Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves

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Abstract
The interrelationship among water-stress-induced abscisic acid (ABA) accumulation, the generation of reactive oxygen species (ROS), and the activities of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) was investigated in leaves of detached maize (Zea mays L.) plants exposed to −0.7 MPa water stress induced by polyethylene glycol (PEG 6000). Time-course analyses of ABA content, the production of ROS, and the activities of antioxidant enzymes in water-stressed leaves showed that a significant increase in the content of ABA preceded that of ROS, which was followed by a marked increase in the activities of these antioxidant enzymes. Pretreatment with an ABA biosynthesis inhibitor, tungstate, significantly suppressed the accumulation of ABA, and also reduced the increased generation of ROS and the up-regulation of these antioxidant enzymes in water-stressed leaves. A mild oxidative stress induced by paraquat, which generates O$_2^-$ and then H$_2$O$_2$, resulted in a significant enhancement in the activities of antioxidant enzymes in non-water-stressed leaves. Pretreatment with some ROS scavengers, such as Tiron and dimethylthiourea (DMTU), and an inhibitor of NAD(P)H oxidase, diphenyleneiodonium (DPI), almost completely arrested the increase in ROS and the activities of these antioxidant enzymes induced by water stress or ABA treatment. These data suggest that water stress-induced ABA accumulation triggers the increased generation of ROS, which, in turn, leads to the up-regulation of the antioxidant defence system.

Key words: Abscisic acid, antioxidant enzymes, oxidative stress, reactive oxygen species, water stress, Zea mays.

Introduction
Water stress is one of the most important environmental factors that regulate plant growth and development, and limit plant production. Plants can respond and adapt to water stress by altering their cellular metabolism and invoking various defence mechanisms (Bohnert and Jensen, 1996). Survival under this stressful condition depends on the plant’s ability to perceive the stimulus, generate and transmit the signals, and initiate various physiological and chemical changes (Bohnert and Jensen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). The plant hormone abscisic acid (ABA), as a stress signal, increases as a result of water stress and plays important roles in the regulation of plant responses from the whole plant level (Davies and Zhang, 1991) to the cellular level (Shinozaki and Yamaguchi-Shinozaki, 1997). How the ABA signal is transduced into a physiological or biochemical response has been an interesting research subject in the recent years.

Increasing evidence indicates that one mode of ABA action may be related to its role in the oxidative stress in...
plant cells. It has been documented that ABA can cause an increased generation of O\textsubscript{2} (Jiang and Zhang, 2001) and H\textsubscript{2}O\textsubscript{2} (Guan et al., 2000; Pei et al., 2000; Jiang and Zhang, 2001; Murata et al., 2001; Zhang et al., 2001), and induce the expression of antioxidant genes encoding Cu, Zn-SOD (Sakamoto et al., 1995; Guan and Scandalios, 1998a; Kaminaka et al., 1999), Mn-SOD (Zhu and Scandalios, 1994; Bueno et al., 1998; Kaminaka et al., 1999), Fe-SOD (Kaminaka et al., 1999), and CAT (Williamson and Scandalios, 1992; Anderson et al., 1994; Guan and Scandalios, 1998b; Guan et al., 2000). Meanwhile, ABA also increases the activities of antioxidant enzymes such as SOD, CAT, APX, and GR in plant tissues (Anderson et al., 1994; Prasad et al., 1999; Bueno et al., 1998; Kaminaka et al., 1999), and CAT (Williamson and Scandalios, 1992; Anderson et al., 1994; Guan and Scandalios, 1998b; Guan et al., 2000). Meanwhile, ABA acts upstream of ROS is controversial because a contrary hypothesis has been proposed that an oxidative burst might function as one of the triggers of the water-stress responses and ABA might function downstream of ROS to regulate gene expression as well as physiological and biochemical responses during water stress (Shinozaki and Yamaguchi-Shinozaki, 1997). A recent study has shown that ROS induced by water stress is involved in water-stress-induced ABA biosynthesis in root tips of wheat seedlings (Zhao et al., 2001). Moreover, there also might exist an ABA-independent or ROS-independent pathway during the process of antioxidant response to water stress (Guan et al., 2000). However, to date, no single study as far as is known has critically investigated the interrelationship among ABA, ROS and antioxidant defence in a single species under water stress.

