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Direct measurement of paraquat in leaf protoplasts indicates vacuolar paraquat sequestration as a resistance mechanism in *Lolium rigidum*

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ABSTRACT

Sequestration of paraquat away from its target site in the chloroplast has been proposed as a mechanism of paraquat resistance. However, no consensus has been reached as to where paraquat is sequestered. This study quantifies paraquat in leaf protoplasts of paraquat resistant (R) and susceptible (S) *Lolium rigidum*. Intact protoplasts were prepared from plants treated with commercial dose of paraquat for 2 h. Paraquat absorbed by the leaf protoplasts was determined by light absorption of reduced paraquat following concentration and purification using a cation-exchange resin. Leaf protoplasts from treated paraquat resistant plants contained 2- to 3-fold more paraquat than leaf protoplasts isolated from susceptible plants. Since paraquat is not metabolised in *L. rigidum* and paraquat readily enters chloroplasts of both R and S plants, this greater amount of paraquat in leaf protoplasts of R plant must be kept away from the target site (chloroplast). This result indicates that paraquat resistance in *L. rigidum* is associated with a cytoplasmic mechanism, most likely a greater rate of vacuolar sequestration.

1. Introduction

The non-selective herbicide paraquat has been used worldwide since 1960 and remains an important herbicide for broad-spectrum weed control. Evolved resistance to paraquat has been rare, although resistant biotypes of at least 24 weed species have been documented [1,2]. Studies to identify the biochemical basis of evolved paraquat resistance have been conducted with resistant weed biotypes of a number of species. One resistance mechanism that has been identified is reduced paraquat movement (translocation) within resistant plants [1,3–5]. Reduced paraquat movement in paraquat resistant plants has been demonstrated in *Erigeron philadelphicus* and *E. canadenisis* [6], *Conyza bonariensis* [7,8], *Hordeum leporinum and H. glaucum* [9–11], *Arctotheca calendula* [12], and *Lolium rigidum* [13,14]. To be effective, paraquat must enter leaves and be translocated throughout the plant to reach and enter chloroplasts where it disrupts photosynthetic electron transport resulting in paraquat catalysed over-production of reactive oxygen species (which devastate plant membranes). In paraquat resistant plants, reduced paraquat movement within a leaf limits amount of paraquat translocating to other established and developing leaves.

The biochemical and molecular basis of the reduced paraquat movement resistance mechanism remains unknown. It has been suggested that the restricted mobility of paraquat in R plants is primarily due to sequestration into metabolically inactive compartments [1,3,4]. Photosynthetic measurements (CO_2-dependent O_2 evolution, CO_2 fixation and chlorophyll fluorescence) and fluorescence imaging have proved to be valuable tools for following the appearance of paraquat within chloroplasts. Using photosynthetic fluorescence imaging of intact leaves, reduced presence of diquat at the active site was observed in a resistant biotype of *A. calendula* [15]. Similarly, inhibition of photosynthesis was limited and delayed in R biotypes of *H. glaucum* and *H. leporinum*, compared to S biotypes [10]. Likewise, a transient inhibition followed by recovery of photosynthesis was reported in R biotypes of *Conyza canadensis* [16] and *C. bonariensis* [17]. These limited, delayed, or transient inhibition of photosynthesis in R biotypes by paraquat, together with other lines of evidence, further implicate a paraquat sequestration mechanism.

Potential sites for paraquat sequestration in plant leaves are the cell wall or the vacuole. No difference in cell wall binding of paraquat was found between R and S biotypes of *C. bonariensis* [7], *H. glaucum* [18] or *A. calendula* [15]. However, we are aware that it is difficult to measure total paraquat content in the cell wall space, because cell wall paraquat comprises both a bound and a free fraction. Only the bound paraquat fraction is measured by traditional cell wall preparation methods. No difference was found in paraquat uptake across the plasmalemma between paraquat resistant and susceptible *H. glaucum*, using isolated leaf protoplasts or intact roots [19–20]. Interestingly, isolated protoplasts from both R and S *Hordeum* spp. exhibit surprisingly low sensitivity to [^{14}C]-paraquat,

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indicating isolated protoplasts do not readily take up paraquat [19]. A recent study by Jóri et al. [21] with paraquat resistant *C. canadensis* showed that 97% of applied paraquat is located in the leaf cytosol + vacuole fraction, and that a greater amount of paraquat was measured in this fraction in R plants than in S plants.

