Plant & Cell Physiol. 24(2): 281–288 (1983)

Observation of Stomatal Movements of Intact Plants Using an Image Instrumentation System with a Light Microscope

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In order to observe the stomatal response of intact plants to environmental changes under growing conditions, we developed a remote-control image instrumentation system with a light microscope. This system is composed of: (1) a light microscope with a wide working distance (13 mm) at high magnification (ca. 1,600-fold magnification on a TV monitor); (2) a movable microscope stage designed to permit the passage of conditioned air along both sides of a fixed leaf and for illuminating from above and below; (3) a high-sensitivity SIT camera (S20 type spectral response) and a monochromatic TV monitor with high resolution and small distortion, used to observe the microscope image in a separate room (stomata can be observed with single reflected or transmitted light above ca. 0.1 mW/cm^2); (4) remote controllers to adjust camera sensitivity, microscope focus and movement of the visual field of the microscope image from the separated observation room. This system solved the problems encountered with an ordinary light microscope in observing stomatal movement of intact growing plants. This system also can be used to observe many intact stomata because of its easy and rapid operation. Furthermore, the stomatal aperture and the ratio of transpiration from the cuticle to that from the stomata can be accurately determined using this system.

Key words: Observation of stomatal movements — Intact plants — Image instrumentation — Light microscope — Remote control.

Stomatal movement is greatly influenced by the plant's environment (Meidner and Mansfield 1968, Burrows and Milthorpe 1976, Raschke 1979, Pospíšilová and Solárová 1980, Jarvis and Mansfield 1981). The stomatal conductance or resistance, measured using porometers (Meidner and Mansfield 1968, Meidner 1981) and a thermal image instrumentation system (Omasa et al. 1981a, 1982) has been used to evaluate the stomatal response of intact leaves to environmental changes of growing plants (Meidner and Mansfield 1968, Kaufmann 1976, West and Gaff 1976, Omasa et al. 1981b, c). However, direct observation of the stomatal movement of growing intact plants has been very difficult (Meidner and Mansfield 1968, Meidner 1981). Although the scanning electron microscope (Turner and Heichel 1977, Shiraishi et al. 1978) and the light microscope in which a piece of leaf is immersed in water or liquid paraffin (Monzi 1939, Stålfelt 1959, Meidner 1981) can provide a clear image at high magnification, observation of intact stomata under their growing conditions is impossible. An ordinary light microscope can provide a clear image of the large stomata of an intact plant at

Abbreviations: l_a, width of a stomatal pore; l_b, length of a stomatal pore; l_{bmax}, l_b in the case of an opened stoma, i.e., maximum value of l_b; g_s, stomatal conductance; n_s, density of the stomata; $E(l_a)$, mean value of l_a; $E(l_{bmax})$, mean value of l_{bmax}; k_l, degree of the stomatal aperture expressed by l_a/l_{bmax} or $E(l_a)/E(l_{bmax})$; working distance, distance between the leaf and the microscope objective during observation; RH, relative humidity; SIT, silicon intensifier target.

low magnification with transmitted or reflected light. Heath (1959) obtained a clear image of a large stoma below 400-fold magnification with transmitted light from a condenser and a dry objective corrected for the lack of a cover glass. However, observation of the small stomata, which requires high magnification, was very difficult (Heath 1959). Furthermore, observation with an ordinary light microscope under a plant's growing conditions poses some problems (Meidner and Mansfield 1968). First, visual observation under weak light is very difficult. Second, the environment of the lower side of the leaf can not be controlled because the leaf is directly held on the microscope stage; the environment is also affected by human manipulation of the microscope. And third, the working distance, that is, the distance between the leaf and the objective during the observation is very narrow at high magnification, thus subjecting the leaf to the danger of sticking to the objective during focusing and destroying the plant's environment between the leaf and the objective.

We, therefore, developed a remote-control image instrumentation system using a new light microscope with a wide working distance at high magnification and an SIT camera with high sensitivity, and observed the stomatal response of intact plants to environmental changes under their growing conditions using this system.