In this study, an effort was made to discover a possible interrelationship among ABA, ROS and antioxidant enzymes in plants exposed to water stress. First of all, the time-course of ABA accumulation was monitored as well as the increased generation of ROS such as O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}, and the induction of several antioxidant enzymes such as SOD, CAT, APX, and GR in the leaves of maize plants exposed to water stress. The effects of pretreatment with the ABA biosynthesis inhibitor, tungstate, on the levels of ROS and the activities of antioxidant enzymes in water-stressed leaves were also investigated. Tungstate was shown to block the formation of ABA from ABA-aldehyde by impairing ABA-aldehyde oxidase (Hansen and Grossmann, 2000). Several other ROS-manipulators were then used, such as DPI, a well-known inhibitor of NAD(P)H oxidase (O\textsubscript{2} synthase) (Levine et al., 1994; Bolwell et al., 1998; Papadakis and Roubelakis-Angelakis, 1999; Orozco-Cárdenas and Ryan, 1999; Pei et al., 2000; Orozco-Cárdenas et al., 2001), Tiron, a specific O\textsubscript{2} scavenger (Wise and Naylor, 1987; Kawano et al., 1998), DMTU, a trap for H\textsubscript{2}O\textsubscript{2} (Levine et al., 1994; Rao et al., 1997; Casano et al., 2001), and paraquat, which generates O\textsubscript{2} and its decomposition product H\textsubscript{2}O\textsubscript{2} (Babbs et al., 1989). The manipulation of ROS levels in stressed or non-stressed leaves may help to assess their possible link between water-stress-induced ABA and stress-enhanced antioxidative defence.

Materials and methods

Plant materials and treatments

Seeds of maize (Zea mays L.) were sown in trays of sand in a greenhouse at a temperature of 25±30 °C, with photosynthetic active radiation (PAR) of 400 μmol m\textsuperscript{-2} s\textsuperscript{-1} (enhanced with high-pressure sodium lamps) and a photoperiod of 14/10 h (day/night), and watered daily. When the second leaf was fully expanded, the plants were collected and used for all investigations.

The plants were excised at the base of the stem, rinsed in distilled water, and the cut ends of the stems were placed in beakers wrapped with aluminium foil containing 250 ml PEG solution at –0.7 MPa for 24 h at 25 °C with a continuous light intensity of 200 μmol m\textsuperscript{-2} s\textsuperscript{-1}. In order to study the effects of inhibitors and scavengers, the detached plants were pretreated with sodium tungstate (1 mM and 5 mM), 5–100 μM DPI, 10 mM Tiron and 5 mM DMTU for 12 h and then exposed to water stress treatment (–0.7 MPa) or ABA treatment (100 μM) for 12 or 24 h under the same conditions as described above. To test whether the effects of tungstate could be overcome by exogenously supplied ABA, the detached plants were pretreated with 1 mM tungstate+100 μM ABA or 5 mM tungstate+100 μM ABA for 12 h, and then exposed to water stress treatment (–0.7 MPa) or ABA treatment (100 μM) for 12 or 24 h under the same conditions as described above. To test whether the effects of tungstate could be overcome by exogenously supplied ABA, the detached plants were pretreated with 1 mM tungstate+100 μM ABA or 5 mM tungstate+100 μM ABA for 12 h, and then exposed to water stress treatment for 24 h. For oxidative stress treatments, the detached plants were incubated with 1 μM or 50 μM paraquat for 12 h under the same conditions as described above. To investigate the effects of ROS scavengers on oxidative stress treatments, the detached plants were pretreated with 10 mM Tiron and 5 mM DMTU for 12 h, and then exposed to paraquat treatments for 12 h. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. After treatment of detached maize plants, the second leaves were sampled and immediately frozen under liquid N\textsubscript{2}, and then stored at –80 °C for further analysis.

Enzyme assays

Frozen leaf segments (0.5 g) were crushed into fine powder in a mortar and pestle under liquid N\textsubscript{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 the addition of 1 mM ascorbic acid (ASC) in the case of the APX assay. The homogenate was centrifuged at 15 000 g for 20 min at 4 °C and the supernatant was used for the following enzyme assays. Protein content was determined according to the method of Bradford (1976) with BSA as standard.
Total SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Giannopolitis and Ries (1977). The 3 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μl enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 5000 lx. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

CAT (EC 1.11.1.6) activity was determined by following the consumption of H₂O₂ (extinction coefficient 39.4 mM⁻¹cm⁻¹) at 240 nm for 3 min (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 10 mM H₂O₂ and 200 μl of enzyme extract in a 3 ml volume.

