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Isolation of Paraquat-tolerant Mutants from Tomato Cell Cultures

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Summary. Tomato callus clones selected for the ability to grow at paraquat concentrations lethal to wild-type cells were found at an approximate frequency of 5×10^{-8} per viable cell. Diploid plants were regenerated from nine of the nineteen paraquat-tolerant callus clones isolated. Although some of these plants appeared normal, others had altered morphology and reduced vigor and fertility. New callus cultures initiated from these regenerated plants typically had at least a 30-fold increase over the wild type in tolerance to paraquat. Tests on callus from sexual progeny showed that the paraquat-tolerant phenotypes of clones PQT^4, PQT^6, and probably also PQT^{13} resulted from dominant nuclear mutations, but the number of loci involved is not yet known. Paraquat spray experiments indicated that slight paraquat-tolerance was expressed at the plant level in PQT^{13}, but not in any of the other clones tested.

Key words: Tomato – Cell cultures – Herbicide-tolerant mutant – Methyl viologen – Paraquat

Introduction

Plant cell cultures have been used to isolate mutants resistant or tolerant to various toxic chemicals (Maliga 1980; E. Thomas et al. 1979). Some substantial technical problems, such as epigenetic phenotypes (Dix 1977; Maliga et al. 1976; Meins and Binns 1979; Miller and Hughes 1980), increase in ploidy (D'Amato 1978; Bayliss 1980) and loss of totipotency (Reinert et al. 1977; Murashige 1974) complicate these experiments. Epigenetic events can be very difficult to distinguish from mutations when studies are limited to the cell level. But regeneration of fertile plants and transmission of the phenotype to sexual progeny provides definitive proof for mutation. Regeneration also allows both basic plant-level studies and agricultural application. Fertile diploid regenerants are most likely to be gotten if the presumptive mutants are isolated from newly initiated cell cultures and then promptly regenerated into plants (Maliga 1981; Flick et al. 1981). In addition to this, the best possible regeneration potential for any particular species can be achieved through design of cell culture media (Padmanabhan et al. 1974), selection of the best plant genotype (Malmberg 1979) or through plant breeding (McCoy and Bingham 1977).

The only report of mutant selection experiments using tomato cell cultures is that of Meredith (1978). These clones, selected for resistance to aluminium ion, remained resistant after 2–4 months of growth in the absence of the stress. No plants were regenerated from these stable clones so it was impossible to do a progeny test to determine whether or not the resistance was due to a mutation (Meredith, personal communication).

Use of plant cell cultures to select for mutants resistant or tolerant to various herbicides, reviewed recently by Meredith and Carlson (1982), is of particular interest because of potential agronomic applications and research applications in plant physiology and herbicide chemistry. Mutants tolerant to the herbicide paraquat were isolated as a model system to test our mutant isolation methods and the special plant genotype that we have developed for this purpose.

Materials and Methods

Stocks

Tomato genotype L2, with ancestry 75% *Lycopersicon peruvianum* and 25% *L. esculentum,* was used for mutant isolations. L2 is a hybrid produced in our lab and selected for self-fertility, high rate of callus growth, and high efficiency regeneration of shoots from callus (Thomas and Pratt 1981 b).

Sexual transmission of paraquat-tolerance was shown either by self-fertilization of the plant regenerated from the tolerant cell clone or by crosses in which pollen from the regenerated plant was used to fertilize plants of VFNT × LA1283-4, an interspecific hybrid between *L. esculentum* and *L. peruvianum* (Thomas and Pratt 1981 a).

