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# Dominant pleiotropy controls enzymes co-segregating with paraquat resistance in *Conyza bonariensis*

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**Summary.** The genetics of paraquat-resistance in *Conyza bonariensis* was studied. Reciprocal crosses were prepared between resistant and sensitive individuals. The enzymes of the pathway that detoxifies superoxide to innocuous oxygen species involved in resistance were evaluated in the $F_1$ and $F_2$ generations. All $F_1$ plants were as resistant as the resistant parent, irrespective of parental sex, demonstrating dominance and excluding maternal inheritance. The activities of superoxide-dismutase, ascorbate-peroxidase and glutathione-reductase in the $F_1$ were constitutively as high as in the resistant parent. Resistance in the $F_2$ generation was distributed in a 3:1 ratio (resistant to sensitive). Leaves from $F_2$ plants were removed for a resistance assay and enzyme immuno-assays of single plants were performed. The high levels of superoxide-dismutase and glutathione-reductase, the two enzymes for which antibodies were available, were similar in resistant individuals to the levels in the resistant parent; the levels were low in the susceptible individuals. These results indicate either a very tight linkage, or more probably, that one dominant nuclear gene controls resistance by pleiotropically controlling the levels of enzymes of the active-oxygen detoxification pathway.

**Key words:** *Conyza bonariensis* – Superoxide-dismutase – Glutathione-reductase – Ascorbate-peroxidase – Paraquat resistance – Oxygen-radical detoxification

## Introduction

Paraquat-resistant *Conyza* biotypes evolved in Egypt after several years of multi-annual paraquat treatments. The resistant biotype of *Conyza bonariensis* has a 100-fold higher $I_{50}$ to paraquat than the wild type (Fuerst et al. 1985; Shaaltiel and Gressel 1986). A biotype of *Erigeron philadelphicus* L. (synonym *Conyza philadelphicus*) that evolved resistance also has a 50–100 times $I_{50}$ shift (Watanabe et al. 1982). Photosynthetic system I (PSI), the target of paraquat disruption, was equally inhibited in isolated thylakoids of plastids from resistant and sensitive plants (Fuerst et al. 1985). Three enzymes of the metabolic pathway detoxifying oxygen radicals (Foyer and Halliwell 1976; Nakano and Asada 1981) are elevated in the intact Class A chloroplasts of the resistant biotype (Shaaltiel and Gressel 1986). These three enzymes of the "Halliwell-Asada" pathway act as follows: superoxide-dismutase dismutates superoxide forming hydrogen-peroxide; ascorbate peroxidase detoxifies the peroxide preventing the formation of hydroxyl radicals after reaction with iron salts, glutathione reduces the dehydroascorbate formed back to ascorbate; glutathione-reductase uses NADPH to reduce the oxidized glutathione back to the reduced form. These enzymes are usually localized in chloroplasts, although isozymes of superoxide dismutase are also found in the cytoplasm. Ascorbate-peroxidase and glutathione-reductase were found exclusively in chloroplasts (Gillham and Dodge 1986).

Resistance to atrazine, a herbicide specifically inhibiting photosynthetic system II, is maternally inherited on the chloroplast genome (Souza-Machado 1982; Goloubinoff et al. 1984). We therefore performed reciprocal crosses and selfed the $F_1$ plants to ascertain dominance, lack of maternal inheritance and that a single gene was involved. As other mechanisms have been proposed to explain paraquat resistance (Fuerst et al. 1985; Vaughn and Fuerst 1985), it was necessary to ascertain that the constitutively elevated enzyme

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levels co-segregated with resistance through the $F_1$ and $F_2$ generations, although only two of the enzymes could be checked in the $F_2$ generation. This information may be crucial for developing protocols to transfer this resistance via genetic engineering to crops.

## Materials and methods

### *Plant material*

Achenes of *Conyza bonariensis* (L.) Cronq. (synonym *C. linifolia*) were originally gathered near Alexandria and achenes of the resistant type were gathered from the Tahrir irrigation district in Egypt where paraquat-resistance evolved (Gressel et al. 1982). Paraquat-resistance was confirmed by Dr. M. Parham, ICI (UK) who propagated achenes that he kindly provided. Taxonomic classification was according to Feinbrun-Dothan (1978). Seeds were germinated in a growth room under a 14 h light period at 25 °C ± 2 °C.

