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ARTICLE

Evidence that Norflurazon Affects Chloroplast Lipid Unsaturation in Soybean Leaves (*Glycine max* L.)

Ouzna Abrous-Belbachir,[†] Rosine De Paepe,[‡] Antoine Trémolières,[‡] Chantal Mathieu,[‡] Fatiha Ad,[†] and Ghouziel Benhassaine-Kesri*,[§]

[†]Université des Sciences et de la Technologie Houari Boumedienne, Faculté des Sciences Biologiques, Laboratoire de Biologie et Physiologie des Organismes, BP 39, El Alia, Bab Ezzouar, Alger, Algeria, [‡]Université Paris-Sud XI, UMR CNRS 8618, Institut de Biotechnologie des Plantes, 91405 Orsay, France, and [§]UPMC Univ Paris 06, Unite de Recherche 5, Centre National de Recherche Scientifique, Equipe d'Accueil Conventionnée, Laboratoire de Physiologie Cellulaire et Moléculaire des Plantes, F-75252 Paris cedex05, France

Norflurazon is a bleaching herbicide known to block carotenoid biosynthesis by inhibiting phytoene desaturase activity. Soybean plants were treated with norflurazon, and we examined the effects on the desaturation of lipid molecular species in leaves using ammonium [1-^{14}C] oleate labeling. In monogalactosyldiacylglycerol (MGDG), the main chloroplast lipid, a decrease in 18:3/18:3 molecular species and an increase in its precursors 18:2/18:3 and 18:2/18:2 were observed suggesting that the ω^3 FAD7 desaturase activity in planta was inhibited by norflurazon. The in vitro activity of MGDG synthase was also inhibited by 69%. In contrast, the amount of 18:3/18:3 molecular species of phosphatidylcholine (PC) in the extraplastid compartment increased. The observed increase in in vitro lysoPC-acyltransferase activity and activation of desaturation of [1-^{14}C] oleate suggest that extraplastid ω^3FAD3 desaturase was activated. Analysis of the expression of ω^3 *FAD3* and ω^3 *FAD7* genes in norflurazon treated plants indicate that ω^3 FAD7 and ω^3 FAD3 desaturases are controlled at the post-transcriptional level.

KEYWORDS: Norflurazon; lipid metabolism; ω^3 desaturases; FAD7 and FAD3 transcripts

INTRODUCTION

Norflurazon (4-chloro-5-methylamino-2α,α,α-trifluoromethylphenyl-3-(2*H*)-pyridazinone; San 9789) is used as a preemergence herbicide to control the development of annual grasses and broadleaf weeds in various crops such as cotton, soybean, peanut, sugar cane, and many fruit trees (*1*). Norflurazon is a potent noncompetitive inhibitor of phytoene desaturase, a key enzyme in carotenoid biosynthesis (*2*, *3*), which is located in the plastid membranes (*4*, *5*). A lack of carotenoids impairs photoprotection of the photosynthetic apparatus against oxidants including free radicals (*6*, *7*) and, depending on the light intensity, may result in chlorophyll degradation, which leads to the characteristic bleaching symptoms. As carotenoids act as stabilizers of the lipid phase in thylakoid membranes (*8*), the decrease in the number of long chain carotenoids alters the membrane structure and impairs the stability of pigment proteins and lipids (*9*).

In previous work, we showed that the lipid and fatty acid composition of soybean leaves was altered after norflurazon treatment (*10*). We hypothesized that in addition to inhibiting the desaturation of phytoene norflurazon could have an effect on lipid desaturases which synthesize polyunsaturated fatty acids from saturated fatty acids. The reaction converting single bonds to double bonds is a dehydrogenation reaction very similar to phytoene dehydrogenation. Our current understanding of lipid desaturation in plants has come from the characterization of *Arabidopsis* mutants deficient in the activity of membrane-bound desaturases (*11*). Two types of desaturases localized in two different cell compartments are involved in the biosynthesis of linolenic acid from oleic acid: ω^6 FAD6 and ω^3 FAD7 desaturases and FAD8, a cold-inducible isoenzyme, are localized in plastids, while ω^6 FAD2 and ω^3 FAD3 desaturases are specific to the endoplasmic reticulum (*12*). Linolenic acid is the main component of monogalactosyldiacylglycerol (MGDG), the major plant membrane lipid. Plants can be classed into two groups according to the way in which they desaturate MGDG fatty acid and their molecular species composition. The 18:3 plants, such as soybean, only desaturate 18-carbon fatty acids by a cooperative plastid/endoplasmic reticulum (eukaryotic) pathway. The 16:3 plants, such as *Arabidopsis*, desaturate 16-carbon fatty acids by the plastid (prokaryotic) pathway and 18-carbon fatty acids by the eukaryotic pathway (*4*).

