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The Ascorbic Acid Redox State Controls Guard Cell Signaling and Stomatal Movement

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H_2O_2 serves an important stress signaling function and promotes stomatal closure, whereas ascorbic acid (Asc) is the major antioxidant that scavenges H_2O_2. Dehydroascorbate reductase (DHAR) catalyzes the reduction of dehydroascorbate (oxidized ascorbate) to Asc and thus contributes to the regulation of the Asc redox state. In this study, we observed that the level of H_2O_2 and the Asc redox state in guard cells and whole leaves are diurnally regulated such that the former increases during the afternoon, whereas the latter decreases. Plants with an increased guard cell Asc redox state were generated by increasing DHAR expression, and these exhibited a reduction in the level of guard cell H_2O_2. In addition, a higher percentage of open stomata, an increase in total open stomatal area, increased stomatal conductance, and increased transpiration were observed. Guard cells with an increase in Asc redox state were less responsive to H_2O_2 or abscisic acid signaling, and the plants exhibited greater water loss under drought conditions, whereas suppressing DHAR expression conferred increased drought tolerance. Our analyses suggest that DHAR serves to maintain a basal level of Asc recycling in guard cells that is insufficient to scavenge the high rate of H_2O_2 produced in the afternoon, thus resulting in stomatal closure.

INTRODUCTION

Of the antioxidants found in plants, ascorbic acid (Asc) is the most abundant. Asc is present in plant species in millimolar concentrations that range from 10 to 300 mM (Smirnoff, 2000) and serves as the major contributor to the cellular redox state. In its antioxidant role, Asc is used by ascorbate peroxidase (APX) to convert H_2O_2 to water, and Asc can directly scavenge superoxide, hydroxyl radicals, and singlet oxygen. Asc can serve as an enzyme cofactor, for example, for violaxanthin de-epoxidase (VDE) (Eskling et al., 1997), which catalyzes the conversion of violaxanthin to zeaxanthin (the xanthophyll cycle), which is required for the dissipation of excess excitation energy during nonphotochemical quenching. Asc also has been implicated in the regulation of cell elongation and progression through the cell cycle (reviewed in Horemans et al., 2000).

Reactive oxygen species serve an important signaling function, providing information about changes in the external environment. H_2O_2 has been implicated to play a signaling role in guard cells that permit gas exchange (e.g., entry of CO_2 and loss of water through the stomatal pore). Stomatal pores in many species open in the morning but close in the afternoon to limit water loss (Assmann, 1993; Assmann and Wang, 2001). Stomatal pores also close in response to water stress through the action of abscisic acid (ABA), which causes an increase in cytosolic Ca^{2+} concentration from H_2O_2-activated Ca^{2+} channels and from release from intracellular stores (Price et al., 1994; McAinsh et al., 1996; Grabov and Blatt, 1998; Guan et al., 2000; Hamilton et al., 2000; Pei et al., 2000; Zhang et al., 2001; Kohler and Blatt, 2002). Activation of plasma membrane–localized anion channels results in guard cell depolarization, potassium efflux, and loss of guard cell turgor and volume, which causes stomatal closure (Ishikawa et al., 1983; Thiel et al., 1992; Blatt and Armstrong, 1993; MacRobbie, 2000; Blatt, 2000; Schroeder et al., 2001a, 2001b). ABA causes an increase in H_2O_2 production, which serves as a signaling intermediate to promote stomatal closure (Price et al., 1994; Pei et al., 2000; Murata et al., 2001; Schroeder et al., 2001a, 2001b; Zhang et al., 2001), although a recent study has suggested that H_2O_2 may also function in a divergent pathway that controls stomatal movement (Kohler et al., 2003). H_2O_2-induced stomatal closure was reversed by exogenous application of ascorbate, which is consistent with its role as a scavenger of H_2O_2 (Zhang et al., 2001). Consequently, plants with increased ascorbate might be predicted to exhibit reduced responsiveness to ABA or H_2O_2 signaling.

Asc biosynthesis differs from that in mammals and is made after the oxidation of L-galactose to L-galactono-1,4-lactone, which in turn is oxidized to Asc. Once used, Asc is oxidized to the monodehydroascorbate radical that is reduced to Asc by monodehydroascorbate reductase (MDHAR) or disproportionates to Asc and dehydroascorbate (DHA). DHA undergoes irreversible hydrolysis to 2,3-diketogulonic acid or is reduced to Asc, a reaction catalyzed by dehydroascorbate reductase (DHAR), which requires glutathione. Thus, DHAR allows the plant to recycle DHA, thereby recapturing the Asc before it is lost. Because Asc is the major reductant in plants, DHAR serves to regulate the intracellular redox state. DHAR is expressed in

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rate-limiting amounts as overexpression of DHAR in tobacco (*Nicotiana tabacum*) leaves increased the Asc redox state (Chen et al., 2003). Plants overexpressing DHAR grew and flowered normally under well-watered conditions. However, given the role of Asc as a scavenger of H_2O_2, changes in the Asc redox state might be expected to alter stomatal movement, particularly under conditions in which ABA or H_2O_2 signaling activates stomatal closure.

Water stress results in the depletion of the Asc pool and triggers ABA-induced stomatal closure (Smirnoff and Pallanca, 1996; Leung and Giraudat, 1998; Pastori and Foyer, 2002). Such closure limits CO_2 assimilation and increases the concentration of NADPH as a consequence of a reduction in Calvin cycle activity. Under normal growth conditions, photoactivated chlorophyll transfers its excitation energy to the photosynthetic reaction centers. However, under conditions in which NADP is limiting (e.g., after stomatal closure in a water-stressed leaf), the excitation energy of the photoactivated chlorophyll is transferred to triplet (i.e., ground state) oxygen and excites it to the singlet form. Consequently, water stress increases the production of activated oxygen species (Bowler et al., 1992; Scandalios, 1993).

In this study, we have examined the role that the Asc redox state plays in regulating stomatal function. Plants in which DHAR expression was increased exhibited an increase in the Asc redox state in the leaf as a whole, in the apoplast, and in guard cells, whereas suppression of DHAR expression lead to the opposite effect. Guard cells with increased Asc redox state exhibited greater stomatal opening, both in the percentage of stomata that were open and in the degree of openness. They also exhibited reduced levels of H_2O_2, whereas those with a decreased Asc redox state had an elevated level of H_2O_2. Increasing the Asc redox state resulted in an increase in transpiration rate and stomatal conductance under normal growth conditions. Guard cells with a higher Asc redox state were less responsive to H_2O_2 or ABA signaling, and the plants exhibited enhanced water loss after the imposition of drought conditions. Decreasing the Asc redox state by repressing DHAR expression enhanced stomatal closure under normal growth conditions and after water stress. These observations suggest that the redox state of ascorbate plays an important role in controlling H_2O_2-mediated stomatal closure.

RESULTS

H_2O_2 and the Asc Redox State Are Diurnally Regulated

H_2O_2 has been implicated to serve as a signaling intermediate that promotes stomatal closure (McAinsh et al., 1996; Pei et al., 2000; Chen et al., 2003). Given that stomata close in the afternoon, the concentration of foliar H_2O_2 might be predicted to be higher in the afternoon than in the morning when stomata are open. To investigate this, the level of H_2O_2 was determined from expanded tobacco leaves at 2-h intervals during the course of a day. The concentration of H_2O_2 was low in early morning and increased during the afternoon, peaking in midafternoon before decreasing again throughout late afternoon and evening (Figure 1A). The diurnal increase in H_2O_2 concentration was approximately twofold.

The level of ascorbic acid, glutathione, and tocopherols is diurnally regulated in *Arabidopsis thaliana*, spruce (*Picea abies*), and other species (Schupp and Rennenberg, 1988; Esterbauer et al., 1990; Wildi and Lutz, 1996; Tamaoki et al., 2003). The maximum accumulation of these antioxidants corresponded with maximum daylight intensities and correlated with photosynthetic activity. Whether there is a diurnal change in the redox state of any of these antioxidants has not been examined in detail. Because Asc scavenges H_2O_2 either directly or through the action of APX, the level of Asc and DHA in leaves was measured. The level of Asc remained relatively constant throughout the day, whereas the concentration of DHA was low in early morning and increased approximately twofold during midafternoon, after which it decreased throughout the remaining time points (Figure 1B). Therefore, the Asc redox state was constant during early morning but decreased twofold by midafternoon, after which it recovered by night (Figure 1B). The diurnal changes in the Asc redox state inversely correlated with the changes in H_2O_2. No consistent change in the GSH redox state was observed (Figure 1B), suggesting that the changes were specific to the Asc redox state. These results indicate that the level of H_2O_2 and the Asc redox state are diurnally regulated.

Diurnal regulation of antioxidant enzymes also has been reported. Expression from *Cat3*, one member of the catalase gene family, exhibits circadian rhythm in maize (*Zea mays*) and Arabidopsis (Redinbaugh et al., 1990; Zhong and McClung, 1996; Polidoros and Scandalios, 1998). Circadian regulation from Arabidopsis *Cat2* also was observed and required light as the synchronizer (Zhong et al., 1998). Diurnal changes in expression of superoxide dismutase (SOD) and glutathione reductase (GR) also has been reported in *Aster tripolium* (Erdei et al., 1998). Moreover, L-galactono-γ-lactone dehydrogenase, which catalyzes the final step during Asc biosynthesis, is diurnally regulated in Arabidopsis (Tamaoki et al., 2003). To investigate whether the activity of antioxidant enzymes exhibits diurnal regulation, the foliar activities of DHAR, MDHAR, APX, GR, catalase (CAT), and SOD were determined at 2-h intervals. The activity of DHAR, MDHAR, GR, and CAT was low in the morning, rose as the day progressed to a maximum during the afternoon, and decreased again during the evening (Figure 2). APX activity was high from early morning to midafternoon, after which it declined. In contrast with these changes, SOD activity remained largely unchanged throughout the day (Figure 2). The increase in activity of some of these antioxidant enzymes was unlikely a daily stress response because the plants were watered twice a day and experienced a maximum daytime temperature of 25.9 ± 0.6°C and an afternoon light intensity of 1191 ± 244 $\mu mol^{-2}\ s^{-1}$. Leaf water content was unchanged between the morning and the afternoon (Table 1), supporting the conclusion that growth of the plants under these conditions did not result in water stress. The diurnal increase of DHAR, MDHAR, GR, CAT, and APX activities, all of which are involved in H_2O_2 scavenging either directly or indirectly, suggests that the afternoon increase in H_2O_2 is not a consequence of a reduction in H_2O_2-scavenging enzymes. Moreover, the increase in these enzyme activities was not sufficient to prevent the diurnal increase in H_2O_2 during the afternoon.

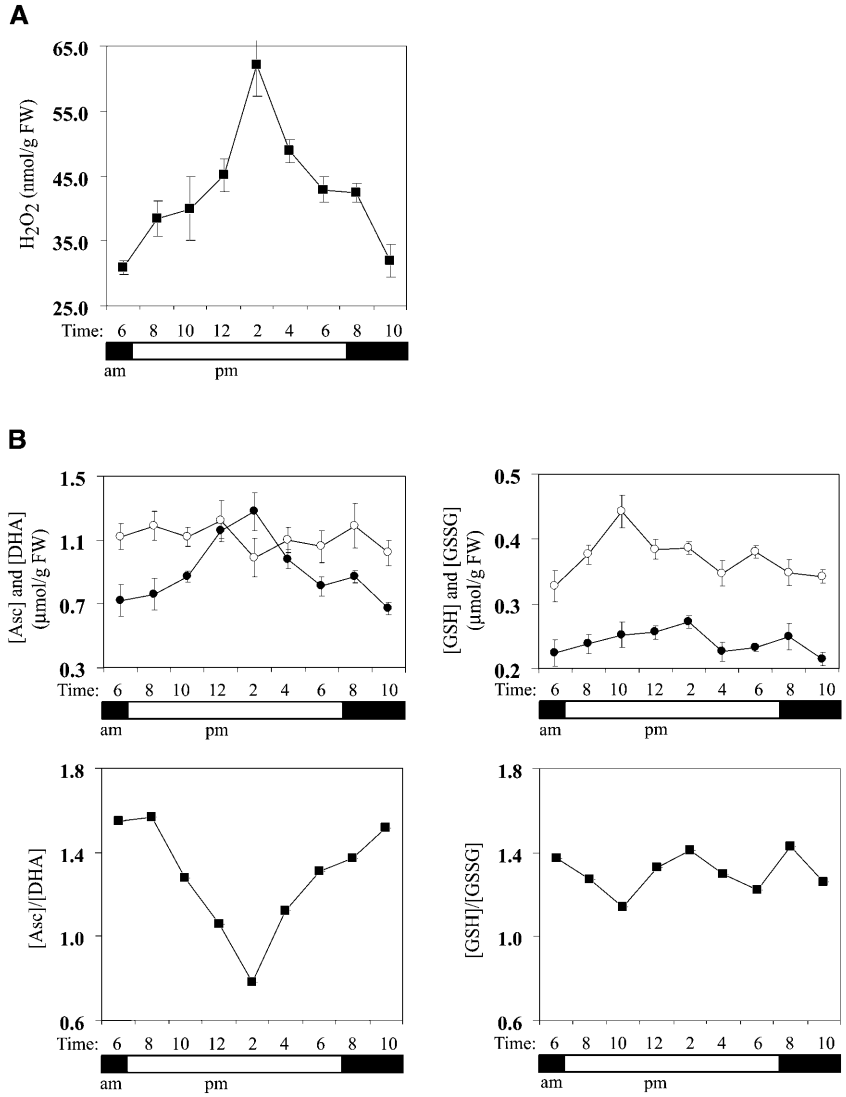


Figure 1. Diurnal Regulation of H_2O_2, Asc, DHA, GSH, and GSSG.