Materials and Methods

Plant materials—Sunflower (*Helianthus annuus* L. cv. Russian Mammoth) and tomato (*Lycopersicon esculentum* Mill. cv. Fukuju No. 2) plants were grown in an environment-controlled greenhouse (Aiga et al. 1982) at 25/20°C day/night temperature and 70% RH under natural light for 5 to 6 weeks after being sown in pots. Broad bean plants (*Vicia faba* L. cv. Otafuku) were grown at 20/15°C day/night temperature and 70% RH for 6 to 7 weeks. The pots were filled with a 4 : 2 : 4 : 1 (v/v) mixture of vermiculite, perlite, peat moss, and fine gravel, which was moistened with nutrient solution. The plants were irrigated daily. After the test plants had been moved to an environment-controlled chamber (Aiga et al. 1982) and acclimatized to the new conditions, mature leaves of the intact plants were used in the experiments.

Remote-control image instrumentation system with a light microscope—Fig. 1 shows a diagram of our remote-control image instrumentation system with a light microscope and Fig. 2 a photograph of the system. This system has a light microscope with a wide working distance (ca.

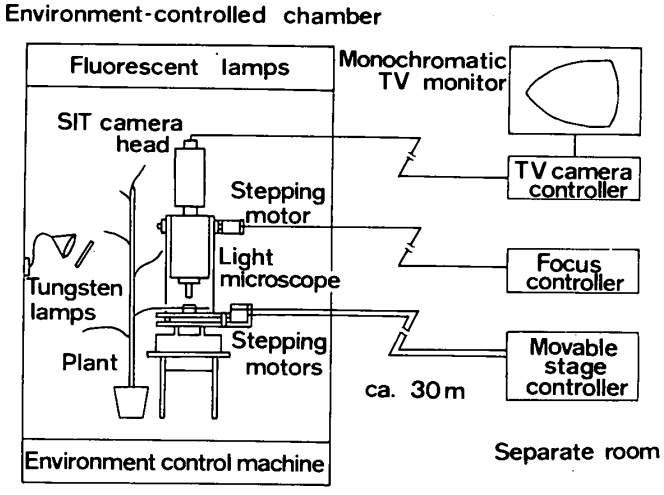

Fig. 1

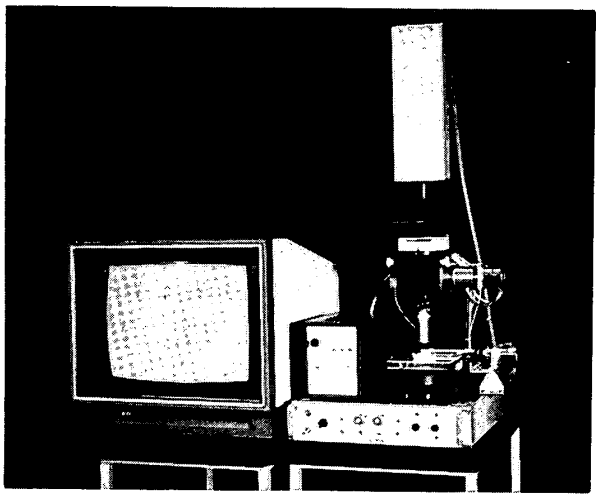

Fig. 2

Fig. 1 Diagram of the remote-control image instrumentation system with a light microscope.
Fig. 2 Photograph of the image instrumentation system.

13 mm) at high magnification (a 50× objective, 1.5× and 2× amplifiers and a TV adapter lens; ca. 1,600-fold magnification on a TV monitor) (Bausch & Lomb, MicroZoom), an SIT camera with high sensitivity of S20 type spectral response, image resolution above 600 TV lines, distortion within ±2% and shading within 20% (Hamamatsu TV, Model C1000-12) as a detector of the microscope image, a monochromatic TV monitor with image resolution of ca. 1,000 TV lines and distortion within 3% (Chuomusen, Model MD2002A) and remote controllers for adjusting camera sensitivity, microscope focus, and movement of the microscope stage.

Observation of stomatal movements of intact plants under their growing conditions—The microscope with the SIT camera head was set in the environment-controlled chamber. Air temperature in the chamber was maintained at 20.0±0.1°C (broad bean plants) or 25.0±0.1°C (sunflower and tomato plants) and humidity was kept at 70±1% RH. Illumination (irradiance) from fluorescent lamps was varied with SCR electric manipulators and that from tungsten lamps with autotransformers through the heat-absorbing glass filters. Illumination was measured with a photometer/radiometer (LI-COR, Model LI-185).

