Abscisic Acid is a Key Inducer of Hydrogen Peroxide Production in Leaves of Maize Plants Exposed to Water Stress

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The histochemical and cytochemical localization of water stress-induced H2O2 production in the leaves of ABA-deficient vp5 mutant and wild-type maize (Zea mays L.) plants were examined, using 3,3-diaminobenzidine and CeCl3 staining, respectively, and the roles of endogenous ABA in the production of H2O2 induced by water stress were assessed. Water stress induced by polyethylene glycol resulted in the accumulation of H2O2 in mesophyll cells, bundle-sheath cells and vascular bundles of wild-type maize leaves, and the accumulation was substantially blocked in the mutant maize leaves exposed to water stress. Pre-treatments with several apoplastic H2O2 manipulators abolished the majority of H2O2 accumulation induced by water stress in the wild-type leaves. The subcellular localization of H2O2 production was demonstrated in the cell walls, xylem vessels, chloroplasts, mitochondria and peroxisomes in the leaves of wild-type maize plants exposed to water stress, and the accumulation of H2O2 induced by water stress in the cell walls and xylem vessels, but not in the chloroplasts, mitochondria and peroxisomes, was arrested in the leaves of the ABA mutant or the ABA biosynthesis inhibitor (tungstate)-pre-treated maize plants. Pre-treatments with the apoplastic H2O2 manipulators also blocked the apoplastic but not the intracellular H2O2 accumulation induced by water stress in the leaves of wild-type plants. These data indicate that under water stress, the apoplast is the major source of H2O2 production and ABA is a key inducer of apoplastic H2O2 production. These data also suggest that H2O2 generated in the apoplast could not diffuse freely into subcellular compartments.


Abbreviations: APX, ascorbate peroxidase; CAT, catalase; DAB, 3,3-diaminobenzidine; DPI, diphenylene iodonium; GR, glutathione reductase; PEG, polyethylene glycol; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Even under optimal conditions, reactive oxygen species (ROS), such as the superoxide radical (O2•−), hydrogen peroxide (H2O2) and the hydroxyl radical (•OH), are generated as by-products of normal metabolism in different subcellular compartments including the chloroplasts, mitochondria, peroxisomes and plasma membrane-linked electron transport systems (Mittler 2002, Neill et al. 2002, Vranová et al. 2002, Foyer and Noctor 2003, Apel and Hirt 2004, Bartoli et al. 2004). Furthermore, the imposition of biotic and abiotic stress conditions, such as drought, salinity, chilling, heat shock, heavy metals, ultraviolet radiation, air pollutants, mechanical stress, nutrient deprivation, pathogen attack and high light stress, can give rise to excess concentrations of ROS, resulting in lipid peroxidation, protein oxidation, enzyme inhibition, and DNA and RNA damage. On the other hand, increasing evidence indicates that ROS as signaling molecules control various processes including pathogen defense, programmed cell death, stress defense, hormonal responses, photosynthesis regulation, and growth and development in plants (reviewed in Mittler 2002, Neill et al. 2002, Vranová et al. 2002, Foyer and Noctor 2003, Apel and Hirt 2004, Laloi et al. 2004, Mittler et al. 2004, Foyer and Noctor 2005, Torres and Dangl 2005).

Water stress is one of the most important environmental factors that affect plant growth and development, and limit plant production. Plants can respond and adapt to water stress by altering their cellular metabolism and invoking various defense mechanisms (Zhu 2002, Boudsocq and Laurière 2005). 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 biochemical changes. The plant hormone ABA, as a stress signal, increases as a result of water stress and plays crucial roles in the regulation of plant water balance and osmotic stress tolerance (Zhu 2002).

An increasing body of evidence indicates that one mode of ABA action is associated with ROS production in plant cells. Exogenously applied ABA can cause the generation of H2O2 in plant cells or tissues.