Leaf samples were taken at 2-h intervals from 6 AM to 10 PM and assayed for H_2O_2 **(A)**, Asc **(B)**, GSH (open circles), and DHA and GSSG (closed circles). Each was assayed in three independent replicates of leaves pooled from three independent plants, and the average and standard deviation were reported. The Asc and GSH redox state was determined from the Asc/DHA and GSH/GSSG ratios. The bar below each graph indicates dark and light periods.

Generation of Tobacco with Altered Asc Redox State

The observation that the diurnal changes in the Asc redox state and concentration of H_2O_2 are inversely correlated suggested the possibility that they are in balance, such that altering the concentration of one would affect the concentration of the other. To investigate whether alteration of the Asc redox state would affect the concentration of H_2O_2 and thus affect the diurnal closure of stomata, the expression of DHAR was modified, an approach shown previously to alter the Asc redox state in tobacco and maize (Chen et al., 2003). Tobacco was transformed with the wheat (*Triticum aestivum*) DHAR cDNA under the control of the 35S promoter of *Cauliflower mosaic virus* (CaMV), and DHAR-overexpressing regenerants were identified by protein gel blot analysis (D1, C4, and B4 in Figure 3A). Overexpression of DHAR was confirmed by assaying for DHAR activity in which more than a 10-fold increase was measured (Figure 3A). Tobacco transformants suppressed for DHAR also were identified by protein gel blot analysis after transformation with the tobacco DHAR cDNA under the control of the CaMV 35S promoter (R_i11 in Figure 3A) and confirmed by DHAR assay, which revealed a reduction to just 30% of wild-type DHAR activity.

An increase in DHAR expression results in an increase in Asc (Chen et al., 2003). Consistent with this, the level of Asc increased in expanding, expanded, and presenescent leaves of

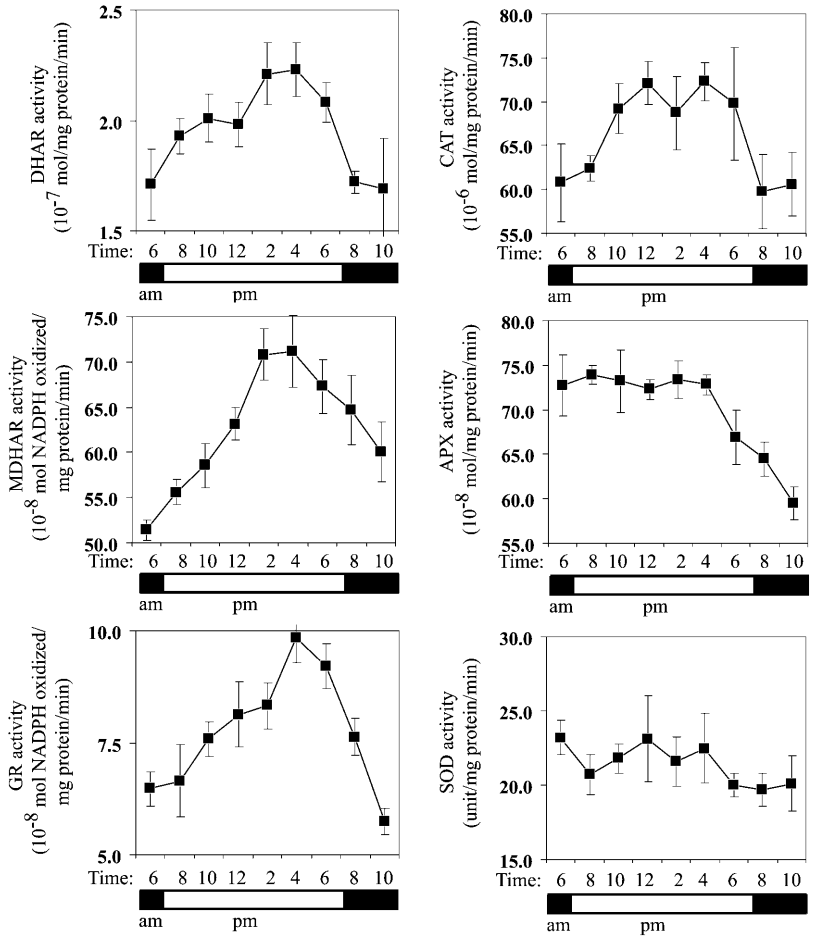


Figure 2. Diurnal Regulation of DHAR, GR, CAT, APX, and SOD Activities.

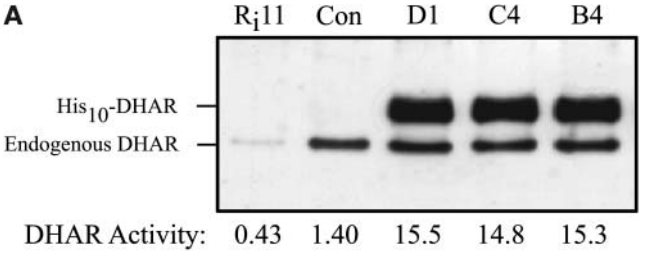
Leaf samples were taken at 2-h intervals from 6 AM to 10 PM and assayed for DHAR, MDHAR, GR, CAT, APX, and SOD. Enzyme activities were assayed in three independent replicates of leaves pooled from three independent plants, and the average and standard deviation were reported. The bar below each graph indicates dark and light periods.

DHAR-overexpressing plants compared with control ($P < 0.05$, $P < 0.05$, $P < 0.005$, respectively) and DHAR-suppressed leaves ($P < 0.05$, $P < 0.05$, $P < 0.005$, respectively) as did the Asc redox state compared with control ($P < 0.001$, $P < 0.05$, $P < 0.005$, respectively) and DHAR-suppressed leaves ($P < 0.001$, $P < 0.05$, $P < 0.005$, respectively) (Figure 3B). No significant change was observed in the level of DHA in expanded or presenescent leaves of DHAR-overexpressing plants compared with the control ($P = 0.210$ or $P = 0.079$, respectively). The level of DHA in expanding leaves of DHAR-overexpressing plants was significantly lower than in DHAR-suppressed leaves ($P < 0.005$), whereas the level of DHA in expanding leaves of DHAR-overexpressing plants was significantly lower than the control for some DHAR-overexpressing individual lines but not others. Similar results were reported previously (Chen et al., 2003). Despite not being consistently significantly different, the level of DHA in expanding DHAR-overexpressing leaves was consistently lower than the control (Figure 3B; Chen et al., 2003). A decrease in the level of DHA in expanding leaves can be understood by the higher level of DHAR expressed in young leaves compared with expanded leaves (Chen et al., 2003), which would be expected to result in more efficient ascorbate recycling and thus reduce the level of DHA present.

Table 1. Relative Leaf Water Content

	Water Absorbed (mg/g Dry Leaf Weight)	
	Morning	Afternoon
Expanding leaf	316 ± 85	324 ± 102
Expanded leaf	97 ± 29	112 ± 20
Presenescent leaf	116 ± 33	138 ± 37

Reducing DHAR expression would be expected to limit its ability to reduce DHA to Asc and thus result in a decrease in the Asc redox state from either an increase in DHA and/or a reduction in Asc. This prediction was borne out in DHAR-suppressed plants in which a significant reduction in the Asc redox state was



B

	R_i11	Con	D1	C4	B4
Expanding leaf					
Asc (μmol/g FW):	1.15±0.07	1.18±0.04	2.84±0.14	2.22±0.13	2.33±0.12
DHA (μmol/g FW):	1.60±0.06	0.79±0.13	0.66±0.03	0.46±0.03	0.50±0.03
Asc/DHA:	0.72	1.49	4.30	4.83	4.57
Expanded leaf					
Asc (μmol/g FW):	0.53±0.03	0.64±0.06	2.51±0.11	1.62±0.04	1.44±0.07
DHA (μmol/g FW):	0.68±0.01	0.69±0.03	0.75±0.08	0.71±0.05	0.79±0.06
Asc/DHA:	0.78	0.93	3.35	2.28	1.82
Presenescent leaf					
Asc (μmol/g FW):	0.47±0.04	0.50±0.04	1.1±0.04	0.97±0.04	0.91±0.06
DHA (μmol/g FW):	0.84±0.03	0.62±0.05	0.57±0.13	0.54±0.08	0.55±0.11
Asc/DHA:	0.56	0.81	1.93	1.80	1.65

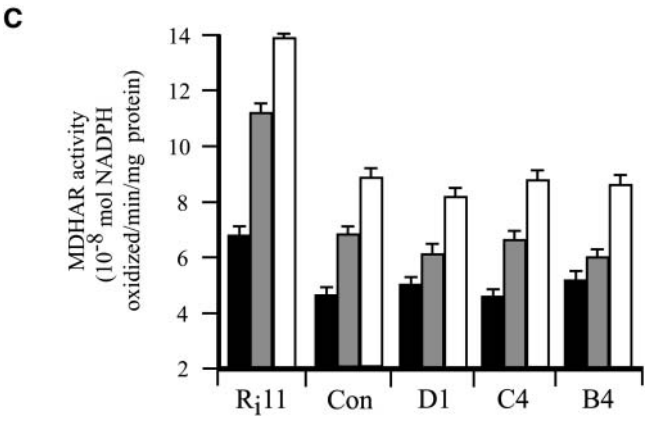


Figure 3. Generation of DHAR-Overexpressing and DHAR-Suppressed Tobacco.

(A) Protein gel blot analysis of DHAR in tobacco suppressed for endogenous DHAR (R_i11), vector-only control (Con), and overexpressing wheat DHAR (D1, C4, and B4). The positions of the His-tag wheat DHAR (His_{10}-DHAR) and endogenous tobacco DHAR are indicated. DHAR activity measured in each leaf type is represented below each lane.

(B) The amount of Asc and DHA was measured in expanding leaves, expanded leaves (which exhibited maximal photosynthetic activity), and presenescent leaves of DHAR-suppressed, control, and DHAR-overexpressing transgenic lines. Measurements were made in the afternoon from three independent replicates of leaves pooled from three independent plants, and the average and standard deviation were reported. The Asc redox state is indicated as the Asc/DHA ratio. FW, fresh weight.

(C) MDHAR activity (10^{-8} mol NAPDH oxidized/min/mg protein) was measured in expanding leaves (black histograms), expanded leaves (gray histograms), and presenescent leaves (white histograms) of DHAR-suppressed (R_i11), control (Con), and DHAR-overexpressing (D1) plants. The enzyme activity was assayed in three independent replicates of leaves pooled from three independent plants, and the average and standard deviation were reported.

observed in expanding, expanded, and presenescent leaves of DHAR-suppressed plants compared with the control ($P < 0.001$, $P < 0.05$, $P < 0.05$, respectively). No significant difference was observed in Asc in expanding, expanded, and presenescent leaves of DHAR-suppressed plants relative to the control ($P = 0.472$, $P = 0.109$, $P = 0.582$, respectively) but was significantly lower than DHAR-overexpressing plants ($P < 0.05$, $P < 0.05$, $P < 0.005$, respectively). DHA was significantly higher in expanding and presenescent leaves of DHAR-suppressed plants relative to the control ($P < 0.05$ and $P < 0.05$, respectively) and DHAR-overexpressing plants ($P < 0.005$ and $P < 0.01$, respectively) but was not significantly different in control or DHAR-overexpressing expanded leaves ($P = 0.777$ and $P = 0.082$, respectively), suggesting that DHA might be lost more quickly in expanded leaves than in expanding and presenescent leaves of DHAR-suppressed plants.

Decreasing DHAR expression resulted in an increase in MDHAR activity (Figure 3C), whereas no change was observed in DHAR-overexpressing plants. The increase in MDHAR activity in DHAR-suppressed plants may be in response to the decrease in DHAR-mediated Asc recycling and the reduction in Asc redox state, which would increase this alternative pathway for the recycling of oxidized ascorbate. These data confirm that DHAR expression controls the Asc redox state and that its wild-type level of expression is rate limiting.