GR (EC 1.6.4.2) activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 mM⁻¹cm⁻¹) for 3 min in 1 ml of an assay mixture containing 50 mM potassium phosphate buffer (pH 7.8), 2 mM Na₂EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and 100 μl of enzyme extract. The reaction was initiated by adding NADPH. Corrections were made for the background absorbance at 340 nm, without NADPH (Schäadle and Bassham, 1977).

APX (EC 1.11.1.11) activity was determined by following the decrease in A₂90 (extinction coefficient 2.8 mM⁻¹cm⁻¹) for 1 min in 1 ml of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ASC, 0.1 mM H₂O₂, and 200 μl of enzyme extract. The reaction was started by enzyme extract. Correction was done for the low, non-enzymatic oxidation of ASC by H₂O₂ (Nakano and Asada, 1981).

**Determination of ROS**

O₂⁻ production was measured as described by Able et al. (1998) by monitoring the reduction of XTT in the presence of O₂⁻, with some modifications. Leaves (1 g) were homogenized with 5 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 5000 g for 10 min. The reaction mixture (1 ml) contained 50 mM Tris–HCl buffer (pH 7.5), 50 μg of 5000 g supernatant proteins, and 0.5 mM XTT. The reduction of XTT was determined at 470 nm for 5 min. Corrections were made for the background absorbance in the presence of 50 units SOD. O₂⁻ production rate was calculated using an extinction coefficient of 2.16×10⁻³ M⁻¹ cm⁻¹.

The content of H₂O₂ was measured by monitoring the A₄₁₅ of the titanium-peroxide complex following the method described by Brennan and Frenkel (1977). Absorbance values were calibrated to a standard graph generated with known concentrations of H₂O₂.

**ABA analysis**

ABA analysis was carried out using the radioimmunoassay (RIA) method as described by Quarrie et al. (1988). The highly specific monoclonal antibody (AFRC MAC 225) was provided by Dr SA Quarrie (Cambridge Laboratory, IPSR, John Innes Centre, UK). 100 mg freeze-dried and powdered leaf mass was shaken in 5 ml distilled water for 24 h at 4 °C in darkness. The supernatant was obtained as a crude extract after centrifuging. 50 μl of crude extracts was mixed with 200 μl phosphate-buffered saline (pH 6.0), 100 μl diluted antibody solution and 100 μl ³H-ABA (about 8000 cpm) solution. The reaction mixture was incubated at 4 °C for 45 min and the bound radioactivity was measured in 50% saturated (NH₄)₂SO₄- precipitated pellets with a liquid scintillation counter. The extraction efficiency and immunoreactive contamination in crude extracts of maize samples were tested earlier by Zhang and Davies (1990), and proved to be reliable.

**Statistical analysis**

The results presented were the mean of six replicates. Means were compared by one-way analysis of variance and Duncan’s multiple range test at the 5% level of significance.

**Results**

**Effects of water stress on ABA, ROS and antioxidant enzymes**

The effects of water stress on ABA content, the production of O₂⁻ and H₂O₂, the activities of several important antioxidant enzymes such as SOD, CAT, APX, and GR in maize leaves are shown in Figs 1 and 2. Water stress led to a continuous increase in the content of ABA within the 24 h of water stress treatment (Fig. 1A). A significant increase in the content of ABA occurred within the first 3 h of water stress treatment (Fig. 1A). A significant increase in the content of ABA occurred within the first 3 h.
of treatment. After 12 h and 24 h of treatment, the content of ABA increased 3.8-fold and 5.9-fold, respectively, compared with the control values. Water stress also caused an increase in the generation of O$_2^-$ and H$_2$O$_2$ (Fig. 1B, C). A significant increase in the production of O$_2^-$ and H$_2$O$_2$ occurred within the first 6 h of treatment. After 12 h of treatment, the production of O$_2^-$ and H$_2$O$_2$ reached the maximum values, and increased by 41% and 46%, respectively, compared with the control value. After 12 h of treatment, the generation of O$_2^-$ and H$_2$O$_2$ decreased and returned to the levels of the control at 24 h.

