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Communication

Kinetic Analysis of Resistance to Paraquat in *Conyza*[1]

EVIDENCE THAT PARAQUAT TRANSIENTLY INHIBITS LEAF CHLOROPLAST REACTIONS IN RESISTANT PLANTS

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ABSTRACT

Paraquat resistance has been claimed to be due to a sequestration of the herbicide before it reaches chloroplasts. This is based on the sensitivity of photosystem I in isolated thylakoids to paraquat, and autoradiographic analyses showing label from paraquat near veins 4 hours after treatment of a resistant biotype. Conversely, the enzymes of the superoxide detoxification pathway were found to be at constitutively elevated levels in intact class A chloroplasts of the resistant biotype of *Conyza bonariensis* (L.) Cronq. Evidence is presented here that physiologically active levels of paraquat rapidly inhibit chloroplast function in both the resistant and sensitive biotype, before the first sequestration was visualized. This inhibition is transient (completed in 2 hours) in the resistant biotype and irreversible in the sensitive type. Intact class A chloroplasts of the resistant biotype with or without paraquat are less susceptible to photoinduced membrane damage than the sensitive biotype without paraquat, as measured by ethane evolution. These data support a hypothesis that the ability to prevent superoxide damage keeps the resistant biotype viable while paraquat or its metabolites are being sequestered.

Paraquat interacts with PSI of photosynthesis, accepting an electron, and the activated paraquat immediately reacts with dioxygen forming toxic superoxide (9, 17). Two theories have been promulgated for the mode of resistance to paraquat that has recently evolved in *Conyza* spp. and *Hordeum* sp. These theories need not be mutually exclusive and may be complimentary in a sequential manner. Based on two types of observation with paraquat resistant biotypes of *Conyza* bonariensis (6, 19), *C. canadensis* and *C. philadelphicus* (18) and *Hordeum glaucum* (1) it was suggested that the primary mode of resistance is a sequestration of paraquat that occurs before it reaches the chloroplasts. The evidence supporting sequestration is as follows: (a) PSI activity in the isolated thylakoids of the resistant biotypes is as sensitive to paraquat as the wild type; (b) radioactivity measured 4 h after application of paraquat to cut-petioles is localized near the vascular bundles (1, 6) or the leaf base (18) in the resistant biotypes, and throughout the leaf in the sensitive biotypes. These findings were interpreted as a sequestration of paraquat which prevents its transport to the chloroplast. No evidence was presented showing that intact Class A chloroplasts were not resistant to paraquat, or that radioactivity was still in paraquat and not in a catabolite of paraquat. No one has described a cell wall fraction that bound paraquat, but it was presumed to be bound to walls. Paraquat is usually sprayed on plants and not applied through cut stems. Furthermore, the light fluence rates used (6, 18) were one-tenth or less than those in field conditions. Paraquat requires high light intensity for phytotoxicity and is less active on cloudy days (17). Sequestration studies with *Conyza* spp. were without kinetics, at 4 h after application. Paraquat sensitive plants are usually in the 'throes of death' or are dead by that time.

The other explanation for resistance to paraquat (14) is that there is an elevation of the enzymes of the superoxide detoxification pathway proposed by Halliwell and Asada (5, 10) based on theirs and previous work (8, 12). Class A chloroplasts of the resistant biotype had constitutively higher activity levels of superoxide dismutase, ascorbate peroxidase, and glutathione reductase (14), enzymes that catalyze parts of this pathway. The latter two enzymes of this pathway can assist in the detoxification of other active oxygen species that are quenched by ascorbate and glutathione, by continually recycling them to the reduced form. The reductant is photogenerated NADPH. Resistance is nuclearly, monogenically, dominantly inherited, and the high levels of superoxide dismutase and glutathione reductase proteins did not segregate away from resistance in the F_2 generation (13; Y Shaaltiel, N Chua, S Gepstein, J Gressel, unpublished data). The paraquat resistant *Conyza bonariensis* biotype had a modicum of cross-tolerance to other active-oxygen generating herbicides such as atrazine and acifluorfen as well as to SO_2 (13; Y Shaaltiel, A Glazer, J Gressel, unpublished data). Selective sequestration of all these xenobiotics is a less logical primary explanation than a general enzymatic ability to detoxify the active-oxygen species generated. Evidence that paraquat and oxidant tolerances are related to levels of the enzymes of the superoxide detoxification pathway come from many other sources: enhanced glutathione reductase levels have been correlated with both water-stress and paraquat tolerance in cotton (2). *Escherichia coli* with superoxide-dismutase genes deleted has extreme hypersensitivity to paraquat (3), and human cells with an added superoxide dismutase gene have increased tolerance to paraquat (4). Plant cell cultures selected with elevated paraquat tolerance had higher levels of superoxide dismutase (7).