The leaf water potentials, measured for control, DHAR-overexpressing, and DHAR-suppressed leaves in the morning and the afternoon, were all well within nonstress values (Table 2), supporting the conclusion that the plants did not experience water stress under the growth conditions used and that changes in antioxidant enzyme activity observed in Figure 2 were a result of diurnal regulation.

The Asc Redox State Controls Stomatal Function

To determine whether changes in the Asc redox state altered stomatal movement, we examined the stomata of leaves from DHAR-overexpressing and DHAR-suppressed plants and compared them to those of vector-only control plants to determine whether the observed change in redox state resulting from alterations in DHAR expression affected guard cell function. Abaxial epidermal strips were stained with 0.2% toluidine blue O and destained, and the stomata were imaged using light microscopy. The effect of the Asc redox state on stomatal behavior might be expected to affect the degree to which a stomata is open of those that are open and affect the number of stomata that are open versus the number that are completely closed. Therefore, three types of data were collected to accurately determine the effect of the Asc redox state on stomatal behavior. The width and length of the aperture of only those stomata that remained open were measured and used to

Table 2. Leaf Water Potential

	Water Potential (MPa)[a]	
	Morning	Afternoon
Control	−0.35 ± 0.022	−0.39 ± 0.032
D1[b]	−0.34 ± 0.041	−0.39 ± 0.027
R_i11[c]	−0.32 ± 0.022	−0.41 ± 0.050

[a] Determined in expanding leaves as described by Gómez-Cadenas et al. (1996).
[b] DHAR-overexpressing leaves.
[c] DHAR-suppressed leaves.

calculate the average stomatal aperture (width/length). In addition, the percentage of stomata that remained open was determined. Finally, the width and length of open stomatal apertures was used to calculate the average stomatal aperture area, and together with the percentage of stomata that remained open, the total open stomatal area per unit leaf area containing 100 stomata [i.e., (average stomatal aperture area) × (percentage of stomata that remained open) × 100] was calculated. The total open stomatal area represents the effect of the Asc redox state on all stomata collectively.

During the morning when stomatal pores are open, no significant difference in the average stomatal aperture was observed between expanding leaves of DHAR-overexpressing (D1 in Figure 4) and vector-only control plants (Con in Figure 4), which was supported by quantitative measurements (Figure 5) ($n = 32$, $P = 0.305$). In addition, the average stomatal aperture in expanding leaves of DHAR-suppressed plants (R_i11 in Figure 5) was not significantly different from that of control plants ($n = 34$, $P = 0.089$), whereas it was significantly smaller than DHAR-overexpressing plants ($n = 34$, $P < 0.05$). The morning stomatal aperture in expanded and presenescent leaves of DHAR-overexpressing plants was significantly greater than that of control plants ($n = 47$, $P < 0.005$ and $n = 53$, $P < 0.01$, respectively), but the stomatal aperture in expanded and presenescent leaves of DHAR-suppressed plants was not significantly different from that of control plants ($n = 47$, $P = 0.275$ and $n = 53$, $P = 0.546$, respectively) (Figure 5). Greater than 80% of all stomata were open in expanding and expanded leaves in the morning, with only small differences in the percentage of stomata that were open among leaves of control, DHAR-overexpressing, or DHAR-suppressed plants. Fewer stomata were open in presenescent leaves in the morning compared with expanding or expanded leaves, but more stomata were open in presenescent DHAR-overexpressing leaves and fewer open in presenescent DHAR-suppressed leaves relative to the control (Figure 5).

During the afternoon, stomatal pores normally close in leaves at all developmental stages as illustrated in control plants where <15% of stomata remained open in leaves of any age (Figures 4

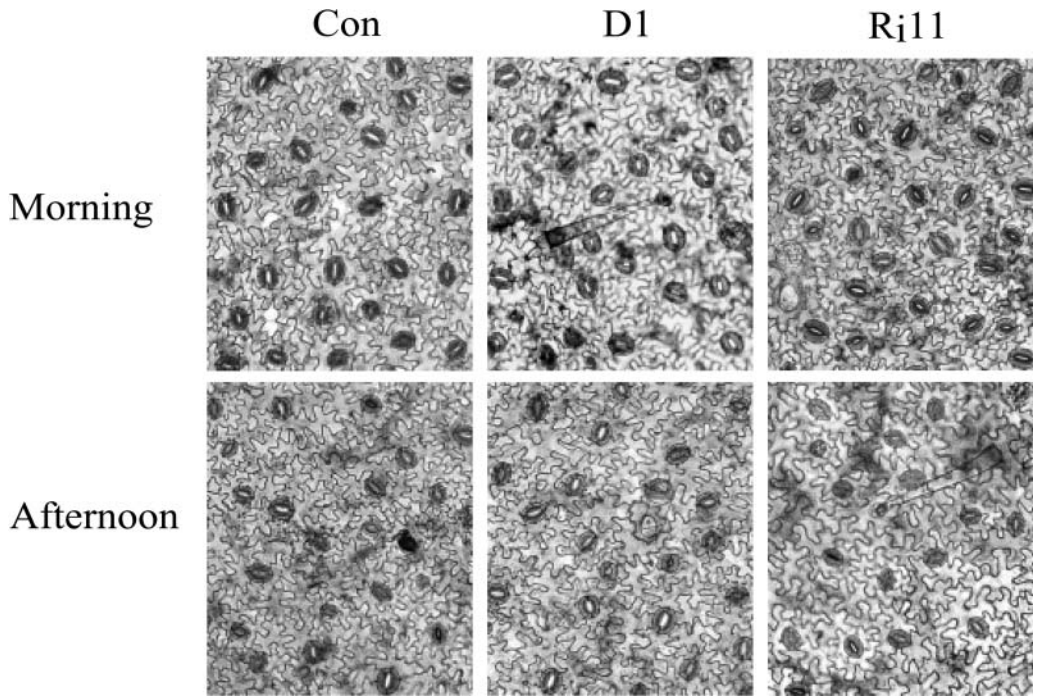


Figure 4. Stomata in Tobacco with Altered Asc Redox State.

Abaxial epidermis was collected in the morning and afternoon from expanding leaves of control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco and stained with 0.2% toluidine blue O before imaging using a compound microscope. A representative region from each line is presented.

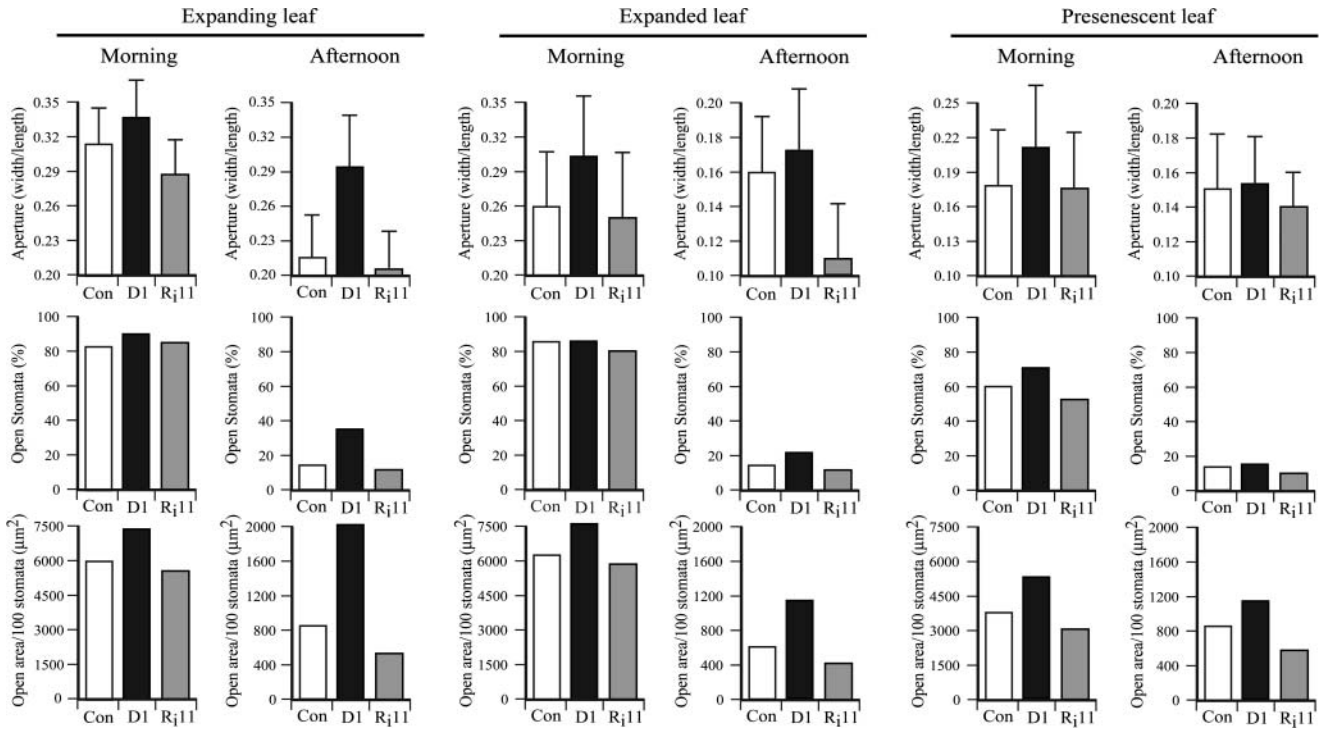


Figure 5. The Asc Redox State Controls Stomatal Movement.

Stomatal bioassay experiments were performed as described (Pei et al., 1997). Abaxial epidermis was collected from expanding, expanded, and presenescent leaves of control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves. Epidermal strips were stained and imaged as described in Figure 2. The width and length of at least 30 stomatal apertures were measured and used to determine the stomatal aperture (width/length) with the standard deviation indicated. The percentage of stomata that were open (defined as having a width >1 μm) was determined from at least 400 stomata. The total stomatal open area was calculated from the average stomatal aperture area and percentage of stomata that were open and reported as the total area (μm^2) per 100 stomata.

and 5). The stomatal aperture in expanding DHAR-overexpressing leaves in the afternoon was significantly greater than the control ($n = 31$, $P < 0.001$), and more than twice as many stomata were open, resulting in a 133% increase in the total open stomatal area relative to the control and a 263% increase in total open stomatal area compared with DHAR-suppressed leaves (Figure 5). The stomatal aperture in expanding control leaves in the afternoon was not significantly different from DHAR-suppressed leaves ($n = 34$, $P = 0.183$) but had more stomata that were open, resulting in 55% more open stomatal area relative to DHAR-suppressed leaves. Although the aperture of those stomata that remained open in expanded DHAR-overexpressing leaves in the afternoon was not significantly different from that of the control ($n = 35$, $P = 0.513$), more stomata were open, resulting in an 89% increase in the total open stomatal area relative to the control and a 172% increase in total open stomatal area compared with DHAR-suppressing leaves (Figure 5). The stomatal aperture in expanded control leaves in the afternoon was significantly larger than DHAR-suppressed leaves ($n = 35$, $P = 0.005$) and had more stomata that were open, resulting in a 45% increase in total open stomatal area relative to DHAR-suppressed leaves. In presenescent leaves, the aperture of those stomata that remained open in DHAR-overexpressing leaves in the afternoon was not significantly different from that of the control ($n = 32$, $P = 0.838$), but more stomata were open, resulting in a 31% increase in the total open stomatal area relative to the control and a 94% increase in total open stomatal area compared with DHAR-suppressing leaves. The stomatal aperture in presenescent control leaves was not significantly different from that of DHAR-suppressed leaves ($n = 37$, $P = 0.392$) but had more stomata that were open, resulting in a 48% increase in the total open stomatal area relative to DHAR-suppressed leaves. These data illustrate that overexpression of DHAR in guard cells results in an increase in total open stomatal area in leaves of all ages, whereas decreasing DHAR expression results in a reduction in total open stomatal area in leaves of all ages, suggesting that changing the Asc redox state in guard cells alters stomatal movement.

