Fatty Acid Alteration of Plastidic and Extra-Plastidic Membrane Lipids in Metribuzin-Resistant Photoautotrophic *Chenopodium rubrum* Cells as Compared to Wild-Type Cells

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The fatty acid compositions of plastidic and extra-plastidic membrane lipids of two metribuzin-resistant cell lines L4 and L7 of *Chenopodium rubrum* were determined after growth in the absence and in the presence of the herbicide and compared with those of wild type cells. Fatty acid biosynthesis was markedly affected in all cell lines by metribuzin treatment. In the absence and in the presence of metribuzin alterations of the fatty acid composition of the various lipid classes were, as compared to wild type cells, generally lower in the highly resistant L4 cells than in the less resistant L7 cells. The two resistant cell lines demonstrated a higher degree of unsaturation within the plastidic monogalactosyldiacylglycerols (L4 cells also within plastidic digalactosyldiacylglycerols) and, particularly, within the predominantly extra-plastidic phosphatidylcholines (L7 cells also within the predominantly extra-plastidic phosphatidylethanolamines), whereas the degree of unsaturation was slightly altered in the plastidic phosphatidylglycerols. Within the two metribuzin-resistant cell lines, the highly resistant L4 cells differed from the less resistant L7 cells by increased α -linolenic acid/palmitic acid ratios in both the plastidic and extra-plastidic membrane lipids suggesting that particularly in L4 cells higher proportions of linolenate are formed as a result of selection pressure. On the other hand, the proportion of linoleate was increased predominantly in extra-plastidic membrane lipids of both L4 and L7 cells which explains a raise in linoleic acid/palmitic acid ratios in both cell lines as compared to wild-type cells. Moreover, in the absence of metribuzin decreased proportions of *trans*-3-hexadecenoic acid were found in phosphatidylglycerols of L4 and, particularly, of L7 cells as compared to the wild type cells. It is suggested that L4 and L7 cells – having multiple mutations in the *psbA* gene as observed earlier – are additionally characterized by increased degree of unsaturation of acyl moieties in various polar lipids, e.g. linoleoyl moieties in L4 and L7 cells as well as linolenoyl moieties particularly in highly resistant L4 cells. This increase gives rise to a change in membrane fluidity and may finally lead to increased metribuzin resistance.

Introduction

Photoautotrophic plant cell cultures are versatile systems for basic and applied plant research.

Abbreviations: GC, gas chromatography; DGD, digalactosyldiacylglycerols; Mbz, metribuzin (4-amino-6-*t*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one); MGD, monogalactosyldiacylglycerols; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerol; TLC, thin-layer chromatography; WT, wild type. Fatty acids are indicated by number of carbon atoms : number of double bonds, e.g. 16:0, palmitic acid; c-16:1, *cis*-9-hexadecenoic acid; t-16:1, *trans*-3-hexadecenoic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, α -linolenic acid.

Established fields of application are, e.g. elucidation of regulatory steps in photosynthetic carbon metabolism, elicitor-induced expression of plant antimicrobial reactions and metabolic evaluation of lipids and phytotoxic reagents (Beimen *et al.*, 1992; Hüsemann *et al.*, 1980; Thiemann *et al.*, 1989; Weber and Mangold, 1983).

Recently, eight photoautotrophic cell suspension cultures of *Chenopodium rubrum* had been selected for resistance against metribuzin (Mbz, 4-amino-6-*t*-butyl-4,5-dihydro-3-methylthio-1,2,4-triazin-5-one) via multiple step selection procedure (Thiemann and Barz, 1994a). Mbz, an *as*-triazinone herbicide, inhibits the photosynthetic elec-

tron transport by displacing the secondary electron acceptor Q_B from its binding site at the D1 protein of photosystem II. Sequence analyses of the relevant part of the *psbA* gene coding for the amino acids 209–291 of the D1 protein revealed different double and triple mutations within the eight resistant lines (Schwenger-Erger *et al.*, 1993; Schwenger-Erger *et al.*, 1999). Quite remarkably, all cell lines carry a mutation at position 219 which suggests that this is a decisive mutation site for the expression of Mbz resistance. None of the resistant cell lines carries a mutation at position 264, which is the sole mutation site found so far in herbicide resistant higher plants (Goloubinoff *et al.*, 1984; Hirschberg and McIntosh, 1983; Shigematsu *et al.*, 1989). Furthermore, none of the D1 proteins of the resistant lines has a single amino acid exchange only which suggests that a mutation at position 219 is not sufficient for Mbz resistance. It was also surprising that various cell lines showed identical mutation sites in the sequenced *psbA* gene fragment, yet their growth behavior was quite different (Thiemann and Barz, 1994a; 1994b).

