Plant Cell Physiol. 47(11): 1484–1495 (2006)
doi:10.1093/pcp/pcl014, available online at www.pcp.oxfordjournals.org

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 H_2O_2 production in the leaves of ABA-deficient *vp5* mutant and wild-type maize (*Zea mays* L.) plants were examined, using 3,3-diaminobenzidine and $CeCl_3$ staining, respectively, and the roles of endogenous ABA in the production of H_2O_2 induced by water stress were assessed. Water stress induced by polyethylene glycol resulted in the accumulation of H_2O_2 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 H_2O_2 manipulators abolished the majority of H_2O_2 accumulation induced by water stress in the wild-type leaves. The subcellular localization of H_2O_2 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 H_2O_2 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 H_2O_2 manipulators also blocked the apoplastic but not the intracellular H_2O_2 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 H_2O_2 production and ABA is a key inducer of apoplastic H_2O_2 production. These data also suggest that H_2O_2 generated in the apoplast could not diffuse freely into subcellular compartments.

Keyword: Abscisic acid — Cytochemistry — Histochemistry — Hydrogen peroxide — Water stress — *Zea mays*.

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 (O_2^-), hydrogen peroxide (H_2O_2) 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 H_2O_2 in plant cells or tissues

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(Guan et al. 2000, Pei et al. 2000, Jiang and Zhang 2001, Zhang et al. 2001, Kwak et al. 2003, Hu et al. 2005). It has been shown that there are two sources of H_2O_2 in *Vicia faba* guard cells in response to ABA: one is the light reaction in chloroplasts, which might be the main regions of H_2O_2 production, and another is the plasma membrane NADPH oxidase (Zhang et al. 2001). Using molecular genetic and cell biological analyses, a recent study has directly demonstrated that the AtrbohD and AtrbohF NADPH oxidases are required for the production of H_2O_2 during ABA-induced stomatal closure in *Arabidopsis* guard cells (Kwak et al. 2003). In mesophyll and bundle sheath cells of maize leaves, H_2O_2 accumulation induced by ABA only occurred in the apoplast in the two types of cells, and the greatest accumulation of H_2O_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_2O_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_2O_2 accumulation in plant cells, the relative contribution of ABA-induced H_2O_2 production to water stress-induced H_2O_2 accumulation has not been studied yet. Furthermore, chloroplasts, mitochondria and peroxisomes have been believed to be important sources of H_2O_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_2O_2 in these organelles. In this report, using the ABA-deficient *vp5* 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_2O_2, the histochemical and cytochemical localization of H_2O_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_2O_2 production and the effects of ABA accumulation on the production of H_2O_2 in chloroplasts, mitochondria and peroxisomes of maize leaves exposed to water stress.

Results

Water stress induces H_2O_2 accumulation in maize leaves and major H_2O_2 production occurs at extracellular sites

To check in situ the accumulation of H_2O_2 in leaves of maize plants exposed to water stress, the reaction of 3,3-diaminobenzidine (DAB) with H_2O_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_2O_2 in leaves of *Vp5* wild-type maize plants (Fig. 1A). H_2O_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_2O_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_2O_2, and catalase (CAT), the enzyme eliminating H_2O_2, were applied. Pre-treatments with these inhibitors or scavengers abolished the majority of H_2O_2 accumulation induced by water stress (Fig. 1C), indicating that extracellular H_2O_2 is a major H_2O_2 source in the leaves of maize plants exposed to water stress.

ABA is a key regulator of H_2O_2 production under water stress

In order to determine the relative contribution of endogenous ABA in water stress-induced H_2O_2 production, the ABA-deficient maize *vp5* mutant was used. The *vp5* mutant leaves were fully developed under control conditions, but were completely photobleached (Fig. 1B). Under control conditions, no visible H_2O_2 accumulation was observed within the 4 h treatment. Water stress led to an increase in the production of H_2O_2 in the mutant leaves (Fig. 1B). However, the extent of H_2O_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 100 μM ABA substantially increased the accumulation of H_2O_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_2O_2 production in leaves of maize plants exposed to water stress.

Subcellular localization of water stress-induced H_2O_2 production in maize leaves

To investigate the subcellular localization of H_2O_2 accumulation in the leaves of maize plants exposed to water stress, a cytochemical technique with $CeCl_3$, which reacts with H_2O_2 to produce electron-dense deposits of cerium perhydroxides (Bestwick et al. 1997), was used. In the control leaves of *Vp5* wild-type maize plants, $CeCl_3$ deposits, indicative of the accumulation of H_2O_2, were not observed in the mesophyll cells (Fig. 2A). At 1 h after the beginning of water stress treatment, H_2O_2 accumulation was visible in the cell walls of mesophyll cells. At 2 and 4 h of water stress treatment, H_2O_2 accumulation was

