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11. Immediately after collection, the *p*H of the sample was measured and aliquots were separated for later analyses. Major metal cations were determined by atomic absoption spectrophotometry and major anions by ion chromatography. Both NH_4^+ and CH_2O were determined by standard colorimetric methods. Ultrex grade HNO_3 was added to preserve the trace metals; SO_3^{2-} was stabilized by reaction with CH_2O at *p*H 4.
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Longevity of Guard Cell Chloroplasts in Falling Leaves: Implication for Stomatal Function and Cellular Aging

Abstract. *Guard cell chloroplasts in senescing leaves from 12 species of perennial trees and three species of annual plants survived considerably longer than their mesophyll counterparts. In* Ginkgo biloba, *stomata from yellow leaves opened during the day and closed at night; guard cell chloroplasts from these leaves showed fluorescence transients associated with electron transport and photophosphorylation. These findings indicate that guard cell chloroplasts are highly conserved throughout the life-span of the leaf and that leaves retain stomatal control during senescence.*

Deciduous trees shed their leaves in autumn as an adaptive strategy for survival at low temperatures. Before leaf abscission, many compounds are catabolized and transported into perennial branches for further reutilization in the next growing season (*1*). Having a high nitrogen content, mesophyll chlorophyll is degraded and recycled; loss of chloroplast integrity is one of the earliest events in leaf senescence (*1, 2*). Because of chlorophyll degradation, leaves turn yellow and photosynthetic capacity declines as senescence progresses, with abscission occurring over a period of several days or even a few weeks, depending on the species and environmental conditions.

The fate of guard cell chloroplasts during leaf senescence is unknown (*1*). Chloroplasts are a highly conserved feature of guard cells, and guard cell chloroplasts are the only green plastids in the epidermis of the leaves of most higher plants (*3, 4*). Using fluorescence microscopy, we monitored chlorophyll degradation in senescing leaves of *Ginkgo biloba* and found that guard cell chloroplasts were brightly fluorescent in leaves where mesophyll fluorescence was no longer detectable (*5*).

We quantified the degradation of mesophyll and guard cell chloroplasts at different stages of senescence by measuring the chlorophyll fluorescence intensity at 685 nm. We found that chloroplast fluorescence in single guard cells remained stable throughout the senescing process, whereas mesophyll fluorescence declined steadily (Fig. 1). Furthermore, yellow leaves collected at the time of abscission (*6*) and kept in the laboratory in a humidified Plexiglas chamber under ambient light and at room temperature retained fluorescing guard cell chloroplasts for 10 days.

A survey of yellowing leaves of trees from several perennial species growing in the vicinity of the laboratory showed that prolonged survival of guard cell chloroplasts was common. Yellow leaves from *Citrus limonia* (lemon), *Diospyrus virginiana* (persimmon), *Populus* sp. (poplar), *Prunus armeniaca* (apricot), *P. cerasifera* (cherry plum), *P. domestica* (plum), *P. glandulosa* (almond), *P. persica* (peach), *P. persica nucipersica* (nectarine), *Punica granatum* (pomegranate), and *Quercus* sp. (oak) exhibited fluorescing guard cell chloroplasts whereas the chloroplasts of mesophyll cells had lost all or most of their fluorescence. Furthermore, observations of old, senescing leaves in growing plants from *Commelina communis* (dayflower), *Vicia faba* (faba bean), and *Phaseolus vulgaris* (bean) showed that guard cell chloroplasts in these annuals also retained their fluorescence much longer than their mesophyll counterparts. Thus, the extended longevity of guard cell chloroplasts appears to be a widespread phenomenon in senescing leaves from both annual and perennial plants.

In isolated mesophyll chloroplasts, chlorophyll fluorescence intensity and electron transport rates decline steadily with age (*7*). The stability of fluorescence levels in guard cell chloroplasts from yellow leaves thus constitutes evidence of their integrity. Their functional competence was further ascertained by measurements of chlorophyll *a* slow fluorescence transients, which are correlated with electron transport and photophosphorylation (*8, 9*).