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of maize plants exposed to water stress, the reaction of stress-induced H$_2$O$_2$ production and the effects of ABA elucidate the contribution of endogenous ABA to water stress. The aim of this work was to induced by water stress was examined in leaves of the Arabidopsis mutant and its wild type. A recent study has demonstrated that the AtrbohD and AtrbohF NADPH oxidases are required for the production of H$_2$O$_2$ during ABA-induced stomatal closure in Arabidopsis guard cells (Kwak et al. 2003). In mesophyll and bundle sheath cells of maize leaves, H$_2$O$_2$ accumulation induced by ABA only occurred in the apoplast in the two types of cells, and the greatest accumulation of H$_2$O$_2$ was observed in the walls of mesophyll cells facing large intercellular spaces (Hu et al. 2005). It has been shown that ABA-induced H$_2$O$_2$ production is involved in the regulation of stomatal closure (Pei et al. 2000, Zhang et al. 2001, Kwak et al. 2003), antioxidant defense (Jiang and Zhang 2002a, Jiang and Zhang 2002b, Jiang and Zhang 2003, Hu et al. 2005, Zhang et al. 2006), and seed germination and root elongation (Kwak et al. 2003).

Although both water stress and ABA can induce H$_2$O$_2$ accumulation in plant cells, the relative contribution of ABA-induced H$_2$O$_2$ production to water stress-induced H$_2$O$_2$ accumulation has not been studied yet. Furthermore, chloroplasts, mitochondria and peroxisomes have been believed to be important sources of H$_2$O$_2$ in plant cells under water stress (Mittler 2002, Noctor et al. 2002, Foyer and Noctor 2003, Bartoli et al. 2004, Luna et al. 2004, Mittler et al. 2004). However, it is unknown whether water stress-induced ABA accumulation affects the production of H$_2$O$_2$ in these organelles. In this report, using the ABA-deficient v$p5$ maize mutant, which blocks carotenoid synthesis and, thereby, interrupts ABA biosynthesis early in the biosynthetic pathway (Guan and Scandalios 1998, Sharp 2002), and in vivo detection of H$_2$O$_2$, the histochemical and cytochemical localization of H$_2$O$_2$ production induced by water stress was examined in leaves of the mutant and its wild type. The aim of this work was to elucidate the contribution of endogenous ABA to water stress-induced H$_2$O$_2$ production and the effects of ABA accumulation on the production of H$_2$O$_2$ in chloroplasts, mitochondria and peroxisomes of maize leaves exposed to water stress.

Results

Water stress induces H$_2$O$_2$ accumulation in maize leaves and major H$_2$O$_2$ production occurs at extracellular sites

To check in situ the accumulation of H$_2$O$_2$ in leaves of maize plants exposed to water stress, the reaction of 3,3-diaminobenzidine (DAB) with H$_2$O$_2$, producing a brown polymerization product in the presence of peroxidases (Thordal-Christensen et al. 1997, Fryer et al. 2002), was studied. Water stress (–0.7 MPa) led to a rapid accumulation of H$_2$O$_2$ in leaves of V/p5 wild-type maize plants (Fig. 1A). H$_2$O$_2$ is detectable as early as 1 h after water stress, with the color deepening for 4 h. The color mainly appeared in mesophyll cells, bundle-sheath cells and vascular bundles of maize leaves (Fig. 1A).

To investigate whether the sources of H$_2$O$_2$ in the leaves exposed to water stress are extracellular or intracellular, diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, superoxide dismutase (SOD), the enzyme catalyzing the dismutation of O$_2^-$ to O$_2$ and H$_2$O$_2$, and catalase (CAT), the enzyme eliminating H$_2$O$_2$, were applied. Pre-treatments with these inhibitors or scavengers abolished the majority of H$_2$O$_2$ accumulation induced by water stress (Fig. 1C), indicating that extracellular H$_2$O$_2$ is a major H$_2$O$_2$ source in the leaves of maize plants exposed to water stress.

ABA is a key regulator of H$_2$O$_2$ production under water stress

In order to determine the relative contribution of endogenous ABA in water stress-induced H$_2$O$_2$ production, the ABA-deficient maize v$p5$ mutant was used. The v$p5$ mutant leaves were fully developed under control conditions, but were completely photobleached (Fig. 1B). Under control conditions, no visible H$_2$O$_2$ accumulation was observed within the 4 h treatment. Water stress led to an increase in the production of H$_2$O$_2$ in the mutant leaves (Fig. 1B). However, the extent of H$_2$O$_2$ accumulation induced by water stress in the mutant leaves is far lower than that in the wild-type leaves (Fig. 1A, B). The application of 100μM ABA substantially increased the accumulation of H$_2$O$_2$ in the leaves of mutant maize plants exposed to water stress. These results clearly suggested that water stress-induced ABA is a key inducer of H$_2$O$_2$ production in leaves of maize plants exposed to water stress.

