Enhanced Inducibility of Antioxidant Systems in a *Nicotiana tabacum* L. Biotype Results in Acifluorfen Resistance*

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Levels of non-protein thiols (mostly glutathione, GSH), ascorbic acid (AA), and activities of the enzymes ascorbate peroxidase (AP), glutathione reductase (GR) and GSH S-transferase (GST) were determined in cell-free leaf extracts of acifluorfen-resistant and -sensitive tobacco plants. These parameters were examined also in detached leaves of the above plants exposed to acifluorfen stress. In leaves of untreated plants the AA content was by 40% higher in the resistant biotype as compared to the sensitive ones, but the levels of GSH, AP, GR, and GST did not differ significantly in the two biotypes. However, in the resistant leaves stressed by acifluorfen the activity of AP readily increased while in the sensitive leaves it did not change. The levels of GSH and the activities of GR and GST markedly increased in both biotypes after acifluorfen stress, but the induction in the resistant leaves was consistently stronger in each case. The AA contents were increased equally in both biotypes. These parameters were much less affected by paraquat stress. The only significant changes were observed at low concentrations of this herbicide (8×10^{-9} M): when the thiol content and the activity of GST increased in the resistant leaves.

Enhanced inducibility of antioxidant systems seems to be involved in resistance of tobacco to acifluorfen stress.

Introduction

The biochemical mode of action of the herbicide acifluorfen (sodium 5-[2-chloro-4-(trifluoromethyl)-phenoxy]-2-nitrobenzoate) has been recently discovered [1]. In addition to its effects on the secondary metabolism of plants [2], acifluorfen inhibits the biosynthesis of chlorophyll thereby leading to an accumulation of photooxidative tetrapyrroles [3–6]. Plant cell death in the light is a consequence of oxidative stress (increased production of reduced, active oxygen derivatives) [7]. Leaf cells contain superoxide dismutase (SOD, EC 1.15.1.1) to decompose superoxide radical anion and an efficient ascorbate (AA) – glutathione (GSH) system in the chloroplast to scavenge hydrogen peroxide [8, 9]. Ascorbate peroxidase (AP, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) participate in this system. Higher levels of these two enzymes and that of SOD were found in the chloroplast stromal extracts (but not in total cell extracts) from the paraquat resistant biotype of *Conyza bonariensis* [10]. Paraquat (1,1′-dimethyl-4,4′-bipyridilium dichloride) generates also oxidative stress in green plants and as a consequence it causes membrane damage [11].

Treatment of cucumber leaf disks with acifluorfen led to a strong decrease of GSH content and GR activity [12]. In contrast, bean leaves responded to acifluorfen treatment by increasing the concentration of GSH and by elevating the activity of GR [13]. The increased level of antioxidant systems was suggested to be a general strategy to limit toxic peroxidation [13, 14].

The reactions of GSH with many xenobiotics and electrophilic metabolites are catalyzed by GSH S-transferase enzymes (GST, EC 2.5.1.18). These conjugation reactions, with few exceptions, are considered as detoxication processes. In addition to this role, GST enzymes may also function as GSH peroxidases by catalyzing the reaction between GSH and lipid hydroperoxides [15]. The inducibility of GST isoenzymes by herbicide safeners has been described in higher plants [15, 16].

Elevated SOD activity was found in a superoxide resistant tobacco biotype which had been regenerated from calluses after *in vitro* selection by

Abbreviations: R, resistant leaves; S, sensitive leaves.

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paraquat [17]. In the present study the H_2O_2 scavenging systems (levels of AA, non-protein thiols, and the activities of AP, GR, and GST) have been examined in a superoxide-resistant biotype of tobacco. In order to gain a deeper insight into the mechanism of herbicide resistance the effects of acifluorfen and paraquat on these redox systems have also been investigated.