We have established that paraquat resistance in two *L. rigidum* biotypes is due to reduced paraquat translocation [13–14], and we propose that this is likely due to increased paraquat sequestration. Considering all the factors cited above, the approach we have taken has been to treat whole plants with paraquat at normal field dose and isolate leaf protoplasts and quantify the paraquat content in the protoplasts of R *versus* S plants. In these experiments we mimic agricultural conditions by treating whole plants at a commercial paraquat dose and then directly quantify the paraquat that reaches and enters leaf protoplasts.

2. Material and methods

2.1. Plant growth and treatment

This study compared a paraquat resistant (AFLR1) with a known susceptible (VLR1) *L. rigidum* biotype (hereinafter referred to as R and S biotype, respectively) [13]. Seedlings of R and S biotypes were grown during the normal growing season for this species in a glasshouse at 20–25 °C under optimum growth conditions. When at the 2–3 leaf stage, seedlings (30–40 plants per pot) were sprayed with a sprayer simulating field application with the normal field rate of a commercial paraquat formulation (Gramoxone® at 200 g paraquat ha^{-1}). The treated plants were immediately placed in a controlled environment room at 20 °C at moderate light (50–100 μmol quanta m^{-2} s^{-1}). After 2 h, shoot tissue was harvested, washed with DI water, and placed on ice for immediate protoplast isolation.

2.2. Leaf protoplast isolation

Leaf protoplast isolation and purification was performed according to Nagata and Ishii [22] and Leegood and Walker [23] with modifications. Harvested leaves from paraquat sprayed plants (10–20 g) were cut into 1 mm segments under dim light (<8 μmol quanta m^{-2} s^{-1}) and soaked in 50–100 mL digest solution containing 0.5 M sorbitol, 1 mM $CaCl_2$, 0.1% BSA, 0.05% PVP, 10 mM ascorbate, 5 mM MES, 1.33% Cellulase "Onozuka" R-10 (Yakult, Tokyo, Japan), 0.07–0.1% Pectolyase Y-23 (Yakult, Tokyo Japan) at pH 5.5 adjusted by 1 N KOH. Leaf segments were digested in the dark for 50–70 min at 30 °C. After digestion, the enzyme solution was removed and the digested leaf segments were washed 3 times with ice cold washing solution containing 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM MES (pH 6.0) and filtered through a 200 μm nylon mesh. The crude protoplast preparations were transferred into 50 mL centrifuge tubes and centrifuged at 100*g* for 5 min at 4 °C using swing bucket (SW) centrifugation (model GS-6R, Beckman). The supernatant was discarded and the pellet re-suspended in 5 mL of ice cold solution containing 0.5 M sucrose, 5 mM MES, 1 mM $CaCl_2$ (pH 6.0). The suspension was layered by 2 mL of solution containing 0.4 M sucrose, 0.1 M sorbitol, 5 mM MES, 1 mM $CaCl_2$ (pH 6.0) and then by 1 mL of washing solution. After centrifugation at 250*g* (SW) for 5 min at 4 °C, the purified protoplasts were collected from the sucrose–sorbitol interface. The interface protoplasts were diluted with washing solution and centrifuged at 250*g* for 3 min at 4 °C, and the protoplast pellet stored at −20 °C. For each experiment, 15–20 protoplast preparations were combined for paraquat quantification and the experiment was conducted 4 times.

2.3. Leaf protoplast number and intactness

Protoplast number was counted using a hemocytometer and intactness determined by fluorescence staining of fluorescein diacetate (FDA) [24] and propidium iodide (PI) [25]. For viability staining with FDA, 50 μL of protoplast suspension was mixed with 1 μL of FDA stock solution (5 mg mL^{-1} in acetone). FDA freely permeates the plasma membrane and is cleaved in the cytosol by non-specific esterases to produce fluorescein which is not freely permeable across the intact plasma membrane. Therefore, fluorescein accumulates in viable cells but not dead cells [24]. To stain non-viable protoplasts with PI, 45 μL of protoplast suspension was mixed with 5 μL PI stock solution (2.5 μg mL^{-1} in water). PI can enter cells through leaky membranes and bind to nucleic acids to form bright dots inside cells [26] (Fig. 1E). Intactness of prepared protoplasts was estimated by comparing protoplast number under bright and fluorescent fields using epifluorscence microscopy (Zeiss Axioscope II Microscope).

