RESEARCH PAPER

Cadmium-induced plant stress investigated by scanning electrochemical microscopy

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Abstract

In vivo oxygen evolution above single stomata in Brassica juncea has been used to investigate, for the first time, the effect of Cd-induced stress as imaged by scanning electrochemical microscopy (SECM). SECM images showed a clear stomatal structure—a pore, whose aperture is modulated by two guard cells, serving as the conduit for the oxygen produced. Lower stomatal density and larger stoma size were found in plants treated with 0.2 mM CdCl₂ compared with control plants. Either the introduction of Cd caused a slower cell replication in the plane of the epidermis, hence fewer stomata, and/or the number of open stomata was reduced when plants were under Cd-stress. Oxygen evolution above individual stomatal complexes in Cd-treated plants was lower than that from control plants, as determined from the electrochemical current above the middle of each stoma. All guard cells under illumination were swollen, indicating that the stomata were open in both control and treated plants. Thus, decreased oxygen evolution in response to Cd cannot be attributed to simple closing of the stomata, but to a lower photosynthetic yield. SECM provides an excellent tool for monitoring the effects of Cd on photosynthetic activity at the scale of individual stomata.

Key words: Cd-induced plant stress, oxygen evolution, photosynthesis, phytochemistry, scanning electrochemical microscopy, stomatal complex.

Introduction

Brassica juncea (L.) Czern. cv. AC Vulcan (Indian mustard) can accumulate heavy metals, including cadmium (Cd) (Kumar et al., 1995), from environments polluted by industrial wastes or mining activities. Phytoextraction and bioremediation of Cd-contaminated soils with metal-accumulating plants can be used as a novel strategy to concentrate Cd in the shoots (up to 58-fold) from a substrate (Kumar et al., 1995). While some research efforts have been directed at the physiology and chemistry of Cd-accumulating plant species (reviewed in Prasad, 1995; Sanita di Toppi and Gabrielli, 1999; Clemens, 2001), the mechanisms of Cd-toxicity on leaf photosynthetic reactions have not yet been firmly established. It is clear that Cd-toxicity causes leaf chlorosis and reduced photosynthetic rate, probably due to its detrimental effects on chloroplast replication and cell division (Baryla et al., 2001), chloroplast structure (Barcelo et al., 1988), as well as the water-splitting apparatus of photosystem II and photosynthetic electron transport (Mallick and Mohn, 2003). In addition, Cd causes decreased stomatal density and conductance to CO₂ (Baryla et al., 2001) and reduced number of open stomata (Barcelo et al., 1988), which would further affect photosynthetic rates. The mechanisms of Cd-induced changes in stomata are not well understood but probably arise from Cd-induced water deficit (reviewed in Sanita di Toppi and Gabrielli, 1999; Poschenrieder and Barcelo, 2004).

Because many functions of living cells and tissues are tightly related to photosynthesis, it is desirable to measure non-invasively the effects of Cd on photosynthetic reactions. While net photosynthesis can be estimated via gas exchange, and photosynthetic electron transport can be estimated from non-invasive measurements of chlorophyll fluorescence (see, for example, Baryla et al., 2001; Mallick and Mohn, 2003), the scale of measurement is relatively large, yielding gross measurements from square centimetres of plant tissue or cubic millilitres of algal culture.
In the case of plant leaves, the density of chloroplasts may influence the measurements taken. One can either normalize the readings to a ‘per chloroplast’ measurement, or one can seek alternative methods that are less affected by organellar density.