### *Hybridizations*

The composite flowers of *Conyza bonariensis* contain an inflorescence composed of easily distinguishable female flowers on the outside and hermaphrodite flowers in the center. The hermaphrodite flowers were excised while immature in the female parent. The effectivity of emasculation was checked by covering the inflorescence in a bag and checking the lack of germination of the resulting achenes. The outer female flowers were pollinated with a ripe inflorescence from the desired biotype by touching the two. Reciprocal crosses between resistant and sensitive biotypes were performed to obtain the $F_1$ generation. Fifty $F_1$ plants from each of the reciprocal crosses were selfed and left to produce $F_2$ achenes. The achenes of each reciprocal cross were separately bulked.

### *Paraquat treatment*

Fully expanded, 8–10 cm long leaves from plants in the rosette stage were used in all experiments. Commercial paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) ICI Plant Protection Ltd was formulated with 0.1% Tween-20 for incubation with 7 mm diameter leaf discs placed with their upper surface down in 24-well cluster-dishes with 1 ml paraquat solution in each well. The leaf discs were incubated for 1 h in the dark and then 4 h in 0.3 mE $m^{-2}$ $s^{-1}$ (PAR) light from a Mazda 400 watt metal-halide lamp at 25 °C.

### *$CO_2$ fixation*

The cluster dishes containing leaf discs were placed, after removing the solution, in a sealed transparent plastic chamber. Tartaric acid was remotely mixed with $^{14}C$ sodium bicarbonate (5 μCi) to release $^{14}CO_2$. Leaf discs were illuminated with 0.15 mE $m^{-2}$ $s^{-1}$ (PAR) for 30 min and transferred into vials containing 80% acetone and then put under strong light to photobleach pigments and evaporate the acetone. A scintillation mixture of Lumax:xylene (45:55) was added to the leaves and remaining water. The samples were counted and the data expressed as percent of control.

### *Isolation of intact (Class A) chloroplasts*

As we are dealing with soluble stromal enzymes and this species contains high levels of phenolic compounds that preclude measuring enzyme activities in crude extracts, it was necessary to first isolate intact chloroplasts. Chloroplasts were isolated from 10–20 plants in the rosette stage of growth and kept in the dark for 48 h beforehand to decrease the starch levels. The method of of Slovacek and Hind (1977) was used except that 50 m*M* MES[2-(N-morpholino)-ethane-sulfonic acid] containing 0.1% bovine serum albumin and 10 μ*M* *p*-chloromercuri-benzenesulfonic acid replaced the pyrophosphate in the chloroplast isolation medium. The chloroplasts were re-suspended in 2 ml of 0.36 M sorbitol brought to pH 7.5 with solid Tris-HCl (Sigma) as per Nakatani and Barber (1977) and centrifuged at 240 × g for 10 s. The pellet was re-suspended in the same sorbitol-Tris-HCl buffer. Intactness was assayed in an aliquot of the chloroplasts by measuring ribulose-bis-phosphate-carboxylase activity in the chloroplast fraction and in the supernatant (Bjorkman 1968). Chlorophyll content was measured in 80% acetone (Arnon 1949). The washed chloroplasts were broken with a French pressure cell for enzyme measurements immediately after isolation. Protein was determined in the stromal fraction by the method of Bradford (1976).

### *Enzyme activity assays in parents and $F_1$ offspring*

*Superoxide-dismutase (EC 1.15.1.1).* Stromal extracts were separated on 10% polyacrylamide gels by the method of Laemmli (1970), but without sodium dodecyl sulfate on 2 mm thick and 20 cm long gels. The gels were run at a constant voltage of 120 mV for 14 h at 4 °C. The gels were permeated with riboflavin and illuminated to generate superoxide that was visualized by nitro-blue tetrazolium not turning blue where there was superoxide-dismutase activity (Beauchamp and Fridovich 1971). Superoxide-dismutase activity was quantified by running known levels of superoxide-dismutase (ex bovine blood, Sigma) on the same gel. Superoxide-dismutase units were defined as per McCord and Fridovich (1969). The gels were scanned in a Beckman DU-8 spectrophotometer at 560 nm. The peak areas were measured and related to the superoxide-dismutase standard curve, which was linear up to 0.2 enzyme units.

*Glutathione-reductase (EC 1.6.4.2)* activity was spectrophotometrically measured in stromal extracts by following the decrease in absorption at 340 nm due to the specific oxidation of NADPH by oxidized glutathione using the method of Foyer and Halliwell (1976).

*Ascorbate-peroxidase* activity was measured by following oxidation of ascorbate to dehydroascorbate in a spectrophotometer at 290 nm as outlined by Nakano and Asada (1981). No change in absorption was seen in the absence of ascorbate.