2.4. Paraquat treatment of isolated leaf protoplasts

To determine the sensitivity of isolated protoplasts to paraquat, the protoplasts from untreated plants were prepared, challenged with paraquat and paraquat induced damage quantified by monitoring changes (bleaching) in chlorophyll absorbance at 652 nm. Protoplasts (100 μL) were added to a glass test tube containing 3 mL of reaction solution (5 mM MES, 0.5 M sorbitol, 50 mM sucrose, pH 6.0), supplemented with paraquat (0, 100, 500, 1000 μM). The initial absorbance at 652 nm was recorded by inserting the tube directly into the measuring well of a spectrophotometer (model: Spectronic 21; Bausch & Lomb). The test tubes containing protoplasts in solution, with or without paraquat, were either kept in the dark for 2 h before exposure to light or directly exposed to light for different periods at 18–20 °C under 100, 200 and 250 μmol quanta m^{-2} s^{-1}. After 1, 2, 4, and 6 h of light exposure, the absorbance at 652 nm was again recorded and compared with the initial readings.

2.5. Paraquat quantification in leaf protoplast isolated from intact paraquat-treated plants

Frozen protoplast samples prepared from leaves of paraquat-treated plants were thawed and 15–20 protoplast preparations from each experiment were pooled and diluted with DI water. The chlorophyll content was determined by the method of Arnon [27] and the protein content by the Bradford method [28]. Samples were refluxed in 1 N H_2SO_4 at 90 °C for 2 h to release total paraquat, followed by centrifugation at 10,000*g* for 10 min to remove debris. The pellets were washed with DI water, centrifuged and the supernatant combined and vacuum-filtered through a 0.22 μm polypropylene membrane. Quantification of paraquat was modified from a method established by Calderbank and Yuen [29]. This method was used by Preston et al. [10] to determine the amount of paraquat translocated to the young unexposed leaf tissue 24 h after intact plants of paraquat resistant *H. glaucum* and *H. leporinum* being treated with a commercial dose and formulation of paraquat. The method depends on the measurement of light absorption of reduced paraquat that has been concentrated and purified by cation-exchange chromatography. Amberlite IR-120 was used in this study for its availability and higher recovery [30]. The cation-exchange resin columns were prepared by filling a 25 mL burette with 3.5 g of Amberlite IR-120 resin (Catalog No. 22,435–9, Aldrich) in DI water. The resins were activated by passing 50 mL of saturated NaCl through the column followed by a wash with 50 mL DI water at a flow rate of 3–4 mL min^{-1}. The digested samples were added to the column at a flow rate of 7–8 mL min^{-1}. The columns were then

