Plasma Membrane Lipid Composition and Herbicide Effects on Lipoxygenase Activity Do Not Contribute to Differential Membrane Responses in Herbicide-Resistant and -Susceptible Wild Oat (*Avena fatua* L.) Biotypes

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Plasma membrane lipid composition of herbicide-resistant (R) and -susceptible (S) wild oat biotypes was analyzed to determine the basis for the differential effect of diclofop on the transmembrane electrogenic potential between the two biotypes and reduced herbicide uptake into protoplasts of the R biotype. In addition, lipoxygenase (LOX) activity was examined in herbicide-treated and untreated R and S plants to determine its involvement in herbicide action and resistance. Overall, no significant differences in lipid composition were found between the two biotypes. Glycolipids represented 41 and 36%, phospholipids 29 and 37%, and free sterols 30 and 27% of the total plasma membrane lipid in the R and S biotypes, respectively. No differences in LOX activity were observed between the herbicide-treated and untreated wild oat biotypes. It was concluded that differences in membrane transport of diclofop and its effect on plasma membrane potential in the R and S biotypes are not related to differences in membrane lipid composition or to differential effects of herbicides on LOX activity in the two biotypes.

Keywords: *Wild oat; herbicide resistance; lipid composition; lipoxygenase; ACCase inhibitors*

INTRODUCTION

Aryloxyphenoxypropionate (AOPP; e.g., diclofop-methyl) and cyclohexanedione (CHD; e.g., sethoxydim) herbicides are used to control grass weeds in some dicotyledonous and cereal crops. In plants, diclofop-methyl is rapidly hydrolyzed to the free acid, which inhibits acetyl-coenzyme A carboxylase (ACCase, EC 6.4.1.2), a key enzyme in acyl lipid biosynthesis (Burton *et al*., 1987; Kobek *et al*., 1988). The tolerance of cereal crops to diclofop is based on their ability to rapidly detoxify it by aryl hydroxylation followed by glycosylation (Shimabukuro *et al*., 1979; Jacobson and Shimabukuro, 1984). In broadleaf species, resistance is based on an insensitive form of ACCase (Burton *et al*., 1989). Similar mechanisms confer tolerance/resistance to CHD herbicides.

Resistance to AOPP and CHD herbicides in grass weeds has become widespread in recent years. In most instances, resistance is conferred by reduced ACCase sensitivity to herbicides (Leach *et al*., 1995; Marles *et al*., 1993; Shukla *et al*., 1997b; Tardif *et al*., 1993). In western Canada, several biotypes of wild oat (*Avena fatua*) have been identified that are resistant to diclofop-methyl and other ACCase inhibitors (Heap *et al*., 1993). Initial results indicated that resistance in one of these biotypes, designated UM1, was related to altered effects on the plasma membrane electrogenic potential (E_m), not to reduced ACCase sensitivity (Devine *et al*., 1993a); the E_m of coleoptile epidermal cells was depolarized in both the resistant (R) and susceptible (S) biotypes upon treatment with 50 μM diclofop, but recovered only in the R biotypes when diclofop was removed from the bathing solution. Similar results have been reported in a resistant biotype of annual ryegrass (*Lolium rigidum*) (Häusler *et al*., 1991). Uptake of diclofop and tralkoxydim, studied using plasma membrane vesicles and isolated protoplasts, was also 15−30% lower in the R wild oat biotype than in the S biotype (Devine *et al*., 1993b). Together, these results suggested a resistance mechanism associated with the cell membrane. Overproduction of fatty acids in the microalgae *Spirulina platensis* and *Porphyridium cruentum* confers resistance to SAN 9785, an inhibitor of fatty acid desaturation (Cohen *et al*., 1993), suggesting that mutations affecting lipid biosynthesis can confer protection against herbicides that interfere with lipid synthesis.

It has also been suggested that the AOPP herbicide haloxyfop inhibits growth by affecting polyunsaturated fatty acid (PUFA) content rather than by inhibiting *de novo* fatty acid biosynthesis (Banas *et al*., 1993a,b); inhibitors of lipoxygenase (LOX; EC 1.13.11.12) and free radical scavengers were able to reverse the effects on PUFA content and on growth. Shimabukuro and Hoffer (1995) have suggested that AOPP herbicide action may involve LOX-catalyzed peroxidation of PUFAs, leading to free radical formation. These free radicals cause membrane degradation and thus may contribute to the toxic effect of the herbicides. The relationship between the various effects on membrane function and inhibition of ACCase has not been elucidated.

