growth and plant water use, including stomatal aperture as indicated by reduced stomatal conductance during drought. The triggering of root-sourced chemical signals can occur at different SWC, depending on the cultivar (Xiong and others 2006a, b). Chemical signaling in the modern cultivar was the earliest and had the widest range, 70.8–46.2% FWC. The old cultivar was the last to produce a chemical signal and with the narrowest range of 64–50.5% FWC. The recent cultivar was of intermediate status at 69.9–50.4% FWC (Figure 3, Table 2). The chemical signal of the modern cultivar was at the highest SWC, an early response to soil drying; this conserved water by reducing leaf expansion and closing stomata. The modern cultivar may have altered root-

sourced chemical signaling such that cues from drying soil trigger the chemical signals earlier, and so the signals are sensed longer than in older cultivars. The TRc appears to indicate drought tolerance. The modern cultivar with a wider threshold range also had better drought tolerance (longer SD and less RP). This result is also supported by previous work with eight old and modern wheat cultivars (Xiong and others 2006a). In the present study, TRc was associated with survival duration and RP. It is possible that wider TRc increases biomass by improving drought tolerance.

Many experiments have demonstrated that the chemical signal can be ABA (Dodd 2003), apoplastic pH (Wilkinson and Davies 2002), cytokinins (Kamboj and others 1998), ethylene/ACC (Sharp and others 2000), and nitric oxide (Palmer and others 1996); all play important roles in plant responses to soil drying. It is not known how these chemical signal compounds, particularly the primary signal molecule ABA, directly relate to plant defense response to drought. Recent work has shown that ROS are essential signals, mediated by ABA-induced stomata closure via the activation of calcium-permeable channels in the plasma membrane (Pei and others 2000). Progressive soil water depletion induced stomatal closure and increased flux through the photorespiratory pathway, and increased oxidative load on tissues, since both processes generate ROS. ROS are generated by normal plant cellular activities and levels increase with abiotic stress, particularly drought. In addition to imposing water stress, ROS production increased among the three wheat cultivars. Interestingly, when chemical signals appeared, the ROS levels were significantly higher than those of controls in all three wheat cultivars (data not shown). ABA can accumulate in the root as chemical signals appear (Liu and others 2006; Wilkinson and Davies 2002). Therefore, drought stress induces ABA accumulation, but can also increase ROS production (Figure 4). However, ROS levels differed among cultivars during drought stress. The modern cultivar had the lowest ROS levels, and the older cultivar the highest. The old cultivar had a narrow TRc (Table 2), high ROS production (Figure 4a, b), and a high accumulation rate of ROS (Figure 4c, d). However, the modern cultivar had a wider TRc, lower generation of ROS, and slower accumulation rate. Those cultivars with narrow TRc tended to have higher production and a higher accumulation rate of ROS (Figure 4, Table 2). Consequently, old cultivars may synthesize more ABA in the root than modern cultivars, and rapid transfer to the leaves may mean more rapid accumulation of leaf ABA. If this signal material cannot be quickly decomposed or sequestrated in the shoot, then the chemical signals alter turgor maintenance and induce hydraulic signals earlier than for the modern cultivar, and plants could quickly generate large amounts of ROS. Therefore, although older cultivars generally responded slowly to drought stress, they may have rapidly synthesized the chemical signals in the root, which quickly induced large ROS production in leaves compared with more modern cultivars.

Intracellular ROS can increase with drought stress (Pastore and others 2007). In general, under mild water stress, chemical signals appear and accumulate, thus triggering ROS generation. There are two possibilities to explain the effect of chemical signals on oxidative stress. The first is that chemical signals enhance recovery in cells where molecules have been damaged by ROS, and the second is that the signals increase ROS elimination. Therefore, the production and accumulation of ROS were mediated by chemical signals and antioxidants (Apel and Hirt 2004).

Overexpression of SOD activity in chloroplasts and mitochondria of drought-affected plants also reflected higher O_2^- generation compared to controls, indicating a degree of extra protection against water deficit (McKersie and others 2000). However, dismutation of O_2^- simply converts one destructive ROS to another. The ability of plants to overcome oxidative stress relies partly on the induction of SOD activity and subsequently on the upregulation of other downstream antioxidant enzymes, and on maintenance of the AsA–GSH pool (Alscher and others 2002). Moreover, H_2O_2 is a strong oxidant that rapidly oxidizes thiol groups and cannot be tolerated in excess (Noctor and Foyer 1998). CAT and APX provide efficient H_2O_2 scavenging, and there is a high degree of redundancy among these enzymes (Rizhsky and others 2002). Both enzymes have an important role in H_2O_2 detoxification by catalyzing the reduction of H_2O_2 to water by AsA; the resulting monodehydroascorbate and dehydroascorbate are reduced back to AsA by monodehydroascorbate reductase and dehydroascorbate reductase plus GR, respectively (Iturbe-Ormaetxe and others 2001).