Materials and Methods

Plant material

Seeds of *in vitro* selected superoxide-resistant and sensitive tobacco plants (*Nicotiana tabacum* L., *cv.* Samsun; obtained from Dr. I. Furusawa, Kyoto University) were planted in individual pots containing sand and soil (1 : 1). Plants were grown in a greenhouse under normal conditions (temperature; 18–23 °C, supplemental light: 160 μE m^{-2} s^{-1} for 8 h per day). Leaves of 65–70 days old plants were excised from identical leaf positions and were treated by placing their petioles in solutions of acifluorfen ($1 \times 10^{-5} - 1 \times 10^{-3}$ M), paraquat ($1.6 \times 10^{-9} - 5 \times 10^{-5}$ M), or in tap water. Leaves were exposed to continuous light (25 or 120 μE m^{-2} s^{-1}) at 22 °C.

Enzyme assays

For enzyme assays cell-free homogenates were prepared at 0–4 °C. Leaf material (1 g) was homogenized in 3 ml cold 0.2 M TRIS/HCl buffer (pH 7.8) containing 3% soluble polyvinylpyrrolidone and 0.1 mM EDTA-NA_2. The homogenate was filtered through muslin and centrifuged at 8000 g for 20 min. The supernatants were used as enzyme source.

AP activity was determined according to ref. [9], except that in our experiments 0.5 mM H_2O_2 and 0.25 mM AA was used. GR [8] and GST [18] activities were assayed spectrophotometrically.

Other assays

Levels of AA, non-protein thiols, and GSH were determined spectrophotometrically according to ref. [19], [20] and [21], respectively. Chlorophyll was extracted by 80% acetone and measured according to [22]. For determination of dry leaf weights, leaves (0.5 g) were dried for 2 h at 105 °C.

Statistical analysis

Results are expressed as the means ± S.D. of 3 independent experiments. Significance of differences was evaluated by Student's t-test.

Results

H_2O_2-scavenging system in untreated plants

The AA content was about 40% higher in the leaves of superoxide-resistant tobacco plants when compared to the sensitive ones. However, the level of non-protein thiols and the activities of AP, GR and GST did not differ significantly in the two biotypes. Glutathione accounts for 76% of the non-protein thiol content both in the resistant and sensitive plants.

Acifluorfen and paraquat resistance of tobacco leaves

The toxicity of acifluorfen and paraquat was tested at various concentrations. The visible symptoms, the chlorophyll contents and the desiccation data of the leaves stressed by the herbicides are compiled in Table I. Leaves of the paraquat resistant plants proved to be cotolerant to acifluorfen. Paraquat caused phytotoxic symptoms at much lower concentrations than acifluorfen. The resistant leaves tolerated 1×10^{-6} M paraquat and 2.5×10^{-4} M acifluorfen for 96 h.

Acifluorfen stress

Acifluorfen (5×10^{-5} M) considerably altered the level of H_2O_2-scavening systems in tobacco leaves under continuous light (25 μE m^{-2} s^{-1}). In leaves stressed by acifluorfen the AA content increased equally in both biotypes after 24 h, then declined to the control level (Fig. 1).

Acifluorfen stress resulted in a significant increase of non-protein thiol content both in the resistant and sensitive leaves. In sensitive leaves the thiol content reached 232% of the untreated controls after 48 h exposure, then declined to the control level after 96 h. In paraquat resistant leaves the thiol content increased more rapidly. Thiols reached significantly higher levels than in the sensitive ones (360% of the control level after 48 h) and their level remained above that of the untreated control throughout the experimental period (Fig. 2).

Table I. Visible symptoms, chlorophyll content and desiccation of superoxide resistant and sensitive tobacco leaves after exposure to acifluorfen and paraquat at 25 μE m^{-2} s^{-1} light flux density.