We have recently shown that, in contrast to the results reported previously, the mechanism of resistance

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in the wild oat biotype described above (UM1) is an altered form of ACCase with reduced herbicide sensitivity (Shukla *et al*., 1997a). Genetic evidence indicates that herbicide resistance in this biotype is controlled by a single, semidominant gene, suggesting a single resistance mechanism (Murray *et al*., 1995). Given these recent results, the research reported here was conducted to address the following hypotheses: (a) that reduced herbicide uptake into protoplasts or plasma membrane vesicles of the R compared to the S biotype, and the altered response of the plasma membrane E_m between the R and S biotypes, was related to differences in the lipid composition of the plasma membrane and (b) that differences in LOX activity following herbicide treatment were correlated with differential herbicide sensitivity in the R and S biotypes.

MATERIALS AND METHODS

Plant Material. Seeds of the R and S wild oat biotypes were germinated in 9-cm Petri dishes lined with moistened filter paper and then transferred to pots containing soil. The plants were grown in a growth chamber at 22/18 °C day/night temperatures in a 16-h photoperiod at 325 μeinsteins m^{-2} s^{-1} and were sampled for lipid analysis when they were 3 weeks old.

Preparation and Characterization of Plasma Membrane Vesicles. Plasma membrane vesicles were purified by partitioning in a two-phase system according to the methods of Larsson *et al*. (1987) and Gallet *et al*. (1989), with minor modifications. Leaves (60 g) were homogenized in 200 mL of medium containing 0.5 M sorbitol, 5 mM EDTA, 1 mM phenylmethanesulfonylfluoride, 2 mM dithiothreitol (DTT), 0.6% polyvinylpolypyrolidone, 5 mM ascorbic acid, and 50 mM HEPES/KOH (pH 7.8). The homogenate was filtered through Miracloth and centrifuged at 10000*g* for 15 min. The supernatant was centrifuged for 35 min at 50000*g* to obtain the microsomal fraction. The pellet was resuspended in 0.33 M sorbitol, 9 mM KCl, 5 mM EDTA, 0.5 mM DTT, and 5 mM potassium phosphate (pH 7.8) to a total volume of 9 mL. This microsomal fraction was added to two tubes containing 13.5 g of two-phase system [6.4% (w/w) Dextran T 500, 6.4% (w/w) poly(ethylene glycol) 3350, 0.33 M sorbitol, 9 mM KCl, 5 mM EDTA, 0.5 mM DTT, and 5 mM potassium phosphate (pH 7.8)]. The tubes were shaken and centrifuged at 1500*g* for 10 min. The upper phase containing the plasma membrane vesicles was removed and repartitioned three times with fresh lower phase, and the lower phase was repartitioned once with fresh upper phase. Purified plasma membrane vesicles were collected by centrifugation for 45 min at 100000*g*. The pellet was resuspended in a medium containing 0.3 M sorbitol, 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 2 mM EDTA, 1 mM DTT, and 50 mM potassium phosphate (pH 7.5), subdivided, frozen in liquid nitrogen, and stored at −80 °C.

The purity of the membrane preparations was checked by assaying for various marker enzymes. ATPase activity was assayed using the method of Ames (1966). Vesicles were resuspended in a medium containing 0.5 M sucrose, 1 mM DTT, and 100 mM Tris/MES (pH 6.5). The reaction was initiated by adding 3 mM ATP to the medium (incubated at 37 °C for 30 min) containing the vesicles (30 mg), 0.5 M sucrose, 3 mM $MgSO_4$, 100 mM KCl, 100 mM Tris/MES (pH 6.5), and Triton X-100 (0.02%, w/v) and stopped by adding a solution containing ammonium heptamolybdate (0.42%), SDS (2.1%), and 1 N H_2SO_4. To measure the amount of phosphate released, ascorbic acid (10%) was added and the absorbance read at 820 nm, and the results were expressed as millimoles of P_i per minute per milligram of protein.

Membrane-specific ATPase inhibitors were used to test for other membrane contamination: 0.25 mM sodium vanadate (plasma membrane ATPase), 50 mM $NaNO_3$ (tonoplast ATPase), 1 mM NaN_3 (mitochondrial ATPase), and 0.1 mM molybdate (acid phosphatase). Cytochrome *c* oxidase was measured according to the method of Hodges and Leonard (1974) in Triton (0.01%), 2.4 mM cyt *c* reduced with sodium hydrosulfite, and 5 mM sodium phosphate (pH 7.2). Absorbance was read at 549 nm, and results were expressed as ΔA_{549} per minute per miiligram of P. Chlorophyll was determined using the method of Arnon (1949). Vesicles were diluted in acetone (80%), and absorbance was read at 645 and 663 nm after the sample was filtered. The results were expressed as milligrams per gram of fresh weight. Protein content was determined according to the method of Bearden (1978); Triton X-100 (0.01%) was used to solubilize membrane proteins, with bovine serum albumin used as a standard.