The prokaryotic and eukaryotic types of galactolipids are generated in the chloroplast envelope by galactosylation of diacylglycerol (DAG) molecular species of distinct origins. Whereas prokaryotic DAG is fully synthesized in plastids, eukaryotic DAG originates from PC formed in the endoplasmic reticulum. Direct import of PC from the endoplasmic reticulum or import of intermediates, possibly lysophosphatidylcholine (*13*),

*Corresponding author. E-mail: ghouziel.benhassaine@upmc.fr. Tel.: 33 (0) 144 27 5916. Fax: 33 (0) 144 27 61 51.

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DAG (*14*), or phosphatidate (*15*, *16*), derived from the endoplasmic reticulum is therefore required.

In higher plants, the final step of MGDG biosynthesis occurs in the plastid envelope and is catalyzed by MGDG synthase, UDP galactose:1,2-diacylglycerol 3-[α]-D-galactosyltransferase (*17*). In *Arabidopsis*, three MGDG synthases are classified into type A (atMGD1) and type B MGD isoforms (atMGD2 and atMGD3). atMGD1 is an inner envelope membrane-associated protein of chloroplasts and is responsible for the bulk of galactolipid biosynthesis in green tissues. Its function is indispensable for thylakoid membrane biogenesis, photosynthesis, and embryogenesis (*18*, *19*). Type B atMGD2 and atMGD3 are localized in the outer envelope membranes and are induced by phosphate starvation (*20*).

In plants and in other organisms, membrane fluidity is modified by changes in unsaturated fatty acid levels, a function provided by the regulated activity of fatty acid desaturases (*21*). Adjustment of membrane fluidity maintains a suitable environment for the function of critical integral proteins in stress conditions. Polyunsaturated fatty acids are thus crucial for cells as they contribute to inducible resistance to abiotic and biotic stresses by the remodeling of membrane fluidity (*22*, *23*). A number of abiotic stresses such as high temperature (*24*), cold (*25*), drought (*26*), and high salinity (*27*) are known to induce changes in membrane lipid unsaturation. However, there is limited information on differential responsiveness to chemical stresses such as those obtained from herbicides.

Here we sought to specify the effects of norflurazon on lipid desaturases in terms of the changes in the molecular species of lipids and their desaturation. We also studied the effect of norflurazon on MGDG synthase and two key enzymes involved in PC biosynthesis (CDP-choline phosphotransferase and lysoPC-acyltransferase). The expression of soybean *FAD7* and *FAD3* genes, which encode plastid and endoplasmic reticulum desaturase enzymes, respectively, was examined to understand how these changes might be regulated at the molecular level.

MATERIALS AND METHODS

Chemicals. Norflurazon 4-chloro-5-methylamino-2α,α,α-trifluoromethylphenyl-3-(2*H*) pyridazinone was used as a selective preemergence herbicide. [1-^{14}C] oleic acid (2.04 GBq mmol^{-1}) and [1-^{14}C] oleoyl CoA (2.04 GBq mmol^{-1}) were purchased from Amersham (Saclay, France). Cytidine 5′-diphosphate-[^{14}C] choline (1.92 GBq mmol^{-1}) and uridine diphosphate-[^{14}C] galactose (12.0 GBq mmol^{-1}) were from NEN.

Plant Growth Conditions and Herbicide Treatment. Soybean seeds (*Glycine max*. L. Var. Weber) were kindly supplied by Rustica Semences (Mondonville, France) and sown in soil wetted with water (control) or with a 100 μM norflurazon solution (treated plants). Seedlings were grown in a greenhouse under a 16 h light/8 h dark regime at 25−18 °C with 60% relative humidity. All in planta analyses were performed on the control and treated plants two weeks after the herbicide treatment (the stage at which leaves had developed).

In vivo Labeling Experiments. Ammonium [1-^{14}C] oleate (30 μL) was deposited in situ on the upper surface of leaves of control or treated plants (in total six plants) per experiment. The radioactivity applied was equivalent to 7 μmol of ammonium [1-^{14}C] oleate per leaf. Total lipids were extracted 2, 6, 12, and 24 h after ammonium [1-^{14}C] oleate application.

Lipid Analysis. Leaves were fixed in boiling water, and lipids were extracted as described previously (*28*). Lipid classes were separated by thin layer chromatography (TLC) (*29*). Spots corresponding to lipid classes of interest were scraped off the TLC plate, and the amount of incorporated radioactivity was determined by liquid scintillation spectrometry. Fatty acids were transmethylated (*30*) and analyzed by radio-GC using a Girdel 300 apparatus equipped with a carbowax column (25 m long, 0.5 mm diameter) at 170 °C coupled to a radiomatic gas proportional counter.