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Media and Growth Conditions

Medium 2D/1P contains the mineral salts of Murashige and Skoog (1962) plus (per liter) 2 mg 2,4-D, 1 mg N^6-(Δ^2-isopentenyl)-adenine, 1 mg thiamine · HCl, 0.5 mg pyridoxine · HCl, 0.5 mg nicotinic acid, 30 gm sucrose, 10 g of agar, and 100 mg inositol. Medium 2D/1P was used at 27 °C in the dark for growth, mutant selection and sensitivity tests using colorless white callus. Callus turned brown when its growth was inhibited due to addition of paraquat to the medium. Green callus was observed only as a necessary intermediate step in shoot development under the regeneration conditions described previously (Thomas and Pratt 1981a). For mutant selection and growth inhibition experiments, commercially formulated "Ortho Paraquat CL" (a gift of Chevron) or purified methyl viologen (paraquat) from Sigma was added to 2D/1P medium either before autoclaving or, as a filter-sterilized solution, after autoclaving. The paraquat concentrations described are based on the concentration of active ingredient as assayed by Chevron. When expressed in this way, commercially formulated paraquat and purified methyl viologen inhibit callus growth to the same extent. – Callus from plant L2 was initiated from young leaflets that were almost fully expanded. Whole leaves were surface-sterilized by ten cycles of alternate dipping in solutions of 95% ethanol and 5% sodium hypochlorite. This was followed by rinsing with two changes of sterile distilled water. Leaflets were cut into pieces of approximately 1 square cm. Four leaf pieces were plated on each 100×15 mm petri plate of 2D/1P agar medium. Callus up to 3 cm in diameter was formed by each leaf piece within three weeks. – Callus from sexual progeny was produced using hypocotyl tissue. This method allowed callus to be produced from small seedlings while saving the plant for further experiments. Dry seeds were efficiently sterilized when dipped in 95% ethanol, then soaked in 5% sodium hypochlorite for 5 min. These sterilized seeds were germinated on MSS agar medium. One to two weeks later, the apical portion of each seedling was excised and placed on a fresh plate of MSS medium; after new roots formed, these plants could be transferred to soil. The hypocotyl of each seedling was cut into pieces approximately 5 mm long and placed on 2D/1P agar medium for callus production.

Mutant Selection Technique

For mutant isolation experiments, we used freshly isolated white callus, generally three weeks after plating leaf tissue on callus medium. Callus cells were suspended in liquid medium and then pipetted onto agar medium for mutant selections (Müller and Grafe 1978). For making the suspension, the callus was placed in a cup-shaped stainless steel screen having a pore diameter of 1.5 mm. The screen was partially submerged in 2D/1P liquid medium. The callus was forced through the screen by stirring with a spatula. Leaf tissue and large pieces of hard callus remained inside the screen and were discarded. Callus that passed through the screen was concentrated to approximately 0.4 g fresh weight cells per ml. Wide-tipped pipettes were used to dispense 3 ml of this suspension onto 100×15 mm petri plates containing 2D/1P agar medium plus paraquat. Either Ortho paraquat or pure paraquat was used (see Table 1). The total number of cells plated was determined using the conversion factor 3.5×10^6 cells per gram fresh weight. Cell number was counted after dispersing cell aggregates using a chromic acid digestion procedure (Street 1977). Approximately 2/3 of the cells in the suspended callus were viable as estimated by the phenosafranin dye exclusion method (Widholm 1972). A plating efficiency of 100% by these viable cells was assumed for calculation of mutant frequency.

Presumptive mutant clones were given tentative designations based on their phenotype (eg. PQT^{14}). When further work identifies one or more gene loci that mutated to produce the phenotype observed, then each clone will be renamed to describe its genotype (eg. *Pqt*-1^{14}) using the rules for nomenclature in tomato genetics (Barton et al. 1955; Clayberg et al. 1966).

Regeneration of plants from callus was done as described previously (Thomas and Pratt 1981a). Ploidy estimates on these plants were made by measurement of stomata length or by counting Feulgen-stained chromoses of pollen mother cells. Considering the gross morphological abnormalities of virtually every known tomato aneuploid (Rick and Barton 1954; Khush and Rick 1966, 1967, 1968, 1969), regenerated plants of normal morphology and vigor are likely to be euploid.

Agar Underlay Technique

An agar underlay technique, used to determine the relative increase in resistance to paraquat among the presumptive mutants, was modified from Rosset and Gorini (1969). Leaf callus from the regenerated plants was initiated and maintained on 2D/1P agar. The growth response to paraquat was tested on callus maintained in culture at least one month. Paraquat was added under the agar of recently inoculated petri plates where small callus pieces had begun to grow on 2D/1P medium. The growth response was scored one month after paraquat addition. Optimum results in this assay depended upon having a callus inoculum which had been maintained in rapidly growing condition by timely subculture.