Lipid Extraction and Separation. Lipids were extracted from the plasma membrane vesicles following the procedure described by Taylor *et al*. (1992). A silica Sep-Pak cartridge was used to separate the total lipids into neutral lipids, glycolipids, and phospholipids (Sandstorm and Cleland, 1989; Hamilton and Comai, 1984). Lipids were extracted from vesicle preparations containing 0.2−0.5 mg of plasma membrane protein using 2 mL of methanol/chloroform/HCl (2:1:0.3, v/v/v). After addition of 3 mL of chloroform and 1 mL of water, the mixture was centrifuged at 1000*g* for 5 min and the chloroform phase, containing the lipids, was removed and dried under nitrogen. The concentrated lipids were dissolved in 2 mL of chloroform/acetic acid (100:1, v/v) and transferred to the Sep-Pak cartridge, previously rinsed with 3 mL of chloroform/acetic acid. Two milliliters of this solvent was used to rinse the tube containing the lipids. The neutral lipids were eluted with 10 mL of the same solvent. Glycolipids were eluted using 10 mL of acetone and 10 mL of acetone/acetic acid (100:1, v/v). Ten milliliters of methanol/chloroform/water (100:50:40, v/v/v) was added to elute the phospholipids. Addition of 1 mL of chloroform and 2 mL of water was necessary to get phase separation. The chloroform phase was collected. All samples were concentrated under nitrogen and dissolved in appropriate solvents in the presence of 0.2% butylated hydroxytoluene to prevent oxidation: chloroform for the neutral lipids, chloroform/methanol (1:1, v/v) for the glycolipids, and chloroform/acetic acid (100:1, v/v) for the phospholipids. Samples were stored at −20 °C prior to analysis.

Sterol Analysis. Free sterols were separated by GC on a 30-m DB-5 column (0.32 mm i.d., 0.25 mm film thickness) under the following conditions: oven temperature, 180 °C (1 min hold) to 290 °C at 8 °C min^{-1} (held for 10 min); injector and detector temperatures, maintained at 280 and 300 °C, respectively; and split ratio, 100:1. Peak areas were measured using an integrator. 5α-cholestane was added as an internal standard; β-stigmasterol was used for response factor calculations. The response factors for the other sterols were assumed to be the same as that of β-stigmasterol. Mass spectra were obtained using a mass spectrometer interfaced with a GC and operated in the electron impact mode. Samples were injected in the split mode onto a 60 m × 0.32 mm DB-1 column, temperature programmed from 200 to 300 °C at 10 °C min^{-1}. The electron energy was 70 eV, and the quadropole was scanned from 45 to 450 amu every 1.5 s.

Phospholipid Analysis. Phospholipids were separated by two-dimensional TLC (silica gel H, 0.25 mm layer thickness). The two solvent systems used were chloroform/methanol/28% aqueous ammonia (65:35:5, v/v/v) followed by chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, v/v/v/v/v) (Rouser *et al*., 1970). Phospholipids were detected by iodine vapor and identified by coelution with known standards, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidic acid (PA). The bands were eluted from the TLC plates with chloroform/methanol/water (5:5:1, v/v/v) and analyzed according to the method of Ames (1966).

Glycolipid Analysis. Glycolipids were separated by one-dimensional TLC (silica gel H, 0.25 mm layer thickness) using chloroform/methanol/acetic acid/water (85:15:10:3.5, v/v/v/v) as the solvent system. The bands were detected by exposure to iodine vapor and identified using known standards (glucocerebroside, steryl glycoside). The glycolipids were then eluted from the silica gel and quantified as described by Roughan and Batt (1968).

