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Effect of salicylic acid on leaf anatomy and chloroplast ultrastructure of barley plants

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Abstract

Light and electron microscopy were used to relate histological and ultrastructural differences of barley leaves treated with different concentrations of salicylic acid (SA, 100 μM-1 mM). Light microscopy revealed that the thickness of all leaf tissue components decreased in SA-treated plants. The effect was most pronounced on the width of the adaxial epidermis and on the size of the bulliform cells. The chloroplast ultrastructure was also affected by SA treatment. Swelling of grana thylakoids in various degrees, coagulation of the stroma, and increase in chloroplast volume were observed. 1 mM SA caused a vast destruction of the whole plastid structure.

Additional key words: abaxial and adaxial epidermis; bulliform cells; chloroplast volume; *Hordeum vulgare* L.; mesophyll thickness; stroma guard cell; thylakoid number and length; vascular bundles.

Introduction

Exogenous application of salicylic acid (SA) to plants exerts diverse physiological effects, such as inhibition of dry mass accumulation (Schettel and Balke 1983), promotion of stomatal closure (Larque-Saavedra 1979), control of ion uptake and transport (Harper and Balke 1981), and inhibition of ethylene synthesis (Leslie and Romani 1986). Evidence for the involvement of SA in induction of an alternative respiratory pathway (Elthon *et al.* 1989) and expression of a nuclear gene encoding the alternative oxidase protein in *Sauromantum guttatum* (Rhoads and McIntosh 1991) have been presented. Pancheva *et al.* (1996) showed that treatment of barley with SA exercised a considerable effect on the growth of the seedlings. Leaf emergence was delayed, blades expanded more slowly over a longer period of time, and mature blades were both narrow and shorter. The rate of photosynthesis and the carboxylating activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) decreased in SA-treated plants. As a result of SA treatment, breakdown in the synthesis of RuBPCO (Pancheva and Popova 1998), an inhibition of the Hill reaction activity, and some changes in the kinetics of flash-induced O_2 evolution (Maslenkova and Toncheva 1998) as well as changes in net photosynthetic rate, carotenoid and sugar contents (Chandra and Bhatt 1998) were reported.

The present study examined the effects of SA on the anatomical organisation and ultrastructure of leaves from barley plants grown for 7 d over a range of SA concentrations (100 μM-1 mM). Effects of SA on the thickness of the adaxial and abaxial epidermes, and on the lamina and mesophyll tissue width were investigated. Reasons for focusing on leaf anatomy were two: firstly, leaf expansion is constrained by the extensibility of epidermis (Kutschera *et al.* 1987). Secondly, the observed inhibition in the rates of transpiration and photosynthesis (Pancheva *et al.* 1996) could partially be due to some alterations of guard cell morphology and stomatal size and aperture.

The ultrastructural observations were focused particularly on chloroplasts, because thylakoids are the membranes which undergo the greatest changes during

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Abbreviations: abE - abaxial epidermis; adE - adaxial epidermis; b - bulliform cells; c - chloroplast; cw - cell wall; I - intercellular spaces; LM - light microscope; m - mitochondrion; mc - mesophyll cells; s - stroma; PPFD - photosynthetic photon flux density; RuBPCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; SEM - scanning electron microscope; TEM - transmission electron microscope; v - vascular bundles.
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adverse environmental conditions, such as drought (Poljakoff-Mayber 1981), heavy metals (Angelov *et al.* 1993), or after exogenous treatment with growth regulators (Wilhelmová and Kutík 1995, Popova and Uzunova 1996). The observed inhibition in the rate of photosynthesis may be related to the changes of the membrane structure of chloroplasts. Primary steps in the electron-transport system of chloroplasts may be disrupted under the influence of SA, and this in its turn leads to changes in the production of sufficient amounts of ATP and NADPH necessary for maintaining the enzyme activity and consequently, a high rate of CO_2 fixation.