Treatment		Visible symptoms[a] after various exposure periods [h]					Chlorophyll content[b] [mg/g dry wt]	Desiccation[c] [%]
		24	48	72	96	120		
Acifluorfen [M]								
1×10^{-3}	R	−	−	+	+	+++	5.9	15.4
	S	−	+	+++	++++	++++	1.8	30.8
2.5×10^{-4}	R	−	−	−	−	++	6.6	9.6
	S	−	−	+++	+++	+++	3.1	11.6
1×10^{-4}	R	−	−	−	−	++	8.3	10.6
	S	−	−	++	+++	+++	5.1	9.4
5×10^{-5}	R	−	−	−	−	++	8.4	10.3
	S	−	−	++	++	++	5.9	10.8
1×10^{-5}	R	−	−	−	−	+	10.9	10.2
	S	−	−	−	++	++	7.5	9.1
Paraquat [M]								
5×10^{-5}	R	−	*	**	****	****	3.6	37.6
	S	**	***	****	****	****	1.8	42.4
2.5×10^{-5}	R	−	*	**	***	***	8.3	11.9
	S	−	***	****	****	****	5.5	14.1
1×10^{-5}	R	−	−	−	**	**	8.8	10.2
	S	−	**	***	****	****	6.3	10.9
5×10^{-6}	R	−	−	−	**	**	11.9	9.7
	S	−	*	***	***	***	6.7	9.9
1×10^{-6}	R	−	−	−	*	*	12.9	8.9
	S	−	−	**	**	**	10.2	9.1
Tap water	R	−	−	−	−	−	9.6	11.2
	S	−	−	−	−	−	9.1	8.9

[a] Visible symptoms:

Acifluorfen treatment
+ water-soaked spots
++ color change to yellow
+++ yellow leaf surface, dark spots
++++ wilting, desiccation

Paraquat treatment
* weak necrosis along major veins
** necrosis along major veins
*** approx. 50% of leaf surface is yellow or brown
**** total surface is brown, desiccation

[b] Measurements were carried out after 96 and 120 h of exposure in the case of paraquat and acifluorfen, respectively.

[c] Desiccation level (%): the dry leaf weight as percentage of the fresh leaf weight, determined after the herbicide treatment (according to ref. [25]).

Significantly increased ascorbate peroxidase activity was found in the resistant, but not in the sensitive biotype when leaves were exposed to acifluorfen. The AP activity in the resistant leaves reached 516% of the control after 96 h of treatment (Fig. 3).

Acifluorfen increased the activity of GR in both biotypes. However the induction was stronger in the resistant leaves during the entire course of treatment than in the leaves of the sensitive biotype. After 96 h of exposure, the GR activity was 890% of the control in the resistant leaves (Fig. 4).

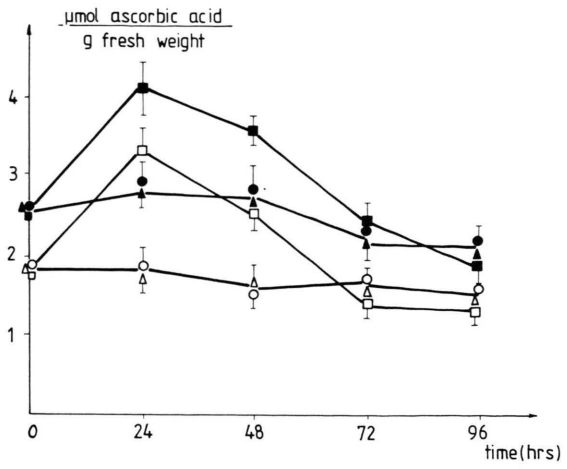

Fig. 1. Changes in the ascorbic acid content of leaves of acifluorfen resistant and sensitive tobacco plants after acifluorfen and paraquat stress. Explanation of symbols:
—○—○— sensitive plant, tap water control;
—□—□— sensitive plant, 5×10^{-5} M acifluorfen;
—△—△— sensitive plant, 1×10^{-6} M paraquat;
—●—●— resistant plant, tap water control;
—■—■— resistant plant, 5×10^{-5} M acifluorfen;
—▲—▲— resistant plant, 1×10^{-6} M paraquat.