Fatty Acid Analysis. Fatty acids from the neutral lipid, glycolipid, and phospholipid fractions were analyzed by GC

after transmethylation. The samples were dried and transmethylated by incubation with 50 μL of 2,2-dimethoxypropane and 0.5 mL of methanolic HCl for 1 h at 80 °C. After cooling, 1 mL of NaCl (0.9%) and 0.5 mL of 3 N hexane were added. The upper layer was removed after centrifugation (2000*g* for 4 min) and the aqueous layer re-extracted with hexane. The combined hexane extracts were dried, redissolved in hexane, and separated by GC on a 30-m DB-23 column (0.32 mm i.d., 0.25 mm film thickness). Temperature was maintained at 150 °C (1 min) and increased to 240 °C at a rate of 4 °C min^{-1} (5 min hold). Injector and detector temperatures were 220 and 250 °C, respectively, and split ratio was 80:1. Fatty acid contents were determined by reference to an internal standard, heptadecanoic acid.

Herbicide Effects on Lipoxygenase Activity. Wild oat plants at 1−2 leaf stage were sprayed with sethoxydim [1.47 or 2.35 kg ha^{-1} (5× or 8× the recommended field rate)], tralkoxydim [1.0 kg ha^{-1} (5× the recommended field rate)], clethodim [0.046 kg ha^{-1} (recommended field rate)], fenoxaprop [0.46 kg ha^{-1} (5× the recommended field rate)], or diclofop-methyl [2.13 kg ha^{-1} (3× the recommended field rate)]. Leaf tissue was harvested 24, 48, and 72 h after herbicide treatment, frozen in liquid N_2, and stored at −80 °C.

Lipoxygenase Extraction and Assay. Lipoxygenase was partially purified using a modified version of the method described by Kato *et al*. (1992). Frozen tissue (5 g) was homogenized in grinding buffer [50 mM sodium phosphate, 1.5% (w/v) Triton X-100, 0.1 mM EDTA, 10% (w/v) glycerol, pH 6.8] and centrifuged at 23000*g* for 15 min. Triton X-100 was removed by adding Amberlite XDA-2 (5 g). The supernatant was subjected to a 35% ammonium sulfate cut, followed by a 75% cut. The pellet after the 75% cut was dissolved in 1.0 mL of elution buffer [50 mM sodium phosphate, 0.1% Tween 20, 0.1 mM EDTA, 10% (w/v) glycerol, pH 6.8]. The samples were then desalted on Sephadex G-25 columns equilibrated with equilibration buffer [10 mM sodium phosphate, 0.1% Tween 20, 0.1 mM EDTA, 10% (w/v) glycerol, pH 6.8]. Protein in the desalted samples was estimated using Bradford's method (Bradford, 1976).

The assay was performed using a modified method of Axelrod *et al*. (1981). To start the reaction, 500 μL of substrate stock (9.98 mM linoleic acid and 0.3% Tween 20), tissue extract (equivalent to 200 μg of protein), and 0.2 M sodium phosphate (pH 6.5) (total volume 3 mL) were mixed in a cuvette. The change in absorbance at 234 nm was recorded every 2 s for 3 min. The rate of reaction in the linear portion of the curve (30−90 s) was used to calculate the LOX specific activity.

RESULTS

Plasma Membrane Lipid Composition. Highly enriched plasma membrane vesicles were obtained from leaf tissue of both the R and S biotypes (Table 1). The chlorophyll content of the vesicles was <1% of that of the microsomal fraction, and cytochrome *c* oxidase activity, a marker for mitochondrial inner membranes, decreased 14-fold during vesicle preparation. Vanadate-inhibited ATPase activities were similar in the R and S biotypes. $NaNO_3$, NaN_3, and molybdate did not significantly inhibit the ATPase activity, indicating little or no contamination from tonoplast or mitochondrial ATPases or from acid phosphatase.

The total amount of lipid was not different between the R and S biotypes (Table 2). In the S biotype, phospholipids and glycolipids represented 37 and 36%, respectively, of the total plasma membrane lipids, whereas free sterols constituted 27% of the plasma membrane lipids. In the R biotype, the glycolipids were the major class of lipids (41%), with lesser amounts of phospholipids (29%) and free sterols (30%) present. The differences between the R and S biotypes were not statistically significant.

Different amounts of protein (on a grams of fresh weight basis) were obtained from plasma membrane preparations of the two biotypes (32.7 and 39.3 μg of protein/g of FW of leaves from the S and R biotypes, respectively). To account for this, the data in Table 2 were also expressed in nanomoles per gram of FW. In this case, equal amounts of phospholipids were found in the R and S biotypes, but slightly more glycolipids and free sterols in the R biotype. However, the percentage of total lipid calculated from these values was equal in the two biotypes.