Plant-Level Tolerance to Paraquat

Plants potted in soil were sprayed with paraquat using a herbicide sprayer/conveyor belt as described in Shaw and Swanson (1951). This machine was calibrated for a spray rate of 370 liters per hectare producing a nearly continuous layer of small droplets on exposed plant surfaces. Ortho Paraquat was diluted in water to give an application rate of 1.1 kg of active ingredient per hectare (1 pound per acre). Ortho DPX-77 surfactant was used at 0.25% (v/v), a non-phytotoxic dosage, to enhance penetration of paraquat into the foliage. Plants were propogated by cuttings because L2 is a hybrid. This limited the number of plants available for paraquat spray experiments and also caused some variability in the size and shape of the plants used. Treated plants were incubated in a growth chamber (day: 12 hr, 12,000 lux, 28 °C; night: 12 hr, dark, 18 °C), for 2 weeks for observation of damage caused by paraquat treatment and subsequent growth of surviving plants via apical meristems or outgrowth of inhibited lateral buds.

Results

Isolation of Mutant Plants

The concentration of paraquat to use for mutant selection experiments was determined by plating suspended callus on 2D/1P agar containing various paraquat concentrations. The cells showed no growth at concentrations greater than or equal to 1×10^{-4} M. 5×10^{-4} M, the highest concentration used in mutant

Table 1. Frequency of paraquat-tolerant clones in plated cells

Experiment	Viable cells plated	Number of tolerant clones	Clones that produced diploid regenerated plants
	Paraquat concentration 1.0×10^{-4} *M*		
I[a]	9×10^7	2	PQT^{13}
	Paraquat concentration 5.0×10^{-4} *M*		
II[a]	2×10^7	2	None
III[a]	1×10^7	1	None
IV[a]	3×10^7	4	PQT^4, PQT^5, PQT^6
V[a]	1×10^8	4	None
VI[a]	5×10^7	2	PQT^{19}, PQT^{20}
VII[b]	1×10^8	4	PQT^{23}, PQT^{24}, PQT^{25}
Totals	4×10^8 viable cells	19 tolerant clones	

[a] Ortho Paraquat CL was used
[b] Purified paraquat (methyl viologen) was used

selection, is thus at least five-fold greater than the minimum inhibitory concentration.

The suspended callus plating method was used for mutant isolation in seven separate experiments shown in Table 1. A total of nineteen localized callus growths, presumably paraquat-tolerant mutant clones, appeared three to six weeks after the cells were plated on media containing various lethal concentrations of paraquat. These tolerant clones were produced from approximately 4×10^8 viable cells, giving an estimated frequency of 5×10^{-8} tolerant clones per viable cell.

Plants regenerated from nine of the nineteen paraquat-tolerant clones obtained were estimated to be diploid by cell size measurements. This was confirmed by chromosome counting for plants of PQT^{13}. The other clones either regenerated only tetraploid plants or no plants at all. All regenerated plants had normal pigmentation; no chlorotic plants were produced on the regeneration plates. The morphology, vigor and fertility of these plants varied widely from clone to clone. PQT^5 was the most abnormal clone, having small leaves, a reduced growth rate and complete sterility. PQT^4 and PQT^6 had similar abnormalities except for occasional fertility as the male parent in outcrosses to VFNT × LA1283-4 plants. Many attempts to set fruits ended in failure, perhaps because of low pollen viability in PQT^4 and PQT^6: a high proportion of their pollen grains was shriveled. PQT^{20} was more normal in morphology, but appeared completely sterile. The remaining paraquat-tolerant clones which regenerated diploid plants (see Table 1) were essentially normal in morphology, vigor and fertility.

Consistent with our hypothesis that they derive from clones, most paraquat-tolerant calli produced regenerated plants that were uniform in morphology and fertility. As expected from the genetic instability of callus, occasional clones produced both diploid and tetraploid plants. PQT^{13} was the only callus isolate that produced regenerated plants that were strikingly different from each other. PQT^{13A} was a plant that had reduced vigor and fertility; PQT^{13} was a more normal plant.