Scanning electrochemical microscopy (SECM) (Bard, 2001) has shown great applications in the area of chemical and biochemical kinetics (Amemiya et al., 2000; Ding et al., 2001; Sun et al., 2002; Fonseca et al., 2003), chemical activity imaging (Tsiomsky et al., 1997; Yasukawa et al., 2000; Lee et al., 2002; Liu et al., 2002; MacPherson et al., 2002; Fernandez et al., 2004; Mauzeroll and Bard, 2004; Turcu et al., 2004; Zhao and Wittstock, 2004), and micrometer scale structuring (El-Giar and Bard, 2004; Turcu et al., 2004; Zhao and Wittstock, 2004), and micrometer scale structuring (El-Giar et al., 2000; Wittstock, 2001; Katemann et al., 2003) at liquid/liquid, liquid/solid, and liquid/membrane interfaces. The most significant advantage offered by SECM is its capability to probe chemical information of electron and ion transfer processes at the interfaces. An SECM probe is usually a platinum (Pt) disc electrode with a diameter <25 μm, insulated by a glass sheath. A constant potential is applied to the electrode to drive quickly an electrochemical reaction of one redox species (the mediator) in a bulk solution. The measured current has to be constant (steady-state current), which is determined by hemispherical diffusion of the reactant at a fixed concentration to the electrode. When the probe is brought down toward a substrate, this constant current is perturbed by the substrate at close proximity through either blockage of the diffusion (on an insulator, negative feedback) or dominant regeneration of the reacted species from the electrode (on a conductor, positive feedback) (Bard, 2001). To detect the feedback better, the probe should get closer to the substrate. The edge of the glass sheath has to be machined down to a small size (sharpened to a tip) to avoid touching a tilted substrate. A small RG (ratio of the outermost glass diameter to Pt disc diameter) is very critical. On the other hand, the disc diameter, then the global tip diameter (outermost diameter), determines the resolution of SECM directly as the feedback reflects the perturbation from a substrate domain as small as the electrode. It is possible to map out the surface topography and/or chemical reactions by monitoring the current perturbation when the tip is rastered above a plane close to the substrate. It is obvious that the quality of SECM images is determined by how accurately the tip is positioned and the current is measured.

Here, Cd-induced stress is investigated by using an SECM probe to monitor oxygen evolution (oxygen is the mediator), hence net photosynthetic activity, through individual stomata in B. juncea. This oxygen arises from photosynthetic electron transport and impaired photosynthetic efficiency is a common initial response to stress. Thus, measures of oxygen concentration profiles provide a means of measuring immediate stress responses. For the first time, the changes to stomatal structure and reactivity caused by introducing Cd-stress is assessed, although Tsionsky et al. (1997) reported using SECM to monitor photoelectrochemistry and in vivo topography of single stomata in unstressed Tradescantia fluminensis Vell cv. Variegata.

Materials and methods

Plant material and growth conditions

Seeds were placed on moist filter paper in Petri dishes in the dark to germinate. Two-day-old seedlings were transferred to pots containing 99.8% quartz sand moistened with half-strength nutrient solution (Table 1) and moved to a growth chamber (300±45 μmol m<sup>−2</sup> s<sup>−1</sup> photon flux and a 16/8 h day/night cycle at 20 °C). Ten-day-old seedlings were transferred to 1.4 l glass jars (two plants per jar) filled with aerated nutrient solution (pH 5.5). After 1 week, plants were transplanted to fresh aerated nutrient solution supplemented with 0 or 0.2 mM CdCl<sub>2</sub> (purchased from Aldrich, Mississauga, Canada); solutions were replaced weekly. SECM analyses were initiated after 5 d of exposure to Cd (3-week-old seedlings). A total of 22 plants (10 control, 12 Cd-treated) were studied.

Electrode fabrication

A borosilicate glass capillary (OD, 2.0 mm; ID, 1.0 mm; Borosilicate, Sutter Instrument Co., CA, USA) was pulled in the middle using a heating coil puller (PP-83; Narishine, Japan). Two pulled tubes were obtained each time. The pulling parameters were adjusted to keep the inner diameter no more than 100 μm. This reduced greatly the workload to polish and sharpen a SECM probe. An ~12 mm length of 2 μm diameter Pt wire (Goodfellow Cambridge Ltd), coated with Ag to a diameter of 100 μm, i.e., Wollaston wire, was placed into the pulled capillary glass tube through the large open end. A hook was made at one end of the wire, which should touch the inner wall of the capillary so that the Pt wire is held with its straight end closest to the pulled end of the capillary. The silver coating on the Pt wire was etched away chemically by dipping the pulled end of the glass capillary into nitric acid (1:1 concentration, v/v) several times. This process was examined with a microscope (Metallographic Microscope, VWR, USA). The etched wire in the capillary was washed with copious deionized water, and then with acetone. The capillary tip was sealed using a Bunsen burner such that the distal end of Pt wire was straight in the tubing.

