Light-Induced H+ Secretion and the Relation to Senescence of Oat Leaves

SHIMON GEPSTEIN
Department of Biology, Technion—Israel Institute of Technology, Haifa 32 000, Israel

ABSTRACT

When abraded oat (*Avena sativa* L. cv Victory) leaf segments are floated on KCl solution, white light causes acidification of the solution external to leaf tissue. The presence of mannitol amplifies the light-induced proton secretion. Mature leaves as well as young ones acidify the medium in light, while senescing leaves (after 3 to 4 days incubated in water in the dark) lose the ability to produce this response to light. The decrease in H+ secretion is already measureable after as little as 30 minutes in darkness, while the increase in proteolysis rate was detected only after 6 hours in dark. The decrease in capacity to secrete protons is one of the symptoms of leaf senescence. Moreover, fusicoccin mimics light in stimulating H+ pumping and delaying the senescence in the dark. On the other hand vanadate, an apparent inhibitor of plasma membrane H+ ATPase, blocks the acidification and promotes the chlorophyll and protein degradation in leaf segments during the 2-day period of incubation. These results, which show a parallel between cessation of H+ secretion and acceleration of senescence, may suggest a regulatory role for H+ secretion in leaf senescence.

According to the acid growth theory, HI+ secretion is a prerequisite for auxin-induced growth during the growth phase of coleoptiles and etiolated seedling stems (5). Green leaf cells which usually do not respond to auxin, expand in response to white light (3, 20). Recently, Van Volkenburgh and Cleland found that light-induced growth in bean leaves is accompanied by light-induced proton excretion (21). These authors suggest that leaf cell enlargement may occur by the same mechanism, as in coleoptiles, but that in this case proton excretion is induced by the light rather than by auxin (21).

Light is also very effective in regulating the last stage of the development of leaves, namely senescence. In the absence of light, the rates of Chl and protein loss (the dominant features of leaf senescence) are accelerated. However, when the leaf segments are placed in light the whole syndrome of senescence is delayed (17).

Evidence for the existence of a plasma membrane proton pump in providing driving force for ion transport and for the auxin function in cell wall loosening has been accumulated (16). However, the process of light-stimulated proton efflux in nonaquatic plants has so far been observed only in bean leaves and only during the cell enlargement stage of leaf development (21). The present report explores and characterizes the light-induced proton secretion by oat leaf segments. This paper will demonstrate that the cessation of H+ secretion is one of the earliest indicators of senescence. Moreover, the promoting effect of fusicoccin on the acidification in darkness and the concomitant delay of senescence might indicate a possible regulative role for the proton secretion during this last stage of development.

MATERIALS AND METHODS

Plant Material. Seeds of *Avena sativa* L. cv Victory were sown and grown on vermiculite for 7 d under continuous illumination (25 w/m²) at 25° ± 1°C. The apical 3-cm segments of the first leaves were used either for Chl and α-amino nitrogen determination (7) or for the measurements of proton secretion.

Measurements of Proton Secretion. The leaf segments were cut into 0.5-cm pieces after being scrubbed by aluminum oxide abrasive (Buchler, Evanston, IL) in order to reduce the effect of the cuticle as a barrier to proton transport (5). Sixty 0.5-cm segments (2.0 g) were incubated in 3 ml solution containing 250 mM mannitol and 1 mM KCl and were shaken on a shaker. The light intensity usually was 20 w/m², and the vials which were to receive a dark treatment were wrapped in aluminum foil. The secretion of protons from leaf segments was measured in the solution external to the leaf tissue by two methods. The first was based on measurements of changes in the media pH. A combination pH electrode connected to a PHM 84 pH meter (Radiometer) was lowered into the solution, and the pH values were determined at frequent intervals or monitored continuously on a chart recorder. The initial pH was adjusted with 10 mM NaOH to pH 6.2 to 6.7.

The second method of monitoring net H+ efflux was based on measuring the amount of hydroxyl ions needed to neutralize the protons released. These measurements were carried out by a pH stat apparatus which was composed of a titrator, autoburet, pH meter, and recorder (Radiometer, Copenhagen). Five g of 0.5-cm abraded leaf segments were incubated in 10 ml of 250 mM mannitol and 1 mM KCl at pH 6.0. The pH of the medium was kept constant by titration with 1 mM NaOH. Measurements were made under light 20 w/m² or in dark conditions at 25° ± 1°C.

