Analysis of Guard Cell Viability and Action in Senescing Leaves of *Nicotiana glauca* (Graham), Tree Tobacco

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ABSTRACT

In an attempt to determine whether low epidermal conductances to water vapor diffusion of senescing leaves were caused by internal changes in guard cells or by factors external to guard cells, stomatal behavior was examined in intact senescing and nonsenescent leaves of *Nicotiana glauca* (Graham), tree tobacco, grown in the field or in an environmental chamber. Conductances of senescing leaves were 5 to 10% of the maximum conductances of nonsenescent leaves of the same plant, yet guard cell duplexes isolated from epidermal peels of senescing leaves developed full turgor in the light in solutions containing KCl and sodium cobaltinitrite staining showed that K⁺ accumulated as turgor developed. Ninety-five per cent of the guard cells isolated from senescing leaves concentrated neutral red and excluded trypan blue. Intercellular leaf CO₂ concentrations of senescing and nonsenescent leaves of chamber-grown inorganic plants were not significantly different (about 240 micromolars per liter), but the potassium contents of adaxial and abaxial epidermes of senescing leaves taken from plants grown in the field were less than half those of nonsenescent leaves. We conclude that guard cells do not undergo the orderly senescence process that characteristically takes place in mesophyll tissue during whole-leaf senescence and that the reduced conductances of senescing leaves are produced by factors external to guard cells.

Leaf senescence may occur as a genetically determined stage of plant development, or it may be induced by a variety of environmental conditions including drought, photoperiod, temperature, or shading. Regardless of how leaf senescence is induced, it is characterized by the orderly, progressive disassembly of mesophyll tissue. In the process, leaf proteins and Chl are catabolized, and the released nitrogen and other elements are mobilized to juvenile tissues of the plant before leaf death and abscission (17).

Stomates of senescing leaves generally open significantly less than those of nonsenescent leaves (4, 11, 13, 20), but there is no evidence that guard cells ever undergo an orderly senescence process like that carried out in mesophyll cells. Guard cells of senescing leaves of *Vicia faba* are capable of responding to changes in CO₂ concentration and to kinetin (21). Guard cells in epidermal peels from senescing leaves of *Gingko biloba* (24) and *V. faba* (21) are capable of developing a certain amount of turgor, but not full turgor, when illuminated in solutions containing KCl. Chloroplasts in guard cells of senescing leaves of *G. biloba* are green and show typical fluorescence transitions associated with electron transport and photophosphorylation at a time when chloroplasts in mesophyll cells of the same leaf do not exhibit signs of functionality (24). As a result, Zeiger and Schwartz (24) have suggested that yellowing leaves retain stomatal control throughout the senescence process.

These studies raise the question of how stomatal movements are controlled in senescing leaves. Stomates of senescing leaves may fail to open fully because properties of guard cells that facilitate turgor production are damaged or modified. Alternatively, stomatal movements in senescing leaves might be controlled solely by factors external to guard cells.

In this paper, we examine the diurnal patterns of transpiration and epidermal conductance, and the water relations of senescing and nonsenescent leaves of *Nicotiana glauca*, tree tobacco, growing in the field. We show that even though stomates of intact senescing leaves do not respond to changing environmental conditions, guard cells isolated from all other tissues and cells of senescing leaves are capable of full turgor production. We conclude that the reduced epidermal conductances of senescing leaves are not produced by impaired processes within guard cells, but by factors external to them.

MATERIALS AND METHODS

Plant Material. A single plant of *Nicotiana glauca* (Graham), tree tobacco, was used for measurements of diurnal epidermal conductance to water vapor diffusion, pressure-volume curve analysis, measurements of leaf water potential, and epidermal peel and impression analyses. The plant was approximately 3 m high and was growing on a western slope on the campus of Pepperdine University, Malibu, CA. Samples for K⁺ analysis were collected at random from plants at two roadside locations 6 and 9 km east of the campus along Malibu Canyon Road. The soil in which all plants were growing was highly compacted, fragmented sandstone.