After an increase in the content of ABA and the production of O$_2^-$ and H$_2$O$_2$ induced by water stress in maize leaves, the activities of several antioxidant enzymes increased (Fig. 2). A significant enhancement in the activities of SOD (Fig. 2A), CAT (Fig. 2B), APX (Fig. 2C), and GR (Fig. 2D) occurred within 9 h of treatment. After 12 h of treatment, the activities of SOD, CAT, APX, and GR increased by 21%, 52%, 33%, and 38%, respectively, compared with the control. However, after 24 h of water stress treatment, the activities of these antioxidant enzymes had a tendency to decrease when compared to the 12 h treatment. No significant changes in the content of soluble protein were observed during water stress treatment of 24 h (data not shown).

**Effects of pretreatment with tungstate on ABA, ROS and antioxidant enzymes**

In order to determine whether the increases in the production of ROS and the activities of antioxidant enzymes were related to the accumulation of ABA in the leaves of maize plants exposed to water stress, the effects of pretreatment with ABA biosynthesis inhibitor, tungstate, which impairs ABA-aldehyde oxidase, on these enzymes were studied.
ROS and antioxidant enzymes under water stress were investigated. Meanwhile, exogenous ABA was added at a final concentration of 100 μM together with tungstate to test whether the effects of tungstate could be overcome by exogenously supplied ABA. Experimental results showed that pretreatment with tungstate significantly inhibited the accumulation of ABA in the water-stressed leaves (Fig. 3A). After 12 h and 24 h, pretreatment with 1 mM tungstate inhibited the increase in ABA content induced by water stress by 35% and 78%, and 5 mM tungstate inhibited the increase by 65% and 92%, respectively. These pretreatments did not affect the content of ABA in the non-stressed leaves. At the same time, pretreatment with 5 mM tungstate significantly suppressed the increase in the generation of O$_2^-$ and H$_2$O$_2$ in the leaves exposed to water stress for 12 h, although 1 mM tungstate only led to a slight reduction in the generation of these ROS in the stressed leaves (Fig. 3B, C). These pretreatments alone did not affect the production of these ROS in the non-stressed leaves. Accordingly, the pretreatments with tungstate significantly reduced the activities of SOD (Fig. 4A), CAT (Fig. 4B), APX (Fig. 4C), and GR (Fig. 4D) induced by water stress in a dose- and time-dependent pattern. Pretreatment with 5 mM tungstate almost completely arrested the increase in the activities of these antioxidant enzymes induced by water stress in the stressed leaves. These pretreatments alone also did not affect the activities of these antioxidant enzymes in non-stressed leaves (Fig. 4A–D). The application of 100 μM ABA substantially restored the level of ABA inhibited by tungstate (Fig. 3A), and fully prevented the reduction in the generation of ROS (Fig. 3B, C) and the activities of SOD, CAT, APX, and GR induced by tungstate in the stressed leaves (Fig. 4A–D).

![Fig. 3](image1.png)

**Fig. 3.** Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the content of ABA (A), the production of O$_2^-$ (B) and H$_2$O$_2$ (C) in leaves of detached maize plants exposed to water stress. The detached plants were treated as follows: 1, distilled water (control); 2, 1 mM sodium tungstate; 3, 5 mM sodium tungstate; 4, water stress (~0.7 MPa); 5, 1 mM sodium tungstate+water stress; 6, 5 mM sodium tungstate+water stress; 7, 1 mM sodium tungstate+100 μM ABA+water stress; 8, 5 mM sodium tungstate+100 μM ABA+water stress. The detached plants were pretreated with sodium tungstate, sodium tungstate plus ABA for 12 h, and then exposed to water stress or distilled water for 12 h (B, C) or 24 h (A). Values are means ±SE (n=6). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan’s multiple range test.

![Fig. 4](image2.png)

**Fig. 4.** Effects of pretreatment with ABA biosynthesis inhibitor, tungstate, on the activities of SOD (A), CAT (B), APX (C), and GR (D) in leaves of detached maize plants exposed to water stress. The detached plants were treated as follows: 1, distilled water (control); 2, 1 mM sodium tungstate; 3, 5 mM sodium tungstate; 4, water stress (~0.7 MPa); 5, 1 mM sodium tungstate+water stress; 6, 5 mM sodium tungstate+water stress; 7, 1 mM sodium tungstate+100 μM ABA+water stress; 8, 5 mM sodium tungstate+100 μM ABA+water stress. The detached plants were pretreated with sodium tungstate, sodium tungstate plus ABA for 12 h, and then exposed to water stress or distilled water for 12 h or 24 h. Values are means ±SE (n=6). Means denoted by the same letter did not significantly differ at $P < 0.05$ according to Duncan’s multiple range test.
Effects of oxidative stress on ABA, ROS and antioxidant enzymes