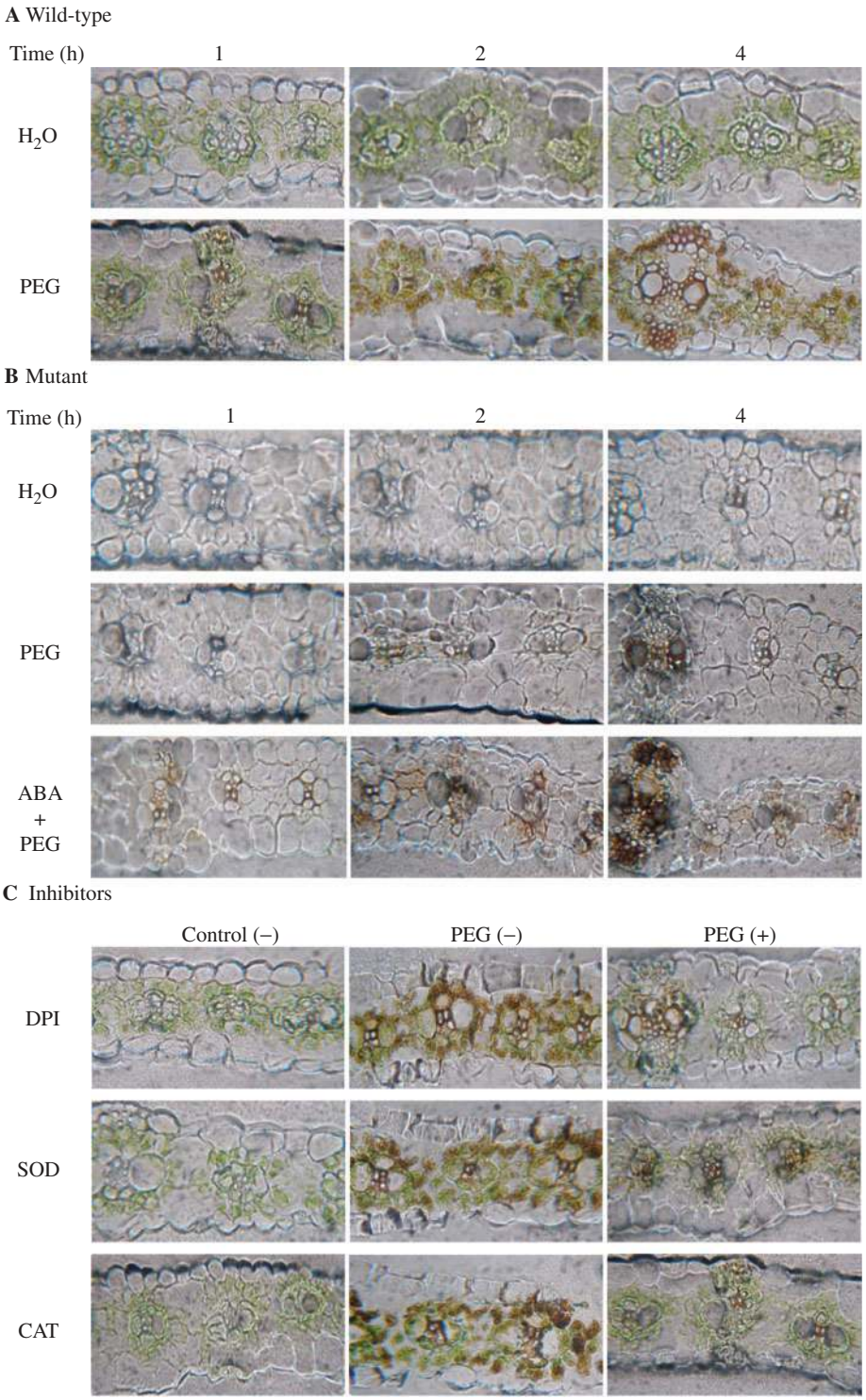

Fig. 1 Histochemical detection of H_2O_2 with DAB staining in the wild-type and the *vp5* mutant maize leaves exposed to water stress. (A) The time course of changes in the production of H_2O_2 in the wild-type maize leaves exposed to water stress. (B) Changes in the production of H_2O_2 induced by water stress and the effect of pre-treatment with ABA on the production of H_2O_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_2O_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.