Molecular Species Analysis. Polar lipids were separated by HPLC (*31*) on a semipreparative silica column of 7.8 mm × 30 cm (Waters, USA) with a linear gradient program of two mixed solvents running from 100% of solvent A, isopropanol:hexane (4:3, v/v), to 100% of solvent B, isopropanol:hexane:water (8:6:1.5, v/v/v). Molecular species were separated by HPLC on a 5 μm ODS 4.6 mm × 25 cm column (Beckman Coulder, Palo Alto, CA, USA). The mobile phase consisted of methanol:water:acetonitrile (90.5:4:2.5, v/v/v) solvent containing 2.9 g·L^{-1} of choline chloride. Molecular species were detected at 205 nm and collected. Radioactivity was measured using a continuous flow apparatus. Fatty acids from each molecular species were prepared by transmethylation in the presence of an internal standard (heptadecanoic acid) and analyzed by radio-GC.

Membrane Preparation. All procedures were carried out at 4 °C. To prepare microsomal membranes, leaves were ground in 100 mM Tris-HCl pH 8.0 containing 500 mM sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1% BSA (w/v), and 4 mM cysteine hydrochloride. The homogenate was filtered through a double layer of Miracloth and centrifuged at 10 000*g* for 20 min. The supernatant was centrifuged again at 100 000*g* for 90 min. The microsomal pellet was suspended in 50 mM Tris-HCl pH 8.0 and stored at −80 °C until use.

Chloroplasts were extracted according to (*32*) and (*33*). Leaves were ground in a buffer containing 50 mM Tris-HCl pH 7.8, 330 mM sorbitol, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA (w/v), 1 mM cysteine hydrochloride. The homogenate was then filtered through two layers of cheesecloth and one layer of Miracloth and centrifuged for 3 min at 3000*g*. The pellet was suspended, and chloroplasts were purified on a Percoll gradient. Intact chloroplasts were disrupted in 20 mL of 10 mM Tris-HCl pH 7.8 containing 4 mM $MgCl_2$. The suspension of broken chloroplasts was then centrifuged for 10 min at 5000*g*. The pellet was discarded, and the pellets rich in chloroplast membranes were suspended in 30 mM tricine−NaOH pH 7.8 and 1 mM dithiothreitol.

Enzymatic Activities. Choline phosphotransferase (CDP-choline: 1,2-diacylglycerol choline phosphotransferase, EC 2.7.8.2) catalyzes the last step of PC biosynthesis by transferring the phosphocholine from CDP choline to 1,2-DAG. It was measured here by incubating microsomal suspensions in 100 μL of Tris-HCl (50 mM pH 8.0) containing 100 mM DTT and 25 mM $MgCl_2$ with 265 pmol of CDP-[^{14}C] choline (1.92 GBq mmol^{-1}). The reaction was started by adding the labeled CDP-choline, incubated in a water bath shaker at 30 °C for 20 min, and stopped by adding 2 mL of boiling methanol.

LysoPC-acyltransferase or acylCoA:lysoPC-acyltransferase (EC 2.3.1.23) catalyzes PC biosynthesis by acylating the oleoyl residue on lysoPC to form PC molecules. The enzymatic activity was measured by incubating microsomal suspensions in 500 μL of 100 mM phosphate buffer (pH 7.2) containing 24 mM $MgCl_2$. The reaction was started by adding 650 pmol of oleoyl [1-^{14}C] CoA (2.04 GBq mmol^{-1}), incubated in a water bath shaker at 30 °C for 6 min, and stopped by adding 2 mL of boiling methanol. MGDG synthase (UDP-galactose:1,2-diacylglycerol-3-α-D-galactosyltransferase, EC 2.4.1.46) activity was measured in a mixture of 10 mM tricine−NaOH pH 7.8 and 82 mM UDP-[^{14}C]galactose (12.0 GBq mmol^{-1}) in a final volume of 500 μL (*33*). The reaction was started by adding an aliquot of membrane fraction, and the suspension was incubated in a shaker water bath at 30 °C for 30 min.

Lipids were extracted, and enzymatic activities of choline phosphotransferase, lysoPC-acyltransferase, and MGDG synthase were measured by determining the radioactivity found in either PC or MGDG expressed as nanomoles or picomoles per milligram of protein per minute.

Protein content was determined as described previously (*34*) using Biorad protein assay reagents with BSA as a standard.

Analysis of Transcript Levels. Leaf tissues from control and treated plants were harvested in liquid nitrogen and stored at −80 °C. Total RNAs were extracted using the Trizol-chloroform procedure (Gibco BRL). Ten micrograms of total RNA was electrophoresed on a denaturing formaldehyde/1.2% agarose gel and blotted onto nylon-based membranes (Appligene) that were hybridized with *FAD7* and *FAD3* probes labeled by random priming (*35*).