Paraquat Tolerance of Callus

The levels of paraquat tolerance were tested using white callus derived from leaves of the regenerated plants. Callus transferred directly to paraquat-containing media gave extremely uneven growth. At many intermediate paraquat concentrations only a fraction of the callus pieces was able to grow. This growth often derived from sectors within the callus pieces even though the cells were presumably uniform for their content of alleles that contribute to paraquat tolerance. This same callus produced uniform growth or growth inhibition only when we applied various amounts of paraquat to cells that had already begun to grow on the medium used for the test. This is shown in Figure 1 for PQT^6, which is able to grow at paraquat concentrations at least 30-fold greater than those needed to prevent growth of the L2 parental strain callus. Table 2 shows the similar high levels of tolerance to paraquat

Table 2. Growth of callus from regenerated plants on paraquat-containing medium

Strains	Paraquat concentration				
	0	1.5×10^{-4}	5×10^{-4}	1.5×10^{-3}	5×10^{-3}
L2	+ + +	− −	− −	− −	− −
VFNT × LA1283-4	+ + +	− −	− −	− −	− −
PQT^4	+ + +	+	+	+	+
PQT_5	N.D.	+ +	+ +	±	±
PQT_6	+ + +	+ +	+ +	+ +	+
PQT^{13}	+ + +	+ +	+ +	+ +	+
PQT^{13A}	+ + +	±	− −	− −	− −
PQT^{19}	+ + +	±	±	− −	− −
PQT^{20}	+ + +	+ +	+	±	±
PQT^{23}	+ + +	− −	− −	− −	− −
PQT^{24}	+ + +	+ +	+ +	+ +	±
PQT^{25}	+ + +	+ +	+ +	N.D.	N.D.

Paraquat was added to growing cells using the underlay technique. Each entry in the table was based on at least two plates. The minimum inhibitory concentration for normal cells was approximately 1×10^{-4} M. N.D. = not determined; − − = no growth; ± = slight growth from some but not all of the callus pieces (usually from sectors); + = slight growth from all of the callus pieces (usually not from sectors); + + = callus pieces grew to less than 1 cm in diameter; + + + = callus pieces grew to greater than 1 cm in diameter