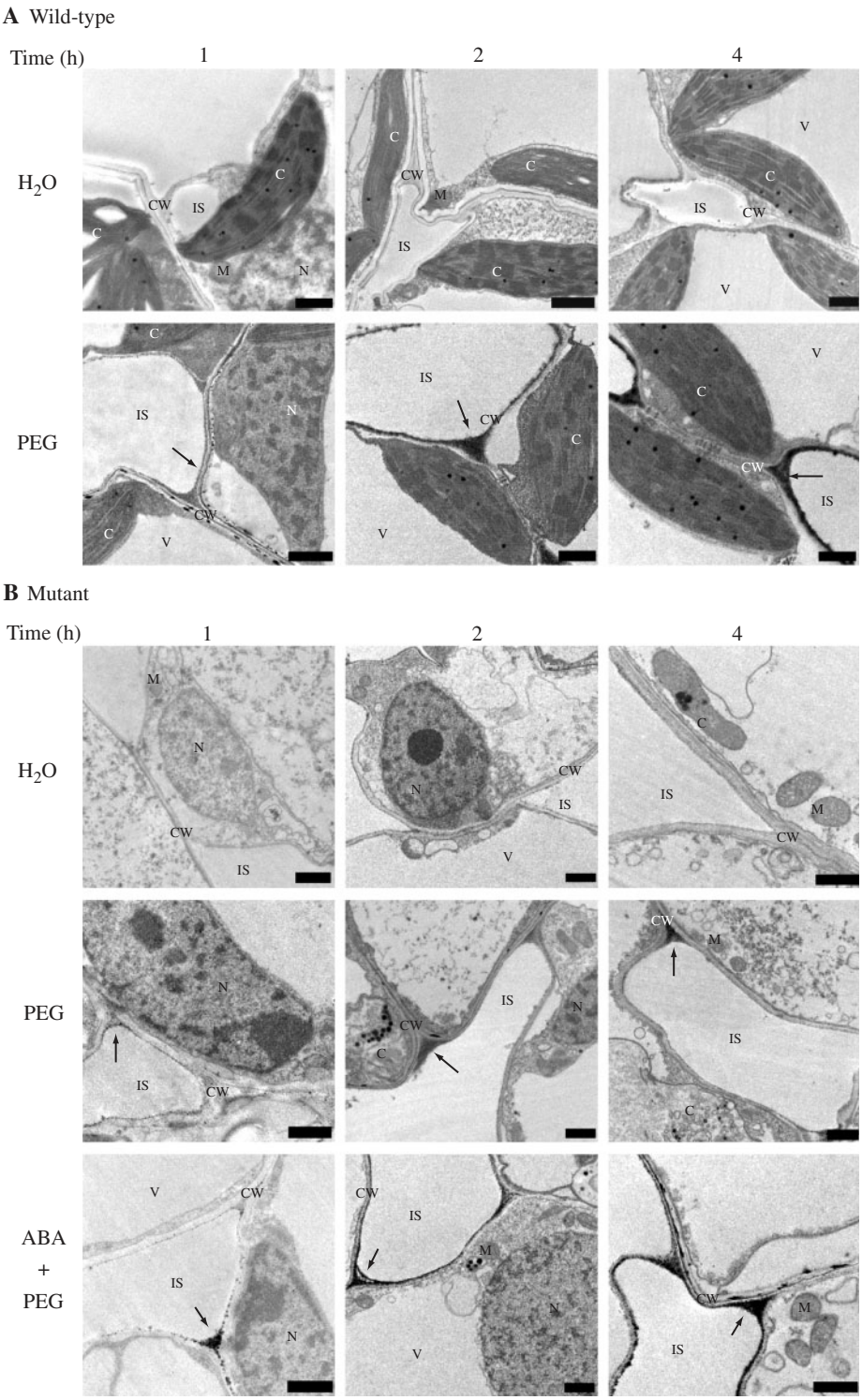

Fig. 2 Cytochemical localization of H_2O_2 accumulation in mesophyll cells of wild-type and *vp5* mutant leaves exposed to water stress. (A) The time course of changes in the production of H_2O_2 in the mesophyll cells of wild-type maize leaves exposed to water stress. (B) Changes in the production of H_2O_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_2O_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_2O_2 was observed in the cell walls facing the intercellular spaces (Fig. 2A). After 6 h of water stress treatment, H_2O_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_2O_2, and the application of 100 μM ABA substantially restored the level of H_2O_2, when compared with that of mesophyll cells in the wild-type leaves (Fig. 2A, B). In vascular bundles, H_2O_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