The Asc Redox State Governs the Level of H_2O_2 in Guard Cells

The diurnal decrease in the Asc redox state observed in Figure 1 may result from the inability of DHAR to recycle Asc at a rate rapid enough to scavenge the increased rate of H_2O_2 production that occurs in the afternoon as a consequence of excess light. To

determine whether alterations in DHAR expression affect the level of guard cell H_2O_2, the level of H_2O_2 was determined in guard cells of DHAR-overexpressing, DHAR-suppressed, and control plants. Epidermal strips from leaves were loaded with 2',7'-dichlorofluorescin diacetate (H_2DCF-DA), and after washing, fluorescence emitted specifically from guard cells was detected at 510 to 530 nm and quantified using confocal microscopy. Much of the H_2O_2 present in guard cells was localized in chloroplasts, in good agreement with previous reports (Zhang et al., 2001). The level of H_2O_2 in guard cells from expanding leaves of DHAR-overexpressing plants collected during the morning was significantly lower than in control guard cells ($n = 70$, $P < 0.001$), whereas it was significantly higher in DHAR-suppressed guard cells ($n = 70$, $P < 0.001$) (Figure 6, Table 3). These differences in H_2O_2 level could not be explained by changes in APX activity, which was not significantly different from the level in DHAR-overexpressing guard cells ($P = 0.075$) or in DHAR-suppressed guard cells ($P = 0.295$) (Table 3). As expected, the level of guard cell H_2O_2 increased during the afternoon. However, the level of H_2O_2 in DHAR-overexpressing guard cells remained significantly lower ($n = 70$, $P < 0.001$ in expanding leaves) than that in the control, and H_2O_2 in DHAR-suppressed guard cells remained significantly higher ($n = 70$, $P < 0.001$ in expanding leaves) (Figure 6, Table 3). As observed in the morning, the changes in guard cell H_2O_2 could not be explained by changes in APX activity (Table 3). APX activity in expanding leaves of DHAR-overexpressing or DHAR-suppressed plants was not significantly different from that in control plants in the afternoon ($P = 0.061$ and $P = 0.507$, respectively). The level of guard cell H_2O_2 in presenescent leaves was higher than that in guard cells of expanding leaves, consistent with previous reports that the level of active oxidative stress increases with leaf age (Havaux et al., 2000; Munne-Bosch and Alegre, 2002). Although the level of guard cell H_2O_2 increased with leaf age, the same differences in guard cell H_2O_2 were observed among the DHAR lines regardless of leaf age (Figure 6, Table 3).

To examine whether changes in guard cell H_2O_2 that were observed after alteration of DHAR expression correlated with changes in CAT or SOD activities, the two enzyme activities were measured in the afternoon in expanding and presenescent leaves of DHAR-overexpressing, DHAR-suppressed, and control plants (Table 4). As expected, CAT and SOD activity increased with leaf age, however, no significant or substantial change in their activity was observed in either DHAR-overexpressing or DHAR-suppressed leaves. SOD activity in DHAR-overexpressing and DHAR-suppressed plants was not significantly different from that in expanding leaves ($P = 0.365$ and $P = 0.073$, respectively), although a small increase was observed in presenescent leaves ($P = 0.027$ and $P = 0.035$, respectively) compared with control plants, whereas CAT activity in DHAR-overexpressing and DHAR-suppressed plants was not substantially different in expanding leaves ($P = 0.045$ and $P = 0.738$, respectively) or in presenescent leaves ($P = 0.195$ and $P = 0.813$, respectively) compared with control plants. In fact, the only significant difference in CAT activity was a decrease in its activity in DHAR-overexpressing leaves compared with control leaves. This suggests that the observed changes in H_2O_2, and thus stomatal movement, were not a result of changes in CAT and SOD activities but, instead, a result of changes in Asc through alterations in DHAR expression.

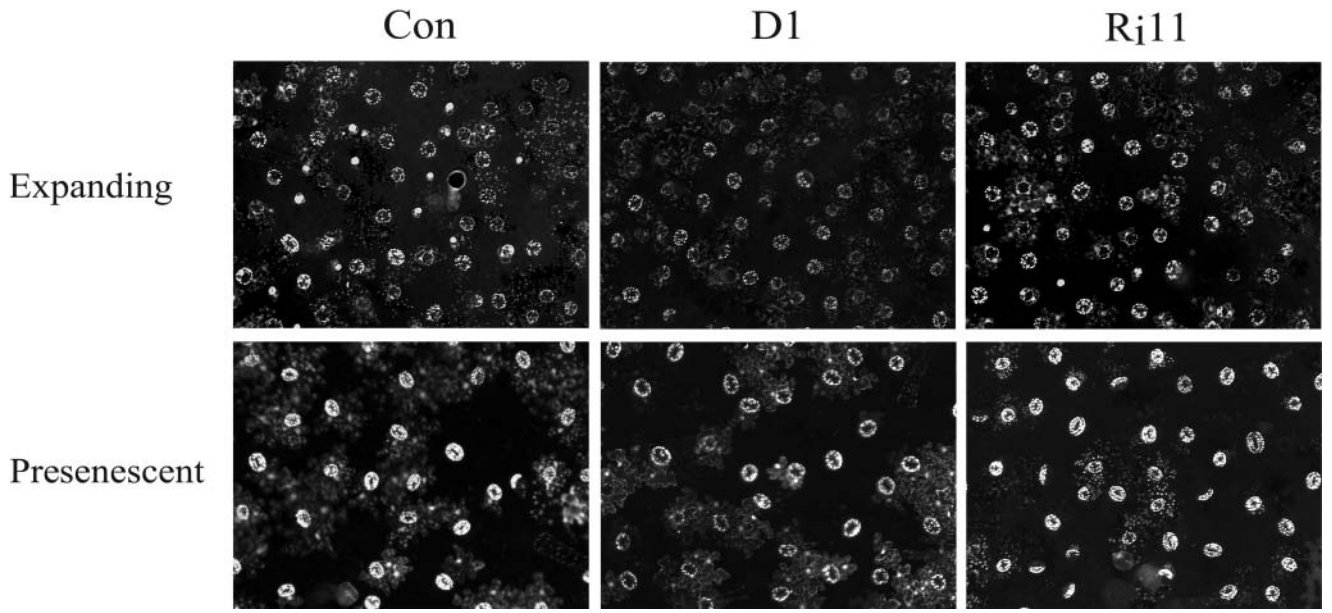


Figure 6. The Asc Redox State Controls H_2O_2 Concentration in Guard Cells.

Abaxial epidermis was collected in the morning from control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves. H_2O_2 production was revealed after loading with H_2DCF-DA. Fluorescence (excitation 465 to 495 nm, emission 510 to 530 nm) was recorded using confocal microscopy. A representative region from each line is presented.

Table 3. Guard Cell H_2O_2 Levels with Altered Asc Redox State

	H_2O_2 Fluorescence (Pixel Intensity)			
	Expanding	Expanded	Presenescent	APX Activity[a]
Morning				
Control	76.3 ± 26.7	84.7 ± 21.3	85.1 ± 19.7	2.65 ± 0.23
D1[b]	59.4 ± 14.6	60.4 ± 16.9	73.4 ± 17.2	2.15 ± 0.10
R_i11[c]	86.3 ± 21.8	93.6 ± 27.7	95.4 ± 15.8	2.89 ± 0.13
Afternoon				
Control	102 ± 21.2	126 ± 27.6	136 ± 18.2	2.99 ± 0.11
D1	76.6 ± 14.6	109 ± 15.9	112 ± 16.9	2.68 ± 0.03
R_i11	118 ± 22.1	144 ± 21.1	147 ± 13.1	3.08 ± 0.13

[a] 10^{-6} Asc oxidized/min/mg protein. Determined from guard cells isolated from expanding leaves as described by Kruse et al. (1989).
[b] DHAR-overexpressing leaves.
[c] DHAR-suppressed leaves.

To determine directly whether the Asc redox state of guard cells was altered in DHAR-overexpressing or DHAR-suppressed leaves, the level of Asc and DHA was measured in guard cells isolated from each line. In the morning, the level of Asc in DHAR-overexpressing guard cells was substantially higher than in control guard cells or DHAR-suppressed guard cells (Figure 7A), consistent with the increase in Asc observed in whole leaves (Figure 3B) and consistent with increased DHAR expression in DHAR-overexpressing guard cells (see below). The level of DHA also increased in DHAR-overexpressing guard cells, which may have resulted from the higher level of Asc available to participate in reactions that consume the compound. The Asc redox state in DHAR-overexpressing guard cells increased to 2.49 from a control value of 1.58 and Asc redox state of 1.48 in DHAR-suppressed guard cells (Figure 7C). The high Asc redox state in DHAR-overexpressing guard cells correlated with the largest total open stomatal area observed in the morning in expanding DHAR-overexpressing leaves in Figure 5, whereas the low Asc redox state in DHAR-suppressed guard cells correlated with the smallest total open stomatal area observed in DHAR-suppressed leaves. The increase in Asc redox state in DHAR-overexpressing guard cells relative to DHAR-suppressed guard cells indicates that, despite an increase in DHA, DHA recycling into Asc is improved in guard cells when DHAR expression is increased. Conversely, the level of Asc decreased in DHAR-suppressed guard cells relative to DHAR-overexpressing guard cells is consistent with a reduced ability to regenerate Asc. The level of DHA also decreased in DHAR-suppressed guard cells relative to DHAR-overexpressing guard cells similar to what was observed in expanded leaves in Figure 3, suggesting that DHA may be lost at a higher rate in DHAR-suppressed guard cells than that observed in control guard cells.

The level of DHA increased in control guard cells in the afternoon relative to its level in control guard cells in the morning (cf. PM to AM values for Con, Figure 7B) at the same time that the level of Asc decreased slightly (cf. PM to AM values for Con, Figure 7A), indicating that Asc is being increasingly consumed in the afternoon. This resulted in a decrease in the Asc redox state from 1.58 in the morning to 1.04 in the afternoon (Figure 7C). In DHAR-overexpressing guard cells, the afternoon level of Asc decreased relative to its morning level (cf. PM to AM values for D1, Figure 7A) at the same time that the level of DHA increased (cf. PM to AM values for D1, Figure 7B), thus resulting in a decrease in the Asc redox state from 2.49 in the morning to 1.44 in the afternoon (Figure 7C). This indicates that, as in control guard cells, Asc is being increasingly consumed in the afternoon. However, compared with control guard cells, the level of Asc remains proportionately greater than the level of DHA; thus, the afternoon Asc redox state of 1.44 in DHAR-overexpressing guard cells remained greater than the afternoon Asc redox state of 1.04 in control guard cells. In DHAR-suppressed guard cells, the level of DHA increased substantially in the afternoon relative to its level in the morning (cf. PM to AM values for R_i11, Figure 7B), thus resulting in a decrease in the Asc redox state from 1.48 in the morning to just 0.77 in the afternoon (Figure 7C). Compared with control guard cells, the afternoon increase in DHA relative to Asc was proportionately greater; thus, the afternoon Asc redox state of 0.77 in DHAR-suppressed guard cells was lower than the afternoon Asc redox state of 1.04 in control guard cells. These observations are consistent with the notion that the Asc redox state is diurnally controlled in guard cells as it is in whole leaves and that DHAR expression in guard cells is rate limiting. The changes in Asc redox state in guard cells resulting from changes in DHAR expression was similar but not identical to those observed in whole leaves, suggesting that the level of Asc biosynthesis or the rate of its consumption in guard cells may differ from that in whole leaves. In contrast with the changes in the redox state of Asc, the GSH redox state did not change substantially in guard cells (Figure 7F), suggesting that alterations in DHAR expression resulted in changes in the Asc redox state specifically. The high Asc redox state in DHAR-overexpressing guard cells correlated with the largest total open stomatal area observed in the afternoon in expanding DHAR-overexpressing leaves in Figure 5, whereas the low Asc redox state in DHAR-suppressed guard cells correlated with the smallest total open stomatal area observed in DHAR-suppressed leaves.

Asc is exported to the apoplast where it functions in cell wall synthesis, protects against exposure to external ROS, and is reimported as DHA. To determine whether the guard cell Asc

Table 4. CAT and SOD Activities in Tobacco Leaves with Asc Redox State

	CAT Activity (10^{-6} mol H_2O_2 Reduced/min/mg Protein)		SOD Activity (Units to Inhibit Nitroblue Tetrazolium Photoreduction by 50%)	
	Expanding	Presenescent	Expanding	Presenescent
Control	68.2 ± 5.96	86.6 ± 8.05	12.8 ± 0.26	33.5 ± 1.05
D1[a]	56.7 ± 3.43	76.9 ± 5.47	12.2 ± 0.82	37.5 ± 1.21
R_i11[b]	66.6 ± 3.64	84.9 ± 7.38	14.6 ± 0.53	37.0 ± 0.30

[a] DHAR-overexpressing leaves.
[b] DHAR-suppressed leaves.