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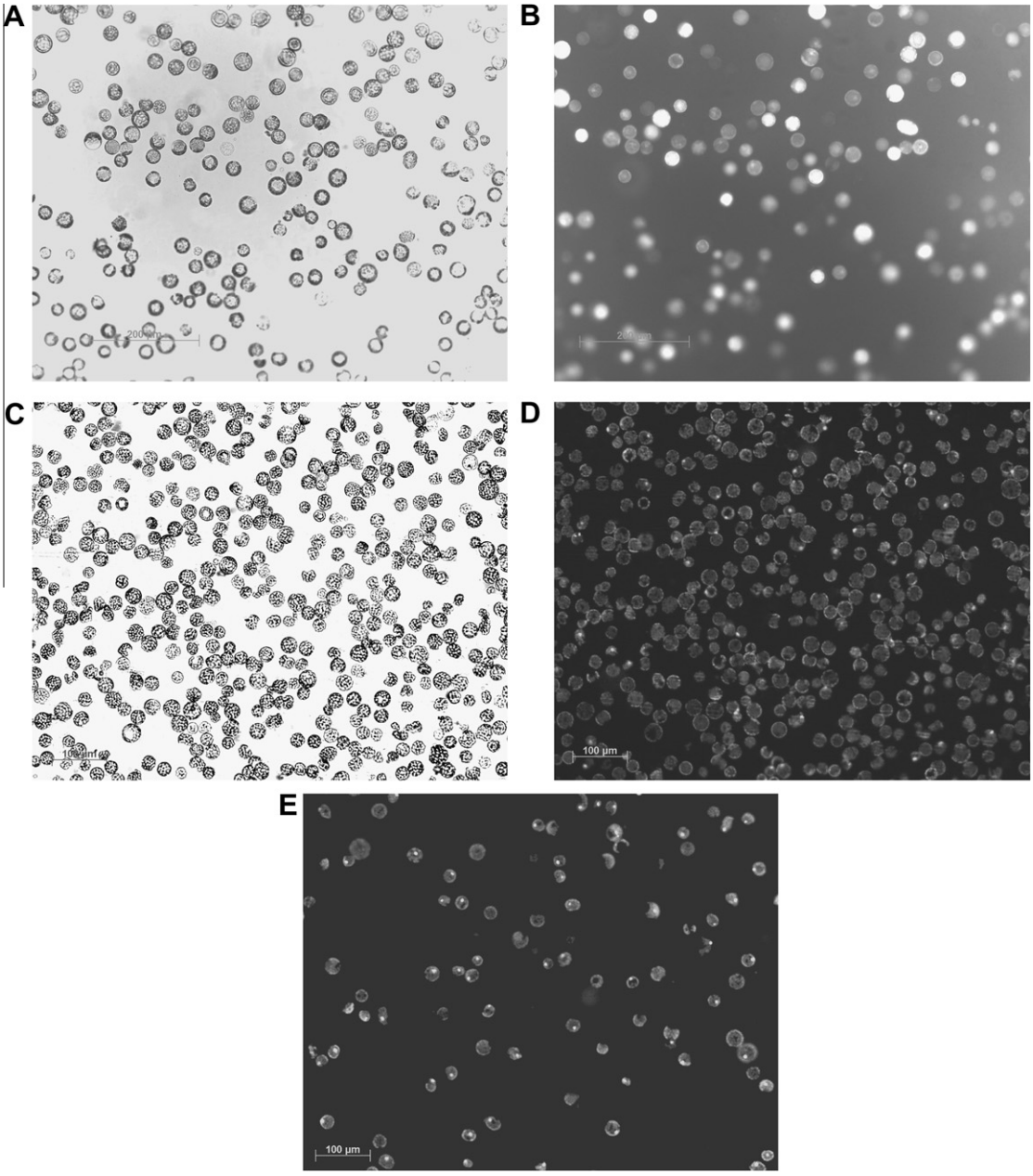

Fig. 1. Representative micrographs of prepared protoplasts from paraquat-treated leaves of *Lolium rigidum*. A and B: bright field *versus* FDA staining images. C and D: bright field *versus* PI staining images. Bright dots inside protoplast indicate PI staining relative to chlorophyll autofluorescence. E: distinctive PI staining of protoplast prepared from paraquat treated leaves after prolonged storage (>4 h).

washed with 100 mL DI water at a flow rate of 3–4 mL min^{-1}. The paraquat bound to resins was eluted with saturated ammonium chloride solution at a flow rate of 1 mL min^{-1} and the first 30 mL of eluent collected. To quantify paraquat content, 5 mL of the eluent and 1 mL of 0.1% sodium dithionite in 2 N NaOH were mixed and the absorbance at 392, 396, and 401 nm recorded with the mixture of saturated ammonium chloride and sodium dithionite as a blank. To cancel background absorption by naturally occurring substances, the absorbance at 396 nm was corrected using equations described by Calderbank and Yuen [29]. This method of background calculation has the particular advantage of rendering unnecessary analysis of control samples. Standard paraquat solutions in the range of 0.025–1 ppm (µg mL^{-1}) were prepared using analytical grade paraquat dichloride dissolved and diluted in saturated ammonium chloride solution. These solutions were treated with sodium dithionite and their absorbances at 396 nm were recorded to obtain a standard curve. Paraquat content was calculated from the standard curve. The paraquat standard curve had linearity of 0.999 and the method had a sensitivity of 0.01 ppm paraquat (µg mL^{-1}). The recovery rate of spiked paraquat at 0.05 ppm in protoplast preparation suspension was 86 ± 2%. Significant differences in chlorophyll or protein based paraquat content between R and S samples and among independent experiments were assessed using ANOVA two-factor without replication analysis at the 5% level.