Subcellular localization of water stress-induced H$_2$O$_2$ production in maize leaves

To investigate the subcellular localization of H$_2$O$_2$ accumulation in the leaves of maize plants exposed to water stress, a cytochemical technique with CeCl$_3$, which reacts with H$_2$O$_2$ to produce electron-dense deposits of cerium perhydroxides (Bestwick et al. 1997), was used. In the control leaves of V/p5 wild-type maize plants, CeCl$_3$ deposits, indicative of the accumulation of H$_2$O$_2$, were not observed in the mesophyll cells (Fig. 2A). At 1 h after the beginning of water stress treatment, H$_2$O$_2$ accumulation was visible in the cell walls of mesophyll cells. At 2 and 4 h of water stress treatment, H$_2$O$_2$ accumulation was
Fig. 1  Histochemical detection of H$_2$O$_2$ with DAB staining in the wild-type and the vp5 mutant maize leaves exposed to water stress. (A) The time course of changes in the production of H$_2$O$_2$ in the wild-type maize leaves exposed to water stress. (B) Changes in the production of H$_2$O$_2$ induced by water stress and the effect of pre-treatment with ABA on the production of H$_2$O$_2$ in the vp5 mutant leaves. The detached maize plants were pre-treated with 100 µM ABA or distilled water for 5 h, and then exposed to −0.7 MPa water stress or distilled water treatment for various times. (C) Effects of pre-treatments with DPI, SOD and CAT on the production of H$_2$O$_2$ induced by water stress in the wild-type leaves. The detached maize plants were treated as follows: distilled water for 9 h (control, −); distilled water for 5 h and then −0.7 MPa water stress for 4 h (PEG, −); DPI (100 µM), SOD (200 U) and CAT (200 U) for 5 h and then −0.7 MPa water stress for 4 h (PEG, +). All experiments were repeated at least three times with similar results.
Fig. 2  Cytochemical localization of H$_2$O$_2$ accumulation in mesophyll cells of wild-type and vp5 mutant leaves exposed to water stress. (A) The time course of changes in the production of H$_2$O$_2$ in the mesophyll cells of wild-type maize leaves exposed to water stress. (B) Changes in the production of H$_2$O$_2$ in the mesophyll cells of vp5 mutant leaves exposed to water stress and the effect of pre-treatment with ABA on the production of H$_2$O$_2$. The detached mutant maize plants were pre-treated with 100 μM ABA or distilled water for 5 h, and then exposed to −0.7 MPa water stress or distilled water treatment for various times. All experiments were repeated at least three times with similar results. Arrows indicate CeCl$_3$ precipitates. C, chloroplast; CW, cell wall; IS, intercellular space; M, mitochondrion; N, nucleus; V, vacuole. Bar = 1 μm.
further increased and the greatest accumulation of H$_2$O$_2$ was observed in the cell walls facing the intercellular spaces (Fig. 2A). After 6 h of water stress treatment, H$_2$O$_2$ accumulation decreased (data not shown). In the control leaves of vp5 mutant maize plants, the chloroplasts were destroyed and the thylakoids had disappeared and mostly only vesicles were present (Fig. 2B). Water stress only led to a slight increase in the production of H$_2$O$_2$, and the application of 100 $\mu$M ABA substantially restored the level of H$_2$O$_2$, when compared with that of mesophyll cells in the wild-type leaves (Fig. 2A, B). In vascular bundles, H$_2$O$_2$ accumulation was detected on the surface of xylem vessels and in the cell walls of adjacent vascular parenchyma cells in leaves of Vp5 wild-type maize plants exposed to water stress, and this accumulation was substantially reduced in the vp5 mutant leaves (Fig. 3). Pre-treatment with tungstate, which was shown to block the formation of ABA from abscisic aldehyde by impairing abscisic aldehyde oxidase (Hansen and Grossmann 2000), also substantially prevented the water stress-induced