Materials and methods

Plants and SA treatment: Caryopses of barley (*Hordeum vulgare* L. cv. *Alfa*) were germinated for 2 d in two layers of moist filter paper in moist vermiculite at 25 °C in the dark. Then they were transferred into Petri dishes containing 40 cm^3 of distilled water or equal amounts of water solution of the required SA concentrations (100 µM, 500 µM, and 1 mM). pH was 4.6-4.8. Stock of SA (*Sigma*) was prepared in a small volume of ethanol (final concentration 1 %), diluted to its final concentration in water, and kept refrigerated until use. The solutions were changed every 24 h. During the experimental period (8 d), the seedlings grew in a growth chamber under "white" fluorescent lamps (160 µmol m^{-2} s^{-1} PPFD), with a 12 h light/dark period. Day/night temperatures were 25/20 °C; relative humidity was about 50 %.

Microscopic observations

Light microscopy: For anatomical studies, all leaf samples were taken at mid-lamina from the second leaf and fixed with 2.5 % glutaraldehyde in phosphate buffer (pH 7.4). The thickness of the lamina between bundles was examined. Cross sections were cut by hand. Ten morphometric measurements were repeated in triplicate and microphotographs were taken using an *Amplival* (*Carl Zeiss*, Jena, Germany) microscope.

Transmission electron microscopy (TEM): Leaves were sectioned and fixed for 12 h with 3 % glutaraldehyde in phosphate buffer (pH 7.4) at 4 °C. After rinsing in buffer, the samples were post-fixed in buffered 2 % OsO_4, again rinsed in phosphate buffer, dehydrated through a graded series of ethanol, and embedded in Spurr's epoxy resin. Thin sections were cut on a *Reichert* (Austria) ultramicrotome at 50 nm, stained with 2.5 % uranyl acetate followed by lead citrate (Reynolds 1963), and examined at 80 kV in a *JEOL 1200 EX* transmission electron microscope. The ultrastructure was assayed for 50 random chloroplast sections photographed at the same magnification. The chloroplast ultrastructure was analysed from the electron micrographs as described by Silaeva and Silaev (1979) and Evans (1986).

Scanning electron microscopy (SEM): The samples were fixed with 2 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), dehydrated in ethanol-isoamyl series, critical-point-dried through carbon dioxide, mounted on stubs, and coated with gold. The material was examined in a *JEOL-JSM 35* scanning electron microscope.

Results

Anatomical observations: Barley leaf cross-sections observed with light microscope were structurally of a typical festucoid type (Fig. 1*A*). As unifacial, the leaves were amphistomatic with clearly expressed wave-like upper surface. Large bulliform cells, almost uniform, fan-shaped, situated in groups of 4 and more rarely of 5 were observed on the adaxial side between bundles. In control leaves, the thickness of the lamina was 39.10±3.17 µm, the average height of the bulliform cells was 12.10±1.16 µm. They formed 31 % of the overall thickness (Table 1). The abaxial epidermis (25 % of the lamina thickness) consisted of cells almost equal in size. The mesophyll tissue was composed of loosely packed mesophyll cells, the average thickness of this tissue was 18.40±2.54 µm. The vascular system was well developed with a distance of 100±8.3 µm between the bundles.

Distinct differences were observed in leaf components of the control and SA-treated barley seedlings. The average thickness of the lamina declined from 39.10±3.17 µm for the control to 26.6±3.2 µm for 1 mM SA. A marked reduction in the width of the adaxial epidermis was observed in all SA-treated variants. The morphometric values showed that treatment of barley with 100 and 500 µM SA caused a uniform decrease in the thickness of adaxial and abaxial epidermes. The mesophyll tissue was also thinner compared to the control, the effect being more expressed at higher applied SA concentrations. Quantitative correlation between the tissues in the architecture of leaves remained approximately the same. The adaxial and abaxial epidermes occupied 23 % each, while for mesophyll this percentage was 54 % of the total thickness (Table 1 and Fig. 1*B*). Application of 1 mM SA