Although an altered D1 protein as the primary genetic and biochemical basis of triazine resistance of certain plant biotypes is rather well known, there are various data which point at the importance of the lipid phase surrounding the herbicide binding protein. For example, interactions between triazine herbicides and acyl lipids in chloroplast membranes may influence sensitivity of weed biotypes to the triazine herbicides (Pillai and St. John, 1981). The binding and inhibitory properties of triazine herbicides undergo substantial changes upon specific depletion by lipase treatment of the thylakoids (Siegenthaler and Mayor, 1992). Certain triazine-resistant biotypes show a higher proportion of unsaturated fatty acids in the thylakoid lipids, especially in the monogalactosyldiacylglycerols (MGD) fraction (De Prado *et al.*, 1992) and an increased level of *trans*-3-hexadecenoic acid (t-16:1) in the plastidic phosphatidylglycerol (PG) (Burke *et al.*, 1982; Chapman *et al.*, 1985; Lehocski *et al.*, 1985; De Prado *et al.*, 1992). The occurrence of t-16:1 acid in PG has been related to the activity of LHCP oligomers (Huner *et al.*, 1989; Kruse *et al.*, 2000). The present investigation was designed to study the alteration of acyl moieties in plastidic (MGD, DGD, and PG) and pre-

dominantly extra-plastidic (PC and PE) membrane lipids of cultured cells of *C. rubrum* resistant to Mbz. For better comparison the highly resistant cell line L4 and the only moderately resistant line L7 were chosen.

Materials and Methods

Chemicals were purchased from E. Merck (Darmstadt, Germany). Fatty acid methyl ester standards were products of Nu Chek Prep (Elysian, MN, USA). Standards of mono- and digalactosyldiacylglycerols as well as sulfoquinovosyldiacylglycerols were generous gifts from Professor E. Heinz, University of Hamburg, Germany.

Eight photoautotrophic Mbz-resistant cell suspension cultures were selected from a Mbz-sensitive wild type (WT) of *C. rubrum* via 'multiple step selection' as described previously (Thiemann and Barz, 1994a). The resistant strains and the wild type cells were grown under photoautotrophic conditions in a mineral medium according to Murashige and Skoog (1962). For investigations of the herbicide effect on the lipid composition the cells were cultivated without or with sublethal concentrations of Mbz. Thus, the culture medium of WT cells contained 10^{-7} M Mbz and that of cell lines L4 and L7 10^{-5} M Mbz.

C. rubrum cell suspension cultures were harvested at the beginning of the stationary growth phase by suction. The cells (average fresh weight 20 g from four culture flasks) were homogenized in 4 ml of dichloromethane:methanol (1:2, v/v) per g fresh weight using an Ultra-Turrax blender (IKA-Werke, Staufen, Germany). After centrifugation, pellets were reextracted twice with 10 ml, each, dichloromethane:methanol (2:1, v/v). The combined extracts were diluted with dichloromethane and water, and the phases were separated by centrifugation (Bligh and Dyer, 1959). The organic phases were dried and concentrated, and total lipids (around 150–200 mg, each) from the cell lines determined by weighing.

The fractions of polar lipids, such as monogalactosyldiacylglycerols and digalactosyldiacylglycerols as well as phosphatidylcholines, phosphatidylethanolamines and phosphatidylglycerols were fractionated by preparative thin-layer chromatography (TLC) on Silica Gel H using acetone:benzene:water (91:30:8, v/v) (Siebertz *et al.*, 1979). The

fractions containing MGD and DGD as well as the origin consisting mainly of the various phospholipids were isolated and extracted from silica gel with dichloromethane:methanol:water (1:2:0.8, v/v). The fractions of MGD and DGD were purified by repeated TLC on Silica Gel H with dichloromethane:methanol (9:1, v/v and 4:1, v/v, respectively).

PC were separated from the other phospholipids by TLC on Silica Gel H with dichloromethane:methanol:acetic acid (70:30:5, v/v). The remaining prefractionated PE and PG were isolated and further purified by TLC on Silica Gel H with dichloromethane:acetone:methanol:acetic acid:water (30:40:10:10:3, v/v) (Kates, 1972). Finally, PE were separated from PG by TLC on Silica Gel H with dichloromethane:methanol:acetic acid (65:25:5, v/v).

All lipid fractions were identified by co-chromatography with standards. In addition, the various fractions of phospholipids were detected on the TLC plates by staining with Dittmer-Lester reagent (Dittmer and Lester, 1964). The fractions of PC were further identified on the TLC plates by staining with Dragendorff reagent (Wagner *et al.*, 1961), the fractions of PE by staining with ninhydrin reagent (Skipski *et al.*, 1962).