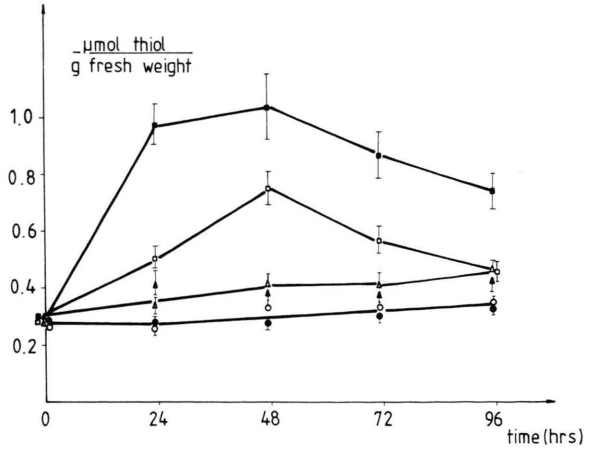

Fig. 2. Changes in the non-protein thiol content of leaves of acifluorfen resistant and sensitive tobacco plants after acifluorfen and paraquat stress. For symbols see Fig. 1.

Massive and rapid induction by acifluorfen of GST was detected in both biotypes. Similar to the results obtained with GR, this induction was more consistent in the resistant leaves (Fig. 5).

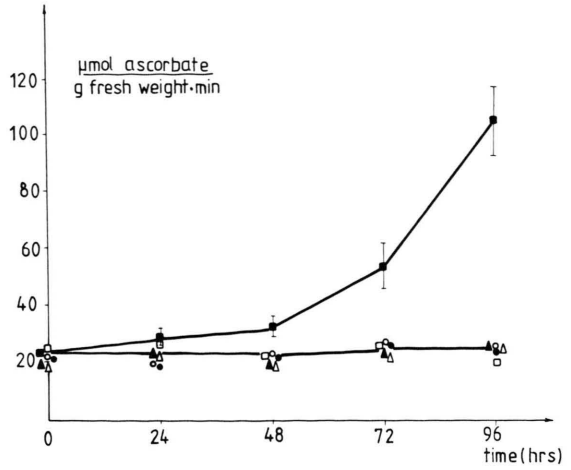

Fig. 3. Induction of ascorbate peroxidase activity in the leaves of an acifluorfen resistant tobacco biotype after acifluorfen stress. For symbols see Fig. 1.

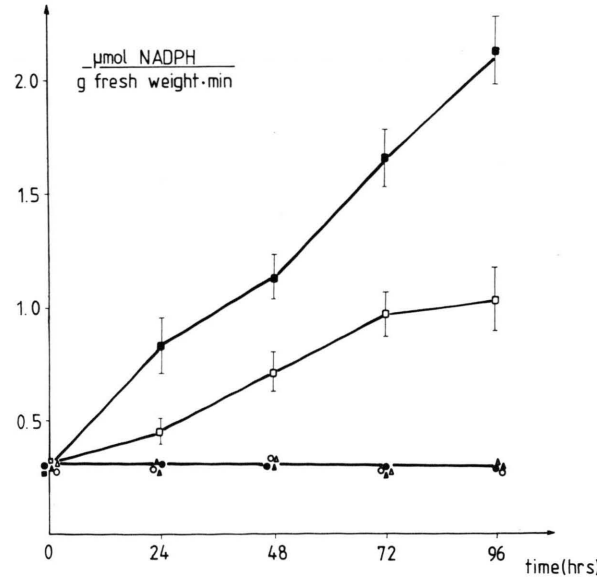

Fig. 4. Induction of glutathione reductase activity in the leaves of acifluorfen resistant and sensitive tobacco biotypes after acifluorfen and paraquat stress. For symbols see Fig. 1.

Paraquat stress

In the first experiments paraquat (1×10^{-6} M) did not exert any influence on the H_2O_2-scavenging systems of tobacco leaves under continuous light