Segments from green or yellow leaves from *G. biloba* were viewed on a microscope slide under low magnification and dark-adapted for several minutes. At time zero, the segments were illuminated with blue light from a mercury lamp of an epifluorescence illuminator, interfaced with an electronic shutter. Fluorescence emission at 685 nm was measured with a microspectrophotometer (*10*). The voltage output from the microspectrophotometer was fed into a microcomputer, sampling every 100 msec. Sampling and opening and closing of the shutter were under software control. The amount of actinic light provided by a ×4 objective

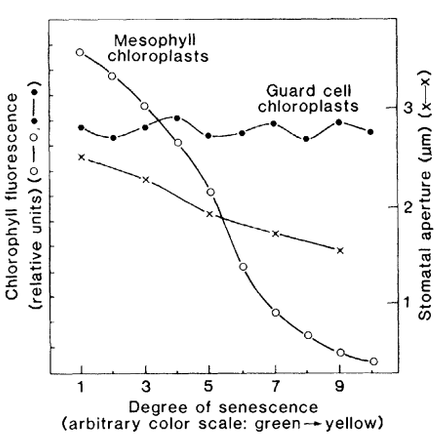

Fig. 1. Chlorophyll fluorescence and stomatal apertures in senescing leaves of *Ginkgo biloba*. Yellow leaves from trees growing near the laboratory were ranked visually by color, and mesophyll and guard cell chlorophyll fluorescence was measured in a Nonospec 10 microfluorospectrophotometer mounted on an American Optical optical microscope. Actinic light was supplied by a 50-W mercury lamp filtered with a BG12 exciting filter, a OG515 barrier filter, and a 500 nm dichroic beam splitter. Relative fluorescence intensity at 685 nm was recorded from the digital readout of the microspectrophotometer within seconds after the opening of an electronic shutter blocking the light path. Mesophyll fluorescence was measured in the adaxial side of the leaf with a ×20 objective. Fluorescence from guard cell chloroplasts was measured in single guard cells from epidermal peels, at ×400 magnification. Each point is the average of 18 readings in three different leaves. Stomatal apertures were measured microscopically with an ocular micrometer in peels made a few minutes after gathering the leaves at midmorning (light intensity, 1.2 to 1.4 mmole m^{-2} sec^{-1}). Each point is the average of 90 measurements; standard errors were less than 20 percent of the means.

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gave the most reproducible transients with minimal photobleaching.

Green leaves of *G. biloba*, illuminated at their adaxial surface, showed typical OPSMT transitions (Fig. 2A) which are associated with electron transport and photophosphorylation (*8, 9*). The abaxial side of yellow leaves, the only leaf surface with stomata in *Ginkgo*, exhibited a single-peak transient (Fig. 2B), similar to the one reported for guard cell chloroplasts from *Chlorophytum* (*9*). Although not as well resolved as the transients reported by Melis and Zeiger (*9*) who used different methods, transients from guard cell chloroplasts measured with a microspectrophotometer show an initial rise and a slow decay, which are indicative of electron transport and photophosphorylation. Restoration of 60 to 80 percent of the transients upon dark readaptation ruled out the possibility of an artifact due to photobleaching; the lack of transients from the adaxial surfaces from yellow leaves, which are devoid of stomata, demonstrated the absence of interference from mesophyll chloroplasts (Fig. 2C). The presence of intact guard cell chloroplasts in yellow leaves lacking contaminating mesophyll chloroplasts thus provides another experimental system for the characterization of in vivo fluorescence transients from guard cell chloroplasts in intact leaves. This analytical tool is proving advantageous for elucidating the role of these organelles in stomatal function (*9*).

Our results demonstrating a functional competence of guard cell chloroplasts from senescing leaves are in apparent contradiction with our observations showing that stomatal apertures in yellowing leaves of *Ginkgo* are rather small (Fig. 1). Low stomatal conductances in old leaves have been documented (*11*), and it has been argued that aging guard cells become sluggish and lose their ability to open. We tested that hypothesis with epidermal peels from yellow and green leaves mounted in microchambers continuously perfused with 20 m*M* KCl (*10*) and illuminated with high-intensity (about 2 mmole m^{-2} sec^{-1}) white light, conditions which maximize stomatal apertures in epidermal peels (*10, 12*). After 90 minutes, stomatal apertures in peels from green leaves averaged 3.02 ± 0.42 μm (N = 90); those in peels from yellow leaves were 1.92 ± 0.27 μm (N = 90). Aperture values at the beginning of the treatment were 0.55 ± 0.2 and 0.40 ± 0.2 μm, respectively. A period of darkness after the light treatment caused stomatal closing, an indication that the opening seen in the light was reversible. These observations show that stomata from yellow leaves can open substantially and that the reduced opening seen in attached senescing leaves was not due to a loss of functional capacity of the guard cells but was most likely a result of regulatory control.