![Fig. 3](image)

Fig. 3 Cytochemical localization of H$_2$O$_2$ accumulation in vascular bundles of wild-type and vp5 mutant leaves exposed to water stress. The detached maize plants were pre-treated with 2 mM sodium tungstate (Tun), 100 $\mu$M ABA or distilled water for 5 h, and then exposed to −0.7 MPa water stress or distilled water treatment for 4 h. All experiments were repeated at least three times with similar results. Arrows indicate CeCl$_3$ precipitates. C, chloroplast; M, mitochondrion; N, nucleus; V, vacuole; XV, xylem vessel. Bar = 1 $\mu$m.
increase in the production of H$_2$O$_2$ on the surface of xylem vessels and in the cell walls of adjacent vascular parenchyma cells in leaves of wild-type maize plants (Fig. 3). The application of ABA in the mutant plants restored the accumulation of H$_2$O$_2$ in vascular tissues of maize leaves exposed to water stress.

To investigate further the intracellular sites of H$_2$O$_2$ production under water stress, the chloroplasts of mesophyll cells and the mitochondria and peroxisomes of bundle-sheath cells were examined. H$_2$O$_2$ accumulation was visible in the thylakoids and stroma of chloroplasts and the inner membrane of mitochondria and the matrix of peroxisomes in the leaves of wild-type maize plants exposed to water stress (Fig. 4A). In the mutant leaves, water stress also led to H$_2$O$_2$ accumulation in mitochondria and peroxisomes of bundle-sheath cells (Fig. 4B), and the extent of H$_2$O$_2$ accumulation was similar to that of wild-type maize leaves (Fig. 4A, B). Pre-treatment with tungstate did not affect the water stress-induced H$_2$O$_2$ accumulation in chloroplasts, mitochondria and peroxisomes of wild-type maize leaves (Fig. 4A), and the application of ABA also did not affect the accumulation of H$_2$O$_2$ induced by water stress in mitochondria and peroxisomes in leaves of $vp5$ mutant maize plants (Fig. 4B).

Pre-treatments with DPI, SOD and CAT do not affect the intracellular H$_2$O$_2$ accumulation induced by water stress

In order to determine whether the intracellular H$_2$O$_2$ accumulation induced by water stress was related to the increase in the apoplastic H$_2$O$_2$ in leaves of maize plants, several apoplastic H$_2$O$_2$ manipulators, such as DPI, an inhibitor of plasma membrane NADPH oxidase, and SOD and CAT, which cannot cross the plasma membrane, were used. Fig. 5 shows that the application of DPI, SOD and CAT almost completely blocked the apoplastic H$_2$O$_2$ accumulation induced by water stress in mesophyll cells of wild-type maize leaves, but did not affect the water stress-induced H$_2$O$_2$ accumulation in the chloroplasts of mesophyll cells and the mitochondria and peroxisomes of bundle-sheath cells in wild-type maize leaves, indicating that the intracellular H$_2$O$_2$ accumulation induced by water stress is not related to the apoplastic H$_2$O$_2$ in leaves of maize plants.

Effect of water stress on the activities of antioxidant enzymes in leaves of maize plants

To determine whether the accumulation of H$_2$O$_2$ in the wild type and the reduction in the mutant under water stress results from the changes in antioxidant defense, the activities of several major antioxidant enzymes such as SOD, CAT, ascorbate peroxidase (APX) and glutathione reductase (GR) were analyzed. No significant differences in the activities of these antioxidant enzymes between the mutant and the wild type were observed under the control conditions, and the 4h water stress also hardly affected the activities of antioxidant enzymes in the leaves of $vp5$ mutant and wild-type maize plants (Table 1), suggesting that the accumulation of H$_2$O$_2$ under water stress results from the increase in H$_2$O$_2$ synthesis.