### *Immunoassays of enzyme levels*

*Purification of chloroplast superoxide-dismutase and preparation of specific antibodies.* Intact chloroplasts from primary leaves of oats (*Avena sativa* cv Victory) were isolated according to the method of Kamieniezki and Nelson (1975). The chloroplasts were lysed in 10 m*M* potassium phosphate buffer pH 7.8 containing 10 μ*M* EDTA. The resultant lysate was centrifuged for 10 min at 900 × g and the pellet was discarded. Purification of the enzyme was carried out as described by Reiss and Gershon (1976). The active fractions of the superoxide-dismutase were pooled, dissolved in 5% sodium-dodecyl-sulfate and 2.5% 2-mercaptoethanol and separated by electrophoresis under denaturing conditions with sodium-dodecyl-sulfate-on polyacrylamide gels (15% separation gel) overnight at 8 mamp/gel. Staining with Coomassie brilliant blue revealed only one band corresponding to the monomeric superoxide-dismutase at ca

16,000 daltons. This band was excised from the gel for protein electroelution as described previously (Nelson 1983). The electroeluted polypeptide (0.3 mg) was mixed with an equal volume of Freund's complete adjuvant and subcutaneously injected into rabbits. A booster injection given 4 weeks post injection contained 0.3 mg of purified superoxide-dismutase. The rabbits were bled 14 days later and the antisera were stored at −20 °C.

Total *Conyza* cell extracts were electrophoretically separated as described above without sodium-dodecyl-sulfate. They were then suspended for 30 min in small aliquots of buffer containing 2.3 g sodium-dodecyl-sulfate, 8 ml glycerol, 0.75 g Tris-HCl, 0.5 ml 2-mercaptoethanol, per 90 ml distilled water, at pH 6.8 to open the native structure of the protein, as was done in the antibody preparation (A. Zilberstein, personal communication). We performed Western blots on nitrocellulose according to Gershoni et al. (1985). The nitrocellulose was treated for antibody reaction according to Gershoni and Palade (1982).

*Antibody to glutathione-reductase* was prepared as follows: Spinach glutathione-reductase was purchased from Sigma and further purified by sodium-dodecyl-sulfate gel electrophoresis. Antibodies were raised in rabbits by injection with purified protein by standard techniques.

Total *Conyza* cell extracts were electrophoretically separated on polyacrylamide gels containing 1% 2-mercaptoethanol in the running buffer according to Halliwell and Foyer (1978). The gel was rinsed in cold water for 2 h and transferred to nitrocellulose as described above. In both cases the nitrocellulose was covered with X-ray film for 6 days on dry ice. The exposed films were scanned with a Bio-Rad 620 video densitometer.

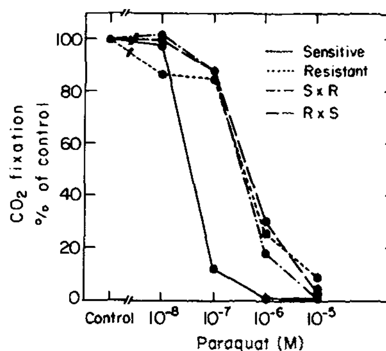


**Fig. 1.** Nuclear dominance of paraquat resistance in reciprocal $F_1$ hybrids of the resistant (*R*) and susceptible (*S*) biotypes. The dose response curves were obtained using leaf discs of the parents and the hybrid offspring floated on paraquat for 4 h. $^{14}CO_2$ fixation was used as a measure of paraquat sensitivity

**Table 1.** Segregation of resistance in *Conyza.* Plants in the rosette stage were sprayed to run-off with the discriminatory dose of 0.1 mM paraquat

| | | | | |
|---|---|---|---|---|
| $F_1$ | | | | |
| S×R | 0 | 50 | ∞ | |
| R×S | 0 | 50 | ∞ | |
| Total | 0 | 100 | ∞ | |
| $F_2$ | | | | |
| S×R | 61 | 170 | 2.8:1 | <0.005 |
| R×S | 52 | 170 | 3.3:1 | <0.005 |
| Total | 113 | 340 | 3.0:1 | <0.005 |

## Results

### *$F_1$ progeny*

The resistance of *Conyza bonariensis* $F_1$ offspring from reciprocal crosses between resistant and susceptible individuals was compared to the parents (Fig. 1). The resistant parent and the progeny of the two reciprocal crosses had very similar $I_{50}$ values that were ten times higher than the value of the susceptible parent. We previously reported a 100-fold $I_{50}$ difference between the two biotypes (Shaaltiel and Gressel 1986). The 10-fold change in $I_{50}$ is due to the fact that the present paraquat exposure was for only 4 h instead of 24 h. $CO_2$ fixation in resistant plants is initially inhibited by paraquat and there is a slow recovery (Shaaltiel and Gressel 1987b). The susceptible plants do not recover, so the $I_{50}$ of the resistant biotype increases with the recovery time.