Plants for gas exchange analysis were grown from seed collected from the plant growing on the campus. Plants were germinated and maintained in sterilized Uni-Gro potting soil (L & L Nursery Supply, Inc., Chino, CA). Following germination, plants were transferred to 8-L clay pots and were watered daily with modified Hoagland nutrient solution. Distilled H₂O was used in place of Hoagland solution for every third watering in order to purge the soil of salts. Plants were maintained in a 1.8 × 1.2 × 0.8 m growth chamber (Scientific Systems, New Orleans, LA) with a 12-h photoperiod, 25°C day, 20°C night air temperatures, and 18.7°C day, 12.7°C night dewpoint temperatures. During the light period, the concentration of CO₂ in the chamber was maintained at 340 ± 2 μL/L using and ADC Series 225 differential IR gas analyzer (Analytical Development Co., Hoddesdon, England) connected to an electronic injection valve (Leeds and Northrup, North Wales, PA) that controlled the release of compressed CO₂ from a cylinder. The chamber was equipped with six 400-w mercury multivapor metal halide lamps and six 400-w high pressure sodium lamps (General Electric, Co., Cleveland, OH). The photosynthetic photon flux density averaged 950

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Diurnal Measurements of Transpiration and Epidermal Conductance to Water Vapor Diffusion. Transpiration, epidermal conductance to water vapor diffusion, vapor pressure deficits, leaf temperatures, and photosynthetic photon flux densities were measured for both adaxial and abaxial surfaces of seven senescing and seven nonsenescing leaves of the field plant using a Li-Cor 1600 porometer. Leaves were considered to be senescing if they showed marked chlorosis compared to nonsenescing green leaves. The degree of chlorosis of senescing leaves was estimated visually and varied from 65 to 90% of the total leaf surface area. Significant decreases in Chl content from morning to late evening were clearly observed for some leaves. Selected leaves were of consistent size (8–10 × 10–12 cm) and free of canopy counter-shading. Measurements were taken at 1- and 2-h intervals between 0400 and 2000 h on July 13, 1983.

Measurements of Bulk Leaf Tissue Water Potential and Pressure-Volume Curve Analysis. Bulk leaf water potentials were measured every 2 h with a Scholander pressure chamber (16) on three senescing and three nonsenescing leaves while diurnal conductance measurements were in progress. At the end of the day, stems of the senescing and nonsenescing leaves were cut under water, covered with a plastic bag, and stored overnight in the dark. Three senescing and three nonsenescing leaves were subjected to pressure-volume curve analysis. Data were analyzed by the method of Wilson et al. (22) to determine the osmotic potentials and turgor loss points of the bulk leaf tissue of samples of each leaf type taken at the end of the day. We did not attempt to account for diurnal adjustments in the turgor loss points of the two leaf types, but such adjustments have been shown to be small (0.1–0.2 MPa) (19).

Estimation of Stomatal Densities. Stomatal densities of adaxial and abaxial surfaces of leaves used for conductance measurements were estimated using a modification of the method of Brown and Rosenberg (2). Leaves were sprayed with Krylon Crystal Clear 1301 acrylic spray coating (Borden, Inc., Columbus, OH), and impressions were lifted from leaf surfaces with Highland brand transparent tape No. 5910 (3M Co., Minneapolis, MN). Six estimates from the adaxial surface and six estimates from the abaxial impressions of the seven senescing and seven nonsenescing leaves were counted microscopically at ×100 using an eyepiece reticle containing 100 small squares and representing 1 mm² of the surface of the leaf.