RESULTS

Effect of Light on H+ Secretion in Oat Leaf Segments—Enhancement by Mannitol. The promotive effect of white light on the acidification of the external solution by leaf segments is shown in Figure 1. Light causes the pH of the medium to drop 0.2 to 0.4 units, while no change can be observed in darkness. However, mannitol, when present in the medium, markedly enhances the light-induced H+ secretion. The maximal degree of this stimulation for a 3-h experiment is caused by 0.25 to 0.5 M mannitol. The same effect could be detected by inclusion of PEG at comparable osmolarity (data not shown). In the presence of 0.25 M mannitol, equilibrium is attained around pH 5.8 to 6.0 in the dark, while upon illumination the pH of the external solution drops and equilibrates around pH 5.0 to 5.2. All the subsequent treatments to be reported here include 0.25 M mannitol in order to amplify the light-stimulated H+ secretion. However, even in the absence of mannitol, the basic effect is significant and reproducible.

Kinetics of H+ Secretion by Oat Leaf Segments. Continuous measurements of H+ secretion as indicated by pH changes of the solution external to leaf tissue show an initial alkalinization upon
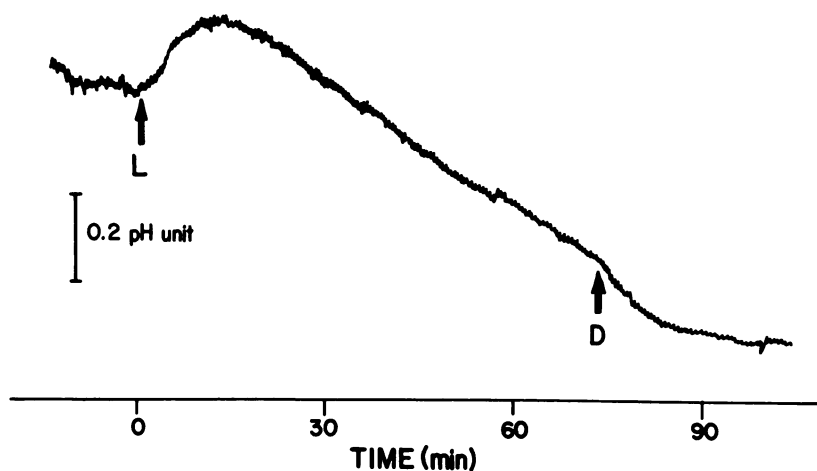
FIG. 2. Tracing of the pH change of the external solution under illumination (L) and after turning off the light (D). The arrows indicate the dark to light (L) and light to dark (D) transition. The starting pH was 6.2.

transition from dark to light followed by a constant acidification (Fig. 2). Removal of the light stimulated a quick acidification for approximately 5 min, and then further acidification was completely inhibited. The initial rise and the drop, upon turning on and off the light, respectively, can be attributed to photosynthetic CO_2 uptake in light and respiratory release in the dark. Bubbling the medium with CO_2 free air causes an immediate alkalinization even in the dark to an extent comparable to the initial pH rise in the light. However, this treatment does not change the acidification rate either in the dark or in the light (data not shown). These results ruled out the possibility that light stimulated acidification via hydration of CO_2.

Measurements of proton efflux with the pH stat apparatus (Fig. 3) are consistent with the pH changes of the external solution. The initial alkalinization is not monitored since the pH stat was adjusted only for acidification measurement in those experiments. However, the constant acidification in light and the transient drop of pH upon removal of the light followed by cessation of H⁺ efflux parallels the pattern of the pH changes of the medium.

Effect of Light Intensities and DCMU on H⁺ Secretion. Tables I and II show the results of experiments designed to test whether the light-stimulated acidification of the external solution is dependent on photosynthesis. It can be seen from Table I that the maximum rate of acidification occurs at low light intensities of 20 w/m². Since the rates of acidification are identical at both light intensities (20 and 200 w/m²), the process is evidently saturated at these light intensities, which are considerably below the saturation values of photosynthesis. Table II shows the effect of DCMU on light-induced H⁺ secretion. In the presence of 10^{-6} M
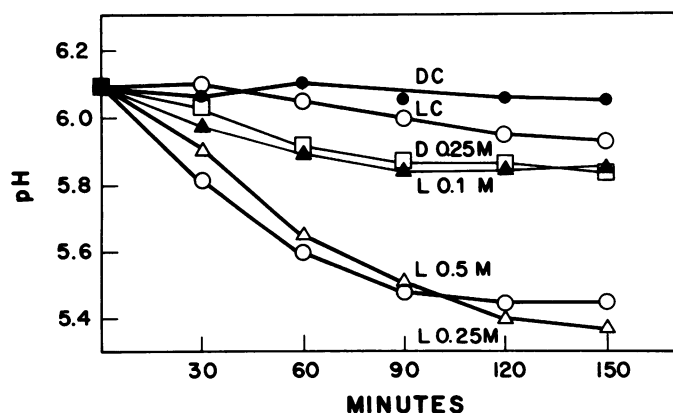