The open end of the capillary of the resulting assembly was connected to a vacuum line for about 30 min to get high vacuum in the capillary. The tip was melted using the heat coil of the puller for approximately 3 s. The capillary was connected to a vacuum line for about 30 min to get high vacuum in the capillary. The tip was melted using the heat coil of the puller for about 3 min. The tip was checked under the microscope. Sometimes the tip appeared misshapen (curls slightly if it is not placed exactly in the middle) but this does not affect the SECM response as long as the distal part is straight. Some tin solder powder and one copper wire were obtained each time. The pulling parameters were adjusted to keep the inner diameter no more than 100 μm. This reduced greatly the workload to polish and sharpen a SECM probe. An ~12 mm length of 2 μm diameter Pt wire (Goodfellow Cambridge Ltd), coated with Ag to a diameter of 100 μm, i.e., Wollaston wire, was placed into the pulled capillary glass tube through the large open end. A hook was made at one end of the wire, which should touch the inner wall of the capillary so that the Pt wire is held with its straight end closest to the pulled end of the capillary. The silver coating on the Pt wire was etched away chemically by dipping the pulled end of the glass capillary into nitric acid (1:1 concentration, v/v) several times. This process was examined with a microscope (Metallographic Microscope, VWR, USA). The etched wire in the capillary was washed with copious deionized water, and then with acetone. The capillary tip was sealed using a Bunsen burner such that the distal end of Pt wire was straight in the tubing.

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were placed in the tubing from the open end and connection of the copper wire to the Pt wire was made using a heating gun.

The electrode tip was cut back to expose the Pt disc cross-section using wet Buehler alumina polish papers [3 μm pre-coated slurries, 4” diameter, PSA backed (self-adhesive); Buehler catalogue no. 69-3102] spinning on a used computer hard disk drive. The pre-coated pads were found to be easier to use than polishing cloths with manually added alumina slurries during the polishing processes. The disc electrode was finely polished consecutively with 0.3 μm and 0.05 μm alumina pads [Buehler, Fibrmet discs, codes 69-3104 (0.3 μm) and 69-3110 (0.05 μm)] on the polishing wheel until the tip end was smooth under the microscope.

Cyclic voltammetry was used to check the quality of the disc electrode. The forward and reverse voltammetric curves should almost overlap if the sealing is good and the electrode has a low capacitive current.

The glass sheath at the tip was sharpened with 0.3 μm pads using the polishing wheel—the electrode was kept rotating in order to trim the tip RG to 10 and keep the Pt disc in the centre of the glass sheath. Because the capillary was pulled prior to sealing, the initial RG should be in the range 10–40. As the polishing wheel spins very quickly, sharpening does not take very long. A lot of attention and patience are needed to carry out this step. An RG of 8–10 is very reasonable and is good enough for the following SECM experiments.

**Characterization of SECM electrodes**

The electrode surface was checked again under the microscope, especially the part around the Pt disc. Good contrast between the Pt disc and the glass sheath represents a good seal and no damage during polishing and sharpening. A cyclic voltammogram was used to inspect if the electrode had a good steady-state current, a reasonable current amplitude, and a low capacitive current (overlapping between forward and reverse scans). The negative feedback current (approach curve) was obtained by moving the tip towards a glass microscope slide. Theoretical approach curves with various RG values are available in the literature (Amphlett and Denuault, 1998; Shao and Mirkin, 1998). The experimental curve should overlap with the corresponding theoretical curve (i.e. one with an RG value similar to that estimated from the video micrograph).