Discussion

For years, chloroplasts, mitochondria and peroxisomes have been considered to be the main sources of H$_2$O$_2$ production in plant cells under water stress (Mittler 2002, Noctor et al. 2002, Foyer and Noctor 2003, Bartoli et al. 2004, Luna et al. 2004, Mittler et al. 2004, Boudsooc and Lauraire 2005). However, many studies used the isolated organelles to detect the production of H$_2$O$_2$. These methods involve cell extraction and extensive tissue disruption. It may be questioned whether the assay can accurately reflect the generation of H$_2$O$_2$ induced by water stress in vivo. In the present study, the histochemical and cytochemical methods which have been widely used for the detection of H$_2$O$_2$ generated in plant tissues in response to biotic and abiotic stresses (Bestwick et al. 1997, Thordal-Christensen et al. 1997, Orozco-Cárdenas and Ryan 1999, Pellinen et al. 1999, Vanacker et al. 2000, Orozco-Cárdenas et al. 2001, Fryer et al. 2002, Pellinen et al. 2002, Romero-Puertas et al. 2004) have been adapted for in vivo and in situ detection of H$_2$O$_2$ induced by water stress. Our results from histochemical detection showed that a mild water stress (~0.7 MPa) led to a rapid, substantial H$_2$O$_2$ accumulation in mesophyll cells, bundle-sheath cells and vascular bundles of $vp5$ wild-type maize leaves (Fig. 1A). H$_2$O$_2$ generated in response to water stress treatment was detected within 1h and maximized at about 4h. The application of several apoplastic H$_2$O$_2$ manipulators, such as DPI, SOD and CAT, abolished the majority of H$_2$O$_2$ accumulation induced by water stress (Fig. 1C), indicating that extracellular H$_2$O$_2$ is a major H$_2$O$_2$ source in the leaves of maize plants exposed to water stress. The results from cytochemical detection further showed that water stress induced H$_2$O$_2$ accumulation in cell walls, xylem vessels, chloroplasts, mitochondria and peroxisomes of maize leaves (Figs. 2-4). Similar results were also obtained from another inbred maize line W64A (data not shown). Our results not only support the traditional concept that chloroplasts, mitochondria and peroxisomes are important sources of H$_2$O$_2$ in plant cells under water stress, but further indicate that the apoplast is the major source of H$_2$O$_2$ production in leaves of maize plants exposed to water stress. In a previous study, however, it was shown that H$_2$O$_2$, detected by CeCl$_3$ staining and transmission electron microscopy, only accumulated in xylem vessels, cell walls and plasma.
Fig. 4  Changes in the production of H$_2$O$_2$ in response to water stress in chloroplasts, mitochondria and peroxisomes of wild-type and vp5 mutant maize leaves. The detached wild-type (A) and mutant (B) maize plants were pre-treated with 2 mM sodium tungstate (Tun), 100 μM ABA or distilled water for 5 h, and then exposed to -0.7 MPa water stress or distilled water treatment for 4 h. The chloroplasts of mesophyll cells and the mitochondria and peroxisomes of bundle-sheath cells were examined. All experiments were repeated at least three times with similar results. Arrows indicate CeCl$_3$ precipitates. C, chloroplast; M, mitochondrion; P, peroxisome. Bar = 1 μm.
ABA-induced H$_2$O$_2$ production under water stress

Fig. 5 Effects of pre-treatments with DPI, SOD and CAT on the production of H$_2$O$_2$ in apoplast, chloroplasts, mitochondria and peroxisomes in leaves of wild-type maize plants exposed to water stress. The detached maize plants were pre-treated with 100 $\mu$M DPI, 200 U of SOD and 200 U of CAT for 5 h, and then exposed to $-0.7$ MPa water stress for 4 h. The apoplast and chloroplasts of mesophyll cells and the mitochondria and peroxisomes of bundle-sheath cells were examined. All experiments were repeated at least three times with similar results. Arrows indicate CeCl$_3$ precipitates. C, chloroplast; CW, cell wall; IS, intercellular space; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 1 $\mu$m.
membranes of mesophyll cells, but not in chloroplasts, mitochondria or peroxisomes in field-grown sage exposed to drought (Munne-Bosch et al. 2001). The difference in the subcellular localization of H2O2 accumulation induced by water stress between the study by Munne-Bosch et al. (2001) and our data may be related to the duration of the water stress (months vs. hours). Water stress for several months can result in an acclimation of plants. In comparison with chloroplasts, mitochondria and peroxisomes, the apoplast has relatively little antioxidant defense and, hence, H2O2 accumulates when H2O2 synthesis is increased (Neill et al. 2002, Pastori and Foyer 2002). This might be the reason that the apoplastic H2O2 accumulated under water stress in the study by Munne-Bosch et al. (2001). It is also possible that different plant species may have different responses to water stress.