Fig. 3 shows a schematic cross-sectional view of the microscope stage for holding a leaf of the intact plant. The leaf (C) was held on a ring (F, 30 mm in inner diameter, 10 mm wide and 10 mm high) fixed to a remote-control movable stage (G) by a holding ring (E, the same diameter and width as F, 3 mm in height) in order to have the conditioned air pass under the surface of the leaf. Furthermore, since the center of the movable stage was cut out in a circle 30 mm in diameter and the distance between the movable stage and a plate (H) fixed on a base (K) was kept at 10 mm, the same temperature and humidity were maintained on both sides of the leaf. The movable stage and the plate were made of transparent acrylic resin in order to allow light from the environment to enter. A shade cover (B) was not used except in the observation with the transmitted light. Although the observation was usually carried out with light from the environment, a halogen lamp (L) was sometimes used as a supplementary light for the observation with the transmitted light.

The microscope image of the leaf held on the movable stage was observed on the TV monitor in the separate observation room. Camera sensitivity, microscope focus, and the movement of the visual field of the microscope image were adjusted with the remote controllers in the separate room. The knobs for focusing and moving the stage were driven by the stepping motors. The stomatal image displayed on the TV monitor was photographed with black and white film.

Experiment 1—The performance of this instrumentation system was examined. We observed

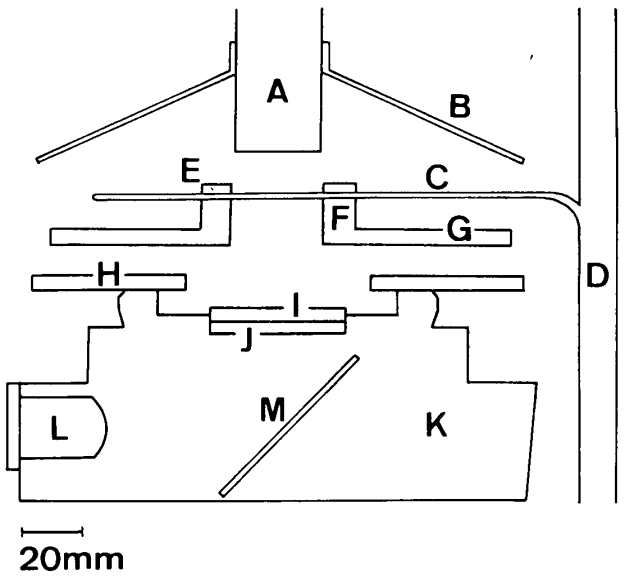

Fig. 3 Schematic cross-sectional view of the microscope stage for holding an intact leaf. A, objective; B, shade cover; C, leaf; D, stem; E, holding ring; F, ring fixed to remote-control movable stage; G, remote-control movable stage; H, plate; I, heat absorbing glass filter; J, diffusing filter; K, base; L, halogen lamp; M, mirror.

the reflection and transmission images of the stomata of intact sunflower plants under various types of illumination from above and below in order to examine the sensitivity of the system. We also checked a test chart for the microscope (Toppan Printing, Toppan resolution test target) to examine the resolution of the system.

Experiment 2—This system was then used for continuous observation of the stomatal response of intact broad bean plant to illumination change under its growing conditions. An intact leaf of the test plant was held on the microscope stage and illuminated at 30 klux (11.9 $mW \cdot cm^{-2}$). After the leaf had been sufficiently acclimatized to the new conditions, the illumination was changed from 30 to 2 klux (0.5 $mW \cdot cm^{-2}$) and then from 2 to 20 klux (7.7 $mW \cdot cm^{-2}$). The microscope image was observed with the reflected light.

Experiment 3—The relationships between stomatal apertures of sunflower, broad bean, and tomato plants and their stomatal conductances were examined. An intact leaf of the test plant was held on the microscope stage under constant illumination. After the leaf had been sufficiently acclimatized to the new conditions, ca. 35 stomata in an area ca. 15 mm in diameter of the leaf were randomly observed with the reflected light, and then the stomatal conductance of the area was quickly measured with a porometer (LI-COR, Model LI-1600). This procedure was repeated for the same area after the illumination was changed.