**Custom-built SECM instrument**

The custom-built SECM instrument is composed of three major parts: the electrochemical system, the positioning system, and the active data acquisition system. In general practice, a potential was applied to the SECM probe and a current was measured by the electrochemical system (electrochemical analyser; CH 832A, CH Instruments, Texas, USA). The positioning and data acquisition systems were used to displace the probe and record the current and position data simultaneously. The data were displayed in situ. For instance, approach curves were obtained by plotting the current versus the displacement in the z direction, and images were acquired by plotting the current versus lateral motion. A spreadsheet file was saved after each experiment for further analysis. The electrochemical and positioning systems were built together in a Faraday cage to isolate external electrical noise.

**Cyclic voltammetry**

Voltammetry was carried out using a 2-μm-diameter SECM electrode versus an Ag/AgCl reference electrode, both immersed in the solution. The reference electrode also acted as the counter electrode, as only <1 nA current passed between the microelectrode and the counter electrode. Cyclic voltammograms were performed on the electrochemical analyser (CH 832A), which polarized the microelectrode with a voltage sweep. In the process of characterizing the electrodes, a solution containing 0.9 mM ferrocenemethanol and 0.1 M KCl as supporting electrolyte was used. The electrode showed a current plateau and the amplitude of the steady-state current should read around 0.27 nA. Equation 1 can be used to calculate the effective electrode radius:

\[
a = \frac{i_{z,0}}{4nFDe_0}
\]

where \(i_{z,0}\) is the steady-state limiting current of the electrode, \(n\) is the number of electrons transferred, \(F\) is the Faraday constant, \(D\) and \(c^0\) are the diffusion coefficient and concentration of ferrocenemethanol, respectively. The diffusion coefficient here was taken as \(7.8 \times 10^{-6}\) cm² s⁻¹ from a previous report (Miao et al., 2002).

**SECM experiments of plant leaves**

During each SECM experiment, one jar (containing two plants) was removed from the growth chamber and placed in close proximity to the SECM electrode. The laboratory air temperature was set to 20 °C and the SECM system was placed near a window so as to maximize ambient light and keep the plant photosynthetically active. While nutrient solutions were not aerated during SECM measurements, the time taken to analyse control and Cd-plants did not differ. An illustrative scheme of the SECM experimental system is shown in Fig. 1. The experimental leaves were illuminated with a deuterium-halogen lamp (model AvaLight-DH-S, Avantes, The Netherlands) on the adaxial (upper) side of the leaf. In probe approach curve (PAC) experiments, the current versus the moving distance toward a leaf was monitored at the electrode, which was biased at a potential to obtain a steady-state current for the reduction of oxygen in the solution. The destination normalized current was set gradually to a low value (e.g. 0.6) to avoid crushing the tip. An air-saturated aqueous solution containing 1 mM KCl as supporting electrolyte was placed on the abaxial (lower) surface of an inverted, attached in vivo leaf, which was sandwiched between a glass slide and a Teflon cylinder to constitute the electrochemical cell. A leaf area of 0.50 cm² was exposed to the solution in the cell (Fig. 1).

In constant-height imaging mode, the current was recorded versus lateral co-ordinates when the electrode was scanned in a plane at a fixed height in the vicinity of the stomata. The scanning speed of the stage was initially set at 100 μm s⁻¹ to move the electrode from one pixel to another pixel. The final scanning speed depended on a set of 0.56 to 0.65 mm and the area exposed to the supporting electrolyte solution (1 mM KCl) was 8 mm in diameter. The height of the electrolyte solution was 10 mm. The SECM probe consisted of a 2 μm Pt wire embedded in a glass sheath with 16 μm in diameter; i.e. the ratio of the sheath diameter to that of the Pt electrode (RG) was 8.