Water stress induces the accumulation of ABA, and ABA can induce the production of H2O2 in plant cells. However, it is not clear what is the relative contribution of water stress-induced ABA accumulation to water stress-induced H2O2 production. It has been suggested that ABA may be a rather weak inducer of ROS (Avsian-Kretchmer et al. 2004). In this study, using the ABA-deficient maize vp5 mutant, the role of endogenous ABA in the production of H2O2 induced by water stress was assessed. Our results showed that the accumulation of H2O2 in mesophyll cells, bundle-sheath cells and vascular bundles (Fig. 1B), in the cell walls of mesophyll cells (Fig. 2B), and in xylem vessels and cell walls of adjacent vascular parenchyma cells (Fig. 3) of vp5 mutant leaves exposed to water stress were substantially blocked, when compared with those in the wild-type leaves exposed to water stress. The application of 100 μM ABA, which increased the content of endogenous ABA in maize leaves to a similar extent to the water stress (Jiang and Zhang 2002a), fully restored the accumulation of H2O2 in the leaves of mutant maize plants exposed to water stress. Moreover, pre-treatment with the ABA biosynthetic inhibitor tungstate, which was shown to block the formation of ABA from abscisic aldehyde by impairing abscisic aldehyde oxidase (Hansen and Grossmann 2000), also substantially prevented the water stress-induced increase in the production of H2O2 in the wild-type leaves exposed to water stress (data not shown; Fig. 3), and the reduction in the production of H2O2 was fully prevented by the addition of ABA (data not shown). These results clearly indicate that ABA is a key inducer of H2O2 production in leaves of maize plants exposed to water stress. However, no differences in the production of H2O2 in mitochondria and peroxisomes of bundle-sheath cells between the mutant and the wild-type under water stress were observed, and the application of ABA did not affect the production of H2O2 in these organelles under water stress (Fig. 4). Pre-treatment with tungstate also did not affect the water stress-induced H2O2 accumulation in chloroplasts, mitochondria and peroxisomes of wild-type maize leaves exposed to water stress. These results indicate that water stress-induced ABA only induces the accumulation of apoplastic H2O2 in leaves of maize plants, which is consistent with the effect of exogenously applied ABA under non-stressed conditions (Hu et al. 2005).

However, the vp5 mutant blocks carotenoid synthesis, resulting in a lack of carotenoids (Guan and Scandalias 1998, Sharp 2002). The mutant leaves, although fully developed, were completely photobleached. The chloroplasts in leaves of the mutant were destroyed and the thylakoids had disappeared, and in the main only vesicles were present. It may be questioned whether such changes affect the antioxidant defense systems, resulting in the reduction in the accumulation of H2O2 in the mutant leaves exposed to water stress. Moreover, it may also be asked whether the accumulation of H2O2 in the wild-type leaves exposed to water stress results from the reduction in the antioxidant defense systems. To answer these questions, the activities of several major antioxidant enzymes such as SOD, CAT, APX and GR were analyzed. Our data showed that there were no significant differences in the activities of these antioxidant enzymes between the mutant and the wild-type under non-stressed conditions, and the water stress also hardly affected the activities of

Table 1 The activities of SOD (U mg⁻¹ protein), CAT (μmol min⁻¹ mg⁻¹ protein), APX (μmol min⁻¹ mg⁻¹ protein) and GR (nmol min⁻¹ mg⁻¹ protein) in leaves of vp5 mutant and wild-type maize plants exposed to water stress

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<th>SOD</th>
<th>CAT</th>
<th>APX</th>
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The detached vp5 mutant and wild-type maize plants were treated with −0.7 MPa water stress or distilled water for 4 h, and the leaves were sampled and analyzed. The values are the means ± SE (n = 6) of three different experiments.