Results and Discussion

The system was first evaluated and Fig. 4 shows microphotographs of an intact stoma observed with reflected and transmitted light using this system. The stomatal image was clear at high magnification (ca. 1,600-fold magnification on the TV monitor). The stoma was observed with reflected light and then rapidly observed with transmitted light, and the stomatal aperture was found to be the same for both. Although this system could provide stomatal images with a mixture of reflected and transmitted lights, the clearest image was obtained with the single reflected or transmitted light. The stomata could be observed with single reflected or transmitted light above ca. 0.1 $mW \cdot cm^{-2}$ [environment illumination; ca. 2 klux (0.5 $mW \cdot cm^{-2}$) with reflected light, ca. 0.5 klux (0.5 $mW \cdot cm^{-2}$) with transmitted light]. If the observation with the single light of 0.1 $mW \cdot cm^{-2}$ is done by naked eye through the eyepiece instead of the SIT camera, the eye must be sufficiently acclimatized in a dark room. Judging

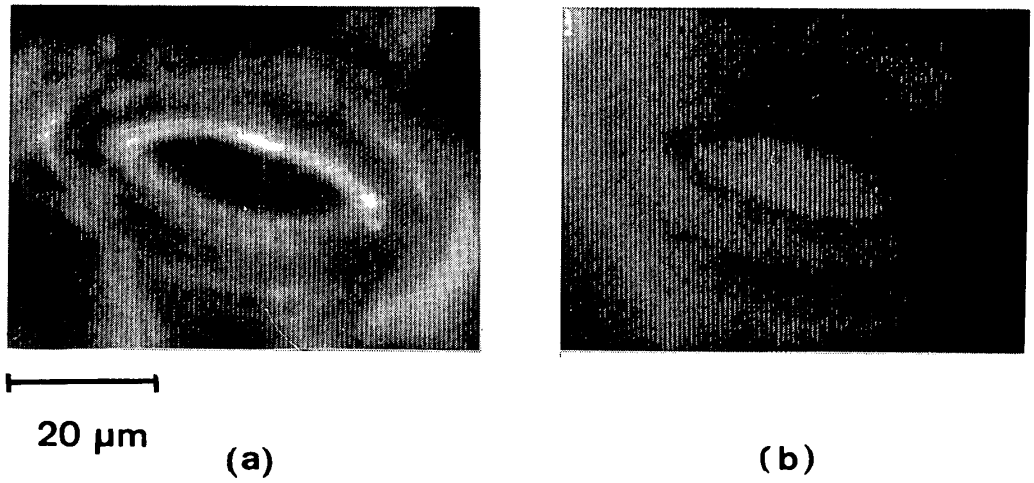

Fig. 4 Microphotographs of an intact stoma observed with reflected or transmitted light using the image instrumentation system. (a) reflection image; (b) transmission image.

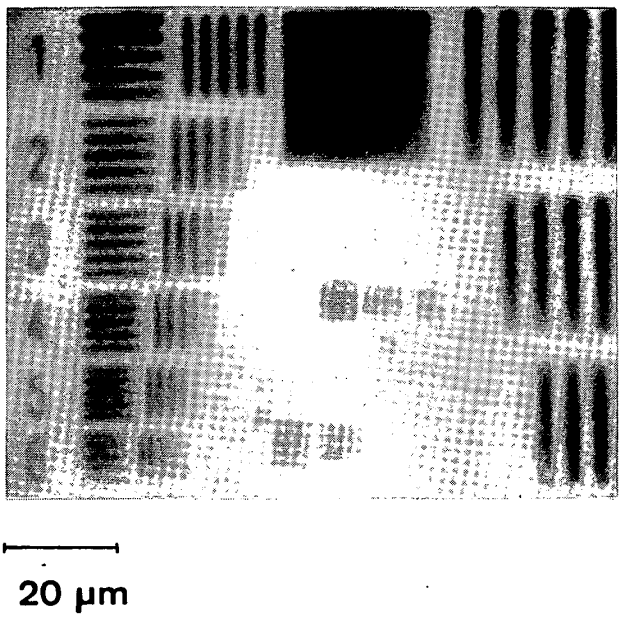

Fig. 5 Microphotograph of test chart measured using the image instrumentation system.

from the microphotograph (Fig. 5) of the test chart measured using this system, we could tell that the resolution of the microscope image was within 1 μm.