FIG. 1. Effect of various concentrations of mannitol on H⁺ secretion in the light (L) and in the dark (D). DC, dark control; LC, light control.

DCMU, the acidification of the medium is inhibited in the light. These data suggest that the light-induced H⁺ secretion in oat leaf segments is tied to (part of) the photosynthetic system, but it is saturated at low rates of photosynthesis.

H⁺ Secretion by Oat Leaf Segments at Different Stages of Development. Table III compares the light-stimulated H⁺ secretion at different stages of leaf development. Young leaves (the first leaves of 6-d-old seedlings) acidify the external solution at a higher rate than mature and senescing leaves in darkness. Under illumination, however, the stimulation by young and mature (leaves of 10-d-old seedlings) is not significantly different, whereas the senescing leaves (after 3 d of incubation of the detached leaves in the dark) failed to show light-stimulated H⁺ secretion.

Leaf segments floated on 3 mg/l kinetin in darkness for 4 d do not senesce as indicated by Chl and protein level. Furthermore, Figure 4 shows that their ability to produce the response to light even after 4 d in dark is virtually identical to the one incubated after 4 d in the light. The decrease in proton secretion is already measureable after as little as 30 min, while the increase in proteolysis rate was detected only after 6 h in dark (17).

Effect of Fusiccocin on H⁺ Secretion and on the Senescence of Oat Leaves. The phytotoxin FC[1] is known to cause acidification in most of the plant tissues studied (11). The effect of 10 μM FC on proton secretion by oat leaf segments is shown in Table IV and Figure 5. At this concentration, FC in darkness was more effective in stimulating H⁺ efflux than the light treatment. Table IV also shows that inclusion of FC in the medium delayed senescence as indicated by slower Chl breakdown. These data confirm and supplement those of Thimann and Sattler (19). The most striking feature is the correlation between the apparent enhancement of proton secretion and the concomitant inhibition of senescence by light and by FC in the darkness. These data may suggest a possible regulative role of H⁺ pumping during this last stage of development.

Effect of Cycloheximide on Light- and FC-Stimulated H⁺ Secretion in Oat Leaf Segments. One, if not the main, difference between FC- and auxin-stimulated H⁺ secretion is the response to protein synthesis inhibitors (1, 9). CHI is known to prevent auxin-stimulated proton secretion, but has no effect on FC-stimulated H⁺ secretion in coleoptiles and etiolated pea stems. This observation suggests that FC and IAA affect acidification via different mechanisms. Inasmuch as the oat leaves respond both to light and to FC but not to auxin, it was of interest to study the effect of CHI on light- and FC-stimulated acidification.

Figure 5 depicts the effect of CHI added to the incubation

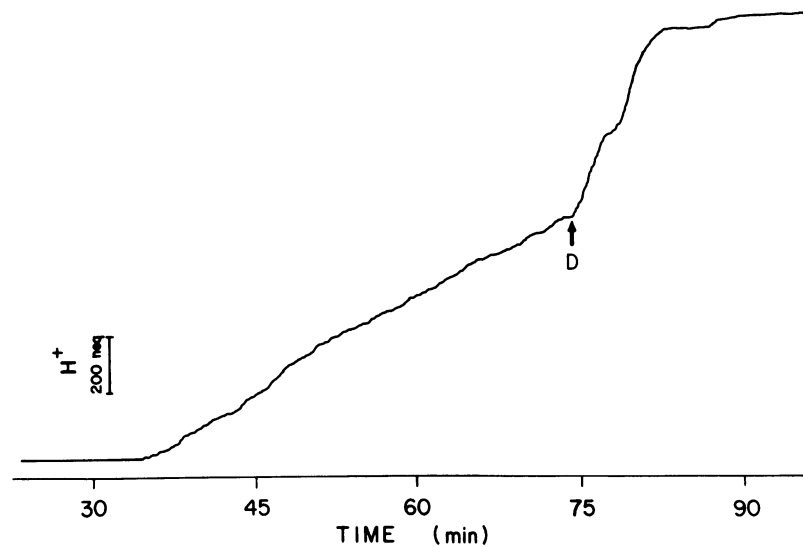[1] Abbreviations: CHI, cycloheximide; FC, fusicoccin.