Paraquat only siphons off part of the electrons transferred in PSI. The detoxification of the superoxide formed is 'expensive' (9). It can be calculated that for every mol of electrons that paraquat 'steals' from PSI, 0.5 mol of photogenerated NADPH is required to regenerate the glutathione and ascorbate utilized in detoxifying each mol of H_2O_2 formed from the superoxide.

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Thus, a plant would be indefinitely inhibited at paraquat levels that trap all electron flow, as NADPH would not be available to reduce the glutathione. Little is published about how, or where, or in what form paraquat is dissipated (17) as there had been no known resistance. We present evidence that paraquat is not immediately sequestered; it rapidly inhibits the chloroplasts of both sensitive and resistant plants. This evidence supports the hypothesis that the high constitutive levels of the enzymes of the active oxygen detoxification pathway (14) keep the resistant plants alive while paraquat is being actively sequestered. Dead plants cannot sequester paraquat. The kinetic data show that paraquat does affect the chloroplasts, but the inhibition is lost with time.

MATERIALS AND METHODS

Plant Material. Seeds of the paraquat sensitive (wild) biotype of *C. bonariensis* (L.) Cronq. (synonym *C. linifolia*) were gathered near Alexandria, and seeds of the resistant biotype were from the Tahrir irrigation district in Egypt where paraquat resistance evolved, as previously described (14). Seeds were germinated in a growth room with a 14 h light period at 0.15 mE m^{-2} s^{-1} at 25 ± 2°C. Commercial paraquat (ICI Plant Protection Ltd) was formulated with 0.25% Tween-20 for spraying. Two plants per treatment were sprayed to run-off with either formulated 0.1 mM paraquat or with formulants. Plants were then placed under relatively cool metal halide lamps with added air circulation in the growth room. The fluence rate at plant level was 0.54 mE m^{-2} s^{-1} (PAR). Controls were sprayed with Tween-20 alone. Whole leaves from paraquat sprayed and mock sprayed plants were put in the $^{14}CO_2$ fixation chamber at each of the times indicated after spraying. Fully expanded, 4 to 8 cm long leaves from plants in the rosette stage were used in all experiments.

CO_2 Fixation. Detached leaves were placed on wet filter paper in a sealed chamber with 3 μCi $NaH^{14}CO_3$ (55 Ci/mol). A vast excess of tartaric acid was remotely dripped on the $NaH^{14}CO_3$ to release $^{14}CO_2$. Leaves were illuminated with 0.15 mE m^{-2} s^{-1} (PAR) for 10 or 20 min. The leaves were then placed in counting vials and 80% acetone was added, and the mixture put under strong light to photobleach pigments and evaporate the acetone. A scintillation mixture of Lumax:xylene (45:55) was added to the bleached leaves and unevaporated water. The samples were counted and the data expressed as radioactivity per unit leaf area. Area was measured with a planimeter (Numonic Digitizer 1224) from photocopies of the leaves made just after photosynthesis measurements.

Stomatal aperture was measured with a LiCor LI 700 transient porometer.

Class A chloroplasts were isolated from leaves of plants placed for 48 h in the dark (to decrease starch grain size) according to Slovacek and Hind (16) except that 50 mM Mes (pH 7.2) was used instead of the pyrophosphate in the chloroplast isolation medium (11). The chloroplast isolation medium contained 40 mM buffered ascorbate to inhibit the effects of the high levels of phenolic compounds in the leaves. The chloroplasts were resuspended in 2 ml of 0.36 M sorbitol brought to pH 7.5 with solid Tris (Sigma) following previously described procedures (11), and centrifuged at 2400*g* for 10 s. The pellet was resuspended in the same sorbitol-Tris buffer.