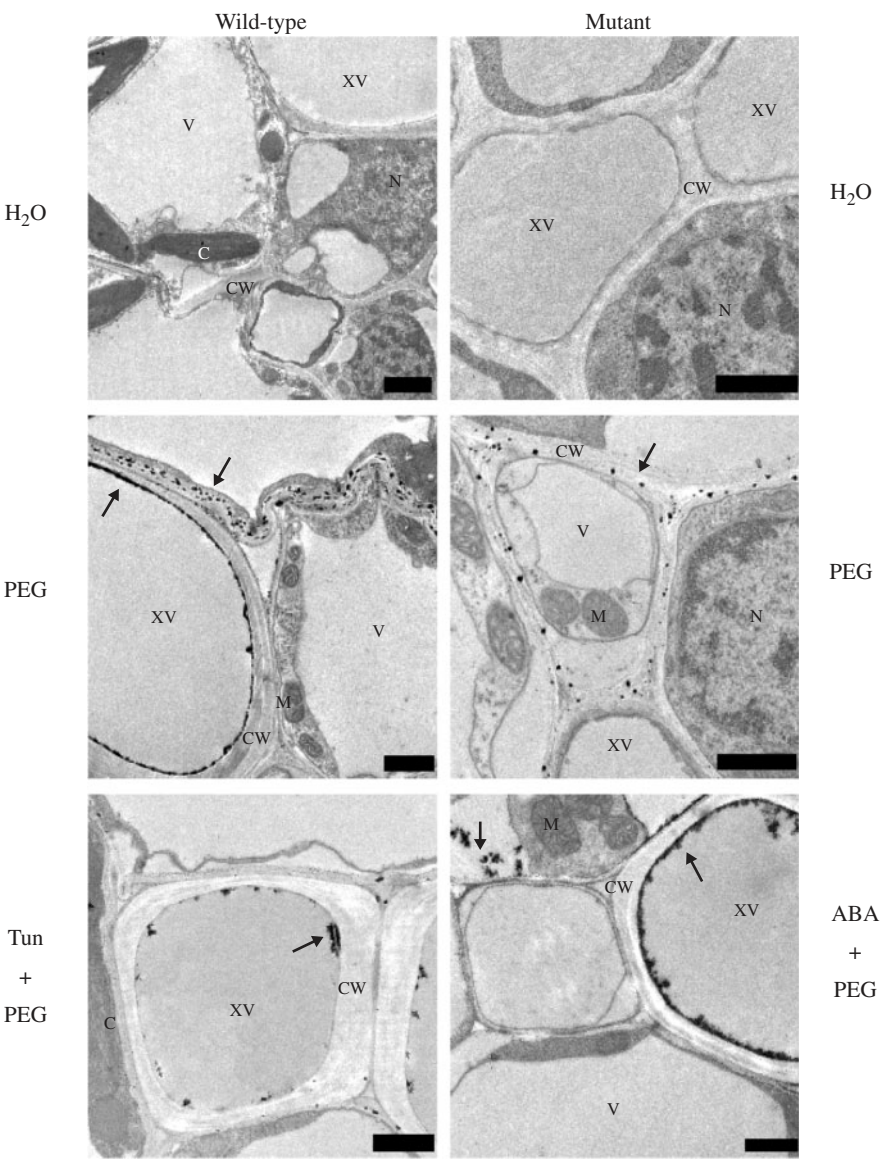

Fig. 3 Cytochemical localization of H_2O_2 accumulation in vascular bundles of wild-type and *vp5* mutant leaves exposed to water stress. The detached maize plants were pre-treated with 2 mM sodium tungstate (Tun), 100 μM ABA or distilled water for 5 h, and then exposed to −0.7 MPa water stress or distilled 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 μm.

increase in the production of H_2O_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_2O_2 in vascular tissues of maize leaves exposed to water stress.

To investigate further the intracellular sites of H_2O_2 production under water stress, the chloroplasts of mesophyll cells and the mitochondria and peroxisomes of bundle-sheath cells were examined. H_2O_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_2O_2 accumulation in mitochondria and peroxisomes of bundle-sheath cells (Fig. 4B), and the extent of H_2O_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_2O_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_2O_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_2O_2 accumulation induced by water stress

In order to determine whether the intracellular H_2O_2 accumulation induced by water stress was related to the increase in the apoplastic H_2O_2 in leaves of maize plants, several apoplastic H_2O_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_2O_2 accumulation induced by water stress in mesophyll cells of wild-type maize leaves, but did not affect the water stress-induced H_2O_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_2O_2 accumulation induced by water stress is not related to the apoplastic H_2O_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_2O_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 4 h 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_2O_2 under water stress results from the increase in H_2O_2 synthesis.

Discussion

For years, chloroplasts, mitochondria and peroxisomes have been considered to be the main sources of H_2O_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, Boudsocq and Laurière 2005). However, many studies used the isolated organelles to detect the production of H_2O_2. These methods involve cell extraction and extensive tissue disruption. It may be questioned whether the assay can accurately reflect the generation of H_2O_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_2O_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_2O_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_2O_2 accumulation in mesophyll cells, bundle-sheath cells and vascular bundles of *Vp5* wild-type maize leaves (Fig. 1A). H_2O_2 generated in response to water stress treatment was detected within 1 h and maximized at about 4 h. The application of several apoplastic H_2O_2 manipulators, such as DPI, SOD and CAT, abolished the majority of H_2O_2 accumulation induced by water stress (Fig. 1C), indicating that extracellular H_2O_2 is a major H_2O_2 source in the leaves of maize plants exposed to water stress. The results from cytochemical detection further showed that water stress induced H_2O_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_2O_2 in plant cells under water stress, but further indicate that the apoplast is the major source of H_2O_2 production in leaves of maize plants exposed to water stress. In a previous study, however, it was shown that H_2O_2, detected by $CeCl_3$ staining and transmission electron microscopy, only accumulated in xylem vessels, cell walls and plasma