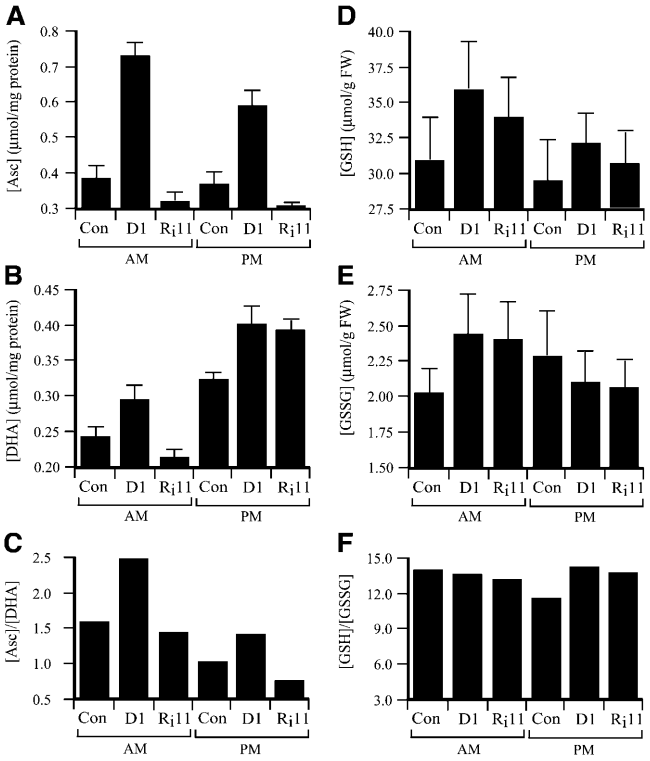


Figure 7. Alteration of the Asc Redox State in Guard Cells.

The level of Asc **(A)**, DHA **(B)**, GSH **(D)**, and GSSG **(E)** was measured from guard cells isolated from control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves collected in the morning (AM) and afternoon (PM). The Asc redox state **(C)** determined by [Asc]/[DHA] and the GSH redox state **(F)** determined by [GSH]/[GSSG] also were determined. FW, fresh weight.

redox state is similar to that of the apoplast, the level of Asc and DHA in the apoplast was measured. No glucose-6-phosphate dehydrogenase activity, used as a cytosolic marker, was detected, suggesting that the apoplastic fluid was contaminated with little if any cytoplasm. DHA (Figure 8B) was present in the apoplast at a concentration substantially higher than that of Asc (Figure 8A), resulting in an Asc redox state of only 0.36 in control leaves (Figure 8C). This is in good agreement with previous reports (Veljovic-Jovanovic et al., 2001) and is in marked contrast with the redox state of 1.58 in control guard cells (Figure 7C). The level of Asc and DHA in the apoplast increased in DHAR-overexpressing leaves, and the Asc redox state increased to 0.7, whereas the apoplastic level of Asc and DHA decreased in DHAR-suppressed leaves, resulting in a redox state of 0.3 (Figure 8C). No significant change in apoplastic-localized ascorbate oxidase activity was observed between DHAR-overexpressing and control plants, and only a small decrease was observed in DHAR-suppressed plants (Figure 8D), neither of which could account for the observed changes in the apoplastic Asc redox state. These observations demonstrate that the redox state of guard cells differs substantially from that of the apoplast, suggesting that the guard cell redox state is established autonomously.

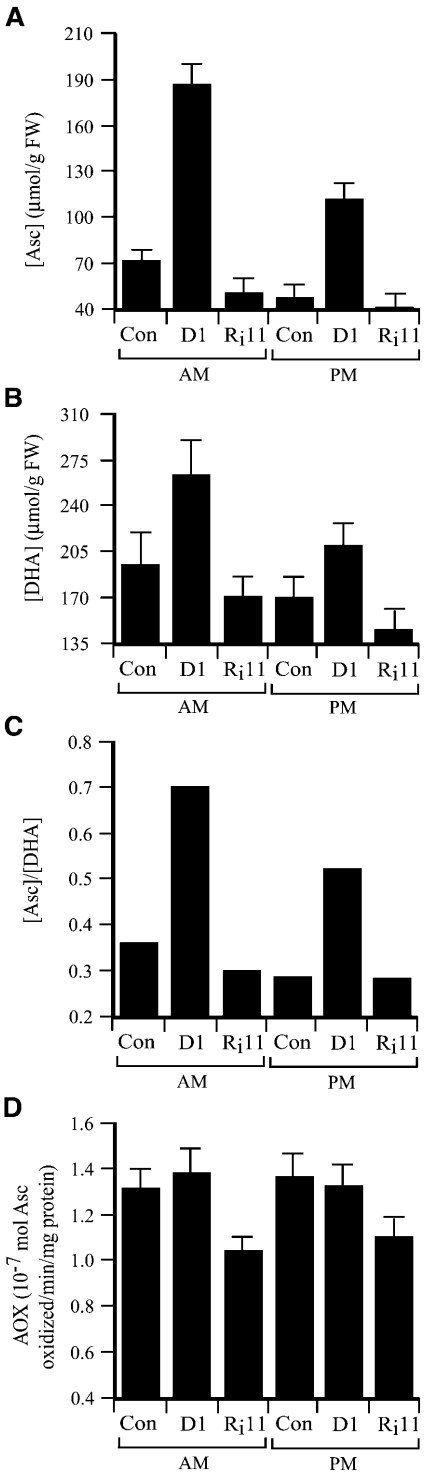


Figure 8. Alteration of the Apoplastic Asc Redox State.

The level of Asc **(A)** and DHA **(B)** was measured from apoplastic fluid from control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves collected in the morning (AM) and afternoon (PM). The Asc redox state **(C)** determined by [Asc]/[DHA] also was determined. FW, fresh weight. Ascorbate oxidase (AOX) was also measured **(D)**.

To determine whether the expression of DHAR is regulated in guard cells in a manner that would explain the diurnal control of the Asc redox state, the level of guard cell DHAR mRNA was quantitated using RT-PCR, which was performed to maintain amplification in the linear range. Amplification of actin mRNA from total RNA isolated from whole leaves and guard cell protoplasts revealed that it is represented in the RNA population to a greater extent in guard cells than it is in the leaf as a whole, whereas the opposite was observed for DHAR, MDHAR, and SOD mRNAs (Figure 9A). Little change was observed in the level of actin mRNA in guard cells between morning and afternoon or in guard cells isolated from DHAR-overexpressing, DHAR-suppressed, or control plants (Figure 9B). DHAR exhibited no diurnal regulation at the RNA level (Figure 9B), suggesting that the diurnal regulation observed at the enzyme activity level (Figure 2) may be attributable to posttranscriptional regulation. However, as expected, an increase in DHAR expression was observed in DHAR-overexpressing plants, and a decrease was observed in DHAR-suppressed plants (Figure 9B). The level of MDHAR mRNA remained unchanged in leaves (Figure 9B), whereas foliar MDHAR activity had increased in the afternoon (Figure 2), suggesting that the diurnal regulation of MDHAR activity may occur posttranscriptionally. The level of MDHAR mRNA in guard cells was higher in the afternoon than in the morning, suggesting diurnal regulation in this cell type. No alteration in MDHAR expression was observed in DHAR-suppressed guard cells compared with the control (Figure 9B), suggesting that the ~40% increase in MDHAR activity observed in DHAR-suppressed leaves in Figure 3C may be attributable to posttranscriptional regulation. SOD expression exhibited no diurnal regulation at the RNA level (Figure 9B) and no diurnal regulation at the enzyme activity level (Figure 2). These results indicate that the diurnal decrease in the Asc redox state in the afternoon is not a result of a diurnal decrease in DHAR (or MDHAR) expression. Taken together, these results demonstrate a correlation between the guard cell Asc redox state, level of H_2O_2, and stomatal movement that suggests that changes to the Asc redox state can affect guard cell functioning through changes in H_2O_2 signaling.

The Asc Redox State Controls H_2O_2 and ABA Signaling in Guard Cells

Treatment with H_2O_2 or ABA triggers stomatal closure (McAinsh et al., 1996; Pei et al., 2000; Zhang et al., 2001). If the changes in the Asc redox state were responsible for the regulation of stomatal movement through changes in guard cell H_2O_2, guard cells with an increased Asc redox state would be predicted to be less responsive to H_2O_2 and ABA signaling. To examine this prediction, epidermal strips were taken from expanding leaves with altered DHAR expression during the morning and incubated for 2 h in CO_2-free buffer at 22 to 25°C under a photon flux density of 200 μmol m^{-2} s^{-1} to promote stomatal opening. The epidermal strips were then treated with 50 μM ABA for 90 min or 1 mM H_2O_2 for 60 min, at which point they were stained with toluidine blue O, and the average aperture of open stomata and the percentage of stomatal pores that remained open were determined.

After treatment to open the stomata, no significant difference in the aperture or percentage of stomata that were open was observed among DHAR-overexpressing, DHAR-suppressed, and control leaves as expected (Figure 10). Treatment with H_2O_2 or ABA promoted stomatal closure in all leaf types (Figure 10). However, a greater percentage of stomata from leaves with

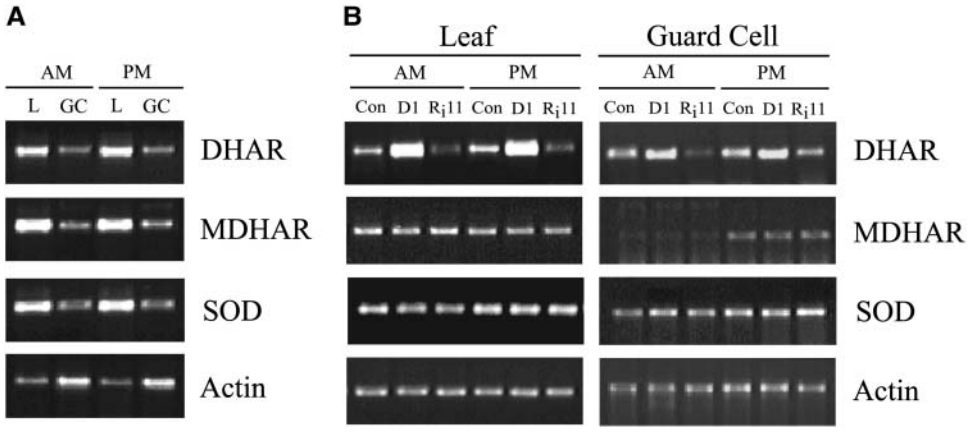


Figure 9. Expression Analysis of DHAR in Guard Cells and Leaves.

(A) Total RNA was isolated from leaves (L) and guard cell (GC) protoplasts isolated from control tobacco leaves collected in the morning (AM) and afternoon (PM). The relative abundance of transcript amounts for DHAR, MDHAR, SOD, and actin were determined using RT-PCR. The number of cycles of amplification required to detect a transcript while maintaining its amplification in the linear range was 28 for DHAR, 35 for MDHAR, 32 for SOD, and 32 for actin for both whole leaves and isolated guard cells.

(B) Total RNA was isolated from leaves and guard cell protoplasts isolated from control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves collected in the morning (AM) and afternoon (PM). The number of cycles of amplification required to detect a transcript while maintaining its amplification in the linear range was 28 and 32 for DHAR, 30 and 35 for MDHAR, 28 and 32 for SOD, and 32 and 28 for actin for whole leaves and isolated guard cells, respectively.

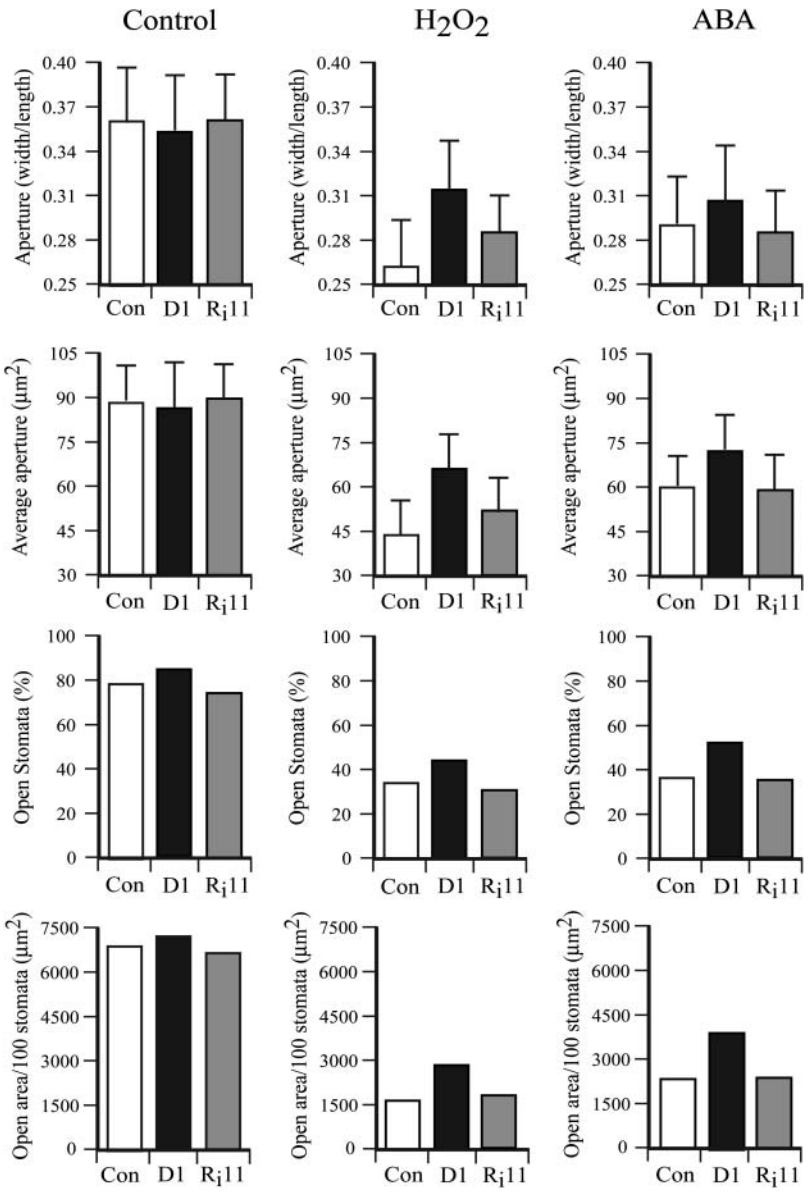


Figure 10. The Asc Redox State Controls Guard Cell Responsiveness to H_2O_2 and ABA Signaling.