2.6. Paraquat metabolism in intact leaves

At the 2–3 leaf stage, two 1 µL droplets of 2 mM paraquat solution, containing 2.2 kBq [^{14}C]-paraquat dissolved in Gramoxone® and 0.06% v/v non-ionic surfactant BS1000, were applied to the youngest expanded leaf. After treatment the plants were maintained at 20 °C under a light regime of 350 µmol quanta m^{-2} s^{-1} and shoots

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were harvested 24 h later and washed in distilled water with 0.1% Triton X100 detergent. Paraquat in shoot tissue was extracted by refluxing the samples with 80% methanol at 70 °C for 3 h, and the residue was further washed with 2 mM EDTA followed by 1 M NaCl and finally by 2 N H_2SO_4. Each fraction was collected separately and concentrated under N_2. Samples were analyzed by thin layer chromatography (TLC) in a mobile phase of methanol: HCl/2: 3 using silica gel plates (Polygram® SIL G, Macheray–Nagel, Germany) or cellulose plates (Polygram® CEL 300). Standard [^{14}C]-paraquat was spiked into untreated plants and the same procedure followed for extraction. The amount of radioactivity in the shoot-washes and each fraction of the extract was quantified by liquid scintillation spectroscopy. Visualisation of the results from TLC analysis was achieved with a phosphor imager (BS 2500, FujiFilm, Japan).

3. Results and discussion

3.1. Protoplast isolation

The efficacy of protoplast preparation from intact leaves is a compromise between length of exposure to digesting enzyme and obtaining reasonable yield. Here, we optimised the enzyme combination of cellulose R-10 (1.33%) and Pectolyase Y-23 (0.07%) to release protoplasts within 50–70 min at 30 °C with a yield of 18–35 μg chlorophyll per gram fresh weight (influenced by growing season and developmental stages).

Intact plants were treated with paraquat at the recommended paraquat field dose (200 g ha^{-1} as l L ha^{-1} of commercial Gramoxone formulation). Treated plants were maintained at moderate light (50–100 μmol quanta m^{-2} s^{-1}) for 2 h to ensure paraquat leaf uptake but to avoid any visual bleaching indicative of leaf paraquat damage. Further steps to minimize any possible damage by paraquat during the protoplast isolation procedure included provision of Na-ascorbate (10 mM) in the digestion solution and incubating in the dark. Under these experimental conditions, good quality protoplasts were obtained from treated leaves. The viability and intactness of prepared protoplasts were examined in a number of experiments by both FDA and PI staining. The averaged intactness of protoplasts from paraquat-treated plants was estimated by PI staining as 84% and 87% and the intactness evaluated by FDA staining was 72% and 78%, for S and R plants, respectively (as represented in Fig. 1). The difference may be due to that PI stains intact cells while FDA stains intact and viable cells. These results indicate that within the given experimental conditions, paraquat treatment at the whole plant level did not cause significant physiological damage to plasma membrane and the protoplasts isolated from leaves of treated plants were largely intact and viable.