In order to investigate whether it is the ROS that induces ABA biosynthesis and the activities of antioxidant enzymes, the detached plants were treated with paraquat, which generates \( \text{O}_2^- \) and then \( \text{H}_2\text{O}_2 \). The treatment with 1 \( \mu \text{M} \) paraquat led to a relatively mild increase in the generation of \( \text{O}_2^- \) (Fig. 5A) and \( \text{H}_2\text{O}_2 \) (Fig. 5B) and a substantial enhancement in the activities of SOD (Fig. 6A), CAT (Fig. 6B), APX (Fig. 6C), and GR (Fig. 6D), but the treatment with 50 \( \mu \text{M} \) paraquat resulted in a steep increase in the production of the ROS and a sharp decline in the activities of these antioxidant enzymes in maize leaves. The pretreatments with Tiron, a specific scavenger for \( \text{O}_2^- \), and DMTU, a trap for \( \text{H}_2\text{O}_2 \), almost fully blocked the increase in the activities of SOD, CAT, APX, and GR induced by 1 \( \mu \text{M} \) paraquat except for DMTU, which did not affect the increase in the activity of SOD due to its specific scavenging for \( \text{H}_2\text{O}_2 \), and significantly alleviated the reduction in the activities of these antioxidant enzymes caused by the treatment of 50 \( \mu \text{M} \) paraquat (Fig. 6A–D).

All treatments with paraquat or \( \text{H}_2\text{O}_2 \) did not affect the content of ABA during the whole period of 12 h treatment (Fig. 7A, B).

Effects of DPI, Tiron and DMTU on water stress- and ABA-induced changes in ROS and antioxidant enzymes

In order to determine whether the enhancement in the activities of antioxidant enzymes induced by water stress or ABA results from the increase in the production of ROS, the detached plants were pretreated with DPI, an inhibitor of NAD(P)H oxidase (\( \text{O}_2^- \) synthase), Tiron and DMTU, the scavengers for \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), respectively, and then exposed to water stress or ABA treatment. Experimental results showed that the pretreatments with DPI, Tiron and DMTU fully prevented the increase in the production of \( \text{O}_2^- \) (Fig. 8A) and \( \text{H}_2\text{O}_2 \) (Fig. 8B). The inhibition of DPI on the accumulation of \( \text{O}_2^- \) induced by ABA or water stress exhibited a high sensitivity, and low concentrations of DPI...
substantially inhibited the increase in the generation of O$_2^-$ (Fig. 8C). Meanwhile, the pretreatments with DPI, Tiron and DMTU completely prevented the enhancement in the activities of SOD (Fig. 9A), CAT (Fig. 9B), APX (Fig. 9C), and GR (Fig. 9D) induced by water stress or ABA treatment except for DMTU, which did not affect the generation of O$_2^-$ and the activity of SOD due to its specific scavenging for H$_2$O$_2$.

**Discussion**

Water stress can induce ABA accumulation and oxidative stress in plant cells (Bowler et al., 1992; Smirnoff, 1993; Shinozaki and Yamaguchi-Shinozaki, 1997). Both ABA and ROS are thought to be involved in the cellular signalling process as secondary messengers to induce antioxidant defences under water stress (Zhu and Scandalios, 1994; Shinozaki and Yamaguchi-Shinozaki, 1997; Bueno et al., 1998; Guan and Scandalios, 1998a, b; Guan et al., 2000). However, the interrelationship between water stress, ABA, ROS, and antioxidant defences in water stress signal transduction cascades is still not clear. The effects have been examined systematically of water stress on ABA content, the generation of O$_2$ and H$_2$O$_2$, and the activities of several main antioxidant enzymes such as...
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Fig. 9. Effects of pretreatment with DPI, Tiron and DMTU on the activities of SOD (A), CAT (B), APX (C), and GR (D) in leaves of detached maize plants exposed to water stress or ABA treatment. The detached plants were treated as follows: 1, distilled water (control); 2, 100 μM ABA; 3, 100 μM DPI+100 μM ABA; 4, 10 mM Tiron+100 μM ABA; 5, 5 mM DMTU+100 μM ABA; 6, water stress (-0.7 MPa); 7, 100 μM DPI+water stress; 8, 10 mM Tiron+water stress; 9, 5 mM DMTU+water stress. The detached plants were pretreated with DPI, Tiron and DMTU for 12 h, and then exposed to water stress or ABA treatment for 12 h. Values are means ±SE (n=6). Means denoted by the same letter did not significantly differ at P<0.05 according to Duncan’s multiple range test.