That the guard cells in senescing leaves are functional was further ascertained by measurements of stomatal conductance to water vapor, obtained with a Li-Cor null balance porometer. In bright sunlight (1.4 mmole m^{-2} sec^{-1}), attached yellow leaves from *Ginkgo* showed an average stomatal conductance of 0.16 ± 0.08 mm sec^{-1} (N = 13). After darkness stomatal conductance in the same leaf population was nil, an indication that the stomata were completely closed. Thus, in spite of their declining photosynthetic capacity, yellow leaves control stomatal function throughout the senescence process. These observations are consistent with the notion that stomatal guard cells play a role in the modulation of senescence (*2*); if that is the case, one would expect that guard cells would survive longer than other cell types in the aging leaf, as was indeed the case in this study.

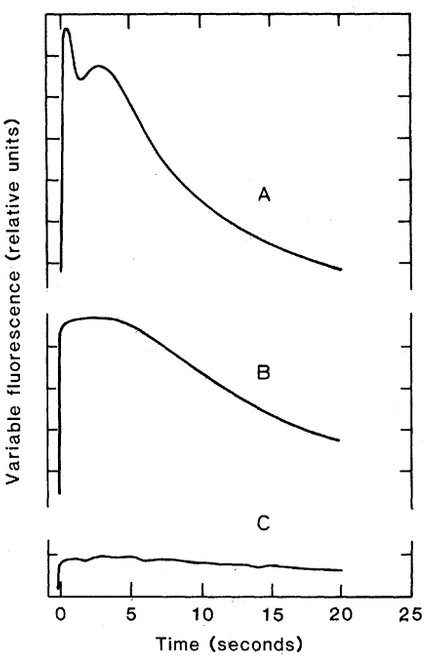

Fig. 2. Chlorophyll *a* fluorescence transients from green and yellow segments of leaves from *Ginkgo biloba*. (A) Typical OPSMT transition from an adaxial side of a green segment. (B) Typical transient from the abaxial surface of a yellow leaf, which had fluorescing guard cell chloroplasts and lacked mesophyll fluorescence. (C) Fluorescence transient from the adaxial surface of a yellow leaf, which is devoid of stomata. Segments on glass slides were placed on the stage of the microscope and focused under a ×4 objective. Illumination was as described in Fig. 1. After several minutes in the dark, fluorescence intensity at 685 nm was measured with a Nanospec 10 microspectrophotometer connected to a Northstar microcomputer through an analog-to-digital converter. Values were stored in floppy disks, and the output was transferred to a chart recorder after the readings were completed. The figures were traced from the chart records.

These results have implications for the understanding of stomatal function and cellular aging. In the absence of photosynthesis, a simple strategy for water conservation in senescing leaves could have been to keep the stomata closed throughout the senescence process. This study, however, indicates that the capacity for stomatal movements is retained until the time of abscission. Gas exchange could be required during senescence for the uptake of O_2, for the extrusion of volatile by-products of catabolism, and for the modulation of turgor in connection with leaf shedding.

The striking conservation of guard cell chloroplasts, contrasting with complete chlorophyll degradation in the mesophyll, is also of interest. The extended longevity of guard cell chloroplasts indicates that these organelles are conserved in time as well as in space (*4*); this finding strengthens the notion of a central role of guard cell chloroplasts in stomatal function. The demonstration of fluorescence transients in guard cell chloroplasts from yellow *Ginkgo* leaves provides further evidence for a role of these organelles in the supply of high-energy equivalents needed to drive ion transport during stomatal opening in daylight (*4, 9, 13*). In senescing leaves lacking a functional mesophyll, guard cell chloroplasts would constitute the only site of photosynthesis.

Investigations on the mechanisms ensuring chloroplast survival in guard cells from leaves actively degrading mesophyll chloroplasts should also be valuable. Since chloroplast degradation appears to be under nuclear and hormonal control (*1*), comparative studies of the metabolism of mesophyll and guard cells during senescence could further our understanding of cellular aging.

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Sensitivity to Carcinogenesis in Nude Mice: Skin Tumors Caused by Transplacental Exposure to Ethylnitrosourea

Abstract. *Female athymic nude mice and their phenotypically normal littermates were exposed transplacentally to ethylnitrosourea. Skin tumors (papillomas and sebaceous adenomas) developed on the nude mice with an almost tenfold greater incidence than on their haired littermates. Skin tumors were also induced on nude mice but not haired controls by direct intraperitoneal treatment with ethylnitrosourea. These results indicate that nude mice have higher than normal susceptibility to carcinogenesis under some circumstances.*

The athymic nude (*nu/nu*) mouse provides a natural model for studying the role of thymus-dependent immune surveillance in tumorigenesis. Contrary to expectations, several carcinogenesis studies have not revealed that nude mice are more susceptible than phenotypically normal (*nu/+*) controls to a variety of spontaneous or chemically induced neoplasms, including epidermal and subcutaneous tumors (*1*, *2*). In the study reported here, BALB/c *nu/nu* mice and their *nu/+* littermates were exposed systemically (transplacentally or intraperitoneally) to the direct-acting alkylating carcinogen ethylnitrosourea (ENU). Transplacentally treated *nu/nu* mice developed significant, dose-dependent numbers of skin tumors; their *nu/+* littermates, by contrast, had a tenfold lower incidence of these tumors. Skin tumors also appeared on *nu/nu*, but not *nu/+*, mice treated intraperitoneally as adults with ENU. These results indicate that the nude mouse is more sensitive than normal to tumorigenesis in certain situations.