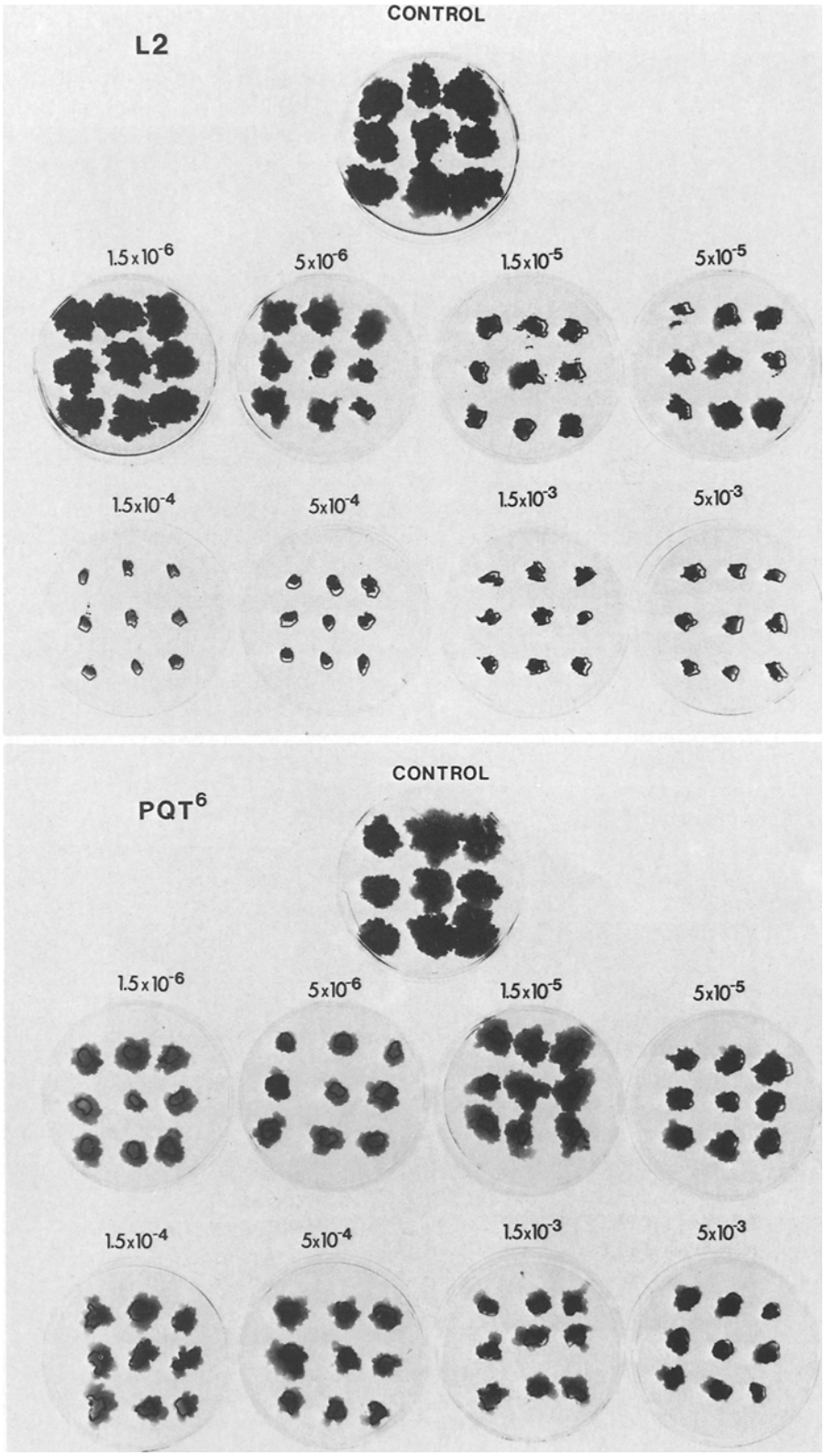

Fig. 1. Growth of callus treated with paraquat using the underlay technique. Cultures of the sensitive parental line (L2), and of one of the tolerant isolates (PQT^6) are shown in the absence (control) and presence of paraquat ($1.5 \times 10^{-6} - 5 \times 10^{-3}$ M)

exhibited by callus from most of the other presumptive mutant clones. All of these clones showed some growth inhibition at 1.5×10^{-4} M paraquat, and thus should be classified as tolerant rather than resistant (Gressel 1979). Little or no paraquat tolerance was observed in callus from regenerated plants of PQT^{13A}, PQT^{19} and PQT^{23}.

The results of testing for paraquat tolerance in callus from sexual progeny are shown in Table 3. A small fraction of the progeny inherited approximately the same level of paraquat tolerance shown by the original regenerated plants. The rest of the progeny were as paraquat-sensitive as the L2 and (VFNT × LA1283-4) genotypes were. No clear example of a progeny with an intermediate or an increased level of tolerance was observed among the few tolerant progeny isolated. Exact determinations were difficult to make because the progeny were also segregating for their callus growth characteristics, especially friability and growth rate. None of the progeny made callus that grew as well as L2 callus, either in the presence or the absence of paraquat.

Table 3. Paraquat tolerance of callus from sexual progeny[a]

Type of progeny	Number of progeny	
	Tolerant	Sensitive
PQT^{4} outcross to sensitive strain	1	8
PQT^{6} outcross to sensitive strain	2	6
PQT^{13} self-pollinated	2	5

[a] Assayed using the underlay technique

Paraquat Tolerance of Plants

Plant-level tolerance to paraquat was tested using small cutting-propagated plants in 10 cm pots. Plants of L2 sprayed with paraquat at 1.1 kg/Ha quickly turned brown or black, followed by rapid dessication of almost 100% of the stems and leaves. As described in Table 4, only 6 out of 19 L2 plants survived, mostly through outgrowth of inhibited lateral buds near the base of stem. Plants of PQT^{4}, PQT^{20}, PQT^{24} and PQT^{25} were just as paraquat sensitive as L2. Plants of PQT^{13} were damaged by 1.1 kg/Ha of paraquat, but the extent of browning and withering was much less than that shown by L2. Approximately half of the PQT^{13} leaf area treated remained green and fully expanded for at least five days after spraying (see Figure 2). Some of this green tissue which was not rapidly killed by paraquat remained healthy and grew noticeably within two weeks after the paraquat treatment. Whenever a paraquat-induced lesion completely encircled a stem,