The detached vp5 mutant and wild-type maize plants were treated with −0.7 MPa water stress or distilled water for 4 h, and the leaves were sampled and analyzed. The values are the means ± SE (n = 6) of three different experiments.
antioxidant enzymes (Table 1). These results suggest that the accumulation of \( \text{H}_2\text{O}_2 \) in the wild type and the reduction in the mutant during the 4 h water stress result from the changes in \( \text{H}_2\text{O}_2 \) synthesis.

There are several potential sources of apoplastic \( \text{H}_2\text{O}_2 \) in plants, including plasma membrane NADPH oxidases, cell wall peroxidases, and apoplastic oxidases and amine oxidases (Mittler 2002, Neill et al. 2002, Pastori and Foyer 2002, Vranová et al. 2002). It has been shown that NADPH oxidase is involved in ABA signal transduction leading to the regulation of stomatal closure in Arabidopsis guard cells (Kwak et al. 2003), antioxidant defense in maize leaves (Jiang and Zhang 2002a, Jiang and Zhang 2003, Hu et al. 2005), and seed germination and root elongation in Arabidopsis (Kwak et al. 2003). Cell wall peroxidase and diamine oxidase have also been suggested to be involved in ABA-induced reduction in root growth of rice seedlings (Lin and Kao 2001). In the present study, our results showed that the accumulation of \( \text{H}_2\text{O}_2 \) in the apoplast induced by water stress occurred in the cell walls of mesophyll cells or vascular parenchyma cells and on the surface of xylem vessels in leaves of wild-type maize plants, and this accumulation was substantially blocked in the leaves of mutant maize plants. These data suggest that there are different enzymatic sources of ABA-induced accumulation of apoplastic \( \text{H}_2\text{O}_2 \) between mesophyll cells and xylem vessels under water stress. In xylem vessels, however, the CeCl\(_3\) deposits also partly occurred in the xylem sap (Fig. 3), as has been shown in field-grown sage exposed to drought (Munné-Bosch et al. 2001). A possible explanation is that \( \text{H}_2\text{O}_2 \) in the xylem sap might result from the transport of \( \text{H}_2\text{O}_2 \) to the nearby generating sites.

In the apoplast, \( \text{H}_2\text{O}_2 \) has several possible fates. \( \text{H}_2\text{O}_2 \) can be used directly in cell wall metabolism, it can be oxidized by ascorbate or it can act directly as a local or systemic signal (Pastori and Foyer 2002). \( \text{H}_2\text{O}_2 \) has been considered to be a mobile signal molecule (Mittler 2002, Neill et al. 2002, Pastori and Foyer 2002, Vranová et al. 2002). It has been speculated that water channels (aquaporins) may also serve as conduits for trans-membrane \( \text{H}_2\text{O}_2 \) transport (Henzler and Steudle 2000). \( \text{H}_2\text{O}_2 \) generated in chloroplasts, mitochondria and peroxisomes may also move into the cytosol (Neill et al. 2002, Shigeoka et al. 2002). However, it is not clear whether the accumulation of apoplastic \( \text{H}_2\text{O}_2 \) induced by water stress can move into the cytosol and subcellular compartments. In the present study, no visible \( \text{H}_2\text{O}_2 \) accumulation was observed in the cytosol during the 4 h water stress. Pre-treatments with DPI, SOD and CAT almost completely blocked the apoplastic \( \text{H}_2\text{O}_2 \) accumulation induced by water stress in the wild-type leaves, but did not affect the accumulation of \( \text{H}_2\text{O}_2 \) in the chloroplasts, mitochondria and peroxisomes under water stress (Fig. 5). In the mutant leaves, the reduction in the production of apoplastic \( \text{H}_2\text{O}_2 \) also did not affect the accumulation of \( \text{H}_2\text{O}_2 \) in the mitochondria and peroxisomes under water stress (Figs. 2, 4). Our results suggest that \( \text{H}_2\text{O}_2 \) generated in the apoplast could not diffuse freely into the cytosol and other subcellular compartments, such as chloroplasts, mitochondria and peroxisomes.