When this system was used for continuous observation of the stomatal movement of growing intact plant, we obtained microphotographs like those in Fig. 6 which show the response of an intact stoma of the adaxial epidermis of broad bean plant to illumination change. The illumination was changed from 30 to 2 klux at 0 min (a) and from 2 to 20 klux at 20 min (e). The movement of the central pore of the stoma could be continuously observed. Fig. 7 shows changes in the width (l_a) and degree of aperture (k_l) of the stomatal pore shown in Fig. 6; l_a began to decrease within 5 min (b) after lowering the illumination (30 to 2 klux) and became 0 μm after ca. 15 min (d). It began to increase again within 15 min after raising the illumination (2 to 20 klux), and after 180 min (i), had recovered to ca. 75% of the value before the illumination change. The degree of the stomatal aperture (k_l) was expressed by the ratio l_a/l_{bmax}, where l_{bmax} was the length (l_b) of the stomatal pore of an opened stoma, that is, the maximum value of l_b (l_{bmax}=28.3 μm).

This system was used to analyze the relationship between the stomatal aperture and the stomatal conductance. Fig. 8 shows the relationships between l_a's of the stomata of the adaxial or abaxial epidermis of the various intact plants and their stomatal conductances (g_s). There was a positive correlation between l_a and g_s measured in the same area of the leaf. However,

Table 1 Density of stomata (n_s) and mean value of l_{bmax} [$E(l_{bmax})$] in the same areas as Fig. 8

Plant species	Kinds of epidermis	Density of stomata (n_s) (pieces/mm^2)	Mean value of l_{bmax} [$E(l_{bmax})$] (μm)
Sunflower	adax	86.6	30.1
	abax	76.8	35.9
Broad bean	adax	17.7	31.1
	abax	34.7	31.9
Tomato	adax	24.8	11.6
	abax	77.8	14.5