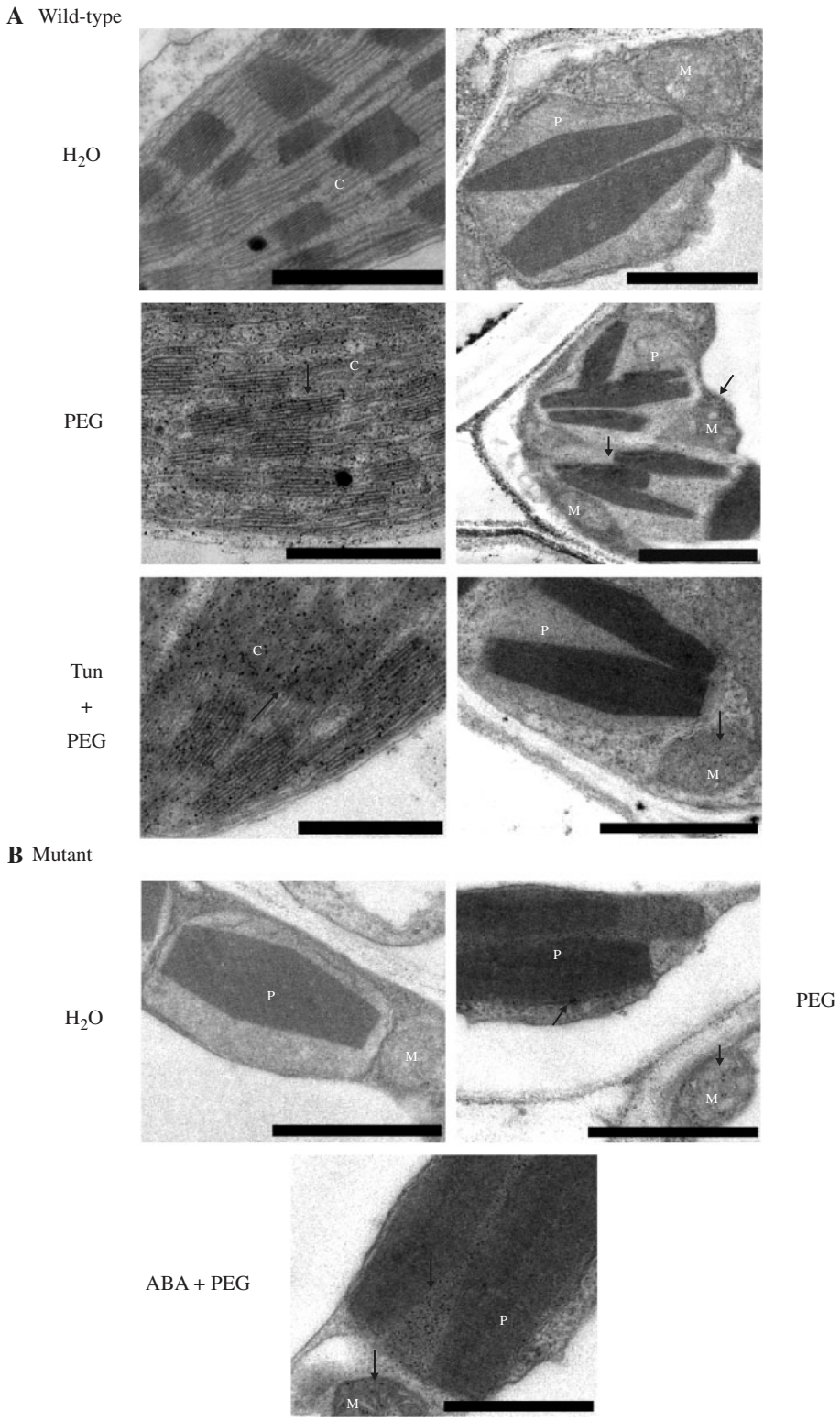

Fig. 4 Changes in the production of H_2O_2 in response to water stress in chloroplasts, mitochondria and peroxisomes of wild-type and *vp5* mutant maize leaves. The detached wild-type (A) and mutant (B) maize plants were pre-treated with 2 mM sodium tungstate (Tun), 100 μM ABA or distilled water for 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.