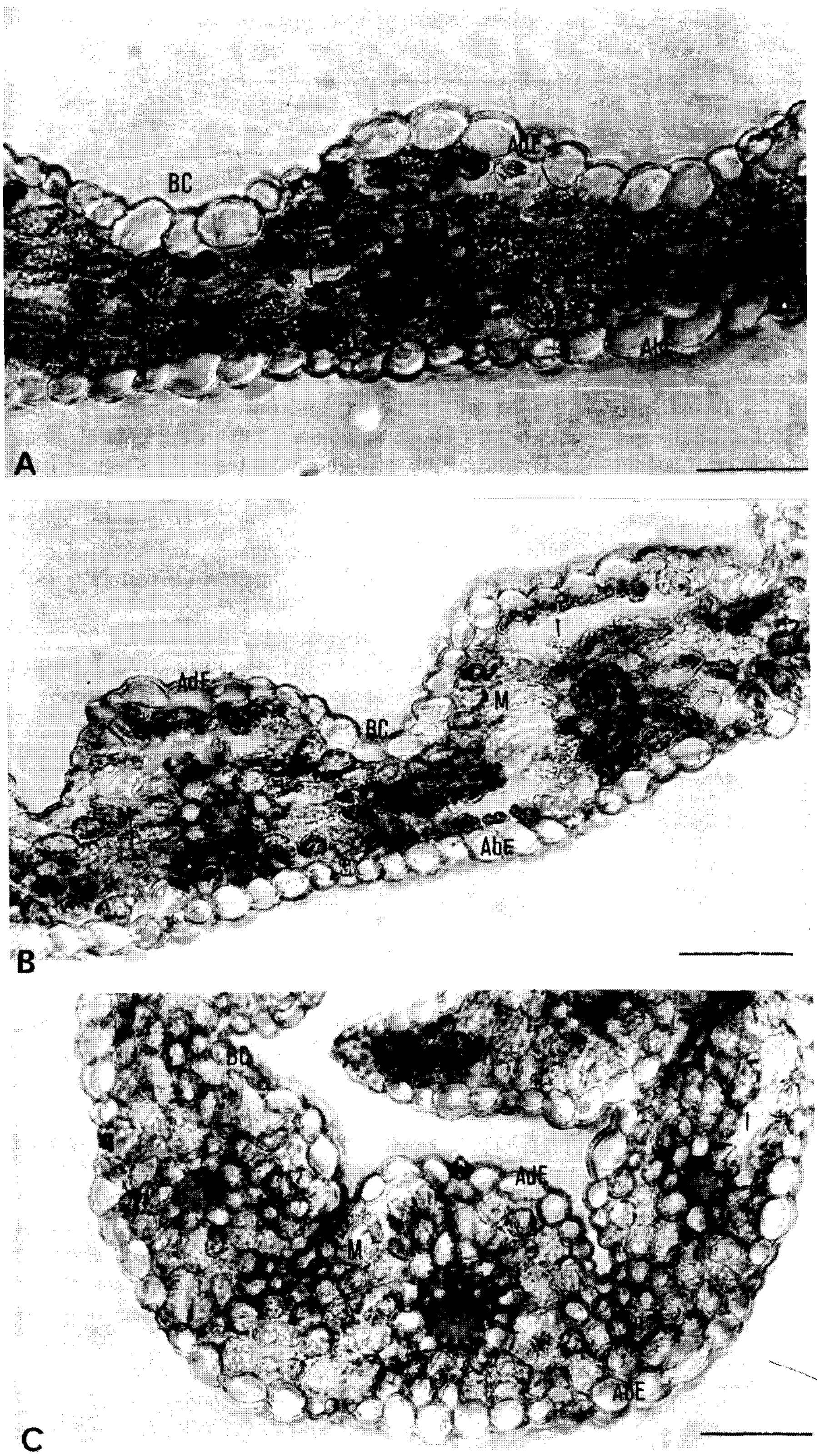

Fig. 1. Effect of salicylic acid (SA) on leaf anatomy of barley plants. Light micrographs of cross sections of barley leaves of control (*A*) or treated with: 500 µM SA (*B*) and 1 mM SA (*C*). Symbols denote various cell types: AbE, abaxial epidermis; AdE, adaxial epidermis; BC, bulliform cells; I, intercellular spaces; M, mesophyll cells; B, vascular bundle. Bars = 25 µm.