FIG. 3. Tracing of H^+ efflux carried out by oat leaf segments and monitored with the pH stat apparatus. Six g of abraded leaf segments were placed in 10 ml standard medium, and the H^+ efflux was measured by automatic titration of the external solution with 1 mM NaOH.

Table I. *Effect of Light Intensity on the Acidification of the External Solution by Oat Leaf Segments*

Abraded leaf segments were incubated in 0.25 M mannitol and 1 mM KCl solution. H^+ secretion was determined by changes of the medium pH. The initial pH was adjusted by 10 mM NaOH to 6.3.

Treatment	pH	
	60 min	120 min
Dark	6.00	6.05
Light, 20 w/m²	5.80	5.45
Light, 200 w/m²	5.75	5.40

Table II. *Effect of DCMU on the Acidification of the External Solution by Oat Leaf Segments*

The segments were incubated in standard medium containing 0.25 M mannitol, 1 mM KCl, in the presence or in the absence of 10^{-6} M DCMU.

Treatment	pH	
	0	120 min
Light	6.3	5.5
Darkness	6.3	6.3
Light + DCMU	6.3	6.2
Dark + DCMU	6.3	6.3

medium of the light and FC treatments. CHI has a negligible effect on FC-stimulated acidification, but it completely inhibits the light-stimulated acidification and even causes slight alkalinization.

This indicates a similarity of light-stimulated H^+ secretion to auxin-stimulated H^+ secretion. On the other hand, FC probably acidifies the medium via a different mechanism.

Effect of Vanadate on H^+ Secretion and on the Senescence of Leaves. Present evidence indicates that a plasma membrane ATPase functions as an electrogenic pump in higher plants (16). Recently, indirect evidence supporting this notion has been presented by Jacobs and Taiz, using sodium vanadate (8). This apparently specific inhibitor of plasma membrane ATPase in *Neurospora* (2) was employed by them to block auxin-induced H^+ secretion and growth in pea stems and coleoptiles. Figure 6 depicts the effect of vanadate on light-stimulated H^+ secretion by oat leaf segments. The inhibitor blocks H^+ secretion. This effect cannot be attributed to its buffer capacity since the control includes phos-

Table III. *pH Changes of the External Solution by Leaf Segments at Different Stages of Development*

Leaf segments were cut from the first leaf of 6-d-old seedlings (young leaves), 10-d-old seedlings (mature leaves), and after 3 d of incubation of detached mature leaves in the dark (senescing leaves).

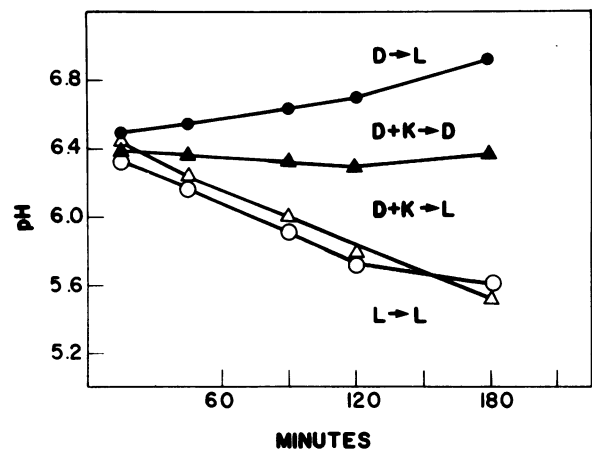| Treatment	pH			
	Dark		Light	
	0	90 min	0	90 min
Young leaves	6.67	6.20	6.69	5.20
Mature leaves	6.70	6.54	6.62	5.45
Senescing leaves	6.70	7.30	6.68	7.35

FIG. 4. pH changes of the external solution induced by oat leaf segments which had been preincubated for 4 d in light (L), in darkness (D), and with 3 mg/l kinetin in darkness. Afterwards, they were transferred and incubated either in light conditions (L) or dark (D) in standard solution (0.25 M mannitol + mM KCl). The pH of the external solution was measured during 180 min after the transfer. The arrows indicate this transfer.

phate buffer at a comparable buffer capacity. The effect of vanadate on the senescence of oat leaves is demonstrated in Figure 7.