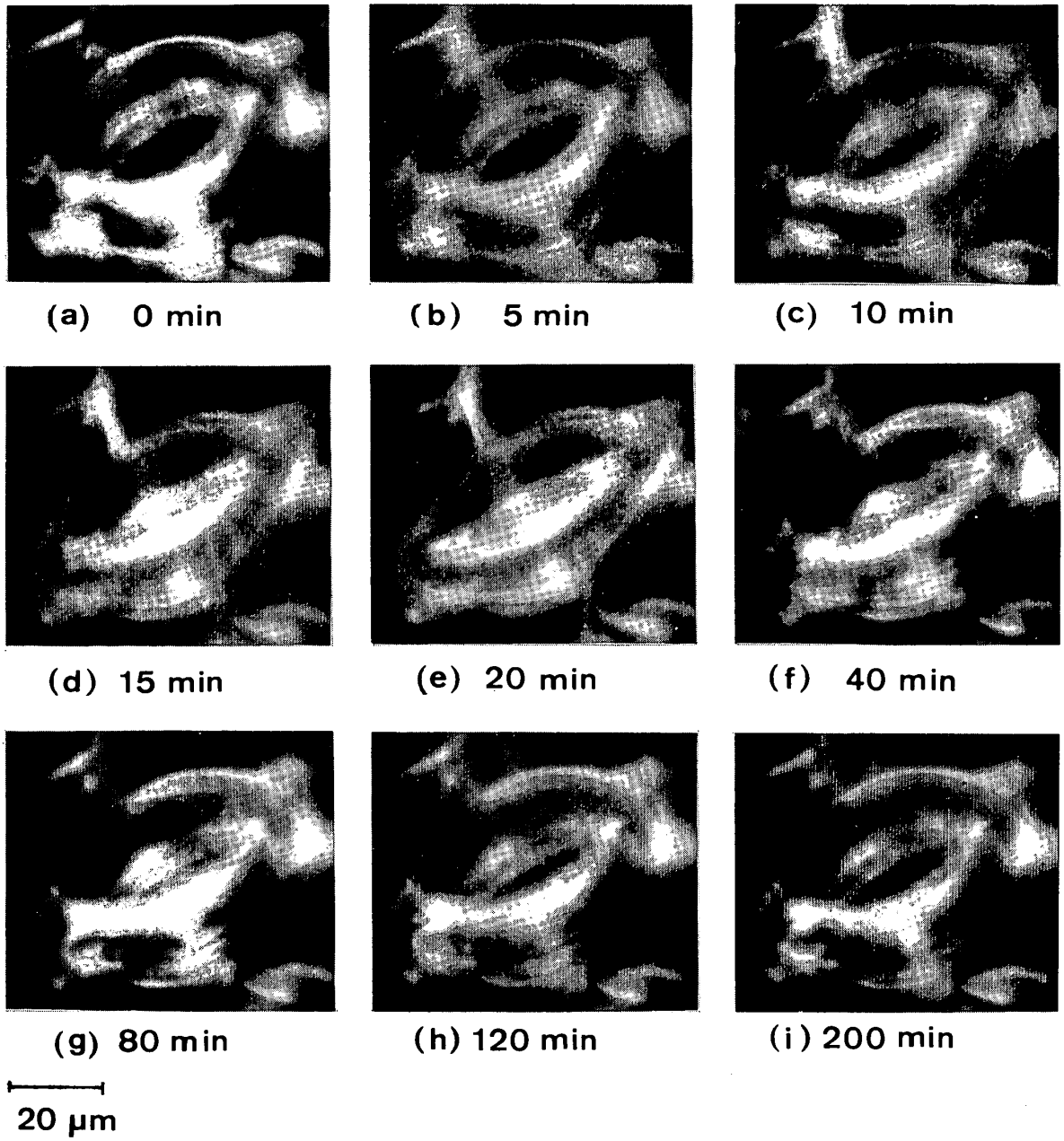

Fig. 6 Microphotographs of responses of an intact stoma of an adaxial epidermis of a broad bean plant to illumination change. The time after the first illumination change is shown under the photographs. The illumination was changed from 30 to 2 klux at 0 min (a) and from 2 to 20 klux at 20 min (e).

the regression curves varied with the kind of plant and epidermis. The maximum values of l_a and g_s also varied. Since all regression curves were concentrated near the origin of the coordinate axes, the transpiration from the cuticle of these plants was negligible in comparison with that from the stomata. Table 1 shows the density of the stomata (n_s) and the mean value of l_{bmax} [$E(l_{bmax})$] in the same areas as Fig. 8. From Fig. 8 and Table 1, we could see that g_s was dependent not only upon l_a but also upon n_s. The maximum values of g_s of the sunflower plants with large n_s and $E(l_{bmax})$ were larger than those of the broad bean and tomato plants with small n_s or $E(l_{bmax})$. Fig. 9 shows the relationships between the degree of stomatal aperture (k_l) and g_s obtained from Fig. 8 and Table 1. From the ratio

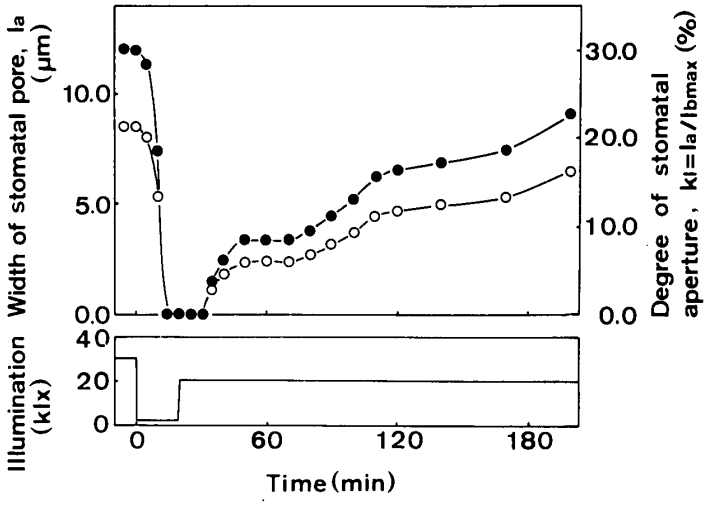

Fig. 7 Changes of the aperture of the stoma shown in Fig. 6. ○, width of stomatal pore (l_a); ●, degree of stomatal aperture expressed by l_a/l_{bmax} (k_l), where $l_{bmax}=28.3$ μm.