Abaxial epidermis was collected in the morning from expanded leaves of control (Con), DHAR-overexpressing (D1), and DHAR-suppressed (R_i11) tobacco leaves. After incubation in CO_2-free buffer under a photon flux density of 200 $\mu mol^{-2}\ s^{-1}$ to promote stomatal opening, stomatal closure was induced by 50 μM ABA or 1 mM H_2O_2. Epidermal strips were stained with toluidine blue O or loaded with fluorescence dye to determine the production of H_2O_2. The width and length of at least 70 stomatal apertures were measured and used to determine aperture area with the standard deviation indicated. The percentage of stomata that were open was determined from at least 400 stomata. The total stomatal open area was calculated by multiplying the area of the average stomatal aperture by the percentage of stomata that were open and reported as the total area (μm^2) per 100 stomata.

an increased Asc redox state remained open and of these, they exhibited a larger average aperture after treatment with H_2O_2 or ABA than did stomata from control leaves (for H_2O_2, $n = 383$, $P < 0.001$; for ABA, $n = 341$, $P < 0.001$). These changes resulted in approximately twice as much total open stomatal area relative to control leaves (Figure 10). The response of guard cells with reduced Asc redox state to H_2O_2 or ABA was similar to that of the control (Figure 10). These results support the conclusion that an increase in the Asc redox state reduces guard cell responsiveness to H_2O_2 and ABA signaling.

The Asc Redox State Controls the Rate of Transpiration and Stomatal Conductance

If a higher Asc redox state results in a substantial increase in the total open stomatal area of a leaf, then greater stomatal

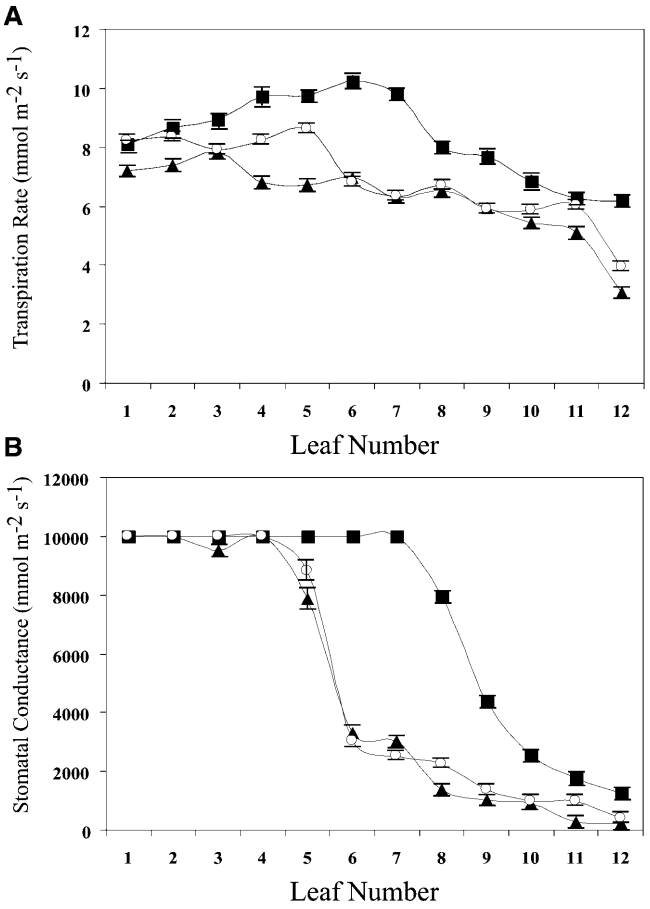


Figure 11. The Asc Redox State Controls Transpiration and Stomatal Conductance.

In situ rates of transpiration **(A)** and stomatal **(B)** conductance were measured in the afternoon from control (open circles), DHAR-overexpressing (closed circles), and DHAR-suppressed (closed triangles) tobacco leaves using a TPS-1 portable photosynthesis system. Transpiration and stomatal conductance were assayed in every other leaf in three independent replicates of leaves pooled from three independent plants, and the average and standard deviation were reported.

conductance and a higher rate of transpiration would be predicted in DHAR-overexpressing leaves compared with control leaves under conditions in which the difference in stomatal area is observed (e.g., during the afternoon when stomata are normally closed). To examine this possibility, the rate of stomatal conductance and rate of transpiration were measured in every second leaf of DHAR-overexpressing, DHAR-suppressed, and control plants during the afternoon. Leaves with an increased Asc redox state exhibited a consistent increase in their rate of transpiration relative to control leaves (Figure 11A), which correlated with the 133, 89, and 31% increase in total open stomatal area observed in Figure 5 for expanding, expanded, and presenescent DHAR-overexpressing leaves, respectively. The increase in transpiration rate was greatest for midexpanding to expanded leaves, which correlated with the largest increase in total open stomatal area. Measurement of stomatal conductance of expanding leaves did not reveal a difference because it exceeded the limit of accurate detection. However, a substantial increase in stomatal conductance of expanded to presenescent leaves was observed (Figure 11B), which correlated with the 89% increase in total open stomatal area in expanded DHAR-overexpressing leaves (Figure 5). The transpiration rate and stomatal conductance from DHAR-suppressed leaves was similar to that of the control (Figure 11). These results support the conclusion that increasing the Asc redox state promotes stomatal opening, resulting in higher rates of transpiration and stomatal conductance.

The increase in total open stomatal area in leaves with an increased Asc redox state would be predicted to increase the rate of transpiration not only during the afternoon but also after imposition of drought conditions and thus lead to increased water loss. To examine this prediction, the rate of water loss was measured in a detached leaf assay in which leaves collected from well-watered plants were allowed to lose water at room temperature. In expanding leaves, increasing the Asc redox state increased the rate of water loss by threefold relative to the control (Figure 12B), and these leaves lost turgor within 30 min of detachment (Figure 12A). In expanded and presenescent leaves with a higher Asc redox state, water loss was more than twice as rapid as from control leaves (Figure 12B). The rate of water loss from leaves with a lower Asc redox state was significantly slower in expanded and presenescent leaves than from control leaves (Figure 12B) and retained turgor after 2 h of detachment when control leaves had not (Figure 12A).

The effect that changes in the Asc redox state had on water loss in a detached leaf assay was then investigated in whole plants by measuring CO_2 assimilation before and after the imposition of a severe water stress as indicated by leaf wilting. Under well-watered conditions, leaves with a higher Asc redox state exhibited a substantial increase in CO_2 assimilation relative to the control (Figure 13A). The increase in CO_2 assimilation was observed in young expanding leaves (e.g., leaves 1 to 3 in Figure 13A), fully expanded leaves (e.g., leaves 4 to 9 in Figure 13A), and presenescent leaves (e.g., leaves 10 to 12 in Figure 13A). By contrast, the rate of CO_2 assimilation was substantially reduced in plants with a lower Asc redox state and was observed in all leaves, particularly in young and fully expanded leaves (e.g., leaves 3 to 9 in Figure 13A). The difference in the rate of CO_2 assimilation for leaves with a high versus low Asc redox state was approximately twofold in all leaves tested.

After the imposition of water stress, the rate of CO_2 assimilation in the control and DHAR-overexpressing leaves decreased up to two orders of magnitude (Figure 13B), whereas it was reduced in DHAR-suppressed leaves only moderately compared with well-watered plants (Figure 13A). These data suggest that reducing the Asc redox state results in partial stomatal closure that reduces their transpiration rate. Thus, the increase in water loss in leaves with a higher Asc redox state is consistent with reduced guard cell H_2O_2, an increase in open stomatal area, and an increase in CO_2 assimilation. Conversely, the conservation of water in leaves with a lower Asc redox state is consistent with an increase in guard cell H_2O_2, a reduction in open stomatal area, and a decrease in CO_2 assimilation.

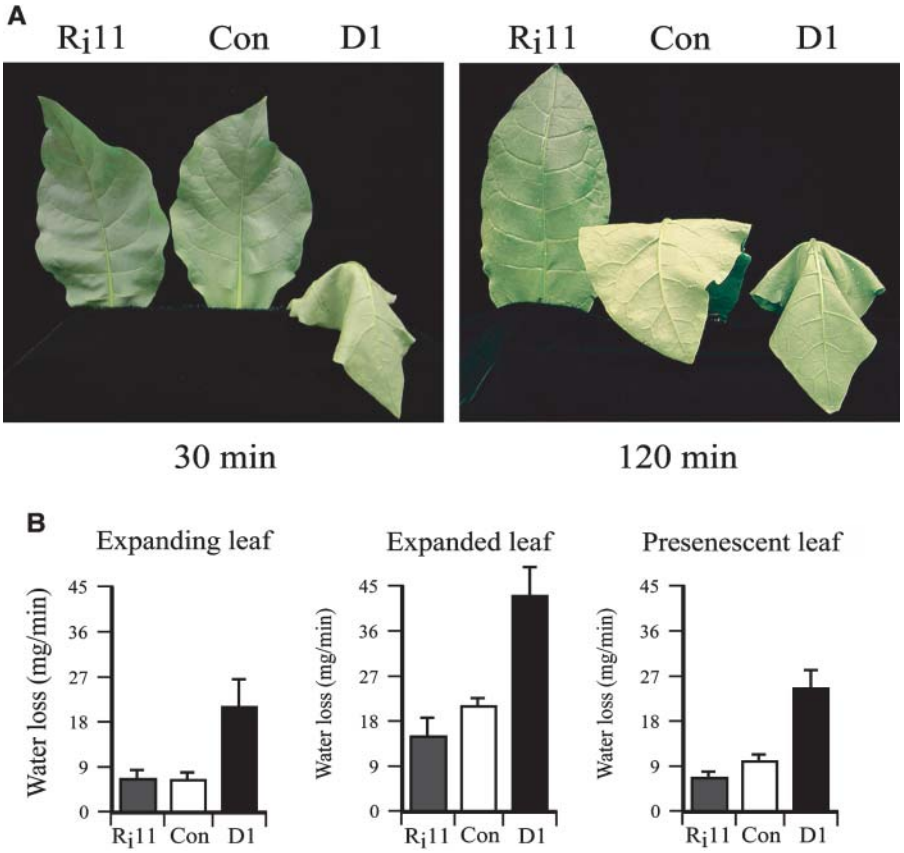


Figure 12. The Asc Redox State Controls Water Loss from Tobacco Leaves.

Expanded leaves were detached from well-watered plants in the afternoon and immediately weighed.
(A) Images of representative expanded leaves were taken at 30 and 120 min after their detachment.
(B) Water loss from the detached leaves held at room temperature was followed by determining leaf weight every 5 min for 40 min. Four leaves from separate plants were used. The rate of water loss as loss in leaf weight was plotted against time, and the average and standard deviation were reported.

DISCUSSION

Ascorbate might be expected to be involved in regulating H_2O_2-induced stomatal closure through its ability to scavenge H_2O_2 (Schroeder et al., 2001a, 2001b; Chen et al., 2003). In support of this, the level of H_2O_2 increased and the Asc redox state decreased in guard cells in the afternoon, which correlated with stomatal closure. In this study, we have investigated the role that Asc plays in regulating stomatal movement. Increasing the Asc redox state was achieved through overexpression of DHAR, whereas reducing it was achieved through suppression of DHAR. The increase in the Asc redox state in DHAR-overexpressing plants can be understood through the recycling function of DHAR, which would increase the likelihood that DHA is converted to Asc before being lost through decay. The increase in the level of DHA in DHAR-suppressed plants would be expected from their reduced ability to convert DHA into Asc. Changes in the level of DHAR expression in guard cells resulted in the alteration of their Asc redox state in that an increase was measured in DHAR-overexpressing guard cells, whereas a decrease was measured in DHAR-suppressed guard cells. Increasing foliar DHAR expression also increased the apoplastic Asc redox state, whereas decreasing DHAR expression had the opposite effect. However, the apoplast and guard cell Asc redox states differed substantially, suggesting that the Asc redox state of guard cells is established autonomously.