SOD, CAT, APX, and GR in maize leaves and the interrelationship among them under a mild water stress (relative water content was lowered by 14.2% during 24 h water stress treatment). The results showed that a significant increase in the content of ABA preceded that of the generation of ROS, and a marked increase in the activities of these antioxidant enzymes followed the increase of ROS in maize leaves under the mild water stress (Figs 1, 2). The reduction in the content of ABA caused by ABA biosynthesis inhibitor, tungstate, was accompanied by the decrease in the production of ROS and the activities of antioxidant enzymes in water-stressed leaves, and the decrease was fully prevented by the addition of ABA, which raised the internal ABA content (Figs 3, 4). A mild oxidative stress induced by 1 μM paraquat resulted in a significant up-regulation in the activities of antioxidant enzymes, which was suppressed by ROS scavengers, Tiron and DMTU (Figs 5, 6). A full block in the increase in the generation of ROS induced by water stress or ABA treatment also completely prevented the enhancement in the activities of antioxidant enzymes, when the plants were pretreated with O2- synthase inhibitor DPI and ROS scavengers such as Tiron and DMTU, respectively (Figs 7, 8). Although the absolute specificity of each inhibitor or scavenger used in this study can always be questioned, these data show clearly that the increase in the capacity of antioxidant defence induced by water stress is due, at least in part, to ABA-mediated metabolic changes leading to an increase in endogenous ROS levels.

Does there exist a two-way regulation between ABA and ROS, i.e. ABA results in the increased production of ROS and ROS can also promote ABA biosynthesis? The general framework of the ABA biosynthesis pathway in plants has been well established. The ABA biosynthetic pathway involves the formation of a 9-cis-epoxycarotenoid precursor. Oxidative cleavage then results in the formation of xanthoxin, which is subsequently converted to ABA under water stress (Schwartz et al., 1997; Hansen and Grossmann, 2000; Taylor et al., 2000). There is only limited information about the effects of ROS on ABA biosynthesis under water stress. In a recent study, the results indicated that water-stress-induced ABA accumulation was affected neither by ROS scavengers, tested with dimethyl sulphoxide (DMSO) and melatonin, nor by the direct treatment with O2 or H2O2 in excised maize leaf and root tissues (Jia and Zhang, 2000). However, in rapid dehydrated root tissues of wheat seedlings, the induction of ABA by dehydration was strongly blocked by ROS scavengers such as Tiron or ascorbic acid, and the ROS generators diethyldithiocarbamic acid, xanthine-xanthine oxidase and triazole also induced ABA accumulation (Zhao et al., 2001). These contradictory results may be related to the excised root or leaf tissues and very rapid, severe dehydration. In the present study, using intact plants excised at the base of the stem, the data show that oxidative stress induced by paraquat or H2O2 treatments did not affect ABA content in maize leaves, regardless of either a mild oxidative stress or a severe oxidative stress (Fig. 7A, B). Meanwhile, pretreatment with the ROS scavengers, Tiron and DMTU, and the inhibitor of NAD(P)H oxidase, DPI, also did not affect the content of ABA in the leaves of maize plants exposed to the mild water stress (data not shown). These data suggest that ROS are not involved in ABA biosynthesis in plants under mild water stress. Nevertheless, a more strict examination is required under physiological conditions for elucidating whether ROS is involved in the accumulation of ABA induced by water stress.