Cholesterol and other sterols were identified by their relative gas chromatographic retention times and by GC/MS (data not shown). In all cases, the appropriate ion and diagnostic fragments ions were obtained. There were no significant differences in the free sterol content of the plasma membrane from the two wild oat biotypes (Table 3). Stigmasterol (31% for S, 28% for R) and β-sitosterol (29% for S, 28% for R) were the main sterols detected. Cholesterol and campesterol were found in

Table 1. Distribution of Chlorophyll and Cytochrome *c* Oxidase Activity in the Microsomal Fraction (MF) and Plasma Membrane (PM) Vesicles Extracted from Wild Oat [Mean of Susceptible (S) and Resistant (R) Biotypes], and Effect of Inhibitors on ATPase Activity in S and R PM Vesicles[a]

assay (units)	MF	PM
chlorophyll (mg g^{-1} FW)	173 (100%)	1.3 (0.8%)
cyt *c* oxidase (D_{A549} min^{-1} mg P^{-1})	9.8 (100%)	0.7 (7%)

	ATPase activity (% of control)	
treatment	S	R
control	100	100
+ sodium orthovanadate	11	13
+ $NaNO_3$	96	100
+ molybdate	103	105
+ NaN_3	108	105
− Mg	0	0

[a] 100% ATPase activity corresponded to 1900 and 1880 nmol of P_i min^{-1} mg P^{-1} for the R and S biotypes, respectively. $n = 4$ for chlorophyll and cyt *c* oxidase assays and 3 for ATPase assays.

Table 2. Lipid Composition of Plasma Membrane Vesicles Isolated from Leaves of Susceptible (S) and Resistant (R) Wild Oat Biotypes[a]

	nmol mg^{-1} P		nmol g^{-1} FW		% total lipids	
lipid fraction	S	R	S	R	S	R
total phospholipid	780 (140)	640 (80)	25	25	37	29
total glycolipid	750 (100)	930 (80)	25	36	36	41
total free sterol	560 (30)	680 (100)	18	27	27	30
total lipid	2090	2250	68	88	100	100

[a] Values represent means and standard errors ($n = 4$ for phospholipids, 11 for glycolipids, 6 for sterols in S, and 9 for sterols in R). The results are expressed in nmol mg^{-1} plasma membrane protein and in nmol g^{-1} fresh weight tissue.

Table 3. Sterol Composition of Plasma Membrane Vesicles Isolated from Leaves of Susceptible (S) and Resistant (R) Wild Oat Biotypes[a]

	S		R	
sterols	nmol mg^{-1} P	% total sterols	nmol mg^{-1} P	% total sterols
stigmasterol	170 (11)	31	200 (26)	29
β-sitosterol	160 (10)	28	180 (25)	27
cholesterol	80 (6)	14	100 (13)	15
campesterol	20 (5)	4	20 (8)	3
unknown	130 (5)	23	180 (34)	26
total	560 (30)	100	680 (101)	100

[a] Values represent means and standard errors ($n = 6$ for S, $n = 9$ for R).

Table 4. Phospholipid (PL) Composition of Plasma Membrane Vesicles Isolated from Leaves of Susceptible (S) and Resistant (R) Wild Oat Biotypes[a]

	S		R	
PL	nmol mg^{-1} P	% total PL	nmol mg^{-1} P	% total PL
PE[b]	300 (16)	39	270 (32)	42
PC[b]	260 (23)	34	220 (19)	35
PA[b]	130 (14)	17	90 (19)	14
others	80 (16)	10	60 (13)	9
total	770 (142)	100	640 (80)	100

[a] Values represent means and standard error ($n = 4$). [b] Phosphatidylethanolamine, PE; phosphatidylcholine, PC; phosphatidic acid, PA.

Table 5. Glycolipid (GL) Composition of Plasma Membrane Vesicles from Leaves of Susceptible (S) and Resistant (R) Wild Oat Biotypes[a]

	S		R	
GL	nmol mg^{-1} P	% total GL	nmol mg^{-1} P	% total GL
steryl glucoside	200 (22)	27.0	250 (44)	26.5
glucocerebroside	460 (39)	61.5	590 (63)	63.0
others	89 (21)	11.5	100 (18)	10.5
total	750 (101)	100	940 (80)	100

[a] Values represent means and standard error ($n = 11$).

lower amounts. A relatively high amount (25%) of (an) unidentified sterol(s) was detected in both biotypes.