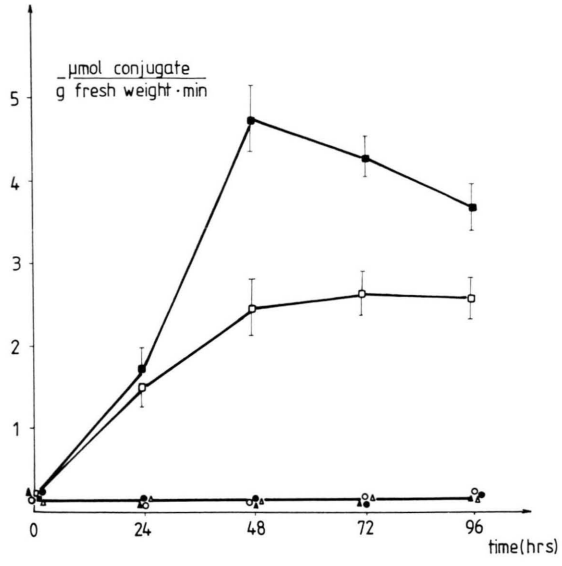

Fig. 5. Induction of glutathione S-transferase activity in the leaves of acifluorfen resistant and sensitive tobacco plants after acifluorfen and paraquat stress. For symbols see Fig. 1.

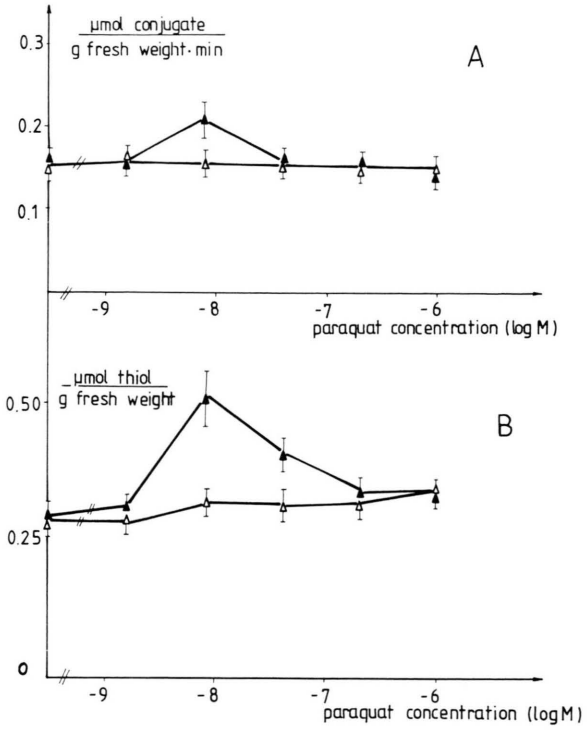

Fig. 6. Effects of paraquat at various concentrations on the glutathione-S-transferase activity (A) and on the non-protein thiol content (B) in the leaves of acifluorfen resistant and sensitive tobacco plants. For symbols see Fig. 1.

(25 µE m^{-2} s^{-1}) (Fig. 1–5) until 96 h, except the non-protein thiols. Thiols were slightly induced by this treatment, but to the same extent in both biotypes (Fig. 2). In subsequent experiments, several paraquat concentrations ($1.6 \times 10^{-9} - 1 \times 10^{-6}$ M) have been applied at the above light flux density. AA, thiols and the detoxifying enzymes were examined after 2 days of exposure. Paraquat slightly increased the non-protein thiol content throughout the whole concentration range in both biotypes. Significant induction of thiols (increased by 59%, at P = 1% significance level) and GST (increased by 31%, at P = 5% significance level) were detected at 8×10^{-9} M paraquat but only in the resistant leaves (Fig. 6). The AA level and the activities of AP and GR did not change significantly at any paraquat concentration in either biotype. Similar results were obtained after 4 days of exposure (at 25 µE m^{-2} s^{-1}) and at higher light flux density (120 µE m^{-2} s^{-1}) after 2 and 4 days of exposure to various paraquat concentrations (data not shown).

The effect of 1 day exposure of the leaves to 5×10^{-5} M paraquat (at 25 µE m^{-2} s^{-1} continuous light) on the antioxidative systems was also examined. This stress effect decreased the levels of AA and of non-protein thiols and decreased activities of AP, GR and GST were found in the sensitive leaves. These parameters did not change significantly in the resistant leaves (Table II).