Analysis of Viability and Turgor-Producing Capacity of Isolated Guard Cell Duplexes. Methods for isolation of guard cell duplexes and analysis of guard cell viability and turgor buildup have been described (6). Briefly, epidermal peels from adaxial or abaxial surfaces of senescing or nonsenescing leaves of plants growing in the field were treated with cellulolytic enzymes so that guard cell duplexes (defined as two guard cells joined at their ends) were the only remaining cellular structures. Guard cells were tested for viability with neutral red (9) and trypan blue (14). Only leaves that were completely yellow to the eye were considered senescent. Absorbances of 80% acetone extracts of such leaves were typically less than 15% of those of nonsenescing leaves when measured at 660 nm. To assess the ability of isolated guard cell duplexes to generate turgor, cleaned peels were subjected to light and dark treatments for 2 h in a solution of salts of Murashige and Skoog medium (12) modified by replacing K⁺ salts with Na⁺ salts followed by the addition of KCl to produce concentrations ranging from 0 to 50 mM. At the end of the incubation period, apertures of isolated guard cell duplexes were measured as described previously (6). All experiments were performed at 23°C in room air. In some experiments, strips were stained for potassium content with sodium cobaltinitrite stain (1).

Estimation of Net Photosynthesis, Total Leaf Conductance to Water Vapor Diffusion, and Concentration of CO₂ in the Intercellular Spaces of Senescing and Nonsenescing Leaves. Net photosynthesis, total leaf conductance to water vapor diffusion, and intercellular concentrations of CO₂ were measured for five senescing and five nonsenescing leaves at insertions one through five from the bottom of another-grown plants. Measurements of water vapor and CO₂ fluxes were performed simultaneously with a miniature, clamp-on assimilation chamber connected to an EG&G Cambridge model 911 dewpoint analyzer (EG&G, Waltham, MA) and an ADC series 225 IR CO₂ gas analyzer. The assimilation chamber and methods of data calculation have been described (3, 4). Total leaf conductances to water vapor diffusion were defined as the sum of the reciprocal values of the stomatal and cuticular resistances to water vapor for the two epidermises added in parallel. Boundary layer resistances were calculated from measurements on moistened Whatman No. 2 hardened filter paper clamped in the cup and subjected to a variety of flow rates. Flow rates were 350 ml/min. For measurements, the assimilation chamber was clamped to each leaf for 3 min in the light and 4 min in the dark. Conductance measurements taken with the porometer after the assimilation cup had been removed from the leaf confirmed that no changes in epidermal conductance occurred in that time.

Estimation of Potassium Contents of Senescing and Nonsenescing Leaf Tissues. Whole leaf tissue, and adaxial or abaxial epidermises of senescing and nonsenescing leaves collected from plants growing in the field were dried, weighed, ashed at 500°C overnight, and analyzed for K⁺ content. Sixteen samples of each type of tissue from both leaf types, each representing pooled material from several leaves, were prepared for atomic absorption analysis by the method of Isaac and Johnson (8) and analyzed using a Perkin-Elmer model 370A atomic absorption spectrophotometer.

RESULTS

Results of diurnal measurements of epidermal conductances to water vapor diffusion, transpiration, bulk leaf water potentials, leaf temperatures, and vapor pressure deficits for seven senescing and seven nonsenescing leaves of a Nicotiana glauca plant growing in the field are summarized in Figure 1. Epidermal conductances of nonsenescing leaves increased during the morning hours and reached a maximum value at 1100 (Fig. 1A). As leaf temperatures and vapor pressure deficits increased over the course of the afternoon (Fig. 1C), epidermal conductances of nonsenescing leaves decreased (Fig. 1A) and leaf water potentials approached the turgor loss point of the bulk leaf tissue, determined graphically from pressure-volume curves to be −1.05 ± 0.02 MPa (se, n = 3). Epidermal conductances of senescing leaves ranged in value between 5 and 10% of the maximum epidermal conductances of the corresponding surfaces of nonsenescing leaves (Fig. 1A) and in the afternoon hours. The basal part of a western slope, peak light levels were not reached until 1500 h (data not shown). Transpiration from adaxial surfaces of nonsenescing leaves was lower than from abaxial surfaces, but both peaked at 1500 h (Fig. 1B), coincident with highest light levels, highest leaf temperatures, and vapor pressure deficits (Fig. 1C). The leaf water potentials of both the bulk leaf tissue at saturation were calculated by extrapolation using linear regression of points along a pressure-volume curve
to be $-0.73 \pm 0.01$ MPa ($n = 3$) for senescing leaves and $-0.88 \pm 0.04$ MPa ($n = 3$) for nonsenescing leaves. Osmotic potentials of the two leaf types were significantly different when tested with a $t$-ratio test ($t = 5.53, P < 0.01$).