The phospholipid fraction of the plasma membranes was composed mainly of phosphatidylethanolamine (PE) (39% for S, 42% for R) and phosphatidylcholine (PC) (34% for S, 35% for R) with a lesser amount of phosphatidic acid (PA) (Table 4). No significant differences were found between the two biotypes. Four other spots were detected on the TLC plates (grouped together in Table 4 under "others"). Since the percentage of each of these was <2.5% of the total phospholipid and was equal in each biotype, they were not identified.

The principal glycolipid identified was glucocerebroside (61.5% for S and 63% for R) (Table 5). Steryl glucoside was identified in both biotypes, but at a lesser amount. Again, there were no statistically significant differences between the two biotypes.

The total amount of fatty acids from each lipid fraction was not significantly different between the R and S biotypes (Table 6). In addition, the fatty acid profiles from the lipid fractions did not vary significantly between the two biotypes. The exception was an unidentified fatty acid in the glycolipid fraction, which was found in higher amounts in the R biotype. This fatty acid represented <11% of the total fatty acid content in the R biotype and 4% in the S biotype. Palmitic acid (16:0) was the predominant fatty acid in the different classes of lipids of both biotypes. In the neutral lipid fraction, 18:0 (stearic acid) and 18:1 (oleic acid) were also prominent. An unidentified fatty acid was found in high amounts (16%) in the neutral lipid fraction of both biotypes. Linoleic acid (18:2) and linolenic acid (18:3) were detected in smaller amounts. In the phospholipid fraction, 18:2, 18:3, and 16:0 were the predominant acyl groups. In the glycolipid fraction, 16:0 constituted 39% (R) and 45% (S) of the acyl groups; this was followed by 18:3, 18:2, 18:0, and 18:1. Unidentified components represented a low percentage of the total fatty acids of the phospholipid and glycolipid fractions. In all classes of lipids of the two biotypes, 20:0, 20:1, 22:0, 22:1, and 24:0 were found in very small or trace amounts.

Table 6. Fatty Acid Composition of Different Lipid Classes of Plasma Membrane Vesicles from Susceptible (S) and Resistant (R) Biotypes of Wild Oat[a]

	neutral lipids		phospholipids		glycolipids	
acyl group	S, % of total	R, % of total	S, % of total	R, % of total	S, % of total	R, % of total
14:0	4.0	4.0	0.8	1.1	1.5	2.0
16:0	20.5	24.9	28.6	27.8	45.3	38.9
18:0	21.6	23.1	5.1	7.1	7.2	9.3
18:1	15.4	11.4	4.4	5.8	5.3	4.7
18:1 (*trans*)	2.9	1.4	*b*	*b*	*b*	*b*
18:2	7.1	6.4	30.0	26.9	12.0	12.5
18:3	7.8	7.7	25.5	24.0	17.7	14.0
20:0	0.3	0.7	*b*	0.5	1.0	1.2
20:1	0.6	0.7	*b*	*b*	0.7	1.0
22:0	1.9	1.8	2.1	2.0	3.7	3.3
22:1	1.5	1.0	*b*	1.3	*b*	*b*
24:0	*b*	*b*	1.8	1.2	2.2	1.6
unknown	16.4	16.9	1.7	2.5	4.0	10.8

[a] Mean fatty acid yields were 530, 475, and 325 μg mg^{-1} P for neutral lipids, phospholipids, and glycolipids, respectively (no differences between R and S biotypes; $n = 5$). [b] Trace amounts.

Table 7. Lipoxygenase (LOX) Activity in Untreated and Herbicide-Treated Susceptible (S) and Resistant (R) Wild Oat Biotypes[a]

			LOX specific activity (pmol min^{-1} μg^{-1} P)	
treatment	rate (kg ha^{-1})	TAT[b] (h)	S	R
control			16.9 (0.3)	14.3 (0.4)
sethoxydim	1.47	24	14.2 (0.4)	13.6 (0.2)
		48	14.3 (0.1)	14.2 (0.4)
		72	14.6 (0.2)	18.7 (0.2)
	2.35	24	14.5 (0.4)	14.1 (0.6)
		48	13.8 (0.8)	14.7 (0.2)
		72	14.5 (1.0)	12.9 (0.2)
clethodim	0.046	24	20.3 (0.4)	12.1 (0.1)
		48	11.8 (0.4)	13.5 (0.1)
		72	15.3 (0.4)	14.9 (0.4)
tralkoxydim	1.00	24	12.8 (0.5)	14.7 (0.4)
		48	13.1 (0.4)	14.5 (0.2)
		72	10.2 (0.4)	14.5 (0.2)
fenoxaprop-ethyl	0.46	24	15.1 (0.5)	16.1 (0.4)
		48	14.1 (0.4)	18.1 (0.2)
		72	14.0 (0.6)	10.1 (0.2)
diclofop-methyl	2.13	24	16.9 (0.2)	14.0 (0.2)
		48	9.1 (0.1)	11.8 (0.4)
		72	13.0 (0.2)	16.6 (0.1)

[a] The values are means and standard error of at least two samples, each assayed three times. [b] TAT, time after treatment.