Table 1. Morphometric values of thickness of leaf lamina and its tissue components [μm] of barley plants after treatment with salicylic acid (SA). Three independent experiments, each with 10 replicates.

SA [μM]	Thickness of lamina between bundles	adaxial epidermis	abaxial epidermis	mesophyll	Distance between bundles
0 (control)	39.10±3.17	12.10±1.16	9.20±1.25	18.40±2.54	100.20±8.27
100	33.60±5.67	8.30±1.14	8.30±1.14	16.80±3.99	84.30±6.93
500	28.40±2.92	6.30±1.50	6.30±0.98	15.40±3.02	74.30±8.38
1000	26.60±3.20	4.00±0.89	7.80±1.66	14.30±1.89	62.30±7.97

resulted in folding of leaves and limitation of their adaxial surface (Fig. 1*C*). This was due to the small size of the bulliform cells. Their average height was only 4.00±0.89 μm and varied in very tight intervals. It was almost three times smaller than the one registered in the control and hardly comprised 15 % of the overall thickness of lamina in the treated plants. The abaxial epidermis had greater relative share (29 %) and its average thickness was 7.80± 1.66 μm. It was twice as big than that established for the adaxial epidermis.

Morphometric values indicated that the epidermal tissue of plants treated with 1 mM SA consisted of smaller cells. These values were confirmed after SEM observations of both leaf surfaces. The analysis showed that the cells of adaxial epidermis were smaller in size in SA-treated plants as compared with the control. Some differences were distinguished between the shape and size of guard cells. Guard cells of control leaves were arranged in parallel rows, typical for *Gramineae*. Guard cells of treated plants were smaller and some of them were sunken relative to the other epidermal cells (Fig. 2*A,B*).

Table 2. Differences in ultrastructural characteristics [μm] of chloroplasts (average values) and thylakoids of barley plants grown in the presence of different concentrations of salicylic acid. Values are means of 50 median chloroplast sections. N.D. – not detectable.

SA [μM]	Chloroplast length (L)	width (H)	L/H	volume [μm^3]	Thylakoid number per granum	length in grana (Lg)	length in stroma (Ls)	Lg/Ls
0 (control)	5.60±0.58	1.90±0.57	2.95	0.84	7.40±1.87	0.58±0.15	0.30±0.16	1.93
100	5.80±0.60	2.20±0.66	2.64	1.17	7.60±1.92	0.60±0.16	0.31±0.19	1.94
500	6.50±0.67	3.40±1.02	1.90	3.13	6.40±2.67	0.68±0.18	0.26±0.10	2.62
1000	4.90±0.51	5.80±1.74	0.84	6.73	N.D.	N.D.	N.D.	-

Ultrastructure of chloroplasts: Chloroplasts of control leaves were elongated with a regular arrangement of grana stacks and a well-developed system of intergranal membranes (Fig. 3*A*). Treatment of barley plants with SA led to significant changes in the internal structure of the plastids, the effect was more pronounced at the higher applied concentrations. In chloroplasts of plants treated with 100 μM SA, more or less typical arrangement of thylakoid membranes was seen (not shown). The morphometric parameters did not show noticeable deviations compared with the control chloroplasts (Table 2). Remarkable structural changes could be distinguished in the chloroplasts of plants treated with 500 μM and 1 mM SA (Fig. 3*B-D*). The chloroplasts of treated plants were slightly swollen, more round, and their relative volume was higher than in the control (Table 2). The grana stacks were lower and broader. The inner structure of chloroplasts was characterised by thylakoid destruction and low electron density of membranes. The electron micrographs showed the presence of many swollen thylakoids, intrathylakoid spaces, and undulating membranes. The loculi of grana and stroma lamellae blistered and swelled, displaying different phases of swelling. Transformation in the inner membrane system was most pronounced for 1 mM SA. As a result, almost no stroma thylakoids remained, and merging of granal thylakoids was observed (Fig. 3*D*).

Discussion

Phenolic compounds are natural plant growth inhibitors (Kefeli and Kutachek 1976). Recently we have shown that treatment of barley seedlings with SA reduced plant growth and rate of photosynthesis (Pancheva *et al.* 1996).