3.2. Paraquat treatment of isolated protoplasts

When protoplasts were prepared from leaves of untreated plants and then the isolated protoplasts incubated in a medium with up to 500 μM paraquat there was no chlorophyll bleaching in either R or S protoplasts (data not shown). An excessive dose of 1 mM paraquat was required to produce measurable damage in both R and S protoplasts as indicated by an 18–25% net decrease relative to untreated samples in chlorophyll absorbance at 652 nm over 4–6 h continuous exposure to light (250 μmol quanta m^{-2} s^{-1}) (Fig. 2). This observation confirmed the results obtained with isolated protoplasts from leaves of paraquat resistant *Hordeum* spp. showing that isolated protoplasts have very low uptake of paraquat, in comparison with the rate of paraquat uptake when leaves of intact plants are treated [19]. In addition, isolated protoplasts could not be maintained in a viable state for lengthy periods since an 18–38% decrease in chlorophyll absorbance in untreated samples was observed over a 4–6 h experiment period (Fig. 2). These results indicate that paraquat resistance in *L. rigidum* is unlikely due to changes in plasma membrane permeability and that isolated protoplasts are not a good system to study paraquat uptake, at least in *L. rigidum* and *Hordeum* spp. This further highlights the biological relevance of working with a commercial dose of paraquat applied by normal commercial spray treatment to intact plants and then isolating protoplasts from the treated leaves to quantify paraquat levels within protoplasts.

3.3. Paraquat metabolism in intact leaves

Experiments with [^{14}C]-paraquat treated intact leaves (2–3 leaf stage plants) revealed an average of 30% of the applied [^{14}C]-paraquat was recovered from the 80% methanol fraction, (0.3% from the 2 mM EDTA fraction), 25% from the 1 M NaCl fraction and 15% from the 2 N H_2SO_4 fraction. Therefore, total recovery was about 70% of applied radioactivity (the remaining radioactivity may be associated with non-extractable residues). The 80% methanol fraction may represent more soluble paraquat while NaCl and H_2SO_4 fractions may represent paraquat bound by proteins and polysaccharides. Using silica or cellulose plates, the TLC profile was found to be similar for the methanol and the NaCl fractions between R and S samples. The H_2SO_4 fraction was not examined. Fig. 3 presents the TLC results of the 80% methanol fraction (A) and the 1 M NaCl fraction (B) as visualised by the phosphor imager, and the radioactivity also co-chromatographed with spiked [^{14}C]-paraquat standard (Fig. 3B lane Rs, Ss). It is evident that, in these whole plant experiments, extractable paraquat was not metabolised to any appreciable extent in either R or S plants, and therefore paraquat metabolism does not contribute to paraquat resistance in this resistant *L rigidum* biotype. This observation is consistent with previous reports on paraquat resistant biotypes of *Lolium perenne* L. [31], *C. bonariensis* [32] and other higher plants [33] which establish that paraquat is not metabolised by higher plants.

3.4. Paraquat quantification in leaf protoplasts isolated from paraquat-treated plants

Table 1 summarises the results of the protoplast paraquat levels measured in four independent experiments. Firstly, chlorophyll con-

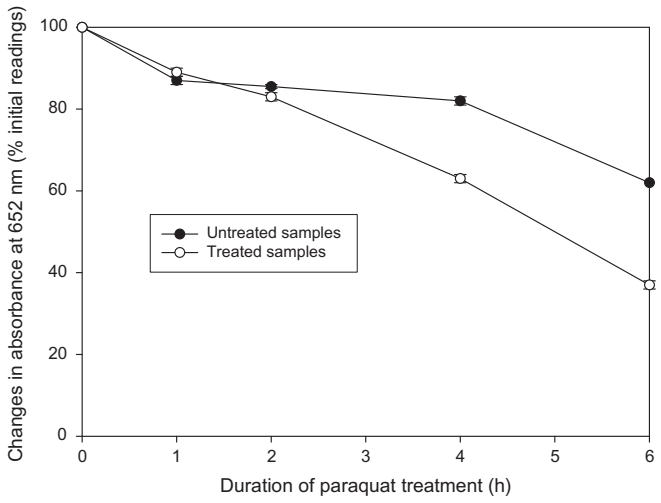

Fig. 2. Comparison of decrease in chlorophyll absorbance at 652 nm of untreated and paraquat treated protoplast preparations. Protoplasts from untreated plants were prepared, then the isolated protoplasts incubated in medium containing 1 mM paraquat under a light intensity of 250 μmol quanta m^{-2} s^{-1} at 18–20 °C. The initial absorbance (100%) at 652 nm in the treated and untreated samples was 0.50 ± 0.01 and 0.52 ± 0.01, respectively. Only the results from S protoplasts are presented here, with the results from R protoplasts similar.