Discussion

Resistance of plants to the herbicides paraquat and acifluorfen has been attributed to alterations in the capability of the plants to detoxify active oxygen species generated by these herbicides [10, 13]. Thus, higher levels of SOD, AP and GR were detected in *Conyza bonariensis* plants that were resistant to paraquat [10]. Increased synthesis of GSH [23] and induction of GR [24] have been observed in plants after various stress effects, including acifluorfen stress [13].

In our experiments we have used paraquat- (superoxide-) resistant tobacco plants selected *in vitro*. These plants proved to be cotolerant to acifluorfen. The leaves of resistant tobacco plants

Table II. Effect of 5×10^{-5} M paraquat on H_2O_2-scavenging systems in the resistant and sensitive tobacco leaves after 24 h of exposure at continuous illumination (25 μE m^{-2} s^{-1}). Significant differences were found between paraquat treatment and control (tap water) only in sensitive leaves (at P = 1% except GST where at P = 5%).

	Sensitive		Resistant	
	Control	Paraquat	Control	Paraquat
AA content (μmol/g F.W.)	1.92 ± 0.21	1.15 ± 0.15	2.43 ± 0.25	1.97 ± 0.22
Non-protein thiols (μmol/g F.W.)	0.31 ± 0.03	0.20 ± 0.02	0.30 ± 0.02	0.29 ± 0.02
AP activity (μmol AA/g F.W. × min)	22.3 ± 2.0	13.7 ± 1.6	24.7 ± 2.2	23.8 ± 3.0
GR activity (μmol NADPH/g F.W. × min)	0.32 ± 0.03	0.20 ± 0.02	0.30 ± 0.02	0.29 ± 0.02
GST activity (μmol conjugate/g F.W. × min)	0.16 ± 0.03	0.11 ± 0.01	0.15 ± 0.02	0.16 ± 0.02

contained more AA (by 40%) than the sensitive ones. This phenomenon can contribute to the resistance against oxidative damage not only because AA participates in the scavenging of H_2O_2 but also because of its role in the direct regeneration of vitamin E under peroxidative conditions [25]. However, the differences in the contents of AA, non-protein thiol, as well as in the activities of AP, GR, and GST in the paraquat resistant and susceptible tobacco biotypes were rather small or insignificant to account for the degree of the observed herbicide resistance.

On the other hand, exposure to herbicides that generate active oxygen derivatives led to changes of sufficient magnitude to explain plant resistance. The non-protein thiol content significantly increased in tobacco leaves exposed to acifluorfen (to a lesser extent in the sensitive leaves) but only slightly to paraquat stress (only in the resistant leaves). GSH was found to be the major thiol in the tobacco biotypes according to earlier reports [26]. Paraquat stress did not alter the AA content of leaves, and the effects of acifluorfen are inconclusive.

Acifluorfen stress markedly increased the activities of AP, GR and especially that of GST. The rate of induction was higher in each case in the resistant leaves. Induction of GST by chloroacetamide and thiolcarbamate herbicides in maize, sorghum and rice [15] and by herbicide safeners in sorghum [16] has been described. The mechanism of induction is unknown. Our findings indicate that oxidative stress may also stimulate GST activity and this response may take place also in a dicotyledonous plant. The results of this study indicate the possibility of decreasing plant susceptibility to acifluorfen or to other types of oxidative environmental stress by preliminary application of low doses of this herbicide for stimulating plant defense mechanisms. Experiments are in progress to investigate this hypothesis.

Interestingly, the above enzymes were not inducible (or very weakly inducible in the case of GST) by paraquat treatment. The difference between the effects of acifluorfen and paraquat reflects that in spite of their common ability to generate oxidative stress their mode of action is different. Furthermore, acifluorfen generates free radicals primarily in the mitochondria while paraquat acts primarily in the chloroplasts [27]. Paraquat is also toxic in the dark with unknown mechanism [27]. Thus it would seem that the mode of action of paraquat is partly independent of the oxidative stress.

The observation that superoxide-resistant tobacco responds to oxidative stress more rapidly and with higher activity than the sensitive one suggests that herbicide resistance is due to the enhanced inducibility of the detoxification systems in the resistant plants.

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