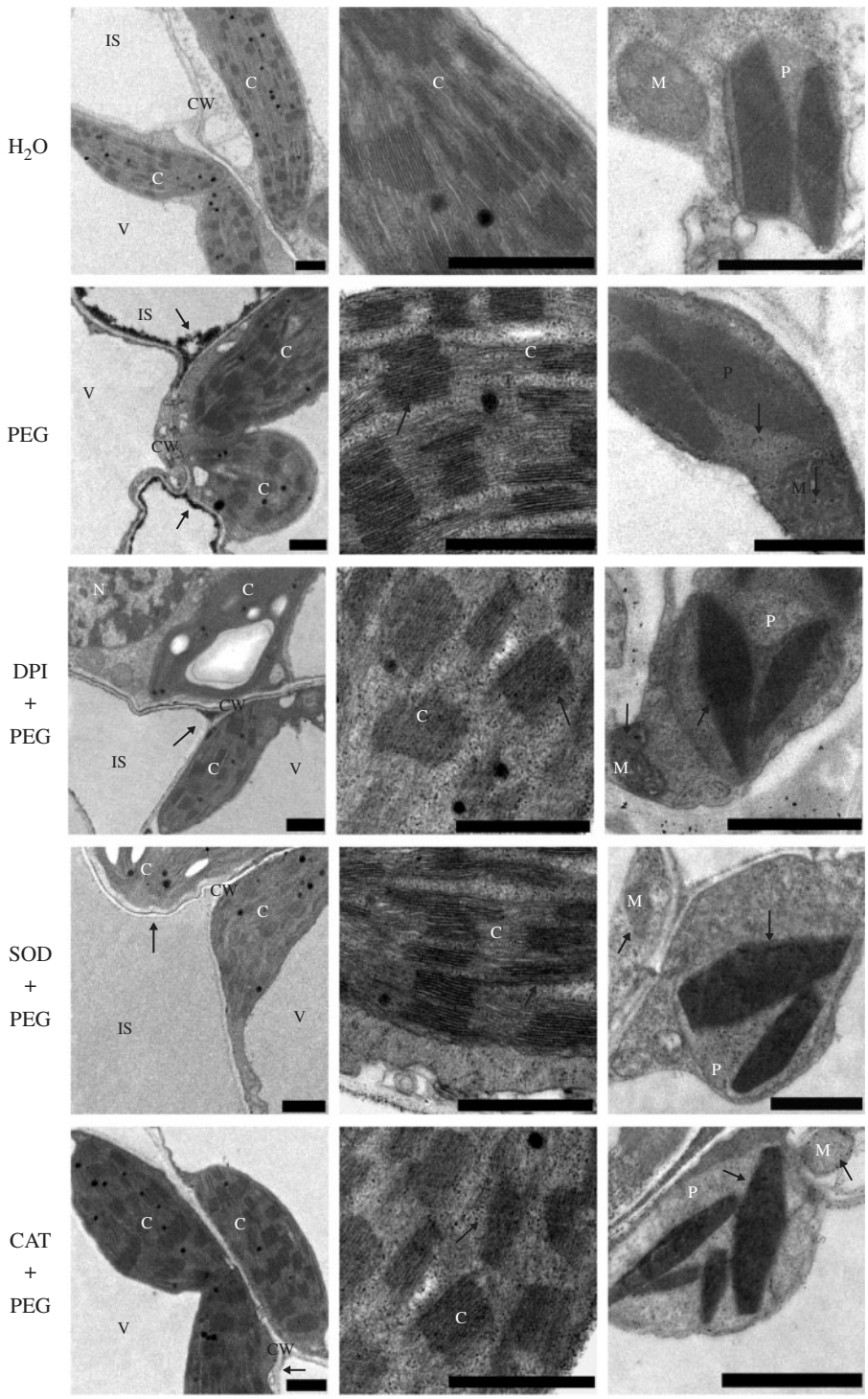

Fig. 5 Effects of pre-treatments with DPI, SOD and CAT on the production of H_2O_2 in apoplast, chloroplasts, mitochondria and peroxisomes in leaves of wild-type maize plants exposed to water stress. The detached maize plants were pre-treated with 100 μM DPI, 200 U of SOD and 200 U of CAT for 5 h, and 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 μm.

Table 1 The activities of SOD ($U\,mg^{-1}$ protein), CAT ($\mu mol\,min^{-1}\,mg^{-1}$protein), APX ($\mu mol\,min^{-1}\,mg^{-1}$protein) and GR ($nmol\,min^{-1}\,mg^{-1}$protein) in leaves of *vp5* mutant and wild-type maize plants exposed to water stress

	SOD	CAT	APX	GR
Wild-type				
Control	36.20 ± 0.87	4.31 ± 0.10	0.353 ± 0.017	21.63 ± 0.68
PEG	37.64 ± 1.71	4.60 ± 0.42	0.374 ± 0.024	23.32 ± 2.17
Mutant				
Control	35.68 ± 1.25	4.25 ± 0.34	0.359 ± 0.012	21.38 ± 1.69
PEG	36.96 ± 2.09	4.47 ± 0.26	0.365 ± 0.019	22.89 ± 1.55

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.

membranes of mesophyll cells, but not in chloroplasts, mitochondria or peroxisomes in field-grown sage exposed to drought (Munnè-Bosch et al. 2001). The difference in the subcellular localization of H_2O_2 accumulation induced by water stress between the study by Munnè-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, H_2O_2 accumulates when H_2O_2 synthesis is increased (Neill et al. 2002, Pastori and Foyer 2002). This might be the reason that the apoplastic H_2O_2 accumulated under water stress in the study by Munnè-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 H_2O_2 in plant cells. However, it is not clear what is the relative contribution of water stress-induced ABA accumulation to water stress-induced H_2O_2 production. It has been suggested that ABA may be a rather weak inducer of ROS (Avsian-Kretcher et al. 2004). In this study, using the ABA-deficient maize *vp5* mutant, the role of endogenous ABA in the production of H_2O_2 induced by water stress was assessed. Our results showed that the accumulation of H_2O_2 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 H_2O_2 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 H_2O_2 in the wild-type leaves exposed to water stress (data not shown; Fig. 3), and the reduction in the production of H_2O_2 was fully prevented by the addition of ABA (data not shown). These results clearly indicate that ABA is a key inducer of H_2O_2 production in leaves of maize plants exposed to water stress. However, no differences in the production of H_2O_2 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 H_2O_2 in these organelles under water stress (Fig. 4). Pre-treatment with tungstate also did not affect the water stress-induced H_2O_2 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 H_2O_2 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 Scandalios 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 H_2O_2 in the mutant leaves exposed to water stress. Moreover, it may also be asked whether the accumulation of H_2O_2 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

antioxidant enzymes (Table 1). These results suggest that the accumulation of H_2O_2 in the wild type and the reduction in the mutant during the 4 h water stress result from the changes in H_2O_2 synthesis.