[Fig. 1. An illustrative scheme of the SECM experiments (not to scale). Leaf thickness ranged from 0.56 to 0.65 mm and the area exposed to the supporting electrolyte solution (1 mM KCl) was 8 mm in diameter. The height of the electrolyte solution was 10 mm. The SECM probe consisted of a 2 μm Pt wire embedded in a glass sheath with 16 μm in diameter; i.e. the ratio of the sheath diameter to that of the Pt electrode (RG) was 8.]
time in a closed-loop to reach a target position. In general, it took 5 min or 12 min to finish a 128×128 or a 256×256 pixel image, respectively.

Results and discussion

Electrochemical behaviour of oxygen at an SECM electrode

The reduction of O$_2$ in the air-saturated supporting electrolyte, containing 1 mM KCl, showed a sigmoidal voltammogram because of its hemispherical diffusion to the electrode. Since the results agree well with those reported by Mancuso et al. (2000), only relevant numbers are presented here. The steady-state current, termed $i_{T,\infty}$ in SECM, read 0.77 nA at an applied potential of −0.6 V. Note that the symbol $\propto$ means that the electrode was far away from the specimen (several electrode diameters). The measured current corresponds to an oxygen concentration of 0.20 mM according to equation 1, assuming that the diffusion coefficient for oxygen in the solution is 2.51×10$^{-5}$ cm$^2$ s$^{-1}$ (Mancuso et al., 2000).

Probe approach curves (PACs)

As the electrode, biased at the potential of −0.60 V in the same electrolyte solution, was driven toward the leaf by an inchworm motor in the $z$ direction, the diffusion-limiting current of the oxygen reduction was monitored versus the distance between the electrode and the substrate (PACs). The current decreased (so-called negative feedback) when the SECM probe moved toward the epidermal surface, but not when moved toward stomatal complexes, because the diffusion of the oxygen from the solution to the tip was blocked (Fig. 2). A theoretical PAC curve (Shao and Mirkin, 1998) is superimposed with the experimental one in Fig. 2. The probe was stopped when a normalized current (the ratio of actual tip current, $i_T$ to $i_{T,\infty}$) of 0.60 was reached. The normalized current corresponds to a tip-to-substrate distance of 1.3 μm according to the theoretical approach curves reported previously (Amphlett and Denuault, 1998; Shao and Mirkin, 1998). It can be observed that the experimental PAC fits very well with the theoretical one. This observation was repeatable in all the measurements (>100 as sample size) and indicates that the bulk leaf acts as an insulator substrate and that the release of oxygen from the bulk leaf is negligible.

SECM imaging of stomata

At a constant height of 1.3 μm, the SECM probe was rastered in the $x$, $y$ plane above the leaf by the closed-loop inchworm positioner system, and the tip current versus the lateral tip positions were recorded to obtain SECM images. Figure 3a shows a typical image of a control B. juncea leaf. The stomata remained open when they were illuminated and in contact with a 1 mM KCl electrolyte solution. The normalized current was observed to be <1 if the SECM tip was above the epidermal surface. By contrast, the current was >1 (positive feedback) when it was positioned above a stoma. This use of the probe to image the oxygen concentration profile generated through the stomata is termed the ‘substrate-generation and tip-collection mode’ in SECM (Bard, 2001).

About five stomata (4.6±1.3 in 10 replicate samples) were detected in an area of 150×150 μm. A typical SECM image is shown in Fig. 3a. These oval-shaped complexes were orientated randomly and were not adjacent to one another. These two characteristics agreed well with Bergmann’s first two rules of stomatal patterning (Bergmann, 2004): the formation through stereotype lineages of asymmetric divisions and local patterning where two stomatal complexes are never adjacent to one another (the one-cell-spacing rule). Baryla et al. (2001) reported a similar density and distribution pattern of stomata on a lower epidermis stripped off from the leaves of Brassica napus L. plants grown under control conditions.