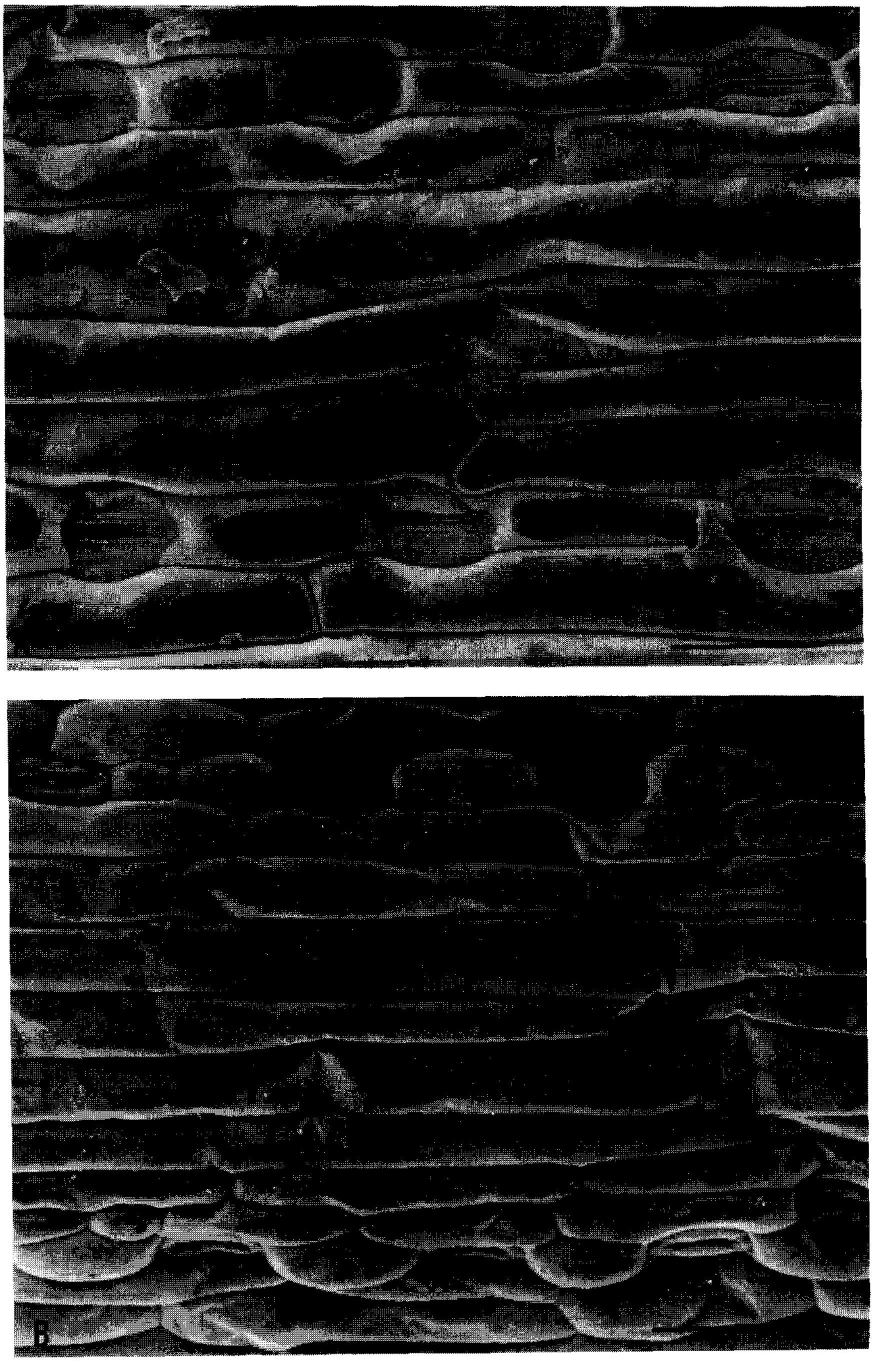

Fig. 2. SEM micrographs of leaf surface (adaxial epidermis) of barley leaves of untreated (*A*) and treated with 1 mM salicylic acid (*B*) plants. Bars = 10 μm.