Table 4. Response of regenerated plants to paraquat spray treatment at 1.1 kg/Ha (1 pound/acre)

Plant	Number of plants		
	Killed	Survived only through growth of inhibited lateral buds	Survived because meristems active at time of spraying were not killed
L2 (sensitive) control	13	5	1
PQT^{4}	4	2	0
PQT^{20}	5	3	0
PQT^{24}	6	2	0
PQT^{25}	2	6	0
PQT^{13}	5	6	7

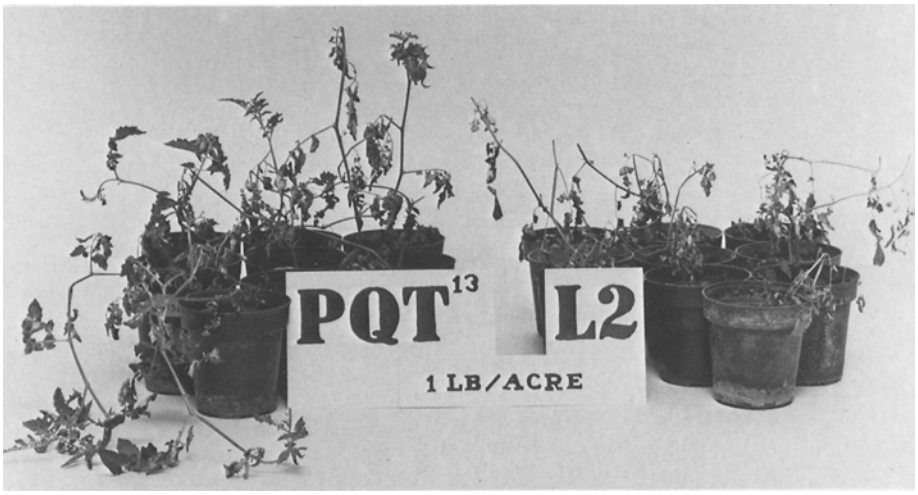

Fig. 2. Plants of L2 and PQT^{13}, 5 days after paraquat spray treatment at 1.1 kg/Ha (1 lb per acre)

any green tissue remaining above that point slowly turned pale, wilted and then became withered. Thus some PQT^{13} plants having leaves and shoot meristems that apparently survived direct exposure to paraquat were later killed as a secondary effect of damage to the stem. In all, 13 out of 18 plants of PQT^{13} survived the paraquat treatment.

Discussion

All available evidence is consistent with the idea that our paraquat-tolerant clones are mutants. Paraquat-tolerant clones were [1] rare in occurrence (approximately 5×10^{-8} per viable cell), [2] stable during many cell-generations of regeneration, growth as a plant and initiation of new callus cultures – all under nonselective conditions, [3] more tolerant to paraquat than strain L2 (30-fold increase in tolerance level as callus), and [4] found among sexual progeny of the three different paraquat-tolerant clones tested.

The mutant frequency described is only an approximation because of the number of assumptions that had to be made. A more exact calculation will require measurements of plating efficiency. All nineteen clones isolated were assumed to be mutants, even though sexual progeny from only three clones have been tested. Clones PQT^{19}, and PQT^{23}, which were relatively paraquat-sensitive, were not subtracted, because of the possibility that regenerants were obtained from a sensitive sector of a chimeral callus. For this calculation, data from all seven experiments were pooled because paraquat was always used at a lethal concentration and because higher paraquat concentrations did not give lower mutant frequencies or higher tolerance levels in the mutants isolated.