There are several potential sources of apoplastic H_2O_2 in plants, including plasma membrane NADPH oxidases, cell wall peroxidases, and apoplastic oxalate 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 H_2O_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 H_2O_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 (Munnè-Bosch et al. 2001). A possible explanation is that H_2O_2 in the xylem sap might result from the transport of H_2O_2 to the nearby generating sites.

In the apoplast, H_2O_2 has several possible fates. H_2O_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). H_2O_2 has been considered to be a mobile signal molecule (Mittler 2002, Neill et al. 2002, Pastori and Foyer 2002). It has been speculated that water channels (aquaporins) may also serve as conduits for trans-membrane H_2O_2 transport (Henzler and Steudle 2000). H_2O_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 H_2O_2 induced by water stress can move into the cytosol and subcellular compartments. In the present study, no visible H_2O_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 H_2O_2 accumulation induced by water stress in the wild-type leaves, but did not affect the accumulation of H_2O_2 in the chloroplasts, mitochondria and peroxisomes under water stress (Fig. 5). In the mutant leaves, the reduction in the production of apoplastic H_2O_2 also did not affect the accumulation of H_2O_2 in the mitochondria and peroxisomes under water stress (Figs. 2, 4). Our results suggest that H_2O_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.) *vp5* mutant and its wild-type *Vp5* were used in these studies. Seeds of the *vp5* mutant and wild-type maize were obtained by selfing 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 μmol 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 MPa for various times up to 4 h at 25°C, with a continuous light intensity of 200 μmol m^{-2} s^{-1}. In order to study the effects of inhibitors and scavengers, the detached plants were pre-treated with 100 μM 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 H_2O_2

H_2O_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 μm) on a frozen cut microtome, mounted on a microscopy slide without a coverslip and observed using a microscope (BH-2, Olympus, Tokyo) with a 'no coverslip' 40× objective lens.

Cytochemical detection of H_2O_2

H_2O_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 H_2O_2 and are visible by transmission

electron microscopy. Tissue pieces (~1–2 mm^2) were excised from the treated and untreated leaves and incubated in freshly prepared 5 mM $CeCl_3$ 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 Eponaraldite (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 H_2O_2 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 A_{340} as oxidized glutathione (GSSG)-dependent oxidation of NADPH.

Acknowledgments

This work was supported by the Major State Basic Research Program of China (grant No. 2003CB114302 to M.J.), the National Natural Science Foundation of China (grant No. 30571122 to M.J.), the Key Project of the Chinese Ministry of Education (grant No. 104100 to M.J.), the Science Foundation of the Doctoral Subject Point of the Chinese Ministry of Education (grant No. 20040307011 to M.J.) and Hong Kong Research Grants Council (HKBU 2149/04M to J.Z.).

References

Apel, K. and Hirt, H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55: 373–399.

Avsian-Kretchmer, O., Gueta-Dahan, Y., Lev-Yadun, S., Gollop, R. and Ben-Hayyim, G. (2004) The salt-stress signal transduction pathway that activates the *gpx1* promoter is mediated by intracellular H_2O_2, different from the pathway induced by extracellular H_2O_2. *Plant Physiol.* 135: 1685–1696.

Bartoli, C.G., Gómez, F., Martínez, D.E. and Guiamet, J.J. (2004) Mitochondria are the main target for oxidative damage in leaves of wheat (*Triticum aestivum* L.). *J. Exp. Bot.* 55: 1663–1669.

Bestwick, C.S., Brown, I.R., Bennett, M.H. and Mansfield, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv *phaseolicola*. *Plant Cell* 9: 209–221.

Boudsocq, M. and Laurière, C. (2005) Osmotic signaling in plants. Multiple pathways mediated by emerging kinase families. *Plant Physiol.* 138: 1185–1194.

Foyer, C.H. and Noctor, G. (2003) Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiol. Plant.* 119: 355–364.

Foyer, C.H. and Noctor, G. (2005) Oxidant and antioxidant signaling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ.* 28: 1056–1071.

Fryer, M.J., Oxborough, K., Mullineaux, P.M. and Baker, N.R. (2002) Imaging of photo-oxidative stress responses in leaves. *J. Exp. Bot.* 53: 1249–1254.

Guan, L. and Scandalios, J.G. (1998) Two structurally similar maize cytosolic superoxide dismutase genes, *Sod4* and *Sod4A*, respond differentially to abscisic acid and high osmoticum. *Plant Physiol.* 117: 217–224.

Guan, L., Zhao, J. and Scandalios, J.G. (2000) *Cis*-elements and *trans*-factors that regulate expression of the maize *Cat1* antioxidant gene in response to ABA and osmotic stress: H_2O_2 is the likely intermediary signaling molecule for the response. *Plant J.* 22: 87–95.

Hansen, H. and Grossmann, K. (2000) Auxin-induced ethylene triggers abscisic acid biosynthesis and growth inhibition. *Plant Physiol.* 124: 1437–1448.

Henzler, T. and Steudle, E. (2000) Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: model calculations and measurements with the pressure probe suggest transport of H_2O_2 across water channels. *J. Exp. Bot.* 51: 2053–2066.

Hu, X., Jiang, M., Zhang, A. and Lu, J. (2005) Abscisic acid-induced apoplastic H_2O_2 accumulation up-regulates the activities of chloroplastic and cytosolic antioxidant enzymes in maize leaves. *Planta* 223: 57–68.