**Materials and Methods**

**Plant material and treatments**

The ABA-deficient maize (Zea mays L.) \( \text{vp}5 \) mutant and its wild-type \( \text{Vp}5 \) were used in these studies. Seeds of the \( \text{vp}5 \) mutant and wild-type maize were obtained by selling plants grown from heterozygous seed (Maize Genetics Stock Center, Urbana, IL, USA). Selfed ears with kernels segregating for the mutation were chosen; mutant kernels were identified by the lack of carotenoid pigmentation. Mutant and wild-type seedlings were grown in trays of sand in a light chamber at a temperature of 22–28°C, photosynthetic active radiation (PAR) of 200 \( \mu \text{mol} m^{-2} s^{-1} \), and a photoperiod of 14/10 h (day/night), and watered daily. When the second leaves were fully expanded, they were collected and used for all investigations.

The plants were excised at the base of the stem, and placed in distilled water for 1 h to eliminate wound stress. After treatment, the cut ends of the stems were placed in beakers wrapped with aluminum foil containing polyethylene glycol (PEG) solution at \(-0.7 \text{MPa}\) for various times up to 4 h at 25°C, with a continuous light intensity of 200 \( \mu \text{mol} m^{-2} s^{-1} \). In order to study the effects of inhibitors and scavengers, the detached plants were pre-treated with 100 \( \mu \text{M} \) DPI, 2 mM sodium tungstate, 200 U of CAT and 200 U of SOD for 5 h, and then exposed to PEG treatment for 4 h under the same conditions as described above. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above.

**In vivo detection of \( \text{H}_2\text{O}_2 \)**

\( \text{H}_2\text{O}_2 \) was detected in the leaves of plants by using DAB as substrate (Orozco-Cárdenas and Ryan 1999). Briefly, plants were excised at the base of stems with a razor blade and supplied through the cut stems with a 1 mg ml\(^{-1}\) solution of DAB (pH 3.8) for 8 h under light at 25°C, and then exposed to various treatments. After these treatments, the second leaves were fixed and prepared for light microscopy according to the method described by Vanacker et al. (2000) with slight modifications. For fixation, tissue segments (~1 to 2 cm\(^2\)) were excised from leaves and were then fixed in 2% (v/v) glutaraldehyde/4% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer, pH 7.2 for 24 h. After fixation tissues were washed twice for 10 min in distilled water and embedded in Jung tissue freezing medium for frozen tissues. Then these frozen tissues were sectioned (12 \( \mu \text{m} \)) on a frozen cut microtome, mounted on a microscope slide without a coverslip and observed using a microscope (BH-2, Olympus, Tokyo) with a ‘no coverslip’ 40× objective lens.

**Cytochemical detection of \( \text{H}_2\text{O}_2 \)**

\( \text{H}_2\text{O}_2 \) was visualized at the subcellular level using CeCl\(_3\) for localization (Bestwick et al. 1997). Electron-dense CeCl\(_3\) deposits are formed in the presence of \( \text{H}_2\text{O}_2 \) and are visible by transmission
electron microscopy. Tissue pieces (~1–2 mm²) were excised from the treated and untreated leaves and incubated in freshly prepared 5 mM CeCl₃ in 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) at pH 7.2 for 1 h. The leaf sections were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer, pH 7.2, for 1 h. After fixation, tissues were washed twice for 10 min in the same buffer and post-fixed for 45 min in 1% (v/v) osmium tetroxide, dehydrated in a graded ethanol series (30–100%; v/v) and embedded in Eponaldrite (Agar Aids, Bishop’s Stortford, UK). After 12 h in pure resin, followed by a change of fresh resin for 4 h, the samples were polymerized at 60 °C for 48 h. Blocks were sectioned (70–90 nm) on a Reichert-Ultracut E microtome, and mounted on uncoated copper grids (300 mesh). Sections were examined using a transmission electron microscope at an accelerating voltage of 75 kV.

Enzyme assays

Frozen leaf segments (0.5 g) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM ascorbate in the case of APX assay. The homogenate was centrifuged at 15,000 g for 20 min at 4 °C and the supernatant was immediately used for the following antioxidant enzyme assays.

The total activities of antioxidant enzymes were determined as previously described (Jiang and Zhang 2001). Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. Total CAT activity was assayed by measuring the rate of decomposition of H₂O₂ at 240 nm. Total APX activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. Total GR activity was measured by following the change in Δ340 as oxidized glutathione (GSSG)-dependent oxidation of NADPH.

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References