Stomatal densities (per mm$^2$) were $123 \pm 1.4$ (SE, $n = 42$) and $113 \pm 1.5$ (SE, $n = 42$) for the abaxial and adaxial surfaces of senescing leaves while the corresponding surfaces of nonsenescing leaves contained $120 \pm 1.2$ (SE, $n = 42$) and $112 \pm 1.5$ (SE, $n = 42$) stomates.

There were no apparent differences between cleaned epidermal peels of senescing and nonsenescing leaves when examined by light microscopy. Guard cell chloroplasts in cleaned peels from senescing leaves were green and appeared intact. Ninety to 95% of guard cells isolated from both leaf types concentrated neutral red and excluded trypan blue. Duplexes from both adaxial and abaxial surfaces of senescing and nonsenescing leaves became turgid upon illumination in solutions containing KCl. In all cases examined, guard cells accumulated K$^+$ as turgor developed, as judged by sodium cobaltinitrite staining. Photographs of duplexes given light and dark treatments and stained for K$^+$ have been published (6). Light and dark KCl response curves were virtually identical for cleaned strips from the adaxial surfaces of both types of leaves (Fig. 2A), except that dark responses were greater in 40 and 50 mM KCl for senescing than nonsenescing leaf duplexes. The responses of abaxial duplexes from both leaf types were similar but not identical (Fig. 1B). Maximum apertures achieved by duplexes isolated from abaxial surfaces of senescing leaves and given light treatment were about 90% of those isolated from nonsenescing leaves. However, abaxial duplexes from senescing leaves reached maximum apertures in 10 mM KCl in the light while those from nonsenescing leaves reached their maximum apertures at a concentration of 30 mM (Fig. 2B). The differences in the responses of duplexes isolated from senescing leaves and nonsenescing leaves may have been caused by differences in the permeabilities of membranes of the guard cells of the two types of leaves to ions, but we did not attempt to test this hypothesis.

Results of gas exchange analyses for senescing and nonsenescing leaves are summarized in Table I. Total leaf conductances to water vapor diffusion and net rates of photosynthesis of senescing leaves were 1 to 3% those of nonsenescing leaves. In some senescing leaves, Chl degradation was uniform and evenly distributed, while in others only a localized region of the leaf was chlorotic. Regardless of the distribution pattern, leaf conductances were reduced in all areas of the epidermis of senescing leaves, including those over green mesophyll. The concentrations of CO$_2$ in the intercellular spaces of the two leaf types were not significantly different.

Results of K$^+$ analyses of whole leaf tissue, adaxial epidermes, and abaxial epidermes of senescing and nonsenescing leaves are shown in Table II. The K$^+$ content of whole leaf tissue of

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**Table I. Net Photosynthesis, Total Leaf Conductance to Water Vapor Diffusion, and Intercellular Leaf CO$_2$ Concentrations for Five Senescing and Five Nonsenescing Leaves of Nicotiana glauca Grown in an Environmental Chamber**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Net Photosynthesis (μmol/m$^2$·s)</th>
<th>Total Leaf Conductance to Water Vapor (mmol/m$^2$·s)</th>
<th>Intercellular Concentration of CO$_2$ (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsenescing</td>
<td>11.3 (1.1)</td>
<td>784.2 (91.3)</td>
<td>239.2 (5.7)</td>
</tr>
<tr>
<td>Senescing</td>
<td>0.4 (0.3)*</td>
<td>7.7 (1.5)*</td>
<td>214.8 (52.1)</td>
</tr>
</tbody>
</table>