Aliquots of the various polar lipid fractions were converted to the methyl esters by sulfuric acid-catalyzed methanolysis. Fatty acid methyl esters were purified by TLC on Silica Gel H with hexane:diethyl ether (4:1, v/v), isolated, and analyzed by gas chromatography (GC). GC of fatty acid methyl esters was carried out using a Hewlett-Packard HP-5890 Series II gas chromatograph equipped with a 0.17 μ m Permabond FFAP-DF fused silica column (Macherey-Nagel, Düren, Germany) of 25 m length and 0.25 mm i.d. Fatty acid methyl esters were separated using nitrogen as the carrier gas and a temperature program from 160 to 240 °C at 4 °C per min; finally the temperature was held at 240 °C for 5 min. The split ratio was 1:10, the injector and the FID temperatures were 270 °C. GC peaks were identified by comparison of their retention times with those of known standards. Peak areas and percentages were calculated using a Kontron PC Integration Pack (Kontron Instruments, Neufahrn, Germany). Data on fatty acid composition of lipid classes from the various cell lines are means $\pm$ SEM of three gas chromatographic analyses ($n = 3$).

Results

Fatty acid compositions of three plastidic (MGD, DGD and PG) and two predominantly extra-plastidic lipid classes (PC and PE), isolated from the Mbz-resistant cell lines L4 and L7 of *C. rubrum*, grown in the absence of Mbz, were determined. These results were compared with those obtained from the corresponding lipids of WT cells. In addition, fatty acid compositions of the plastidic and extra-plastidic lipids of the above cell lines were determined after growth in the presence of sublethal concentrations of Mbz. Thus the herbicide effects on the fatty acid composition of the various polar lipid classes of both Mbz-resistant and *C. rubrum* WT cells were studied.

Plastidic membrane lipids in the absence of Mbz

The proportions of the constituent acyl moieties of plastidic MGD, DGD and PG isolated from photoautotrophic *C. rubrum* cells grown in the absence of Mbz are shown in Tables I-III. The fatty acid composition of MGD (Table I) isolated from highly Mbz-resistant L4 cells showed an increase in the proportions of α -linolenoyl (18:3) moieties as compared to the WT cells, whereas the proportions of linoleoyl (18:2) moieties remained almost unchanged. Increased proportions of 18:2 moieties together with almost unchanged proportions of 18:3 moieties, however, were found in the MGD of the less resistant cell line L7. Concomitantly, the proportions of palmitoyl (16:0) moieties decreased in both L4 and L7 cells. As a consequence, the degree of unsaturation – defined as 18:2+18:3/16:0 ratio – was increased about twofold in both L4 and L7 cells as compared to the WT cells (Table I). The degree of unsaturation was highest in MGD of all three cell lines as compared to the other polar lipids studied. Looking at the 18:2/16:0 and 18:3/16:0 ratios of MGD (Fig. 1, left column) it is obvious that the rise of the degree of unsaturation in L4 and L7 cells was caused predominantly by the increase in 18:3/16:0 ratio, whereas 18:2/16:0 ratios increased only moderately.

Within the plastidic DGD fraction (Table II) both Mbz-resistant cell lines showed similar tendencies as observed within the MGD fraction in the absence of Mbz (cf. Table I). The proportions of 18:3 moieties were, however, markedly reduced in the DGD of the less resistant L7 cells. Particularly,

Table I. Fatty acid composition of monogalactosyldiacylglycerols isolated from photoautotrophic wild type and Mbz-resistant *Chenopodium rubrum* suspension cells cultured in the absence or presence of metribuzin.

Cell lines	Fatty acid composition (%)							
	16:0	c-16:1	16:2	18:1	18:2	18:3	others ^a	$\frac{18:2 + 18:3^b}{16:0}$
WT	5.2 ± 0.2	2.0 ± 0.1	1.6 ± 0.1	3.0 ± 0.1	15.3 ± 0.2	70.0 ± 0.4	2.9	16.4
WT + Mbz	3.1 ± 0.1	1.5 ± 0.1	1.7 ± 0.1	1.3 ± 0.2	13.2 ± 0.2	76.8 ± 1.1	2.4	29.0
L4	2.6 ± 0.2	0.9 ± 0.2	0.7 ± 0.1	1.6 ± 0.3	16.8 ± 0.5	76.0 ± 1.2	1.4	35.7
L4 + Mbz	3.6 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	1.1 ± 0.4	16.9 ± 0.5	76.2 ± 1.2	0.7	25.9
L7	2.8 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	2.6 ± 0.2	21.9 ± 0.2	69.0 ± 0.7	2.1	32.5
L7 + Mbz	4.6 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	2.3 ± 0.1	20.5 ± 0.2	68.6 ± 0.6	2.2	19.4

Abbreviations: Mbz, metribuzin; L4 and L7, Mbz-resistant cell lines; WT, wild type.