The levels of three oxygen-radical detoxifying enzymes of the pathway were measured. The activities of superoxide-dismutase, ascorbate-peroxidase and glutathione-reductase in the $F_1$ were high, i.e., similar to those of the resistant parent (Fig. 2; Table 1).

### *$F_2$ progeny*

Fifty plants from each $F_1$ reciprocal cross were self-pollinated. More than 200 $F_2$ plants resulting from each reciprocal cross were sprayed to run off with 0.1 m*M* paraquat. The same paraquat concentration was used to discriminate between the sensitive and resistant parents. The plants were exposed out-of-doors for 24 h from noon, in summer sunlight of approx. 1.5 mE $m^{-2}$ $s^{-1}$. The number of dead and living plants in each group was counted. Three-fourths of the $F_2$ plants were resistant (Table 1) suggesting that a single, completely dominant gene controls resistance.

We could not measure the enzymatic activities in isolated chloroplasts of individual $F_2$ plants because it was not possible to obtain enough class A chloroplasts from single plants to ascertain whether enzyme activity levels co-segregated with resistance. We could have bulked resistant and sensitive plants separately after single leaf-disc analyses for paraquat resistance and tested enzyme levels of each group, however, we would have run the risk of losing sensitivity and thereby missing segregants. Thus, we used only single plant enzyme assays, using immunoblotting techniques. We were limited by the available amount of antibody for superoxide-dismutase and had no antibody for ascorbate-peroxidase. Three leaves from each rosette were

**Table 2.** Inheritance of oxygen-radical detoxifying enzyme levels in parental $F_1$ and $F_2$ generations. Parental and $F_1$ data activity measurements are on stromal extracts of intact chloroplasts and $F_2$ data are from immunoblotting (Western gels) of whole cell extracts of single plants compared to parent generation extracts. The enzyme levels in the $F_2$ plants were analyzed after assaying for paraquat resistance or susceptibility using excised leaf discs. The results and standard errors were calculated in absolute values and then transformed to the percent of the susceptible parent

| Type of biotype | Measurement | Enzyme activity (% of susceptible parent ± SE) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Superoxide-dismutase | range | *n* | Ascorbate-peroxidase | *n* | Glutathione-reductase | range | *n* |
| Parents | | | | | | | | | |
| S (susceptible) | activity | 100±11 | | 3[a] | 100±8 | 3[a] | 100±12 | | 3[a] |
| S | Western | 100± 3 | | 2[b] | | | 100± 7 | | 2[b] |
| R (resistant) | activity | 159±17 | | 3[a] | 252±17 | 3[a] | 320±21 | | 3[a] |
| R | Western | 170± 5 | | 2[b] | | | 340±14 | | 2[b] |
| F (all) | activity | 182±23 | | 2[a] | 312±38 | 3[a] | 370±22 | | 2[a] |
| F (S offspring) | Western | 100± 8 | \| 92–108 \| | 2[b] | | | 100± 6 | \| 95–105 \| | 6[b] |
| F (R offspring) | Western | 170±12 | \| 162–195 \| | 6[b] | | | 280±38 | \| 225–320 \| | 6[b] |

[a] *n* in the parental and $F_1$ generations refers to the number of experiments using triplicate determinations on gels. The parental data include the experiment presented in Shaaltiel and Gressel (1986)

[b] *n* refers to the number of individual plants extracted and assayed in replicate determinations in two separate experiments. Note that the ranges of the resistant $F_2$ individuals do not overlap and the values are considerably higher than susceptible $F_2$ individuals and susceptible parents