$E(l_a)/E(l_{bmax})$, where $E(l_a)$ was the mean value of l_a, k_l could be calculated and was found to decrease at a given value of g_s in the order of: tomato adaxial epidermis > broad bean adaxial epidermis > broad bean abaxial epidermis > tomato abaxial epidermis > sunflower adaxial epidermis > sunflower abaxial epidermis.

To overcome the difficulty of directly observing the stomatal movement of intact plants under their growing conditions, we developed a new remote-control image instrumentation system with a light microscope and used it for continuous observation of the stomatal response to environmental changes and for analysis of the relationship between stomatal aperture and conductance. This system is composed of (1) a light microscope with a wide working distance (13 mm) at high magnification (ca. 1,600-fold magnification on a TV monitor); (2) a movable

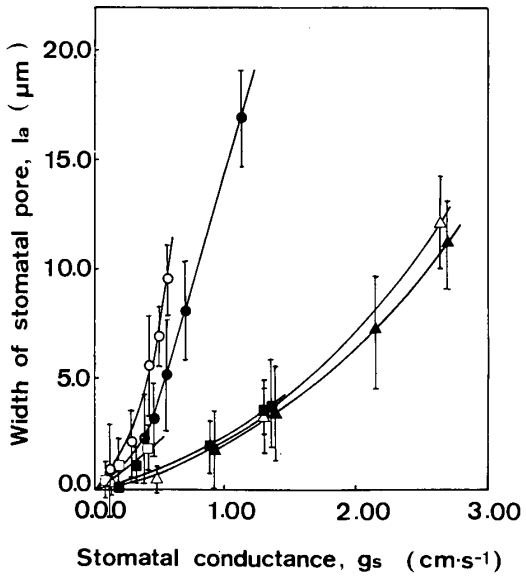

Fig. 8

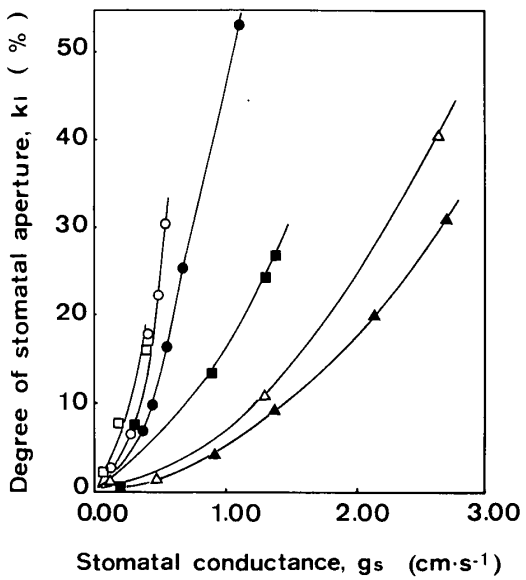

Fig. 9

Fig. 8 Relationships between l_a's of the stomata of adaxial or abaxial epidermis of various intact plants and their stomatal conductances (g_s). Symbols represent mean values of the l_a and vertical bars indicate ± standard error. ○, broad bean adaxial epidermis; ●, broad bean abaxial epidermis; △, sunflower adaxial epidermis; ▲, sunflower abaxial epidermis; □, tomato adaxial epidermis; ■, tomato abaxial epidermis.

Fig. 9 Relationships between the degree of the stomatal aperture (k_l) and g_s obtained from Fig. 8 and Table 1. Symbols are the same as those in Fig. 8.

microscope stage designed to permit the passage of conditioned air along both sides of a fixed leaf and for illuminating from above and below; (3) an SIT camera with high sensitivity (S20 type spectral response) and a monochromatic TV monitor with high resolution and small distortion, to allow observation of the microscope image in a separate room (the stomata can be observed with single reflected or transmitted light above ca. 0.1 mW/cm^2); and (4) remote controllers for adjusting camera sensitivity, microscope focus, and visual field movement of the microscope image. Thus, we were able to solve the problems of the ordinary light microscope in observing the stomatal movement of intact plants under growing conditions. This system is also effective for observing many intact stomata because of its easy and rapid operation. Furthermore, the stomatal aperture and the ratio of the transpiration from the cuticle to that from the stomata can be accurately determined with this system.

We wish to thank the members of the Engineering Division, The National Institute for Environmental Studies, who maintained the equipment and cultivated the plants used in these experiments.

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(Received July 30, 1982; Accepted January 6, 1983)