Membrane deterioration was measured after incubation of Class A chloroplasts in the presence or absence of 10 μM paraquat. Eighteen ml of (40 μg ml^{-1} Chl) chloroplasts were constantly stirred in 20 ml tubes and illuminated with 1.3 mE m^{-2} s^{-1} (PAR) light in a cooled water bath at 20°C for 3 h. Membrane degradation products were estimated as ethane using a Packard 419 gas chromatograph with a 152 × 3 mm, 120 mesh activated alumina column. One ml was injected. The temperatures were as follows: oven, 30°C; detector, 100°C; injector, 110°C. The N_2 flow rate was 5 ml min^{-1}. This system completely resolved pure standards of ethane (evolving at 2.32 min), and ethylene (at 3.04 min), in these conditions. The data were calculated from a standard curve derived by serial dilution of pure ethane into air under atmospheric pressure.

RESULTS AND DISCUSSION

A kinetic analysis was performed to ascertain whether paraquat applied to leaves reaches the chloroplasts, using photosynthetic $^{14}CO_2$ fixation as the indicator. This was done by spray applications to plants and measuring photosynthesis of excised leaves at various times after application (Fig. 1). The differentiating dose of 0.1 mM paraquat partially inhibited photosynthetic CO_2 fixation in both sensitive and resistant leaves as rapidly as could be measured (Fig. 1). The sensitive leaves irreversibly stopped their photosynthesis within 2 h (Fig. 1). This same paraquat dose rapidly (within 0.5 h) and severely inhibited whole leaf photosynthesis in resistant plants. The resistant leaves later recovered. This indicates that paraquat must have penetrated leaf tissue and affected the chloroplasts within this half-hour. $^{14}CO_2$ fixation could have been suppressed if paraquat caused stomatal closure of the resistant plants. We therefore measured stomatal aperture at the critical times during the experiment. In no case was the stomatal aperture resistance of the resistant plants more than 9 s cm^{-1} showing that the stomates were never sufficiently closed to impede CO_2 diffusion (Fig. 2). The only case of closure was one of the severely inhibited sensitive plants: the resistivity at 2 h and later was more than 11 s cm^{-1}. The dying sensitive plants

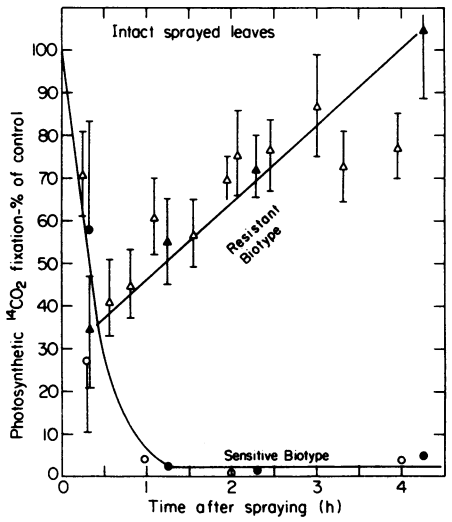

FIG. 1. Rapid and transient effect of paraquat on CO_2 fixation in leaves of paraquat resistant *Conyza bonariensis*. Both paraquat sensitive (○, ●) and resistant (△, ▲) biotypes of *Conyza bonariensis* were sprayed with 0.25% Tween 20 solution (mock control) or with this solution containing 0.1 mM paraquat. The time points are at the half-time of the incubation with $^{14}CO_2$. Open and closed symbols denote two separate experiments. Photosynthetic $^{14}CO_2$ fixation was measured for 20 min in the experiment with closed symbols and for 10 min in the experiments with open symbols. Control and treated leaves of both biotypes were placed in the chamber for measurement at each of the time points and the data are given as percents of the control of that time point. The control values varied slightly for each time point and averaged 4300 dpm cm^{-2} leaf surface in the 10 min experiment and 9900 dpm cm^{-2} leaf surface in the 20 min experiment. There were no significant differences between the Tween-20 sprayed resistant and sensitive controls, nor was there any significant effect of the Tween-20 alone, with time, on either biotype.