Some conclusions can be made about the genetics of paraquat tolerance. The paraquat-tolerant allele(s) from PQT^{4} and PQT^{6} must be nuclear in origin and dominant or semidominant to the wild-type allele(s), as the phenotype was transmitted through pollen to the progeny of an outcross to a paraquat-sensitive genotype. The observation of paraquat-sensitive segregants in these crosses shows that PQT^{4} and PQT^{6} are heterozygous for the allele(s) that confer paraquat tolerance, as would be expected for mutations isolated in a single step. The selfing of mutant PQT^{13} also gave rise to some tolerant and some sensitive progeny, suggesting that again the mutant phenotype is based on dominant or semi-dominant nuclear allele(s) and that the original regenerated plant was a heterozygote. Outcrossing will be needed, however, to rigorously exclude some form of maternal inheritance. The number of loci that must mutate to produce a paraquat-tolerant cell has not yet been determined. Among the small numbers of sexual progeny plants that have been tested there is a clear shortage of tolerant relative to sensitive progeny in comparison with the ratios expected for a one-gene model. The mode of inheritance should eventually be solved by increasing the numbers of sexual progeny tested, including some plants from generations subsequent to the one reported here.

The paraquat tolerance observed in plant spray experiments with PQT^{13} defines a second class of paraquat tolerant mutants which is different from all the other mutants whose tolerance is expressed only in cultured cells. This suggests that some of the mutant plants isolated from cell cultures may have agronomic applications. Although PQT^{13} was extensively damaged by the minimum dose needed to kill most L2 plants, higher levels of tolerance might be achieved in plants homozygous for the tolerance allele(s) described above, in new mutants isolated in the future, or in plants that combine the tolerance alleles from different mutants.

Previous work on paraquat toxicity and the paraquat tolerance of mutants suggests that more than one mechanism could account for the paraquat tolerance of our mutants. The biology and chemistry of paraquat was reviewed recently by Summers (1980). The mechanism proposed to account for paraquat toxicity involves [1] reduction of paraquat by electron transport chains and [2] the subsequent reoxidation of the paraquat radical in a reaction with molecular oxygen, which produces superoxide radical as a product. Paraquat is especially toxic to plants because it is reduced more rapidly by photosynthetic electron transport chains than by respiratory electron transport chains. A chlorotic mutant carrot plant tolerant to paraquat (Miller et al. 1980) and some seedling-lethal mutants of maize that were tolerant to the related herbicide diquat (Miles 1976) probably derive their tolerance from the inability to reduce the herbicides efficiently due to various defects in their photosynthetic electron transport chains.

The subsequent chemistry and biochemistry of paraquat toxicity do not depend so heavily upon whether or not the cells are photosynthetic. Although superoxide itself may be the product responsible for paraquat toxicity, other potentially toxic molecules may be formed from superoxide. Many reactions involving superoxide and its products are either spontaneous or are catalyzed by enzymes, such as superoxide dismutase and catalase, which are found in virtually all aerobic organisms. Once reduced, the subsequent steps in paraquat toxicity may therefore be the same for callus, roots, leaves, etc.

Various mechanisms that provide paraquat tolerance in non-photosynthetic cells might also be effective in photosynthetic cells and vice versa. In addition to the electron transport mutants described above, increased levels of superoxide dismutase, catalase and peroxidase can also confer paraquat tolerance. These enzymes which convert superoxide to harmless products, were formed at slightly increased levels in members of a wild population of perennial ryegrass that was tolerant to paraquat (Harper and Harvey 1978). Cultured tobacco cells selected for paraquat tolerance have been regenerated into plants from which

paraquat-tolerant callus able to grow at 1×10^{-4} M paraquat has been reisolated. One-third of these plants was tolerant to paraquat, based on a leaf disc assay (Miller and Hughes 1980). These clones had either reduced uptake of paraquat, increased superoxide dismutase levels or increased peroxidase levels. Transmission of the phenotype to sexual progeny was also observed recently (K. W. Hughes personal communication).

The paraquat-tolerant tomato mutants described here are normal in pigmentation and viable, if not uniformly vigorous, as plants so alterations in electron transport are not likely mechanisms of tolerance. Future work will concentrate on paraquat permeability and enzymes of superoxide degradation as possible tolerance mechanisms.

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