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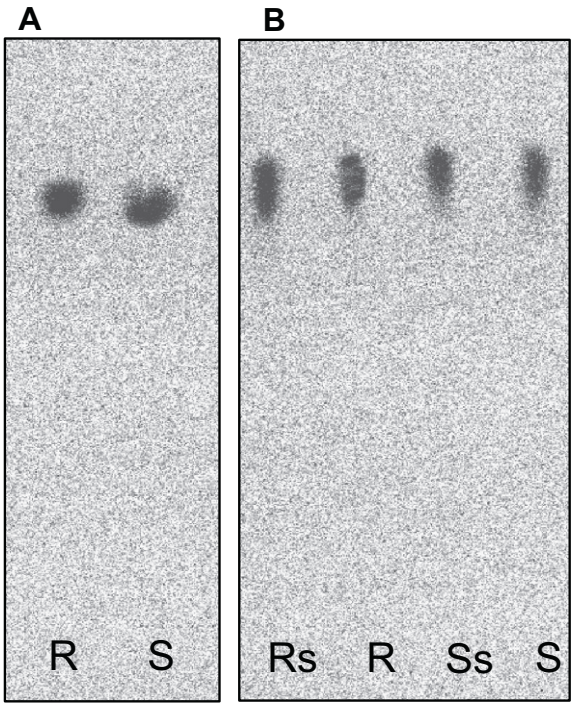

Fig. 3. Thin layer chromatography analysis of leaf extracts from R and S biotypes of *L. rigidum*. The radioactivity on the TLC plate was visualised by a phosphor imager. A: the 80% methanol soluble fraction. B: the 1 M NaCl soluble fraction. Rs: [C^{14}]-paraquat standard spiked in R samples, Ss: [C^{14}]-paraquat standard spiked in S samples.

tent in the leaf protoplasts prepared from R and S leaves was very similar in each experiment. Furthermore, the average number of leaf protoplasts per microgram of chlorophyll was found to be very similar between R and S samples ($3.0 \pm 0.3 \times 10^4$ and $3.05 \pm 0.5 \times 10^4$ for R and S plants, respectively). Therefore, the paraquat content in R *versus* S protoplasts isolated from paraquat-treated plants can be directly compared on a chlorophyll basis (E factor not significant, $P = 0.5$, Table 1). In addition, the protein content was also measured in each preparation and used as a basis for comparing paraquat content ($P = 0.04$). By treating intact plants with the field commercial rate of paraquat in the normal way that paraquat is applied in agriculture and then isolating leaf protoplasts it was consistently found that protoplasts isolated from leaves of treated R plants contained 2- to 3-fold more paraquat than was found in leaf protoplasts from S plants (B factor significant, $P < 0.01$, Table 1).

The 2- to 3-fold higher level of paraquat in protoplasts of R leaves is unlikely to be in the cytosol because it has been established that paraquat readily enters chloroplasts in both R and S plants [19,32]. As paraquat readily enters chloroplasts of both R and S plants, and as there was no metabolic degradation of paraquat in R or S plants (Fig. 3), the most likely interpretation of the results in Table 1 is that paraquat entering the leaves reaches the cytosol where it is sequestered at a greater rate into leaf vacuoles of R plants. Paraquat sequestered in vacuoles will take longer to efflux back into external solution than if it were present in the cytosol, when active efflux of paraquat may occur during protoplast preparation (50–70 min). One could argue that in addition to efflux, paraquat influx from the apoplast into protoplasts could occur after the cell wall is digested and paraquat is released from the wall, and this would confound the results. We measured the concentration of paraquat in the digestion solution to be 16 μM, and because isolated protoplasts have surprisingly low permeability to paraquat (this experiment and Powles and Cornic [19]), then significant influx is unlikely to occur. It could also be that R plants have more paraquat bound on the surface of the plasma membrane. This is unlikely because it was demonstrated in *Hordeum* spp. that concentration-dependent total uptake of paraquat into protoplasts was the same between R and S plants (Alizadeh, Preston and Powles, unpublished). There was no difference in paraquat uptake into plasmalemma vesicles derived from leaves of R and S *Hordeum glaucum* [4]. In addition, we examined paraquat binding/uptake using plasma membrane vesicles and found that binding/uptake was rapid and saturated within minutes and there was no difference between R and S biotypes (Yu and Huang, unpublished). Therefore, at least in this paraquat resistant *L. rigidum* biotype, paraquat must be sequestered into a compartment in the cytoplasm and the vacuole is the most likely site for sequestration.