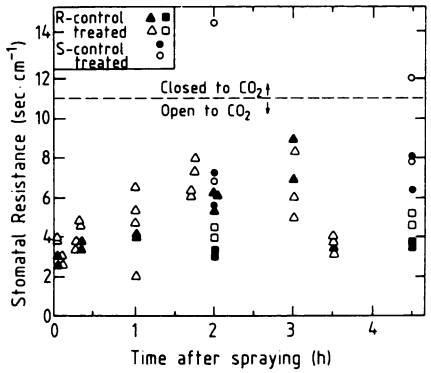

FIG. 2. Lack of effect of paraquat on stomatal aperture in treated paraquat resistant plants. Each point represents an average of the readings of five separate leaves on one plant. The results are of plants from two separate experiments shown in Figure 1.

had decreased stomatal aperture, probably due to membrane damage and loss of water from the guard cells. These experiments clearly demonstrate that (a) paraquat can and does affect the chloroplasts of the resistant plant, and (b) the resistant plants recover from the effect(s) of paraquat. This effect of paraquat on the chloroplasts is in contrast to the predictions of the sequestration hypotheses, if sequestration was the primary mechanism of resistance (1, 6, 18, 19). Primary sequestration should have blocked paraquat or a detoxification product of paraquat, from inhibiting $^{14}CO_2$ fixation (Fig. 1). An integration of the data on sequestration 4 h after treatment (1, 6, 18, 19), and our data (Fig. 1) suggests that paraquat can be sequestered in the leaves of only those resistant plants that managed to stay alive, despite the transient paraquat effect at the chloroplasts.

We measured membrane damage in paraquat treated class A chloroplasts from resistant and sensitive plants to further demonstrate that the inhibited chloroplasts from the resistant plants can withstand the effect of paraquat. Chloroplasts from the paraquat sensitive biotype naturally (without paraquat) evolve three times more ethane than the resistant biotype, 34.9 *versus* 12.2 pl ethane mg Chl^{-1} at 1.3 mE m^{-2} s^{-1} over a 3 h duration. Thus, the plastids from the paraquat-sensitive plants seem to be naturally more susceptible to the natural photodamage occurring as a byproduct of photosynthesis. This indicates that the active oxygen detoxification pathway is constitutively less active in the sensitive biotype, as has been shown by direct enzyme activity assay (14). The results with 10 μM paraquat clearly show that Class A chloroplasts from the resistant plants remain relatively undamaged evolving 24.4 pl ethane mg Chl^{-1}, still a lower level than in the sensitive biotype chloroplasts without paraquat (34.9 pl mg Chl^{-1}). The paraquat treated sensitive plastids evolved three times more ethane (107.8 pl mg Ch^{-1}), than the resistant ones, a sign of considerable damage. This increase indicates again that paraquat reached and affected the resistant chloroplasts but not enough to cause damage. Similar results were achieved in a longer duration experiment (12 h) at a lower fluence rate (0.9 mE m^{-2} s^{-1}). The data on ethane evolution clearly suggest that the superoxide detoxification pathway that is at constitutively higher levels in the resistant biotype chloroplasts (14), can prevent damage by superoxide, or by the hydroxyl radicals generated from it by reaction with superoxide dismutase greatly enhance the effect of paraquat (15), supporting the importance of this pathway. The kinetic data in Figure 1 and the data on ethane evolution together with the autoradiographic evidence (1, 6, 18), indicate that: (a) paraquat sequestration or detoxification can only come about after paraquat temporarily inhibits chloroplast activity, and (b) that the high constitutive levels of chloroplast enzymes (14) protect the plant from paraquat damage, while paraquat is being detoxified. Thus, the internal chloroplast protection reactions have temporal primacy over sequestration. This is by virtue of the high genetic constitutiveness of the enzyme levels in the resistant biotype, which allows rapid protection of resistant plants, until paraquat sequestration, which can only be measured at 4 h (6).

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