Results are expressed as means ± SEM (n = 3) of three GC determinations.

Fatty acids are indicated by number of carbon atoms: number of double bonds, e.g. c-16:1, *cis*-9-hexadecenoic acid.

^a Including small proportions of 14:0, 14:1, 16:3 and 18:0.

^b Degree of unsaturation, defined as (18:2 + 18:3) / 16:0.

Table II. Fatty acid composition of digalactosyldiacylglycerols isolated from photoautotrophic wild type and Mbz-resistant *Chenopodium rubrum* suspension cells cultured in the absence or presence of metribuzin.

Cell lines	Fatty acid composition (%)							
	16:0	c-16:1	16:2	18:1	18:2	18:3	others ^a	$\frac{18:2 + 18:3^b}{16:0}$
WT	19.4 ± 0.5	2.4 ± 0.1	0.9 ± 0.2	3.5 ± 0.7	13.9 ± 0.5	58.5 ± 2.4	1.4	3.7
WT + Mbz	21.9 ± 0.2	2.1 ± 0.1	0.8 ± 0.1	3.9 ± 0.1	15.2 ± 0.1	53.7 ± 0.4	2.4	3.2
L4	16.0 ± 0.1	1.7 ± 0.1	0.4 ± 0.1	3.3 ± 0.1	14.5 ± 0.1	62.7 ± 0.2	1.4	4.8
L4 + Mbz	17.4 ± 0.2	1.6 ± 0.1	0.5 ± 0.1	2.6 ± 0.1	14.7 ± 0.1	59.8 ± 0.4	2.4	4.3
L7	20.7 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	4.8 ± 0.1	21.5 ± 0.1	50.0 ± 0.2	1.5	3.5
L7 + Mbz	18.1 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	3.5 ± 0.2	18.3 ± 0.1	56.2 ± 1.1	2.5	4.1

Abbreviations: as given in Fig. 1.

Results are expressed as means ± SEM (n = 3) of three GC determinations.

Fatty acids are indicated by number of carbon atoms : number of double bonds, e.g. c-16:1, *cis*-9-hexadecenoic acid.

^a Including small proportions of 14:0, 14:1, 16:3 and 18:0.

^b Degree of unsaturation, defined as (18:2 + 18:3) / 16:0.

Table III. Fatty acid composition of phosphatidylglycerols isolated from photoautotrophic wild type and Mbz-resistant *Chenopodium rubrum* suspension cells cultured in the absence or presence of metribuzin.

Cell lines	Fatty acid composition (%)							
	16:0	t-16:1	c-16:1	18:0	18:1	18:2	18:3	$\frac{18:2 + 18:3^b}{16:0}$
WT	45.0 ± 0.7	21.9 ± 0.2	1.2 ± 0.1	1.2 ± 0.1	10.4 ± 0.1	9.6 ± 0.2	7.7 ± 0.4	2.9
WT + Mbz	37.6 ± 0.2	20.9 ± 0.1	1.7 ± 0.2	1.5 ± 0.1	13.0 ± 0.2	12.1 ± 0.2	5.4 ± 0.1	7.8
L4	51.1 ± 0.1	16.9 ± 0.2	1.3 ± 0.1	1.1 ± 0.1	9.3 ± 0.2	11.1 ± 0.2	7.9 ± 0.2	1.1
L4 + Mbz	49.2 ± 0.2	17.5 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	9.7 ± 0.1	10.4 ± 0.1	6.0 ± 0.1	3.4
L7	54.0 ± 0.6	5.9 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	16.7 ± 0.3	11.7 ± 0.3	5.3 ± 0.1	3.4
L7 + Mbz	50.5 ± 0.2	14.4 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	13.1 ± 0.2	9.4 ± 0.1	3.5 ± 0.1	5.5

Abbreviations: as given in Fig. 1.

Results are expressed als means ± SEM (n = 3) of three GC-determinations.

Fatty acids are indicated by number of carbon atoms: number of double bonds, e.g. c-16:1, *cis*-9-hexadecenoic acid; t-16:1, *trans*-3-hexadecenoic acid.

^a Including small proportions of 16:0, 14:1, 16:2 and 16:3.

^b Degree of unsaturation, defined as (18:2 + 18:3) / 16:0.