As demonstrated for one leaf in Fig. 3b–e, four stomata were analysed individually by scanning the corresponding smaller areas (60×60 μm) where stomata were identified in Fig. 3a. Note that hysteresis and non-linearity on the images are minor, as a closed-loop positioning operation was employed, where a system integrated with an encoder in the inchworm motors and its counting circuitry in the controller applies a voltage to move the motors, measures the actual motion, and adjusts to target positions. The typical aperture size of a single stoma in control plants was found to be 21.8±1.1 μm in length and 7.8±0.4 μm in width, averaged from 44 stomata. The guard cells had a narrow ring thickness, indicating a maximum aperture. The stomatal complexes had a width of 15.5±0.5 μm.
SECM is a non-invasive analysis method and offers chemical reactivity images and can also provide the topography of the sample. For instance, Fig. 3f demonstrates the oxygen concentration profile across one stomatal complex (shown in Fig. 3e). While the oxygen concentration reaches a maximum above the centre of the complex, kidney-shaped guard cells surrounding the aperture had a lower current, or a higher topography, relative to the epidermal surface. The guard cells formed two bumps, which blocked oxygen diffusion to the electrode from the solution more than did the epidermal surface. A more negative feedback was observed above the two guard cells, according to the SECM principle. This confirms that guard cells are swollen during the oxygen evolution process and can be seen clearly from a cross-section as shown in Fig. 3f. This point will be returned to later in the paper.

Stomatal density was reduced in plants treated with 0.2 mM CdCl₂; 2.5 ± 0.2 stomata were found per 150×150 μm area with 12 replicates. A typical image with two stomatal complexes is shown in Fig. 4. The fact that Cd-induced stress caused a decrease in the number of stomatal complexes in the SECM images implies two possibilities:

1. The metal may inhibit the division of guard cell precursors, meristemoids, their differentiation into a guard mother cell, and/or the final division of a guard mother cell into two paired guard cells, according to the model of cell replication in the plane of the epidermis (Bergmann, 2004). Barcelo et al. (1988) reported that Cd negatively affected leaf expansion; however, the link between Cd-toxicity and epidermal differentiation to stomata, either along the developmental pathway or in the expression of any of the corresponding genes discussed in a recent review (Bergmann, 2004), is unclear. It has also been reported (Baryla et al., 2001) that Cd caused a decrease in density of mesophyll chloroplasts, organelles containing chlorophyll, and an increase in the size of mesophyll cells (primary site of photosynthesis), which are located below stomata. Interactions between mesophyll cells and the epidermis can influence stomatal development (Bergmann, 2004), but the role of Cd in this interaction is unknown. It is also possible that Cd induces changes in the intracellular environment, which, in turn, may

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Fig. 3. Surface plots of tip current recorded at a 2 μm diameter Pt microelectrode during an image scan of a 150×150 μm area of epidermis (a). (b–e) Zoomed-in images of the individual stomata in areas of 60×60 μm. Relatively high concentrations of oxygen are represented in blue; raised topographical features are in red. The normalized current (\(i_T/i_{T,max} = 0.77 \text{nA}\)) ranges represented by the colour bar profile (0–10) are 0.52–1.22, 0.99–1.12, 0.40–1.19, 0.62–0.89, and 0.23–1.69 for (a), (b), (c), (d), and (e), respectively. (f) A cross-sectional scan across the stoma in (e). The ultramicroelectrode tips were biased at −0.60 V. The tip-to-substrate distance, \(d\), was initially set at 1.3 μm. The solution was 1 mM KCl. Current shown here is normalized by the infinite tip current (\(i_{T,max}\)) when the ultramicroelectrode was far away from the substrate. The scale of \(x, y\) position was in micrometres. It took 5 min to obtain an image.