Herbicide Effects on Lipoxygenase Activity. The specific activity of LOX was equal in the untreated R and S leaf tissue (Table 7). LOX activity in tame oat (*Avena sativa*) plants of similar age was the same as in the two wild oat biotypes (data not shown). Treatment with the ACCase inhibitors sethoxydim, clethodim, tralkoxydim, fenoxaprop-ethyl, or diclofop-methyl had no effect on LOX activity (Table 7); LOX activity remained equal to that in the untreated controls, regardless of the rate of herbicide used and the time between treatment and assay.

DISCUSSION

Overall, the analysis of plasma membrane lipids revealed no significant differences in lipid composition between the S and R biotypes. The lipid/protein ratio reported here for wild oat (2090 and 2250 nmol mg P^{-1} for the R and S biotypes, respectively) was lower than that reported in rye (3400 nmol mg P^{-1}) (Lynch and Steponkus, 1987) but higher than in cultivated oat (1420 nmol mg P^{-1}) (Sandstrom and Cleland, 1989).

The amount of free sterol found in wild oat plasma membranes was close to that found in plasma membranes from barley leaves (35% of the total lipids), oat root (25% of the total lipids), and rye (33% of the total lipids) (Sandstrom and Cleland, 1989; Lynch and Steponkus, 1987; Rochester *et al*., 1987). However, the amount can vary greatly in the plasma membrane, from 7% in spinach leaves to 57% in barley roots (Rochester *et al*., 1987). Stigmasterol and β-sitosterol, the main free sterols in wild oat, have been identified in barley, oat, and rye (Sandstrom and Cleland, 1989; Lynch and Steponkus, 1987; Rochester *et al*., 1987). Although cholesterol is the major sterol in animal cells, it has seldom been identified in plant tissues. However, a small amount was detected in wild oat (14% of total sterols). Cholesterol has previously been reported to constitute a relatively small proportion of the total lipids in plasma membranes from various plant species (Sandstrom and Cleland, 1989; Lynch and Steponkus, 1987; Coupland *et al*., 1991). In those studies, cholesterol was identified on the basis of retention time; our analysis, based on GC retention times as well as mass spectrometry, provides stronger evidence for the occurrence of cholesterol in plants.

The amount of phospholipids recovered (29 and 37% of the total lipids in the R and S biotypes, respectively) was relatively low compared to that in barley leaves (44%), spinach (64%), and cauliflower (53%) (Rochester *et al*., 1987) and in oat roots (79%) (Norberg and Liljenberg, 1991). The major phospholipids identified in wild oat were PC and PE, similar to the plasma membrane phospholipids of other plants. Phosphatidylinositol, not identified in wild oat, has been reported in small amounts in other plant plasma membrane preparations.

A relatively high percentage of glycolipids was found in wild oat plasma membrane (36 and 41% of total lipids in the R and S biotypes, respectively). These values are close to those found in oat (39%) and rye (35%) (Sandstrom and Cleland, 1989; Lynch and Steponkus, 1987), while in barley the amount was lower (21%) (Rochester *et al*., 1987). The relative content of glycolipids may have been overestimated, since it is based on the assumption that each molecule of glycolipid contains a single molecule of sugar (Sandstrom and Cleland, 1989). One characteristic of the plant plasma membrane is the relatively high content of glucocerebrosides. This was reported as the major component of the tonoplast of mung beans (Yoshida and Uemura, 1986) and has also been identified in barley, oat, and rye plasma membranes (Sandstrom and Cleland, 1989; Lynch and Steponkus, 1987; Rochester *et al*., 1987). Glucocerebroside represented 22% (S) and 26% (R) of the total lipids in wild oat plasma membranes. A lesser amount of steryl glycoside was recovered in the membranes. The sterol glycosylation occurs in the plasma membrane and is catalyzed by UDP-glucose:sterol glucosyltransferase, a marker for the plant plasma membrane (Larsson *et al*., 1990). Monogalactosyldiacylglycerol, the most abundant lipid in the chloroplast thylakoid membrane, was not detected, confirming the purity of these plasma membrane preparations.