Fig. 1. The 18:2/16:0 and 18:3/16:0 ratios of monogalactosyldiglycerides (MGD), digalactosyldiglycerides (DGD), phosphatidylglycerols (PG), phosphatidylcholines (PC) and phosphatidylethanolamines (PE) isolated from various photoautotrophic *C. rubrum* cell suspension cultures WT, L4 and L7 in the absence of Mbz (left column) and in the presence of Mbz (right column).

the less resistant L7 cells did not show very distinct changes of the degree of unsaturation of DGD as compared to WT cells (Table II) and 18:2+18:3/16:0 ratios of DGD were much lower than those observed for MGD (cf. Table I). The pattern of 18:2/16:0 and 18:3/16:0 ratios of DGD was similar to that observed in MGD (Fig. 1, left column).

The acyl compositions of plastidic PG isolated from the resistant *C. rubrum* cell lines and from the WT cells grown in the absence of Mbz are given in Table III. These data show increasing proportions of 16:0 moieties in the resistant cell lines L4 and L7 as compared to the WT cells and a simultaneous decrease in the proportions of *trans*-3-hexadecenoyl (t-16:1) moieties which was highest in the less resistant L7 cells. Additionally, an increase in the proportions of 18:1 moieties was

found in the PG of L7 cells as compared to those of WT and L4 cells. The degree of unsaturation of PG (Table III) was very low in all three cell lines (0.3–0.4). The 18:2/16:0 ratio of PG was almost identical in all three cell lines grown in the absence of Mbz, whereas the corresponding 18:3/16:0 ratio was slightly decreased in L7 cells (Fig. 1, left column).

Extra-plastidic membrane lipids in the absence of Mbz

The fatty acid composition of PC isolated from the two herbicide-resistant photoautotrophic *C. rubrum* cell lines and from the WT cells grown in the absence of Mbz are given in Table IV. It is obvious from these findings that the proportion of 16:0 moieties in PC was by far higher in the WT cells than in the two resistant lines L4 and L7. Concomitantly, relatively low proportions of 18:1 and 18:2 moieties were found in PC of the WT cells, whereas PC isolated from Mbz-resistant cell lines L4 and L7 contained by far higher proportions of these acyl moieties than the WT cells. The level of 18:3 moieties, however, was similar in PC of all three cell lines. Altogether, the degree of unsaturation was low (1.4–3.0) in PC of all three cell lines, it was, however, strongly increased in PC of the Mbz-resistant cells as compared to WT cells (Table IV). In contrast to MGD and DGD the increase in 18:2/16:0 ratio of PC was higher than that of 18:3/16:0 ratio of PC in both L4 and L7 cells as compared to WT cells (Fig. 1, left column).

The analyses of the fatty acid composition of PE revealed results similar to those of the PC with a few exceptions (Table V). The proportions of 18:2 moieties were generally higher in PE of all three cell lines and those of 18:3 moieties in PE were generally lower as compared to PC. The differences between the fatty acyl compositions of PE of WT cells and Mbz-resistant cell line L7 showed the same tendency, as was found in PC, i.e. increasing proportions of 18:2 and decreasing proportions of 18:3 moieties in PE of Mbz-resistant L7 cells as compared to WT cells. The proportions of 16:0 moieties were, however, reduced exclusively in PE of L7 cells. The degree of unsaturation increased in PE of L7 cells only (Table V). The 18:2/16:0 and 18:3/16:0 ratios were closely similar in PE of WT cells and highly resistant L4 cells, whereas a dis-

Table IV. Fatty acid composition of phosphatidylcholines isolated from photoautotrophic wild type and Mbz-resistant *Chenopodium rubrum* suspension cells cultured in the absence or presence of metribuzin.

Cell lines	Fatty acid composition (%)							
	16:0	c-16:1	18:0	18:1	18:2	18:3	others ^a	$\frac{18:2 + 18:3^b}{16:0}$
WT	37.5 ± 0.3	2.9 ± 0.1	0.9 ± 0.1	5.2 ± 0.2	27.6 ± 0.1	24.0 ± 0.2	1.9	1.4
WT + Mbz	27.6 ± 0.2	2.4 ± 0.1	0.7 ± 0.1	5.4 ± 0.1	33.8 ± 0.1	28.2 ± 0.1	1.9	2.2
L4	25.0 ± 0.4	1.6 ± 0.1	0.6 ± 0.1	6.2 ± 0.2	39.2 ± 0.1	25.9 ± 0.1	1.5	2.6
L4 + Mbz	29.6 ± 0.5	1.5 ± 0.5	1.0 ± 0.1	6.0 ± 0.2	38.9 ± 0.3	18.7 ± 0.1	4.3	2.0
L7	21.5 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	9.7 ± 0.1	43.1 ± 0.1	22.1 ± 0.1	2.1	3.0
L7 + Mbz	26.5 ± 0.1	1.5 ± 0.1	1.2 ± 0.2	9.7 ± 0.2	38.3 ± 0.2	19.5 ± 0.3	3.3	2.2

Abbreviations: as given in Fig. 1.