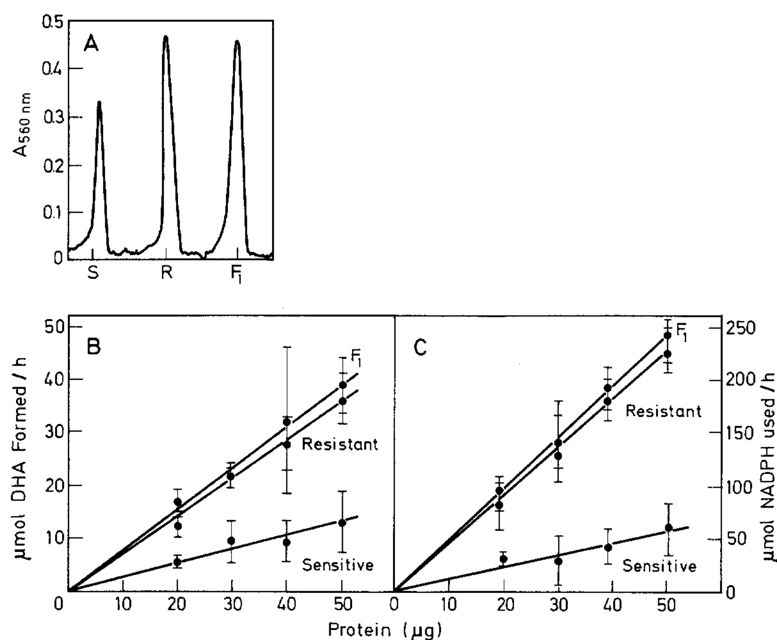


**Fig. 2.** Superoxide-dismutase (**A**), ascorbate-peroxidase (**B**), and glutathione reductase (**C**) activities in stomal extracts of paraquat-resistant and sensitive biotypes and their $F_1$ hybrids. The measurements of these three activities are described in "Materials and methods". *DHA* = Dehydroascorbate

sufficient for each enzyme. We first ascertained that the actual amounts of superoxide-dismutase enzyme protein found in Western blots were comparable to the activity levels. From the parental data in Fig. 3 and Table 2, it is clear that relative enzyme activities and Western blots of enzyme levels directly correlate. The antibody for oat chloroplast superoxide-dismutase reacted only with the *Conyza* chloroplast isoenzyme in whole cell extracts (Fig. 3). Similar results have been reported for antibody specificity to chloroplast superoxide-dismutase in maize (Baum et al. 1983). We performed Western blots for both chloroplast superoxide-dismutase and glutathione-reductase on whole cell extracts of leaves of separate plants (Fig. 4; Table 2). The $F_2$ plants that were paraquat-resistant had the same elevated levels of these two enzymes as the resistant parent, and the paraquat sensitive $F_2$ plants had the same low levels as the sensitive parent. In no case was

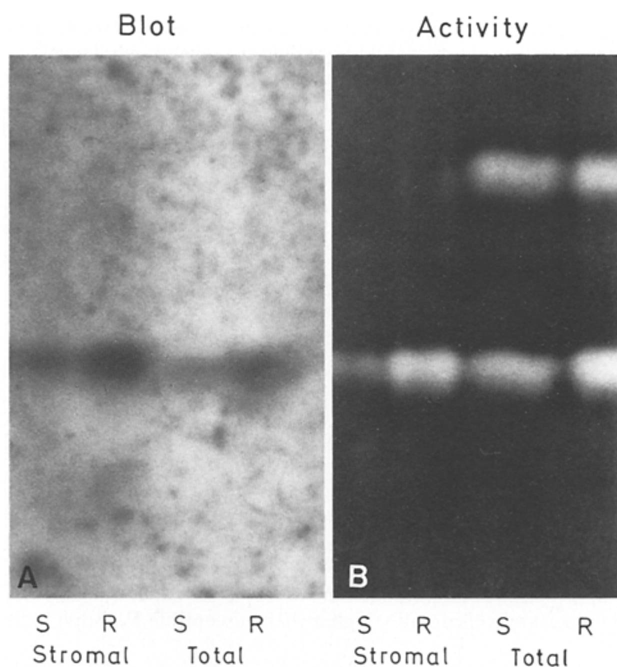


**Fig. 3.** Correlation between superoxide-dismutase activity by specific gel staining (**B**) with levels of the chloroplast isoenzymes by immunoblotting (**A**). Total cell extracts and stromal extracts from paraquat-resistant and sensitive biotypes were separated by electrophoresis, half the gel stained for superoxide-dismutase activity and the other half of the gel immunoblotted (after treating with sodium-dodecyl-sulfate) for superoxide-dismutase levels