During the afternoon, when stomatal closure occurs, increasing the Asc redox state increased the percentage of stomata that remained open and the size of the stomatal aperture. The largest change in stomatal behavior was observed in expanding and expanded leaves that decreased as the leaves aged, suggesting the possibility that signaling in guard cells may become less efficient before entry into the senescence program. Decreasing the Asc redox state reduced the average stomatal aperture and reduced the percentage of stomata that were open. The change in stomatal movement was inversely correlated with the level of H_2O_2 present in guard cells, which was lower in guard cells with a high Asc redox state and higher in guard cells with a lower Asc redox state. These differences in H_2O_2 concentration correlated with the observed differences in stomatal behavior

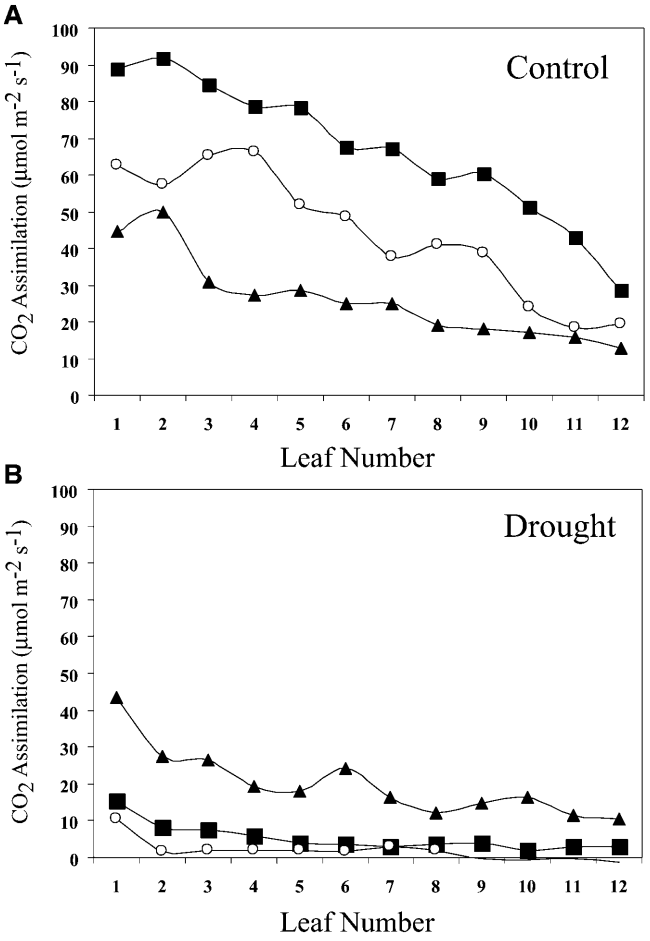


Figure 13. The Asc Redox State Controls Drought Tolerance.

CO_2 assimilation was measured in every other leaf from control (open circles), DHAR-overexpressing (closed squares), and DHAR-suppressed (closed triangles) tobacco leaves **(A)** under well-watered conditions and after the imposition of a severe drought **(B)**. CO_2 assimilation was measured using a TPS-1 portable photosynthesis system. Drought stress was evident in control and DHAR-overexpressing plants by their leaf wilting.

in the afternoon. The fact that the observed differences in H_2O_2 concentration in the morning do not result in similar differences in stomatal behavior suggests that the absolute level of H_2O_2 in guard cells of the lines examined may not be high enough at that time to trigger stomatal closure. The observation that treatment with H_2O_2 promoted stomatal closure to a lesser extent in guard cells with a higher Asc redox state indicates that the increase in Asc redox state reduced guard cell responsiveness to H_2O_2 signaling. The finding that zeaxanthin is involved in blue light sensing that results in stomatal opening (Zeiger, 2000) might suggest that changes in Asc, required for the VDE catalyzed synthesis of zeaxanthin, may affect stomatal opening through changes in the xanthophyll cycle. However, reduction of Asc in the Arabidopsis *vtc-1* mutant did not affect the xanthophyll cycle (Veljovic-Jovanovic et al., 2001), suggesting that the wild-type concentration of Asc is not rate limiting for VDE. The wild-type level of Asc in the DHAR-suppressed plants suggests that the xanthophyll cycle would be unaffected under normal growth conditions. Moreover, the degree of stomatal opening in DHAR-overexpressing and DHAR-suppressed plants in the morning was less affected by changes in the Asc redox state than was the degree of stomatal closure in the afternoon, suggesting that the effects of altering the Asc redox state may be limited to H_2O_2 signaling. In addition, because H_2O_2 serves as an intermediary of ABA-induced stomatal closure, responses elicited by ABA should be similar to those elicited by H_2O_2. This prediction was borne out as treatment with ABA promoted stomatal closure, but guard cells with a higher Asc redox state were less responsive to ABA than were control guard cells.

The effect that changes in the Asc redox state have on stomatal movement would be expected to affect stomatal conductance and the rate of transpiration. Under well-watered conditions, increasing the Asc redox state increased the rate of transpiration and stomatal conductance, whereas decreasing it reduced transpiration. Because ABA-induced stomatal closure is the primary means by which plants respond to conditions of water stress (Leung and Giraudat, 1998), increasing the Asc redox state would be expected to affect the ability of a plant to reduce transpiration during water stress. This was confirmed using whole plant and detached leaf assays. Increasing the Asc redox state increased the rate of water loss threefold, whereas reducing the Asc redox state decreased water loss up to 30%. In addition, after a severe water stress that caused leaf wilting in DHAR-overexpressing and control plants and in which the rate of CO_2 assimilation was virtually abolished, leaves with a lower Asc redox state retained turgor, and their rate of CO_2 assimilation was only slightly reduced relative to well-watered conditions. It should be noted that decreasing the Asc redox state reduced the rate of CO_2 assimilation compared with control plants, which is consistent with the reduction in total open stomatal area observed for DHAR-suppressed leaves. These data suggest that increasing the Asc redox state results in a higher rate of transpiration under normal growth conditions and reduced responsiveness to water stress. This in turn results in an enhanced rate of water loss as a consequence of the increase in the total open stomatal area. Conversely, decreasing the Asc redox state reduced transpiration and CO_2 assimilation from young leaves under normal growth conditions as a consequence of the reduction in the open stomatal area, but it also reduced water loss resulting in increased drought tolerance. These observations support the conclusion that Asc affects leaf function through alterations in stomatal movement.

Previous studies of plants with altered levels of antioxidants have not reported a change in stomatal function. Plants engineered to express higher levels of GR, the enzyme required in GSH recycling, were shown to have elevated Asc levels (Foyer et al., 1995). Although the plants exhibited little change in CO_2 assimilation, the time of day when the measurements were taken or the age of the leaves, both of which are crucial in observing stomatal behavior, were not indicated, making direct comparisons difficult. The Arabidopsis *vtc-1* mutant, which contains only 30% of the wild-type level of Asc but retains an altered Asc redox state, exhibits a normal level of photosynthesis (Veljovic-Jovanovic et al., 2001). This study did not employ plants with

elevated Asc. The DHAR-suppressed plants used in this study are not Asc deficient but rather have a decreased Asc redox state that may explain the differences observed between the present plants and the *vtc-1* mutant. Moreover, the stomatal function of the *vtc-1* mutant was not examined during the course of the day or in response to H_2O_2 or ABA, making it difficult to draw conclusions between a lower level of Asc versus a decrease in Asc redox state. However, one significant difference between the *vtc-1* mutant and plants with altered DHAR expression is that the former affects an enzyme in the Asc biosynthetic pathway, whereas DHAR affects Asc recycling, which may be expected to be a more rapid means to respond to the generation of reactive oxygen species. The observation that DHAR-suppressed plants (in which the level of Asc is unchanged but have a decreased Asc redox state) exhibit greater stomatal closure suggests that it is the Asc redox state rather than the level of Asc that affects guard cell function.

The role of Asc in guard cell functioning can thus be understood through its role as a scavenger of H_2O_2 whereby the balance between H_2O_2 production and the Asc redox state establishes whether the H_2O_2 concentration rises to a level that can trigger stomatal closure. The diurnal increase in H_2O_2 during the afternoon is likely a result of photosynthetic-related processes, such as photorespiration and oxygen photoreduction (the Mehler peroxidase reaction), which serves to maintain electron flow through photosystem I and maintains its correct function. The Mehler reaction comprises the transfer of electrons from photosystem I to oxygen to form superoxides that SOD then disproportionates to O_2 and H_2O_2, the latter of which is reduced to water by APX using Asc as the reductant. Together, the Mehler reaction and the Asc-mediated reduction of H_2O_2 form the water-water cycle in which Asc is consumed to help protect against photoinhibition (Asada, 1999). ABA also can elicit H_2O_2 production as part of the signaling required to promote stomatal closure (Price et al., 1994; Pei et al., 2000; Murata et al., 2001; Schroeder et al., 2001a, 2001b; Zhang et al., 2001). Moreover, peroxisomes generate H_2O_2 as part of photorespiration and may contribute to the level of H_2O_2 present in guard cells. Interestingly, although APX activity could be detected in isolated guard cells, CAT expression or activity, which is normally used to detoxify peroxisome-generated H_2O_2, could not be detected (data not shown), suggesting that H_2O_2 may be detoxified in tobacco guard cells without substantial contribution by CAT. Consumption of Asc in detoxifying H_2O_2 generated by these processes as well as others produces DHA. Because DHAR is present in rate-limiting amounts, the diurnal increase in DHA during the afternoon may reflect a rate of H_2O_2 production and consumption of Asc that exceeds the ability of DHAR to efficiently regenerate Asc. Thus, under conditions of nonsaturating light (e.g., during early morning), the existing Asc redox state may be sufficient to scavenge a basal level of H_2O_2 production and prevent stomatal closure, whereas in excess light, the consumption of Asc by the water-water cycle, the inability of a rate-limiting amount of DHAR to regenerate Asc efficiently, and the resulting decrease in the Asc redox state may allow the concentration of H_2O_2 to increase to a level that signals stomatal closure. Therefore, a model integrating these observations can be proposed in which stomatal closure is triggered when conditions of excess light and the accompanying elevated production of H_2O_2 lower the Asc redox state to a level that it can no longer maintain a low concentration of H_2O_2. Overexpression of DHAR provides a larger reservoir of Asc and more efficient regeneration of Asc that can scavenge higher levels of H_2O_2, thereby preventing its accumulation to a level sufficient to trigger stomatal closure. This would explain the greater open stomatal area, increased stomatal conductance, higher transpiration rate, higher rate of water loss, decreased tolerance to water stress, and reduced guard cell responsiveness to ABA and H_2O_2 signaling that are observed in DHAR-overexpressing plants. Conversely, reducing DHAR expression below wild-type levels reduces the Asc redox state to a level similar to that observed in control plants during the afternoon. The less efficient regeneration of Asc would lead to an elevated accumulation of H_2O_2, which in turn would trigger a greater degree of stomatal closure even under nonstress conditions. Therefore, the guard cell Asc redox state would be expected to control stomatal movement through modulating H_2O_2 signaling.

METHODS

DNA Constructs, Plant Transformation, and Protein Gel Blot Analysis

Full-length wheat (*Triticum aestivum*) and tobacco (*Nicotiana tabacum*) DHAR cDNAs (accession numbers AY074784 and AY074787, respectively) were isolated as described previously (Chen et al., 2003). Transgenic tobacco (*N. tabacum* cv Xanthi) expressing the His-tagged wheat DHAR from the CaMV 35S promoter (in the binary vector pBI101) was generated using *Agrobacterium tumefaciens* as described (Chen et al., 2003). Transgenic tobacco plants suppressed for DHAR were identified after the introduction of a tobacco DHAR construct in pBI101. Plants were grown in a greenhouse supplied with charcoal-filtered air and were used when 13 to 15 weeks old.

Anti-DHAR antiserum raised against DHAR purified from wheat seedlings was used for protein gel blot analysis. Protein extracts were resolved using standard SDS-PAGE, and the protein was transferred to a 0.22-μm nitrocellulose membrane by electroblotting. After transfer, the nitrocellulose membranes were blocked in 5% milk and 0.01% thimerosal in TPBS (0.1% Tween 20, 13.7 mM NaCl, 0.27 mM KCl, 1 mM Na_2HPO_4, and 0.14 mM KH_2PO_4) followed by incubation with primary antibodies diluted typically 1:1000 to 1:2000 in TPBS with 1% milk for 1.5 h. The blots were then washed twice with TPBS and incubated with goat anti-rabbit horseradish peroxidase–conjugated antibodies (Southern Biotechnology Associates, Birmingham, AL) diluted to 1:5000 to 1:10,000 for 1 h. The blots were washed twice with TPBS, and the signal was detected typically between 1 to 15 min using chemiluminescence (Amersham, Buckinghamshire, UK).