Results are expressed as means ± SEM (n = 3) of three GC determinations.

Fatty acids are indicated by number of carbon atoms: number of double bonds, e.g. c-16:1, *cis*-9-hexadecenoic acid.

^a Including small proportions of 14:0, 14:1, 16:2 and 16:3.

^b Degree of unsaturation, defined as (18:2 + 18:3) / 16:0.

Table V. Fatty acid composition of phosphatidylethanolamines isolated from photoautotrophic wild type and Mbz-resistant *Chenopodium rubrum* suspension cells cultured in the absence or presence of metribuzin.

Cell lines	Fatty acid composition (%)							
	16:0	c-16:1	18:0	18:1	18:2	18:3	others ^a	$\frac{18:2 + 18:3^b}{16:0}$
WT	34.6 ± 0.1	1.6 ± 0.1	1.4 ± 0.1	3.7 ± 0.1	35.6 ± 0.1	19.3 ± 0.2	4.8	1.6
WT + Mbz	35.5 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	4.1 ± 0.1	36.4 ± 0.5	15.7 ± 0.3	4.3	1.3
L4	37.5 ± 0.4	1.4 ± 0.2	1.4 ± 0.1	4.4 ± 0.1	40.6 ± 0.4	10.8 ± 0.3	3.9	1.4
L4 + Mbz	36.1 ± 0.9	2.7 ± 0.2	2.4 ± 0.1	5.7 ± 0.2	37.9 ± 0.1	9.7 ± 0.2	5.5	1.3
L7	23.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.1	8.3 ± 0.2	51.4 ± 0.3	10.9 ± 0.9	4.6	2.6
L7 + Mbz	28.8 ± 0.8	3.1 ± 0.1	2.0 ± 0.1	7.8 ± 0.2	41.2 ± 0.8	9.9 ± 0.3	7.2	1.8

Abbreviations: as given in Fig. 1.

Results are expressed as means ± SEM (n = 3) of three GC determinations.

Fatty acids are indicated by number of carbon atoms : number of double bonds, e.g. c-16:1, *cis*-9-hexadecenoic acid.

^a Including small proportions of 14:0, 14:1, 16:2 and 16:3.

^b Degree of unsaturation, defined as (18:2 + 18:3) / 16:0.

tinct increase in 18:2/16:0 ratio was observed in PE of L7 cells (Fig. 1, left column).

Plastidic membrane lipids in the presence of Mbz

The most obvious difference in the composition of 18:2 and 18:3 moieties of MGD of WT cells grown in the presence of Mbz – as compared to the absence of Mbz – was a slight increase in 18:3 moieties, whereas the acyl composition of MGD of both Mbz-resistant *C. rubrum* cell lines was closely similar irrespective of the presence or absence of Mbz (Table I). The degree of unsaturation of MGD was lower in both resistant cell lines, particularly in L7 cells, than in WT cells, both grown in the presence of Mbz (Table I). The 18:2/16:0 ratio in MGD of all three cell lines was almost un-

changed in the presence of Mbz, whereas a slight decrease was found in the corresponding 18:3/16:0 ratio of L4 cells and a distinct decrease in that of L7 cells (Fig. 1, right column).

Slightly increased proportions of 18:3 moieties were observed in the DGD of both L4 and L7 cells – as compared to WT cells – both grown in the presence of Mbz and L7 cells showed additionally a slight increase in 18:2 moieties of DGD under the same conditions (Table II). The degree of unsaturation of DGD markedly rose in both Mbz-resistant cell lines (Table II). The 18:3/16:0 ratio increased in DGD of both resistant cell lines L4 and L7, whereas the corresponding 18:2/16:0 ratio remained almost constant (Fig. 1, right column).

Interestingly, the proportions of t-16:1 moieties increased in PG of less resistant L7 cells grown in the presence of Mbz to a higher degree than in PG of highly resistant L4 cells, whereas they were almost unaffected in PG of WT and L4 cells in the absence or presence of Mbz (Table III). Moreover, the proportions of 16:0 moieties in PG decreased slightly in WT as well as in L4 and L7 cells, each grown in the presence of Mbz, as compared to the absence of Mbz. The degree of unsaturation of PG was low (0.25–0.5) in all three cell lines both in the absence and presence of Mbz (Table II).