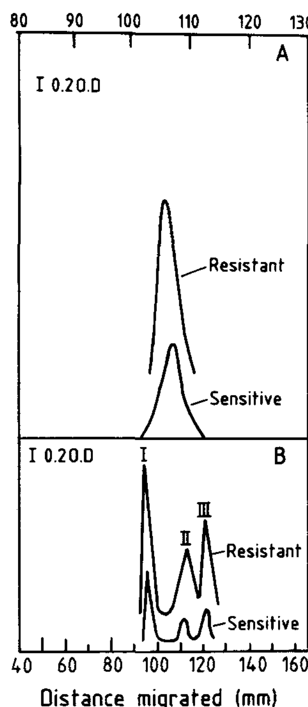


**Fig. 4.** Chloroplast superoxide-dismutase (**A**) and glutathione-reductase levels (**B**) in whole cell extracts of representative individual resistant and susceptible $F_2$ plants. Scans are of immunoblotted gels as outlined in "Materials and methods". The antibody to glutathione-reductase reacted with three protein peaks. It is not clear whether these are isozymes or if some of the peaks are partial degradation products. All three peaks were integrated together for determining relative levels in each plant for the summaries presented in Table 2

there a resistant plant with low levels of either enzyme, nor was there a sensitive plant with high levels of the two enzymes tested (see data on range of enzyme levels in Table 2).

## Discussion

The data from the reciprocal crosses show that, unlike atrazine-resistance, paraquat-resistance is not maternally inherited. The $F_2$ data clearly show that the dominance of resistance is controlled by a single, dominant, nuclear gene. KC Vaughan (personal communication, 1986) also found evidence for a single dominant nuclear gene for paraquat resistance in these *Conyza* biotypes. Similarly, paraquat-resistance was found to be due to a single dominant gene (using $F_1$ and backcrossing to the sensitive biotype) in *Conyza philadelphicus* (Itoh and Miyahara 1984). No enzyme assays were performed using class A chloroplasts in these two studies. Resistance to paraquat in *Conyza* was not polygenic, as was found in evolved resistance in *Lolium* (Faulkner 1974). Hickok and Schwarz (1986) have shown that paraquat resistance, as selected in haploid ferns, is recessively and monogenically inherited in the sporophyte. This resistant mutant in ferns is probably due to some other physiological mechanism than that in *Conyza,* as it lacks the cross-tolerance to other oxidant-generating herbicides and the elevated enzyme levels. More recently, they have isolated an acifluorfen-paraquat cross-tolerant mutant (Hickok et al. 1987), that probably has the same basis for resistance as the *Conyza.*

The activities of all three enzymes of the Halliwell-Asada oxygen radical detoxification pathway are clearly constitutively elevated in the $F_1$ generation to the same levels as the resistant parent. This suggests a pleiotropic co-dominance of the enzymes with resistance. The two enzymes that could be immunologically measured in single plants co-segregated with resistance in all cases in the $F_2$ generation.

Unfortunately, ascorbate-peroxidase could not be measured in the $F_2$ generation, as no antibody was available. Ascorbate-peroxidase acts between superoxide-dismutase and glutathione-reductase in the Halliwell-Asada pathway. Because of its position and ele-

vated levels in the $F_1$, it is fair to presume that ascorbate-peroxidase segregates with the two enzymes that could be measured. All paraquat-resistant $F_2$ plants had high levels of the two enzymes; all sensitive plants had low levels of these two enzymes. This co-segregation of constitutively high levels of two enzymes of the oxygen detoxification pathway in chloroplasts, the organelle affected by paraquat, clearly supports a hypothesis that these enzymes are involved in the resistance mechanism. Paraquat rapidly and irreversibly inhibits photosynthesis in susceptible plants within minutes of spraying and the first visible signs of injury are seen 4 h after spraying. Photosynthesis in resistant plants is transiently inhibited and the plants rapidly recover. Isolated intact, Class A chloroplasts are also more resistant to photo-induced lipoxidation than sensitive chloroplasts (Shaaltiel and Gressel 1987b). Resistance to paraquat can be abolished by treating the plants with inhibitors of superoxide-dismutase and ascorbate-peroxidase (Shaaltiel and Gressel 1987a). It is thus probable that the localization of radioactivity from xylem fed paraquat visualized 4 h after treatment (Fuerst et al. 1985) is due to a secondary sequestration of paraquat or one of its degradation or conjugation products by live plants. The dead plants were unable to metabolize or sequester paraquat.

This pleiotropy of resistance demonstrates the problems that may be encountered in isolating the gene for conferring paraquat resistance by the techniques of molecular biology for genetic engineering. This gene may not necessarily be a structural gene for one of these enzymes. This same gene confers a modicum of cross-tolerance to other oxidant stresses due to herbicides and environmental pollutants (Shaaltiel et al. 1988) and can thus be a very useful gene.

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