The present study showed that SOD, CAT, GR, and APX activity increased with higher ROS levels during progressive soil drying (Figures 4 and 5). Activities of these enzymes differed among cultivars when chemical signals appeared. The modern cultivar generated much more ROS-scavenging enzymes than the old cultivar (H2–H5). In plants, production and removal of ROS is usually strictly controlled, and the equilibrium between ROS generation and scavenging may be perturbed by drought. The modern cultivar triggered early chemical signals, inducing a better balance between ROS and antioxidants compared with older cultivars. These results were correlated with adaptation to drought among cultivars and showed that the modern cultivar may maintain crop production during soil drying.

AsA and GSH redox buffering capacity generates stress tolerance in plants (Apel and Hirt 2004; Foyer and Noctor

2005). It has been suggested that low levels of AsA and GSH allow oxidant signals to persist and accumulate in the apoplast. This may have implications for cell elongation and expansion (Pignocchi and others 2006). Drought-induced oxidation may cause the pool of AsA and GSH to shrink (Lascano and others 2001). In the present study, AsA and GSH content began to decrease under mild water stress (Figure 5e, f). However, the modern cultivar was insensitive to mild drought stress and the decrease of AsA and GSH was later than for older cultivars. Severe drought stress resulted in significantly more decline in the AsA and GSH pool in leaves of the old cultivar; possibly due to the lower biosynthesis of AsA and GSH and lower activities of the AsA–GSH cycle enzymes. Moreover, in the older cultivar the AsA and GSH pool fluctuated widely, possibly providing less oxidative stress protection to the chloroplasts. This is consistent with recent proposals that less fluctuation in AsA and GSH provided protection to chloroplasts (Khanna-Chopra and Selote 2007).

Early and rapid response to water deficit is critical to plant survival during drought (Ober and Sharp 2003). In the present study, the modern cultivar triggered early root-sourced chemical signals, indicated by a better balance of ROS production and removal (Figures 3–5) and by slower oxidation of the AsA–GSH pool (Figure 5e, f). Moreover, interaction between stress and cultivars showed that stress and cultivars differ in their effects on CAT, APX, and GR contents. Therefore, it can be concluded that the level of ROS and oxidation of the AsA–GSH pool among cultivars might rely on antioxidant enzymes, particularly CAT, APX, and GR. The early chemical signal triggering in the modern cultivar (S) may increase ROS generation, upregulate antioxidant enzyme activities (Figure 5a–d), and maintain the AsA–GSH pool (Figure 5e, f) compared with the old cultivar (BM).

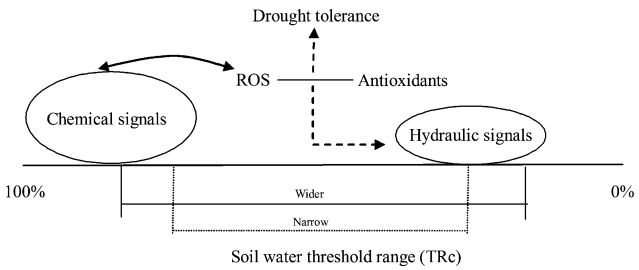

Fig. 6 Mechanism of relationship between soil-water threshold range of chemical signals and drought tolerance mediated by homeostasis between ROS and antioxidants. From left to right represents the reduction trend of soil water content (100 to 0%). When soil-available water decreases, chemical signals are induced which lead to increased ROS production and accumulation, then hydraulic signals appear with further soil drying. The balance between ROS and antioxidants as a regulator could employ a relationship between TRc and drought tolerance

In summary, the modern wheat cultivar with improved drought tolerance triggered early chemical signals, and hydraulic signals appeared later, which can be defined as a wider soil-water threshold range of chemical signals. Furthermore, the modern wheat cultivar had increased enzyme antioxidant activities and smaller decreases in nonenzyme antioxidants, which led to less ROS production during progressive soil drying. It can be speculated that homeostasis between production and removal of ROS could regulate the appearance of hydraulic signals and thus extend the soil-water threshold range of chemical signals. The present work also suggests that homeostasis between ROS and antioxidants may provide a link between the soil-water threshold range of chemical signals and drought tolerance (Figure 6).

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