The successful operation of all photosynthetic reactions is dependent upon the presence of reaction components, their specific organisation within photosynthetic cells, and their being under control of environmental factors and hormonal status of the plant. The assembly of components of the photosynthetic apparatus into a physiologically functional unit is complex, and understanding of anatomical organisation is required to explain fully the processes involved. We hypothesised that the observed photosynthetic differences among untreated (control) and SA-treated plants could be partially due to differences in internal organisation of leaf tissues and by their ability

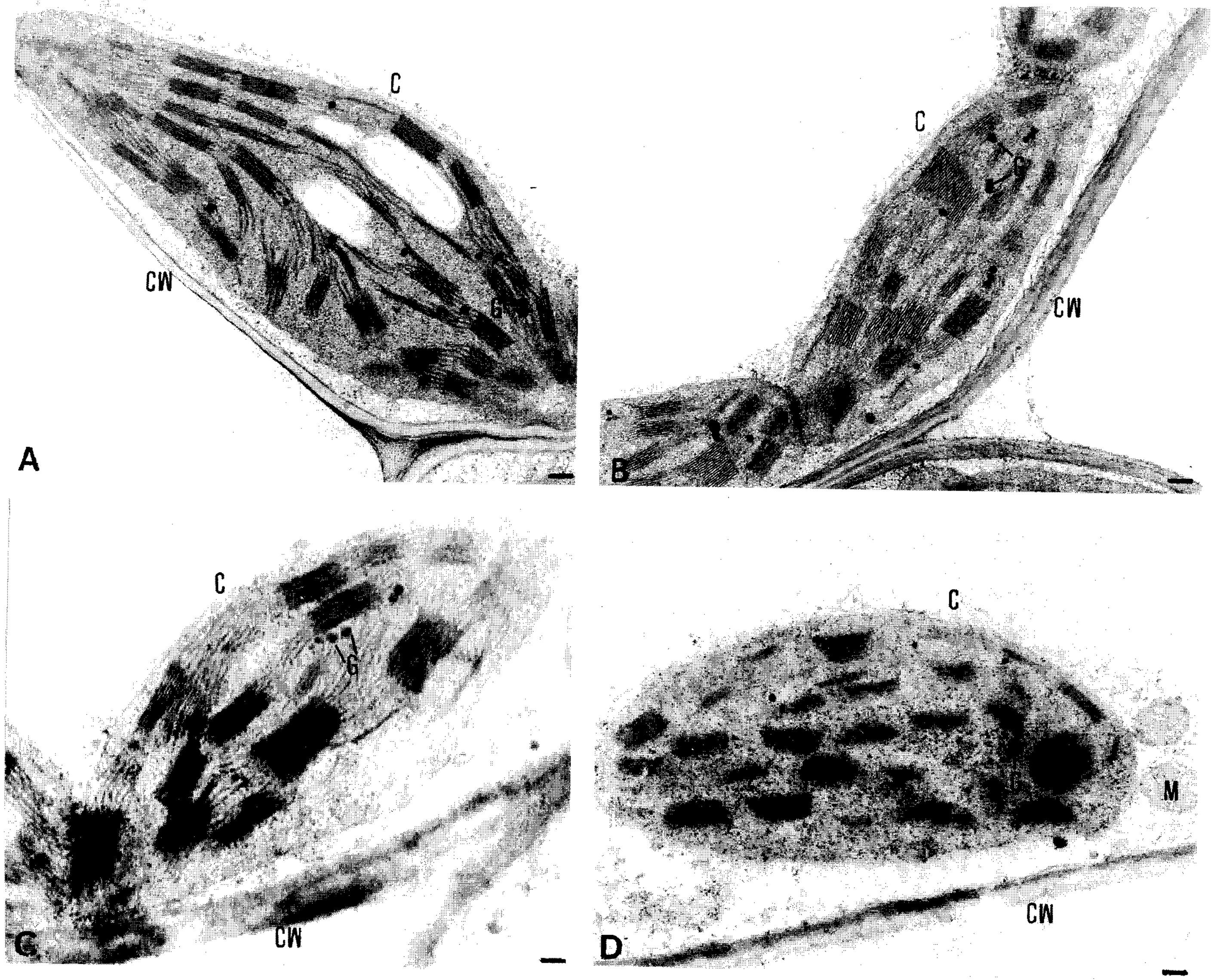

Fig. 3. Electron micrographs of chloroplasts of barley plants grown at different concentrations of salicylic acid (SA). *A*, control untreated plants; *B-D*, plant treated with 500 μM SA (*B*) and 1 mM SA (*C-D*). C, chloroplast; CW, cell wall; G, plastoglobule; M, mitochondrion. Bars = 500 nm.

to produce ATP and reducing equivalents.

Treatment of barley seedlings with SA caused a reduction in lamina thickness and in the dimensions of adaxial and abaxial epidermes. The reduction of epidermal width was more pronounced on the adaxial epidermis. The distance between bundles was also reduced (Table 1). As shown in Fig. 1, the bulliform cells of SA-treated plants were smaller and their volume was lower (Fig. 1*B-C*).

Bulliform cells bring about the rolling or unrolling of mature leaves as a result of their loss or uptake of water. This opinion is consistent with the results shown in Fig. 1. When the highest concentration of SA was applied, the width of the adaxial epidermis was the lowest, the bulliform cells were small, and the leaves were almost rolled (Fig. 1*C*).

Many of the observed plant responses to SA may have adaptive significance because they conserve water. Probably the most significant among the morphological changes is the reduction of leaf expansion. This is a common effect of stress that tends to reduce the total transpiring area. Analogous results were observed as an effect of salinity stress (Miteva and Vaklinova 1991) or after treatment of barley plants with jasmonic acid (Popova *et al.* 1988). The size of epidermal cells decreases after treatment with cadmium (Barceló *et al.* 1988). We suppose that the reduction in size of bulliform cells observed in this study may also be regarded as an adaptive adjustment.