Plant Growth Conditions

All plants were grown in 5-gallon pots with commercial soil in a glasshouse supplied with charcoal-filtered air and were not pot bound for the experiments. Experiments were performed and repeated from November through the winter to avoid excessive heat or light. Plants were watered to saturation twice a day (7 AM and 1 PM) to ensure that the soil was never dry. Plants were grown under natural light conditions in a 10-h-light and 14-h-dark cycle. The average temperature during the day was 25.9 ±

0.6°C and during the night was 20.2 ± 0.5°C. The average light intensity in the morning (9 AM) was 514 ± 206 μmol m^{-2} s^{-1} and in the afternoon (1 PM) was 1191 ± 244 μmol m^{-2} s^{-1}. Plants used in the experiments had produced ~20 leaves and had not yet produced an inflorescence.

Enzyme Assays

DHAR activity was assayed essentially as described (Hossain and Asada, 1984). Tobacco leaves were ground in extraction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 2 mM EDTA, and 1 mM $MgCl_2$), and soluble protein was obtained after a 5-min centrifugation at 13,000 rpm. DHAR was assayed from an equal amount of protein as described (Bradford, 1976) in 50 mM K_2HPO_4/KH_2PO_4, pH 6.5, 0.5 mM DHA, and 1 mM GSH and its activity followed by an increase in absorbance at 265 nm. MDHAR (10^{-8} mol NAPDH oxidized/min/mg protein), SOD (units to inhibit nitroblue tetrazolium photoreduction by 50%), CAT (10^{-6} mol H_2O_2 reduced/min/mg protein), GR (10^{-8} mol NAPDH oxidized/min/mg protein), and APX (10^{-8} mol Asc oxidized/min/mg protein) activities were determined as described (Gainnopolitis and Pies, 1977; Aebi, 1984; de Pinto et al., 2000). Ascorbate oxidase activity present in apoplastic wash fluid (obtained as described below) was determined from the decrease in A_{265} (extinction coefficient of 14 $mM^{-1}cm^{-1}$) at 25°C in a reaction mixture containing 0.1 M sodium phosphate, pH 5.6, 0.5 mM EDTA, and 100 μM Asc as described (Pignocchi et al., 2003).

Guard Cell Isolation

Guard cells and guard cell protoplasts were isolated essentially as described (Kruse et al., 1989). Pieces collected from expanding leaves of 8-week-old plants were transferred to a blender jar in 100 mL of homogenization buffer (10% Ficoll, 5 mM $CaCl_2$, and 0.1% polyvinylpyrrolidone 40) and homogenized at high speed for 1 to 2 min. Epidermal fragments were collected using a nylon mesh (220 μm) and rinsed thoroughly with water. To isolate guard cell protoplasts, the epidermal fragments were incubated in 40 mL of digestion solution (0.25 M D-mannitol, 0.7% cellulysin [Behring Diagnostics, La Jolla, CA], 1 mM $CaCl_2$, 0.1% polyvinylpyrrolidone 40, and 1 μg/mL of pepstatin A, pH 5.5) with shaking for 45 min, collected on nylon mesh, rinsed with 0.25 M mannitol and 1 mM $CaCl_2$, and incubated in fresh enzyme solution for an additional 45 min. Guard cell protoplasts were then released within 3 h in a 50-mL solution of 0.35 M mannitol, 1 mM $CaCl_2$, 1% cellulase Onozuka R-10 (Yakult Honsha, Tokyo, Japan), 0.01% pectolyase Y-23 (Seishin Pharmaceutical, Tokyo, Japan), and 1 μg/mL of pepstatin A, pH 5.5. Epidermal debris were removed using nylon mesh (30 μm), and the protoplasts were collected by centrifugation at 60*g* for 7 min and were washed three times with 0.35 M mannitol and 1 mM $CaCl_2$.

Asc and DHA Measurements

Asc was measured as described (Foyer et al., 1983). Apoplastic levels were obtained essentially as described (Pignocchi et al., 2003) in which leaves from 8-week-old tobacco were collected, the midvein removed, and 2-cm^2 pieces vacuum infiltrated at −70 kPa with chilled 10 mM citrate, pH 3.0, for 10 min. Leaves were blotted dry, rolled, and centrifuged in a prechilled syringe at 2000 rpm for 10 min at 4°C. An equal volume of 2.5 M $HClO_4$ (for the ascorbate assay) or 10% metaphoric acid (for the GSH assay) was added to the apoplastic wash fluid. Guard cells were isolated as described (Kruse et al., 1989). For whole leaf assays, leaves were ground in 2.5 M $HClO_4$ and centrifuged at 13,000 rpm for 10 min. Two volumes of 1.25 M Na_2CO_3 were added to the supernatant, and after centrifugation, 100 μL was added to 895 μL 100 mM K_2HPO_4/KH_2PO_4, pH 5.6. Asc was determined by the change in absorbence at 265 nm after the addition of 0.25 units of ascorbate oxidase. The total amount of reduced and oxidized ascorbic acid (i.e., Asc and DHA) was determined by reducing DHA to Asc (in a reaction containing 100 mM K_2HPO_4/KH_2PO_4, pH 6.5, 2 mM GSH, and 0.1 μg recombinant wheat DHAR protein incubated at 25°C for 20 min) before measuring Asc. The amount of DHA was determined as the difference between these two assays. The level of GSH and GSSG was determined as described (Shimaoka et al., 2000).

Determination of Leaf Water Potential and Leaf Water Content

Leaf water potential was determined at 10 AM and 2 PM using a pressure chamber (Model 3000; Soilmoisture Equipment, Santa Barbara, CA) as described by Gómez-Cadenas et al. (1996). Data are presented as the average and standard deviation of six replicate leaves. Leaf water content was measured at 10 AM and 2 PM. Leaf fresh weight was measured immediately, and their water saturated weight was determined after allowing the leaf to absorb water until saturation. Leaf dry weight was measured after drying at 80°C. The difference in water weight between fresh and water-saturated leaves was then calculated in the morning and afternoon and expressed on a leaf dry weight basis. Data are presented as the average and standard deviation of six replicate leaves.

Stomatal Measurements

Stomatal bioassay experiments were performed as described (Pei et al., 1997). Abaxial epidermis strips were loaded onto glass slides with 100 μL of buffer A (50 mM KCl and 10 mM Mes, pH 6.1). After staining with 0.2% toluidine blue O for 20 s, the sample was rinsed twice with distilled water and imaged using a compound microscope (Leica, Wetzlar, Germany). For each sample, the percentage of stomata that were open (defined as having a width >1 μm) was determined from at least 400 stomata. The width and length of the opening (i.e., aperture) of only those stomata that were open were measured and used to calculate the average stomatal aperture (width/length). The width and length of at least 30 stomatal pores were measured. The width and length of open stomatal apertures also were used to calculate the stomatal aperture area [$\pi \times$ (width/2) $\times$ (length/2)], which together with the percentage of stomata that remained open, were used to calculate the total open stomatal area per unit leaf area containing 100 stomata [i.e., (average stomatal aperture area) $\times$ (percentage of stomata that remained open) $\times$ 100].

Induction of stomatal closure by ABA and H_2O_2 was investigated using epidermal strips that were first incubated in CO_2-free buffer A for 2 h at 22 to 25°C under a photon flux density of 200 μmol m^{-2} s^{-}) to promote stomatal opening. ABA (50 μM final concentration, dissolved in 95% ethanol, with equal volume of ethanol used as a control) or H_2O_2 (1 mM final concentration) was added to the buffer. After the indicated time, samples were stained with toluidine blue O for image recording or loaded with fluorescence dye to determine the production of H_2O_2.

Superoxide and H_2O_2 Measurements

Superoxide was assayed as described (Sutherland and Learmonth, 1997). Leaves were abraded with sandpaper, and 20 to 30 0.5-cm-diameter discs were shaken in 5 mL 10 mM citrate buffer, pH 6.0, to which 50 μM of the tetrazolium dye, sodium, 3′-[1-[phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT), was added. The reduction of XTT in 1 mL of the solution was recorded every 5 min at 470 nm, and the amount of superoxide was determined from the rate of XTT reduction.

Leaf discs (~100 mg) were ground in liquid nitrogen, extracted in 150 μL of 25 mM HCl, and centrifuged at 5000*g* for 5 min at 4°C. Pigments

were removed by vortexing in the presence of activated charcoal and centrifugation at 12,000*g* for 15 min. H_2O_2 was determined by measuring relative fluorescence (excitation at 315 nm, emission at 425 nm) in a reaction containing Hepes-NaOH, pH 7.5, 20 μL of extract, 500 μM homovanillic acid, and 0.5 unit horseradish peroxidase against a standard curve as described (Creissen et al., 1999).

H_2O_2 production from guard cells was examined by loading epidermal peels with H_2DCF-DA as described (Lee et al., 1999). To reduce variation, epidermal strips from control, DHAR overexpressors, and DHAR-suppressed leaves were placed on a single microscope slide loaded with 200 μL of 20 μM H_2DCF-DA for 5 min. After washing with buffer A, dye emission (excitation 465 to 495 nm, emission 510 to 530 nm) from at least 70 guard cells was recorded using an Insight confocal microscope (Kent, WA). Fluorescence intensity, measured as the pixel intensity present specifically within a defined area encompassing a guard cell, was obtained using MCID Elite image software (version 7.0; Imaging Research, St. Catharines, Ontario, Canada). A minimum of 70 guard cell pairs was measured. Representative background pixel intensity was obtained from the average of 10 measurements of similarly sized areas adjacent to the guard cells. The average background value was then subtracted from the average guard cell value, and the difference was presented as the fluorescence intensity of guard cell.

RT-PCR Analysis

Total nucleic acid was isolated from whole leaves and guard cell protoplasts, and the DNA was removed using RQ1 RNase free DNase I (Promega, Madison, WI). The absence of DNA in the samples was confirmed after saturating PCR (38 cycles) with actin-specific primers. One microgram of total RNA was used for cDNA synthesis using Omniscript RT (Qiagen, Valencia, CA) with oligo$(dT)_{20}$ as the primer. PCR reactions contained 1× buffer, 2 μL of the reverse transcription reaction, and 1.5 μg of forward and reverse primers in a total reaction volume of 25 μL using the following conditions: 94°C/5 min; 94°C/50 s, annealing temperature (see below)/50 s; 72°C/1 min, 72°C/5 min. PCR products were visualized on ethidium bromide–stained 1.2% agarose gels.

Primers used were as follows: actin (X63603) (annealing temperature, 52°C), forward, 5′-CGCGAAAAGATGACTCAAATC-3′ and reverse, 5′-AGATCCTTTCTGATATCCACG-3′; CAT (U93244) (annealing temperature, 55°C), forward, 5′-CGGATACCTGAGCGTGTTGTTCATG-3′ and reverse, 5′-GTGATTATTGTGATGAGCACAC-3′; MDHAR (BQ842867) (annealing temperature, 55°C), forward, 5′-ACTTCAAATAGCCGTTTTTAATCCA-3′ and reverse, 5′-AGTTGAACATGTTGATCATTCTC-3′; FeSOD (M55090) (annealing temperature, 53°C), forward, 5′-TGCTTTGGAGCCTCATATGAG-3′ and reverse, 5′-AAGTCCAGATAGTAAGCATGC-3′; tobacco DHAR (AY074787) (annealing temperature, 55°C), forward, 5′-AATTGGATCCCTGATTCTGATGT-3′ and reverse, 5′-GCGAAACAACGGGATTATAATTATG-3′; wheat DHAR (AY074784) (annealing temperature, 59°C), forward, 5′-AATTGGATCCCTGATTCTGATGT-3′ and reverse, 5′-GGATCCAGGGGCTTACGGGTTCACTTTC-3′; to detect wheat and tobacco DHAR (annealing temperature, 59°C), forward, 5′-AATTGGATCCCTGATTCTGATGT-3′ and reverse, 5′-AGATGGTA(G/C)AG(C/T)TTCGGAGCCA-3′.

Leaf Water Loss Assay

Expanded leaves were detached from well-watered plants and immediately weighed. Water loss from the detached leaves held at room temperature was followed by determining leaf weight every 5 min for 40 min. Four leaves from separate plants were used. The rate of water loss was plotted as the loss in leaf weight over time. In situ rates of CO_2 assimilation, transpiration, and stomatal conductance were measured with the TPS-1 portable photosynthesis system (PP Systems, Haverhill, MA). Every other leaf on a plant was measured.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY074784 and AY074787.

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