Extra-plastidic membrane lipids in the presence of Mbz

In all three cell lines the fatty acid composition of mainly extra-plastidic PC revealed small but distinct differences in the presence as compared to the absence of Mbz in the culture medium. In the WT cells the proportions of 16:0 moieties in PC decreased distinctly in the presence of Mbz as compared to cells in the absence of Mbz and a concomitant increase in 18:2 and 18:3 moieties was observed (Table IV); consequently, the degree of unsaturation of PC increased (Table II). In contrast, the proportions of 18:3 moieties of PC were found to decrease in both L4 and L7 cells in the presence as compared to the absence of Mbz; concomitantly, an increase in the proportions of 18:2 moieties of PC was observed as compared to WT cells. The degree of unsaturation of PC did not differ substantially in the three cell lines in the presence of Mbz (Table II), because of low increase of 18:2/16:0 ratios and moderate decrease of 18:3/16:0 ratios in Mbz-resistant L4 and L7 cells as compared to WT cells.

In the presence of Mbz in the culture medium the fatty acid composition of PE in WT cells was almost unchanged as compared to that in the absence of Mbz, with the exception of a decrease in 18:3 moieties. In contrast, the proportion of 18:2 moieties of PE was markedly decreased in L4 and, particularly, in the less resistant L7 cells – both in the presence of Mbz as compared to the corresponding cells in the absence of Mbz (Table V). The degree of unsaturation of PE was decreased slightly in WT cells and moderately in L7 cells, each in the presence of Mbz, as compared to the absence of Mbz, whereas it remained almost un-

changed in the highly Mbz-resistant L4 cells irrespective of Mbz in the culture medium. Both 18:2/16:0 and 18:3/16:0 ratios of PE were closely similar in all three cell lines in the presence of Mbz (Fig. 1).

Discussion

Recently, we have found that the *psbA* gene coding for the D1 protein of photosystem II was altered in eight Mbz-resistant cell lines of *C. rubrum*. Analyses of the growth parameters and photosynthetic capacity of the above cells revealed differences between the various cell lines (Thiemann and Barz, 1994a,b) and also their resistance patterns investigated at the level of thylakoids (Schwenger-Erger *et al.*, 1993; Schwenger-Erger *et al.*, 1999). In the present study, the fatty acid composition of plastidic and extra-plastidic membrane lipids was determined in the highly Mbz-resistant line L4 and the less resistant line L7, carrying the same mutations in the sequenced *psbA* gene fragment in order to study the specific acyl pattern of membrane lipids of cells selected by the herbicide as well as the herbicide effects on fatty acid metabolism.

Fatty acid alterations of membrane lipids of C. rubrum cell lines in the absence of Mbz

The membrane lipids of thylakoids consist of characteristic lipids which constitute mainly of glyceroglycolipids such as MGD and DGD and of a glycerophospholipid, i.e. PG. In contrast, the extra-plastidic membrane lipids of the endoplasmic reticulum consist predominantly of glycerophospholipids such as PC and PE (Dorne *et al.*, 1990). Here we report the fatty acid compositions of membrane lipids of two *C. rubrum* mutants with greatly changed proportions of certain fatty acids either in plastidic or extra-plastidic lipids as compared to WT cells. In addition to the changes described earlier (Schwenger-Erger *et al.*, 1993) it is obvious from our present data that both resistant *C. rubrum* cell lines show different acyl patterns in membrane lipids, particularly in glycerophospholipids such as PC, PE and, to some degree, PG, whereas glyceroglycolipids such as MGD and DGD were less affected (Tables I–V).

One of the most striking results of our studies on the fatty acid compositions of *C. rubrum* mem-

brane lipids is the strong reduction of t-16:1 moieties in the PG fraction of the weakly resistant cell line L7 (Table III). The t-16:1 moieties are typical constituents of plastidic PG; they do not occur in other plastidic and extra-plastidic membrane lipids (Ohnishi and Thompson, 1991). The proportion of t-16:1 moieties in the PG was found to be around 22% in the WT cells in the absence of Mbz, whereas their level dropped in the cell lines L4 and L7 to about 17% and 6%, respectively. Concomitantly, an increase in 16:0 moieties was observed in lines L4 and L7, thus increasing the ratio of 16:0/t-3-16:1 from 2.1 in WT cells to around 3.0 and 9.2 in L4 and L7 cells, respectively. In this context it is of particular interest that good correlation has been observed between t-16:1 concentration and the degree of grana stacking and stabilization of LHCP oligomers (Trémolières *et al.*, 1979; Trémolières *et al.*, 1981; Huner *et al.*, 1989; Dubacq and Trémolières, 1993; Kruse and Schmidt, 1995; Kruse *et al.*, 2000).