Stomata are the main pathway through the epidermis for gas and water exchange. Earlier studies with the same genotype had shown that SA treatment of barley caused a decrease in the size of guard cells and most stomata of treated plants were almost closed. Leaf evaporating surface area was smaller in the treated plants (Pancheva *et al.* 1999). These observations were partially confirmed by this study. The observed reduction in stomatal aperture may also tend to conserve water and restrict CO_2 entry into the leaf (Fig. 2*B*).

An important factor that may affect leaf photosynthetic efficiency is the presence of well-developed system of air spaces in leaf mesophyll tissue that would facilitate rapid gas exchange. The lower intercellular spaces of 1 mM SA-treated plants might be another factor associated with low photosynthetic rate. According to Maksymiec *et al.* (1995), the volume of intercellular spaces in the mesophyll of bean plants was reduced after treatment with copper because of deviation in water balance.

The most obvious effect of SA was not limited only to leaf anatomy, but the plastid ultrastructure was also affected (Fig. 3*B-D*). We showed that treatment of barley seedlings with SA affects chloroplast ultrastructure. The inner structure of chloroplasts was characterized by thylakoid destruction. Gradual changes in chloroplast ultrastructure may result in an inhibition of photosynthetic electron transport around photosystem 2 (PS2), as suggested by Maslenkova and Toncheva (1998). They demonstrated that SA exogenously applied to barley significantly diminished chloroplast PS2 activity. The inhibitory effect of SA on oxygen evolution was correlated with alterations in membrane polypeptide composition showing a liberation of the 33 kDa peripheral protein.

In summary, we show that treatment of barley seedlings with SA causes alterations in leaf anatomy and leads to internal destruction of the plastids. We suggest that the earlier observed low rates of growth and photosynthesis in barley plants treated with SA are primarily associated with alterations in anatomy and ultrastructural organisation of chloroplasts. Similar to other stress factors, the exogenous SA application diminishes chloroplast photosynthetic activity as a result of effects on the thylakoid membranes and light-induced reactions connected to them. The lower leaf photosynthetic area and probably some changes in its water balance could be another possible explanation.

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