Jiang, M. and Zhang, J. (2001) Effect of abscisic acid on active oxygen species, antioxidative defence system and oxidative damage in leaves of maize seedlings. *Plant Cell Physiol.* 42: 1265–1273.

Jiang, M. and Zhang, J. (2002a) Involvement of plasma membrane NADPH oxidase in abscisic acid- and water stress-induced antioxidant defense in leaves of maize seedlings. *Planta* 215: 1022–1030.

Jiang, M. and Zhang, J. (2002b) 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. *J. Exp. Bot.* 53: 2401–2410.

Jiang, M. and Zhang, J. (2003) Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defense in leaves of maize seedlings. *Plant Cell Environ.* 26: 929–939.

Kwak, J.M., Mori, I.C., Pei, Z.-M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D.G. and Schroeder, J.I. (2003) NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. *EMBO J.* 22: 2623–2633.

Laloi, C., Apel, K. and Danon, A. (2004) Reactive oxygen signaling: the latest news. *Curr. Opin. Plant Biol.* 7: 323–328.

Lin, C.C. and Kao, C.H. (2001) Abscisic acid induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. *Plant Sci.* 160: 323–329.

Luna, C.M., Pastori, G.M., Driscoll, S., Groten, K., Bernard, S. and Foyer, C.H. (2004) Drought controls on H_2O_2 accumulation, catalase (CAT) activity and *CAT* gene expression in wheat. *J. Exp. Bot.* 56: 417–423.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7: 405–410.

Mittler, R., Vanderauwera, S., Gollery, M. and Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* 9: 490–498.

Munné-Bosch, S., Jubany-Marí, T. and Alegre, L. (2001) Drought-induced senescence is characterized by a loss of antioxidant defences in chloroplasts. *Plant Cell Environ.* 24: 1319–1327.

Neill, S.J., Desikan, R. and Hancock, J.T. (2002) Hydrogen peroxide signaling. *Curr. Opin. Plant Biol.* 5: 388–395.

Noctor, G., Veljovic-Jovanovic, S., Driscoll, S., Novitskaya, L. and Foyer, C.H. (2002) Drought and oxidative load in the leaves of C_3 plants: a predominant role for photorespiration?. *Ann. Bot.* 89: 841–850.

Orozco-Cárdenas, M.L., Narváez-Vásquez, J. and Ryan, C.A. (2001) Hydrogen peroxide acts as a second messenger for the induction of defense genes in tomato plants in response to wounding, systemin, and methyl jasmonate. *Plant Cell* 13: 179–191.

Orozco-Cárdenas, M.L. and Ryan, C.A. (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. *Proc. Natl Acad. Sci. USA* 96: 6553–6557.

Pastori, G.M. and Foyer, C.H. (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of 'redox' and abscisic acid-mediated controls. *Plant Physiol.* 129: 460–468.

Pei, Z.M., Murata, N., Benning, G., Thomine, S., Klüsener, B., Allen, G.J., Grill, E. and Schroeder, J.I. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. *Nature* 406: 731–734.

Pellinen, R.I., Korhonen, M.-S., Tauriainen, A.A., Palva, T.P. and Kangasjärvi, J. (2002) Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiol.* 130: 549–560.

Pellinen, R., Palva, T. and Kangasjärvi, J. (1999) Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. *Plant J.* 20: 349–356.

Romero-Puertas, M.C., Rodríguez-Serrano, M., Corpas, F.J., Gómez, M., del Río, L.A. and Sandalio, L.M. (2004) Cadmium-induced subcellular accumulation of O_2^- and H_2O_2 in pea leaves. *Plant Cell Environ.* 27: 1122–1134.

Sharp, R.E. (2002) Interaction with ethylene: changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant Cell Environ.* 25: 211–222.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K. (2002) Regulation and function of ascorbate peroxidase isoenzymes. *J. Exp. Bot.* 53: 1305–1319.

Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B. (1997) Subcellular localization of H_2O_2 in plants: H_2O_2 accumulation in papillae and hypersensitive response during the barley–powdery mildew interaction. *Plant J.* 11: 1187–1194.

Torres, M.A. and Dangl, J.L. (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. *Curr. Opin. Plant Biol.* 8: 397–403.

Vanacker, H., Carver, T.L.W. and Foyer, C.H. (2000) Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley–powdery mildew interaction. *Plant Physiol.* 123: 1289–1300.

Vranová, E., Inzé, D. and Van Breusegem, F. (2002) Signal transduction during oxidative stress. *J. Exp. Bot.* 53: 1227–1236.

Zhang, A., Jiang, M., Zhang, J., Tan, M. and Hu, X. (2006) Mitogen-activated protein kinase is involved in abscisic acid-induced antioxidant defense and acts downstream of reactive oxygen species production in leaves of maize plants. *Plant Physiol.* 141: 475–487.

Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W. and Song, C.-P. (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. *Plant Physiol.* 126: 1438–1448.

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annu. Rev. Plant Biol.* 53: 247–273.

(Received June 22, 2006; Accepted September 16, 2006)