Palmitic acid was the major fatty acid in the different classes of lipids. Stearic acid was present in high amounts in the neutral fraction, whereas unsaturated fatty acids, such as linoleic and linolenic, were more prominent in the phospholipid and glycolipid fractions. The fatty acid composition and the degree of unsaturation are usually interpreted in terms of membrane fluidity and bilayer thickness (Carruthers and Melchior, 1986). The results obtained here do not suggest any differences in membrane fluidity or bilayer thickness between the two biotypes.

Differences in lipid composition of the plasma membrane can affect the activity of membrane proteins such as H^+-ATPase (Kasamo, 1990). Phospholipids such as PC and lysophosphatidylcholine (LPC) are required for H^+-ATPase activity, and sterols have also been found to be closely associated with plasma membrane H^+-ATPase (Lynch and Steponkus, 1987). However, the lipid environment did not differ between the two wild oat biotypes. We have reported previously that the plasma membrane H^+-ATPase activity is similar in the two biotypes and is insensitive to diclofop at concentrations up to 100 μM (Devine *et al*., 1993b).

In microalgae, resistance to SAN 9785, a herbicide that inhibits fatty acid desaturation, was associated with a modification of fatty acid composition (Cohen *et al*., 1993). The resistant lines overproduced γ-linolenic acid and eicosapentaenoic acid, both in the presence and in the absence of the herbicide. In our research, no modification of lipid composition was detected in the herbicide-resistant wild oat biotype. Therefore, the differential effect on the plasma membrane E_m and the altered herbicide transport into plasma membrane vesicles and protoplasts from the R and S wild oat biotypes (Devine *et al*., 1993a,b) do not appear to be due to differences in plasma membrane lipid composition. Similarly, differences in the composition of the chloroplast envelope do not appear to be involved in herbicide resistance, since we have found equal herbicide uptake into chloroplasts isolated from the R and S biotypes (Wang and Devine, unpublished results). Analysis of plasma membrane-associated proteins in vesicle preparations has also revealed no differences between the R and S wild oat biotypes (Phelps and Devine, unpublished results), suggesting that the differential response of the two biotypes to herbicides is not a function of modified membrane protein content.

Our results on LOX activity show that AOPP and CHD herbicide action in wild oat does not involve changes in LOX activity. Free PUFA are the preferred substrates for some LOX isozymes; however, methyl esters, triglycerides, phospholipids, and biological membranes can also serve as substrates for LOX (Hilderbrand, 1989; Siedow, 1991; Whitaker, 1991). Recently, Banas *et al*. (1993b) reported an increase in LOX activity in root tips of wheat seedlings upon treatment with haloxyfop and suggested that the toxicity of herbicides may be due to their influence on the activity of LOX, alterations in membrane structure, and/or generation of free radicals and ethylene. More recently, Shimabukuro and Hoffer (1995) suggested that AOPP herbicides may stimulate lipid peroxidation (via LOX activity and the generation of free radicals) rather than inhibition of fatty acid synthesis. However, the results shown here indicate no difference in LOX activity between the R and S wild oat biotypes and suggest that reduced LOX activity is not involved in the resistance mechanism of the R biotype.

Recently, we have shown that the mechanism of resistance in the R wild oat biotype is an altered ACCase with reduced sensitivity to AOPP and CHD herbicides (Shukla *et al*., 1997a). The R/S GR_{50} ratio (value of >152; Heap *et al*., 1993) for sethoxydim correlated well

with the R/S I_{50} ratio (value of 105) at the enzyme level. Similar results have been published for many other resistant weed biotypes [e.g., Devine and Eberlein (1997); Leach *et al*. (1995); and Shukla *et al*. (1997b)]. On the basis of the results reported in this paper, we suggest that the plasma membrane is not involved in the primary mechanism of herbicide action or resistance in these wild oat biotypes. Enhanced lipid catabolism may contribute to the overall toxic effect of the herbicides in sensitive biotypes. However, regardless of the mechanism(s) of toxicity, the results in total point to reduced ACCase sensitivity, not altered membrane properties, as the primary factor responsible for herbicide resistance in this wild oat biotype and most other weed biotypes resistant to these herbicides.

ABBREVIATIONS USED

ACCase, acetyl-CoA carboxylase; AOPP, aryloxyphenoxypropionate; CHD, cyclohexanedione; Em, electrogenic potential; LOX, lipoxygenase; PUFA, polyunsaturated fatty acid.

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