There are many pathways to generate ROS in plant cells, including electron transport, energy transfer, enzymatic and non-enzymatic reactions (Asada, 1999; Foyer and Noctor, 2000). In plasma membranes, a trans-membrane synthase (NAD(P)H oxidase) transfers electrons from NADH or NADPH in the cytoplasm to O2 to form O2- and H2O2. These oxygen radicals can also be generated by photoinhibition responses such as xanthophyll de-epoxidation, the Calvin cycle, and the activity of xanthophylls and oxygenase activity (Schwartz et al., 1997; Grossmann and Noctor, 2000). There is only limited information about the effects of ROS on ABA biosynthesis under water stress. The general framework of the ABA biosynthesis pathway in plants has been well established. The ABA biosynthetic pathway involves the formation of a 9-cis-epoxycarotenoid precursor. Oxidative cleavage then results in the formation of xanthoxin, which is subsequently converted to ABA under water stress (Schwartz et al., 1997; Hansen and Grossmann, 2000; Taylor et al., 2000). There is only limited information about the effects of ROS on ABA biosynthesis under water stress. In a recent study, the results indicated that water-stress-induced ABA accumulation was affected neither by ROS scavengers, tested with dimethyl sulphoxide (DMSO) and melatonin, nor by the direct treatment with O2 or H2O2 in excised maize leaf and root tissues (Jia and Zhang, 2000). However, in rapid dehydrated root tissues of wheat seedlings, the induction of ABA by dehydration was strongly blocked by ROS scavengers such as Tiron or ascorbic acid, and the ROS generators diethyldithiocarbamic acid, xanthine-xanthine oxidase and triazole also induced ABA accumulation (Zhao et al., 2001). These contradictory results may be related to the excised root or leaf tissues and very rapid, severe dehydration. In the present study, using intact plants excised at the base of the stem, the data show that oxidative stress induced by paraquat or H2O2 treatments did not affect ABA content in maize leaves, regardless of either a mild oxidative stress or a severe oxidative stress (Fig. 7A, B). Meanwhile, pretreatment with the ROS scavengers, Tiron and DMTU, and the inhibitor of NAD(P)H oxidase, DPI, also did not affect the content of ABA in the leaves of maize plants exposed to the mild water stress (data not shown). These data suggest that ROS are not involved in ABA biosynthesis in plants under mild water stress. Nevertheless, a more strict examination is required under physiological conditions for elucidating whether ROS is involved in the accumulation of ABA induced by water stress.

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Papadakis and Roubelakis-Angelakis, 1999). These ROS originated from plasma membranes, which is gaining increased interest in the literature, have been served as a signal in plant cells to elicit various defence responses to biological, physical or chemical stress (Levine et al., 1994; Bolwell et al., 1998; Orozco-Cárdenas and Ryan, 1999; Papadakis and Roubelakis-Angelakis, 1999; Pei et al., 2000; A-H-Mackerness et al., 2001; Murata et al., 2001; Orozco-Cárdenas et al., 2001). However, there is no information, as far as is known, about the effects of water stress on ROS generated in plasma membranes of plant cells. DPI, a well-known inhibitor of the mammalian plasma membrane oxidase, has been widely used to inhibit the activity of plant NAD(P)/H oxidase (Levine et al., 1994; Bolwell et al., 1998; Orozco-Cárdenas and Ryan, 1999; Papadakis and Roubelakis-Angelakis, 1999; Pei et al., 2000; Frahry et al., 2001; Orozco-Cárdenas et al., 2001; Schopfer et al., 2001). In guard cells of Arabidopsis, ABA-induced H2O2 production and the H2O2-activated Ca2+ channels are important mechanisms for ABA-induced stomatal closing. DPI blocked the production of ROS and partially inhibited ABA-induced stomatal closure (Pei et al., 2000). The ABA activation of Ca2+ channels requires the presence of NADPH in the cytosol (Murata et al., 2001). These results suggest that plasma membrane NAD(P)/H oxidase is involved in ABA signalling. These data not only showed that DPI could completely prevent the increased production of O2− and H2O2 induced by ABA, but, unexpectedly, also fully inhibited the increase in the levels of these ROS caused by water stress (Fig. 8A, B). The inhibition of DPI on the accumulation of O2− exhibited a high sensitivity (Fig. 8C). Although high concentrations of DPI can affect other enzymes potentially involved in the generation of ROS, including extracellular peroxidases and nitric oxide synthase (Bolwell et al., 1998; Frahry et al., 2001; Orozco-Cárdenas et al., 2001; Schopfer et al., 2001), a high sensitivity of O2− generation to inhibition by low concentrations of DPI (as low as 5 μM) strongly suggest that under the mild water stress, the ABA-dependent generation of ROS originated, at least in part, from the trans-plasma membrane O2− synthase pathway. This matter is presently under further investigation.

In conclusion, these results clearly suggest that, under mild water stress, water-stress-induced ABA accumulation triggers the increased generation of ROS, which may involve a trans-plasma membrane NAD(P)/H oxidase and, in turn, leads to the induction of the antioxidant defence system in plants. Considering the complexity of ROS action, although another possibility in the induction of the antioxidant defence system in response to water stress cannot be ruled out, these results have confirmed an important pathway in plant response to mild water stress.

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