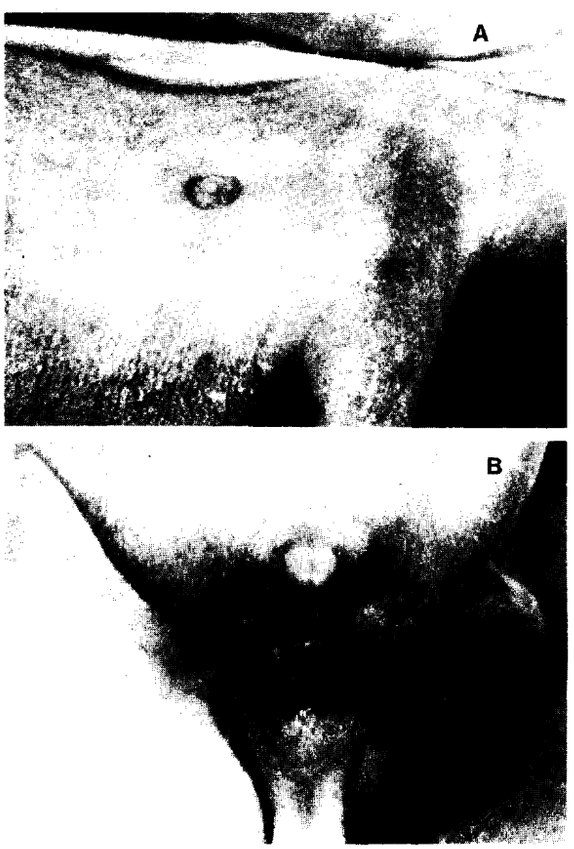

Fig. 1. (A) Sebaceous adenoma on nude mouse 12 months after transplacental exposure to ENU (50 mg/kg). (B) Papilloma 20 months after transplacental exposure to ENU (10 mg/kg).

The BALB/c nude mouse colony was derived from breeders purchased from Gibco Animal Resources Laboratory (Madison, Wisconsin). Skin transplantation experiments confirmed the congenicity of the *nu/nu* and *nu/+* mice. The colony was maintained free of pathogens (*3*) and there were no deaths due to infection. Ethylnitrosourea was synthesized (*4*) and stored at −80°C; solutions in trioctanoin were prepared within 1 hour of injection. On day 15 of gestation *nu/+* females impregnated by *nu/nu* males were injected with ENU (50 or 10 mg/kg) in the high lateral abdomen. In addition, 6-week-old *nu/nu* and *nu/+* females were given two intraperitoneal injections of ENU (50 mg/kg per injection) 1 week apart.

Only female progeny of the treated females were saved, since the responses of males and females were expected to be similar and since BALB/c males are difficult to house because of fighting. All mice were inspected daily and killed when moribund or at the end of the experiment. Most of the moribund animals had neoplasms of lung, lymphoid, or other tissues. At necropsy the skin of *nu/+* mice was searched for tumors by palpation, by wetting with alcohol, and by skinning the mice and examining the skin's lower surface. Since these searches revealed several small tumors comparable to the smallest tumors seen on the *nu/nu* mice, the method ensured discovery of most tumors on *nu/+* mice. The skin tumors were fixed in Bouin's solution, sectioned at 5-μm intervals, and stained with hematoxylin and eosin.

Of the 53 *nu/nu* mice exposed transplacentally to ENU at a dose of 50 mg/kg, 24 (45 percent) developed skin tumors, compared to only 3 of 58 *nu/+* littermates ($P < .01$) (Table 1). At the lower dose of 10 mg/kg fewer tumors appeared, but again the difference was significant ($P < .01$). Average age at death was similar for all mice and for skin tumor bearers in each treatment group, so differences in tumor incidences cannot be attributed to unequal survival times.

Of the 24 *nu/nu* females exposed intraperitoneally to a total of 100 mg of ENU per kilogram, four (17 percent) developed skin tumors (Table 1). No skin tumors were found on *nu/+* mice given

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