Vanadate at the concentration of 0.5 and 1 mM shows a promotion of proteolysis (as shown by the level of amino acids) and

Table IV. *Effect of Fusicoccin on H⁺ Secretion and on the Level of Chl after 3 Days of Incubation*

Leaf segments had been incubated for 3 d in the absence or in the presence of 10 µM fusicoccin. For pH measurements, the leaf segments were transferred and incubated in standard medium (0.25 M mannitol and 1 mM KCl) in the absence or presence of fusicoccin.

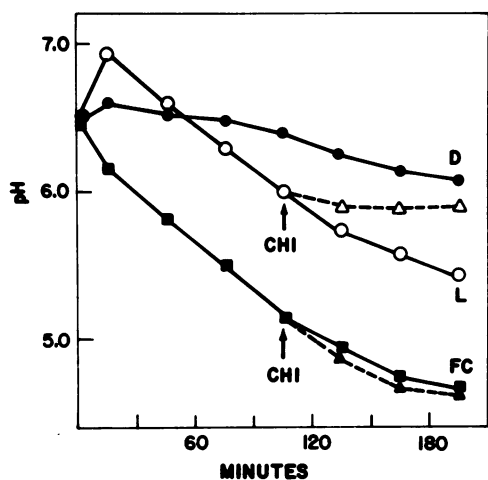| Treatment	pH		Chl
	0	90 min	
			A_{660}
Dark control	6.40	6.00	0.075
Dark + fusicoccin (10 µM)	6.50	5.15	0.175
Light control	6.40	5.55	0.280
Light + fusicoccin	6.45	4.90	0.275

FIG. 5. Effect of cycloheximide (CHI) on light- (L) and on fusicoccin- (FC) induced H⁺ secretion. CHI was added after 100 min (arrows). Concentration of FC was 10 µM and of CHI 10 µg/ml. Light, open symbols; Dark, closed symbols.
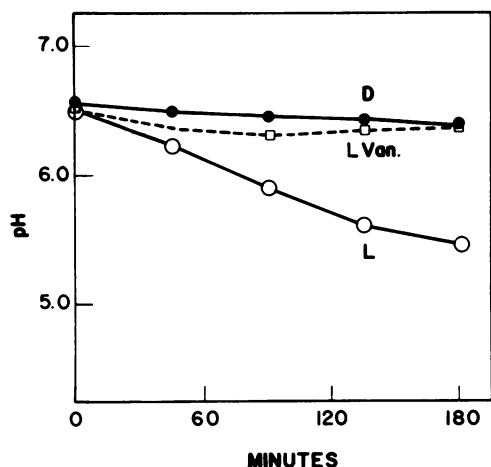

FIG. 6. Effect of 1 mM vanadate on light-induced H⁺ secretion. The solutions of light and dark controls contained 0.25 mM sodium phosphate buffer and 1 mM KCl.

acceleration of Chl loss. Toxic effects usually cause Chl bleaching in light which is not accompanied by an increase in proteolysis. Since the effect of vanadate is similar both in darkness and in light, and since vanadate affects Chl loss and proteolysis at a comparable extent, we may conclude that the effect of vanadate is on the 'true' senescence and not merely bleaching.
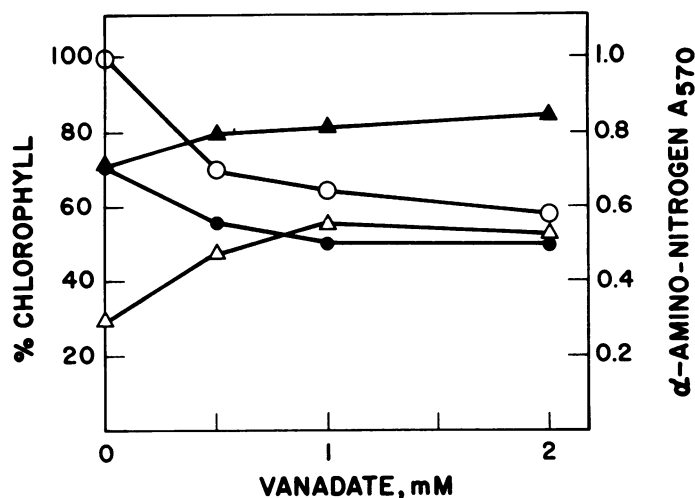

FIG. 7. Effect of various concentrations of sodium vanadate on Chl (O) and on α amino nitrogen (△) content in oat leaf segments after 2 d in the light (open symbols) or in darkness (closed symbols). Sodium vanadate was present throughout the 2-d period. Chl level is expressed as a percentage of Chl extracted from light-incubated segments at the end of the 2 d.