Although different resistant weed species may have different resistance mechanisms, there are a number of studies with various paraquat resistant plant species that suggest the mechanism of paraquat resistance involves vacuole sequestration. No difference in cell wall binding of paraquat was found between R and S biotypes of *C. bonariensis* [7], *H. glaucum* [18,34], *H. leporinum* [34] or *A. calendula* [15]. There was no difference in the permeability of paraquat in isolated leaf protoplasts [19,32] or in the uptake of paraquat across the plasmalemma into intact roots of *H. glaucum* [20]. Additional evidence to support vacuolar sequestration is the compartmentation analysis of paraquat efflux in intact roots of paraquat resistant *H. glaucum*. Both time and concentration-dependent kinetics of paraquat influx across the root cell plasma membrane were similar in R and S biotypes [20]. Compartmentation analysis indicated a greater paraquat accumulation in root vacuoles of the R plants. Cell fractionation studies with paraquat resis-

Table 1
Paraquat in leaf protoplasts isolated from paraquat-treated plants. Four independent experiments were conducted and in each experiment 15–20 protoplast preparations were combined for paraquat quantification.

Experiment (E)	Biotype (B)	Paraquat content (μg)	Chlorophyll content (mg)	Paraquat/Chl (μg/mg)	R/S ratio on Chl basis	Protein content (mg)	Paraquat/Protein (μg/mg)	R/S ratio on protein basis
I								
	R	4.56	3.07	1.5	2.0			
	S	2.74	3.65	0.75				
II								
	R	13.14	7.64	1.72	2.4	63.5	0.207	2.4
	S	5.33	7.41	0.72		60.5	0.088	
III								
	R	9.28	4.37	2.12	3.0	59.3	0.156	2.7
	S	3.14	4.46	0.7		53.3	0.059	
IV								
	R	4.44	2.54	1.75	3.6	34.0	0.131	3.4
	S	1.16	2.41	0.48		30.1	0.037	
E				$P = 0.55$			$P = 0.04$	
B				$P < 0.01$			$P < 0.01$	

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tant *C. canadensis* showed a greater amount of paraquat in the leaf cytosol + vacuole fraction of the R compared to the S plants, and two EST sequences homologous to amino acid and polyamine transporters were identified in the R plants and their role in vacuole sequestration was implicated [21].

In our work we could not harvest enough intact vacuoles from isolated leaf protoplasts for further paraquat quantification and leaf efflux studies were hampered due to the difficulty in removing leaf epidermal layers in *L. rigidum*. Furthermore, the time and concentration-dependent accumulation of paraquat in leaf protoplasts of R and S plants was not examined due to requirement for large scale protoplast preparation. Nevertheless, the result from this study, showing greater accumulation of paraquat in leaf protoplasts of R plants following commercial whole plant paraquat treatment is important because it provides evidence for the possible leaf vacuole sequestration mechanism in resistant *L rigidum* populations. Our genetic inheritance study reveals that reduced paraquat movement based paraquat resistance in this *L. rigidum* population is controlled by a single nuclear gene [35]. We are now undertaking gene expression profiling analysis to examine for the possible mechanistic basis of enhanced paraquat sequestration.

In conclusion, by conducting experiments in which intact paraquat resistant and susceptible plants were treated with paraquat at agronomically relevant dose and conditions and then by isolating leaf protoplasts and measuring paraquat content in the intact protoplasts, we revealed a higher paraquat accumulation in leaf protoplasts of R plants. From this evidence, together with other lines of evidence in other paraquat resistance plant species, we propose that paraquat resistance in this *L. rigidum* biotype is very likely due to a cytoplasmic mechanism, vacuole sequestration of paraquat.

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