* Significant at 0.05 level when compared to nonsenescing by $t$ ratio.
Table II. Potassium Contents of Whole Leaf Tissue, Adaxial Epidermis, and Abaxial Epidermis of Sensing and Nonsensing Leaves of N. glauca

<table>
<thead>
<tr>
<th>Sample</th>
<th>Potassium mg/g dry wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsensing</td>
<td>Senescing</td>
</tr>
<tr>
<td>Whole leaf</td>
<td>12.23 (1.04)</td>
</tr>
<tr>
<td>Adaxial epidermis</td>
<td>16.05 (1.60)</td>
</tr>
<tr>
<td>Abaxial epidermis</td>
<td>14.75 (1.34)</td>
</tr>
</tbody>
</table>

* Significant at 0.05 level when compared to nonsensing by t ratio.

Senescing leaves were approximately 75% that of nonsensing leaves. The K⁺ contents of epidermes from senescing leaves were slightly less than half those from senescing leaves.

DISCUSSION

The stomates of senescing leaves of plants growing in the field do not open in response to light; those of nonsensing leaves do (Fig. 1). Yet guard cell duplexes isolated from senescing leaves of plants growing in the field respond to light by increasing pore apertures to values equal to those measured in duplexes isolated from nonsensing leaves. We conclude that the low epidermal conductances observed in senescing leaves of plants growing in the field are produced by factors external to guard cells themselves.

The results of our gas exchange analysis for senescing and nonsensing leaves are consistent with the hypotheses that epidermal conductance and photosynthesis are either coupled or in concert in both senescing and nonsensing leaves; hence, the high correlation between assimilation rates and total leaf conductances (Table I). Our data also support the hypotheses of Davis and McCree (4) and Wong et al. (23) that throughout the entire process of leaf aging, stomates respond in such a way that intercellular CO₂ concentrations remain constant. We find no evidence that stomates of senescing leaves fail to open because of a rise in intercellular concentrations of CO₂ resulting from declining photosynthesis in the mesophyll. On the contrary, identical intercellular CO₂ concentrations in senescing and nonsensing leaves indicate that intercellular CO₂ concentrations are the combined result of mesophyll photosynthetic rates and stomatal apertures.

Potassium is known to be necessary for guard cell turgor production in a number of species (7), and is mobilized during leaf senescence (17). The saturation osmotic potentials of senescing leaves were significantly less negative, and their K⁺ contents were significantly lower than those of nonsensing leaves. Such differences in osmotic potentials have been correlated with mobilization of nutrients from aging leaves (19), and lowered K⁺ contents have been suggested as a reason for the failure of stomates of senescing leaves to open (18). Nevertheless, the K⁺ level of senescing leaves is most likely sufficient to sustain the guard cell turgor necessary to regulate the small amounts of gas exchange between these leaves and the atmosphere, both in nutrient-limited soil (Table II) and in experiments in which plants are supplied with nutrients (10). Jordan et al. (10) showed that when chamber-grown cotton plants were watered with nutrient solutions, the stomatal conductances of leaves from the top to the bottom of plants decreased, but the K⁺ contents of all but the topmost leaves were identical.

Stomates of both senescing and nonsensing leaves respond to factors external to guard cells, but it is not clear whether guard cells of senescing leaves have the same sensitivities and responses to those factors as the guard cells of nonsensing leaves. Nor is it clear whether guard cells of senescing leaves have exactly the same metabolic and physiological properties as those of nonsensing leaves. To our knowledge, no studies have been published which separate the effects of internal metabolic and physiological changes that accompany guard cell aging from the effects of factors that develop external to guard cells and affect guard cell action as leaves approach senescence. It is clear from our studies and others (4) that gas exchange is regulated in both types of leaves so that their internal leaf CO₂ concentrations are the same. Whether gas exchange is regulated in senescing leaves for the same reasons that it is carefully controlled in nonsensing leaves remains to be determined (5, 15).

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