Reduced proportions of 16:0 moieties are detected in MGD and PC of both Mbz-resistant cell lines (Tables I and IV) as well as in DGD of L4 (Table II) and in PE of L7 cells (Table V). Simultaneously, an increase of the proportions of 18:2 moieties is observed in the various lipid classes of both Mbz-resistant cell lines and, with limitations, of the 18:1 moieties of PC and PE (Tables I–II and IV–V). In contrast, the proportion of 18:3 moieties increases predominantly in the various lipid classes (with the exception of PE) of highly resistant L4 cells, whereas it is less affected in L7 cells (Tables I–V). In accordance with these results Tables I–V as well as Fig. 1 (left column) demonstrate that the degree of unsaturation is generally higher in polar lipids of Mbz-resistant lines L4 and L7 as compared to WT cells and that, particularly, 18:3/16:0 ratios are higher in most of the polar lipids of the highly resistant L4 cells as compared to WT and L7 cells. Finally, these alterations lead to an increased level of 18:2 and 18:3 moieties within the plastidic membrane lipids and the predominantly extra-plastidic PC of Mbz-resistant L4 and L7 cells. Such changes modify the thermotropic behavior of the membranes (Murakami *et al.*, 2000) which may be an adaptive response providing increased Mbz-resistance.

Fatty acid alterations of membrane lipids of C. rubrum cell lines in the presence of sublethal concentrations of Mbz

The data summarized in Tables I–V show that WT cells and Mbz-resistant L4 and L7 cells react in a highly different manner in response to Mbz. It seems that treatment of WT cells and resistant cell lines with the herbicide sometimes causes opposite effects on the composition of acyl moieties – as compared to the corresponding untreated cells – which is particularly true for the acyl changes of PC in the presence of Mbz (Table IV). For example, the levels of 16:0 moieties are markedly decreased in PC of WT cells in the presence of Mbz as compared to the untreated cells; yet in both L4 and L7 cells the presence of Mbz causes an increase in 16:0 moieties in PC. Moreover, a slight increase in the proportions of both 18:2 and 18:3 moieties is observed in PC of the WT cells in the presence of Mbz, whereas the levels of these two acyl moieties of PC decrease in both resistant lines in the presence of the herbicide. Similar effects are found, to some degree, for other membrane lipids, too (Tables I, II and V). Consequently, in the presence of Mbz the degrees of unsaturation of MGD, PG and PC are generally increased in WT cells and decreased in L4 and L7 cells (Tables I, III and IV). This effect is particularly confirmed for 18:3/16:0 ratios of MGD, PG and PC (Fig. 1, right column).

As shown above, the acyl composition of galactolipids is less affected by Mbz than that of glycerophospholipids and the differences found in the less resistant L7 cells are higher than those in highly resistant L4 cells. However, it is conspicuous from the data shown in Tables I–V that an adjustment within the fatty acid composition of both Mbz-resistant cell lines L4 and L7 and the WT cells takes place in the presence of Mbz. This may be explained by opposite effects of the herbicide against enzymes involved in fatty acid desaturation and herbicide resistance (Trémolières *et al.*, 1988; Lehoczi *et al.*, 1985; Burke *et al.*, 1982).

To summarize, our results suggest that fatty acid biosynthesis in *C. rubrum* cells is markedly affected by Mbz treatment. In all the three cell lines the acyl composition of plastidic membrane lipids broadly adjust to each other in the presence of Mbz as compared to the absence of Mbz. Both in

the absence and in the presence of Mbz alterations of the fatty acid composition of the various lipid classes are usually lower in the highly resistant L4 cells than in the less resistant L7 cells. Especially, cells demonstrating increased degree of unsaturation in plastidic and, particularly, extra-plastidic membrane lipids, are discriminated against by Mbz-treatment. As a consequence, cells with increased proportions of unsaturated membrane lipids are selected in culture media containing the herbicide. Within the two Mbz-resistant lines the highly resistant L4 cells are distinguished from L7 cells by increased 18:3/16:0 ratios in most of the polar lipids. Similarly, the proportion of t-16:1 moieties decreases in PG of L4 cells to some extent, whereas the proportion of these acyl moieties is reduced by 73% in PG of less resistant L7 cells as compared to WT cells in the absence of Mbz.

It is suggested that L4 and L7 cells – having multiple mutations in the *psbA* gene – are additionally selected by increasing the degree of unsaturation of acyl moieties of various polar lipids. This gives rise to a change in membrane fluidity,

possibly followed by a decrease in the permeability of cell membranes for Mbz which may finally be responsible for the increased Mbz-resistance. A possible explanation of these alterations in fatty acid composition is that Mbz treatment leads to the selection of cells with different desaturase activities in a similar manner as reported earlier for other herbicides (Harwood, 1996; Ivanova *et al.*, 1999). Moreover, the effect of varying proportions of t-16:0 moieties-containing PG in the presence or absence of Mbz has been observed occasionally for other herbicides, as described above. It needs further investigation to elucidate the particular involvement of these PG species in the assembly of plant photosystem II (Kruse *et al.*, 2000) as well as of desaturases into the fatty acid alterations observed by Mbz treatment.

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