Fig. 4. SECM images (a–c) of a leaf from a plant treated with 0.2 mM Cd. Relatively high concentrations of oxygen are represented in blue; raised topographical features are in red. The normalized current (\(i_T/i_{T,max} = 0.77 \text{nA}\)) ranges represented by the colour bar profile (0–10) are 0.52–1.22, 0.99–1.12, and 0.40–1.19 for (a), (b), and (c), respectively. (d) A cross-sectional scan across the stoma in (c). Other experimental conditions are the same as in Fig. 3.
control stomatal development (Bergmann’s third rule of stomatal pattern; Bergmann, 2004).

(2) Fewer stomata were open in Cd-treated plants. Because the SECM technique allows increased concentrations of oxygen to be used to detect stomatal apertures, closed stomata would not be found by this technique. Indeed, Barcelo et al. (1988) reported that, under control conditions, 42% of stomata in bush bean (Phaseolus vulgaris L.) plants were closed, whereas 67% of the stomata of Cd-treated plants were closed. However, based on light transmission microscopy, Baryla et al. (2001) reported 42% fewer total stomata (open and closed) in Cd-treated B. napus compared with control plants. While closed stomata may have been missed using SECM, it is likely that the Cd-treated plants in the present study did have fewer stomata than the control plants. It may be important to note that it was not tested whether the stomata were functional; SECM was simply used to monitor oxygen profiles above open stomata.

In Fig. 4c, the structural detail of a stomatal complex is clear. The length of the stomatal complex (22.2±1.1 μm; mean ±SE, n=12) appeared unaffected by Cd-treatment (t-test, P=0.87) whereas the width of the stomatal complex (18.5±1.1 μm) increased in response to Cd-treatment (t-test, P=0.07). These results agree well with those obtained with light transmission microscopy of a lower epidermis stripped off from leaves of B. napus (Baryla et al., 2001); Cd induced a lower density of slightly larger stomatal complexes. The width of the aperture (6.2±0.4 μm) decreased in response to Cd-treatment (t-test, P=0.05), agreeing with Barcelo et al. (1988) who reported a Cd-induced decrease in stomatal aperture in P. vulgaris using transmission electron microscopy.

It is worth noting that the steady-state current above the guard cells decreased dramatically relative to the epidermal surfaces as illustrated from the concentration profile across stomatal complex 2 in Fig. 4c. Two electrochemical currents, 0.23 and 0.62, were read when the SECM tip was above a guard cell and above the epidermis, respectively (Fig. 4d). The distance from the tip to the cell (d1, 0.4 μm) and the distance from the tip to leaf (d2, 1.3 μm), corresponding to the two currents, respectively, were read from the theoretical approach curve for negative feedback in Fig. 2. The guard cell height was then the difference of the two distances:

\[ \Delta d = d_2 - d_1 = 0.9 \mu m \]

The difference in oxygen evolution above the stomatal complexes in Cd-treated and control plants was very pronounced as determined from the SECM images. This can be elucidated by determining the current above the centre of a stoma or by estimating the flux of oxygen through a stomatal pore per unit time. Qualitatively, the maximum electrochemical current, which represents the maximum oxygen concentration, above a stoma under Cd stress (normalized current 1.10 shown in Fig. 4d, equivalent to \( i_{\text{max}}=0.85 \text{ nA} \)) was 69% of that above a control stoma (normalized current 1.60 read from Fig. 3f, \( i_{\text{max}}=1.23 \text{ nA} \)), although the current in both cases was higher than that above the epidermal bulk. Using equation 1, the concentration of oxygen above the epidermal surfaces was calculated to be 0.15±0.02 mM, which is apparently lower than that in the bulk solution. This is because the electrode itself partially blocked oxygen diffusion at the probe-to-epidermal distance of 1.3 μm. Concentrations of oxygen above stomatal complexes are shown in Table 2. They were always higher than 0.15 mM due to the extra oxygen flux from stomata to the probe. During the course of this experiment, it was discovered that the readings above a single stoma varied slightly over time, probably due to narrowing (but not closing) of the stomatal aperture. Time dependence of oxygen profiles above the control stomata seemed more evident than above those under Cd stress. Nonetheless, the concentrations of oxygen above stomata from control plants were consistently higher than those from Cd-treated plants. Examination of the raw data reveals that 31 of the 43 control stomata had concentrations of oxygen higher than the mean value for Cd-treated plants (i.e. higher than 0.235 mM). In addition, 14 of the 43 control stomata had concentrations of oxygen higher than any reported for Cd-treated plants (i.e. higher than 0.281 mM). The maximum concentration of oxygen above a Cd-treated stoma was 73% of the maximum concentration above a control stoma (Table 2). A simulation is being developed to obtain quantitative information on the oxygen flux out of a stomatal aperture. Until it is possible to be sure that the readings taken are not being influenced by fluctuations in the width of open stomata while the leaf is in the experimental cuvette, statistics on oxygen concentrations cannot be performed reliably. The authors are confident, however, that the numbers reported herein confirm Cd-induced reduction in oxygen evolution.