DISCUSSION

Light-stimulated H⁺ secretion by oat leaf segments is consistently detectable when the following optimal conditions are employed. (a) Cuticle abrasion or epidermis peeling; H⁺ efflux is hardly detectable in the external medium using intact stem, coleoptile sections (5), or oat leaf segments (data not shown). (b) Inclusion of mannitol at a concentration that causes significant reduction in turgor pressure or even plasmolysis in leaf cells (0.25–0.5 M); this requirement is consistent with that reported by Derlot and Bonnemain (6). Cleland (4) could detect an increasing rate of H⁺ secretion by oat coleoptiles using increasing concentrations of mannitol, but water stress induced by mannitol did not stimulate auxin-induced H⁺ secretion. (c) High tissue to volume ratio shortens the lag period in detecting pH changes in the medium. (d) Shaking or stirring the leaf segments.

The reasons for the amplification of H⁺ secretion by mannitol are not clear. The reduction of turgor pressure under these conditions might affect the activity of a plasma membrane H⁺ ATP-ase. It was noticed earlier that cell turgor affects membrane components that determine membrane potential (12, 23). An alternative interpretation of the mannitol effect is a possible enhancement of K⁺ influx which, in turn, may increase the H⁺ exchange as detected by the acidification of the external medium. The present investigation has further revealed the light-induced proton pumping in leaves of nonaquatic plants (21). Van Volkenburgh and Cleland (21) suggest an important role for this process during the cell enlargement stage of leaf development, whereas the present investigation shows that proton pumping is also active at later stages but deteriorates and stops during the latest phase of development, namely, during the senescence of leaves. Besides its well known role in auxin-induced growth of coleoptiles and stems, the electrogenic pump serves two vital roles (16): first, regulating the cytoplasmic pH and, second, generating an electrochemical potential difference for protons which is responsible for flux of major ions or of sugars and amino acids via symport. According to Raven and Smith (14, 15), the widespread occurrence of active H⁺ transport in plant and animal cells has evolved primarily as a consequence of the requirement for the regulation of cytoplasmic pH. It is suggested that the H⁺ pump functions mainly to eliminate protons from the cytoplasm and prevent acidosis by the so-called 'biophysical pH stat' mechanism.

According to this view, this mechanism may delay the process of senescence by decreasing the activity of acid hydrolases which are known to be involved in leaf senescence (17). Although it is accepted that growth-promoting compounds such as IAA and fusicoccin initiate cell enlargement by causing the cell to acidify the cell wall, it seems more logical to postulate that the role of proton pumping in nonexpanding cells may be primarily to prevent the acidosis of the cytoplasm rather than to acidify the cell wall. A second major function of the electrogenic H^+ pump is to support coupled transport of other solutes. An alternative explanation for the role of active proton pumping in delaying senescence might be the maintaining of an essential solute transport which is coupled to proton pumping. In the absence of this essential solute transport, aging might be initiated.

The existence of H^+ pump ATPase and the demonstrated correlation between the cessation of H^+ pumping and the acceleration of senescence may account for two striking observations made by Thimann et al. (10, 18, 19). Malik and Thimann (10) demonstrated that a low level of ATP is associated with the prevention of senescence, whereas with treatments that enhance the rate of senescence, the level of ATP rises. These authors suggest that the level of ATP at any time is determined by the rate of its utilization rather than by its synthesis. Since it is known that the proton pump is ATP-powered, it is not surprising to discover that, upon accelerated senescence which is accompanied by cessation of proton pumping, the level of ATP rises.

Another observation carries the parallelism between senescence and stomatal aperture (18, 19). Senescence is strongly delayed by treatments that cause stomata to open. It was proposed that the stomatal aperture is the causal factor (19). The present study raises the possibility that stomatal opening and the rate of senescence can be associated with another common process, namely the proton secretion. According to the chemiosmotic hypothesis of stomatal function postulated by Zeiger et al. (22), the ion uptake during stomatal opening is driven by a proton motive force, which is generated by proton extrusion (13, 22).

Taken together, the results reported here can serve as a possible explanation for the relationship between stomatal aperture and senescence with strong indication that H^+ secretion is the causal factor in both processes.

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