The swollen state of all guard cells under illumination indicated that the guard cells were open in both control and treated plants. While the stomatal aperture of Cd-treated plants was 80% that of control plants (hence one would expect ~20% less stomatal conductance), average oxygen evolution above these stomata was reduced by ~30%, indicating that factors other than stomatal aperture

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<th>Table 2. Concentration of oxygen (nM) above control and Cd-treated stomata of Brassica juncea</th>
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contributed to decreased photosynthetic yield. Visible leaf chlorosis of plants treated with Cd supports this conclusion. As previously reported (Baryla et al., 2001), chlorophyll fluorescence emission, representing a direct measure of the efficiency of photosystem II, decreased in intensity while under Cd-stress conditions; however, this deficiency was caused primarily from the decrease in chlorophyll concentration rather than impaired photosynthetic proteins or electron transfer. Combining this result with those from the SECM experiments, it appears that Cd interferes with chloroplast replication as well as epidermal cell division and differentiation (fewer stomata), both of which could lead to reduced photosynthesis. Direct toxic effects of Cd on photosynthetic proteins and enzymes would further reduce photosynthetic efficiency.

The present SECM images are very straightforward in the measurement of net photosynthetic efficiency from single stomatal complexes in terms of oxygen release. In a previous report (Baryla et al., 2001), control plants and Cd-treated plants could not be distinguished on the basis of their oxygen evolution activity per unit leaf area, which was measured with a Clark-type electrode under conditions of high CO₂ and at different photon flux densities (Baryla et al., 2001). SECM provided reactivity and topography information for an individual stoma, while the classic electrochemical method measured global oxygen evolution. It is difficult to miniaturize a Clark-type electrode and to bring it in close proximity to a stoma. Oxygen fluxes in Olea europaea L. leaves and roots were reported (Baryla et al., 2001) using a vibrating Pt microelectrode. Two oxygen concentrations were, in fact, measured by moving the electrode up or down 10 µm against the specimen in one lateral electrode position and the fluxes were calculated using Fick’s first law of diffusion. Baryla et al. (2001) succeeded in mapping and measuring the patterns of net influxes as well as effluxes of oxygen in the plants. However, these authors did not give the details on how to bring the probe close to the substrates. Clearly, SECM provides an excellent tool for examining physiological processes at the cellular level. In those cases where tissue- or organism-level processes are of interest, alternative techniques should be employed. There is no expectation that the results of SECM can or should be extrapolated to larger scales.

**Conclusion**

In summary, in-vivo measurement of oxygen evolution from single stomata in both Cd-stressed and stress-free B. juncea plants has been described. Stomatal density and oxygen evolution was noticeably reduced in Cd-exposed leaves. A Cd-induced decrease in the size of the pore aperture and an increase in the overall stomatal complex have been observed. SECM has provided a simple and more immediate way to assess the net photosynthetic efficiency of individual stomata. Conventional measurement of chlorophyll fluorescence also permits rapid assessment of photosystem II efficiency, but at a much larger scale.

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