To amplify the *FAD3* gene, the forward primer 5′-CATTGCTGGGTGAAGGAC-3′ and reverse primer 3′-ATTCTCCATCCATGRTAWGG-5′ used were based on the *Glycine max FAD3* cDNA, sequence accession no. L22964. To amplify the *FAD7* gene, the forward primer 5′-CATTGYTGGGTCAAGAATCC-3′ and reverse primer 3′-ATTCTCCATC CATGRTAWGG-5′ used were based on the *Glycine max FAD7* cDNA, sequence accession no. L22965 (*36*). The PCR products (about 400 bp) were cloned, sequenced, and used to probe Northern blots.

Table 1. Changes in Molecular Species Distribution of Chloroplast Lipids (MGDG, DGDG, and PG) in Soybean Leaves of Plants Treated with Norflurazon[a,b]

molecular species	MGDG			DGDG			PG		
(mol %)	control	treated	*P*	control	treated	*P*	control	treated	*P*
18:3/18:3	92 ± 1.3	71.9 ± 1.9	**	69.1 ± 1.0	64.0 ± 1.3	*			
18:2/18:3	0.3 ± 0.1	2.7 ± 1.0	**	2.9 ± 0.6	3.9 ± 0.5	NS			
18:2/18:2	3.2 ± 0.6	18.5 ± 0.5	**	0.4 ± 0.1	3.2 ± 1.0	**			
18:0/18:3	0.4 ± 0.1	1.0 ± 0.3	**	5.8 ± 1.4	4.8 ± 1.3	NS			
16:0/18:3	4.4 ± 0.9	5.8 ± 1.6	*	19.8 ± 0.8	21.8 ± 1.2	NS			
18:3/16:0							14.5 ± 0.8	24.5 ± 1.5	**
18:3/16:1t							15.2 ± 1.5	9.2 ± 0.9	**
18:2/16:0							7.2 ± 0 0.9	9.4 ± 1.6	*
18:2/16:1t							3.5 ± 0.4	0.3 ± 0.1	**
18:1/16:0							30.4 ± 0.7	29.0 ± 1.7	NS
18:1/16:1t							29.1 ± 1.5	27.1 ± 1.5	NS

[a] Polar lipids and molecular species were separated by HPLC as explained in the Material and Methods. Fatty acids from each molecular species were analyzed by gas chromatography. [b] Results are the means (±SD) of three independent experiments. *P* value is calculated by ANOVA. *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

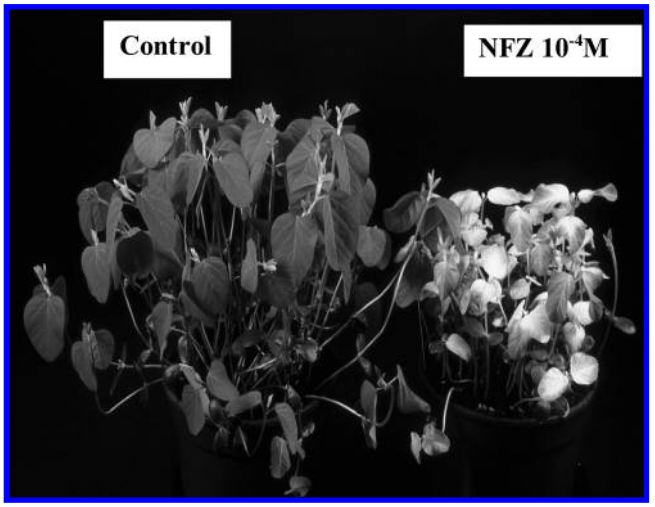

Figure 1. Effect of norflurazon on soybean morphology and growth. Norflurazon (100 μM) was applied to the soil before sowing. Photographs show control and norflurazon-treated plants after two weeks of growth.

The relative abundance of transcripts was quantified by scanning densitometry of autoradiograms using Scion Image software. Results were expressed as the ratio of the integrated density of the signal/the integrated density of the 18S rRNA signal.

Statistical Analyses. Results are the means (±SD) of three independent experiments. The statistical analysis were performed using one-way analysis of variance (ANOVA). The significance of differences between treated and control plants was determined, and *P* values are expressed as *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

RESULTS

Norflurazon Affects the Morphology and Growth of Soybean Seedlings. When soybeans were sown in soil treated with 100 μM norflurazon, seeds germinated normally. Bleaching symptoms were first observed at the base of the leaf in treated plantlets before spreading over the rest of the leaf. About 90% of treated plantlets developed partially or totally bleached leaves. The partially or totally bleached plantlets were noticeably smaller than the controls (**Figure 1**).

Norflurazon Treatment Modifies the Molecular Species Unsaturation of Lipids in Plastidial and Extraplastidial Compartments. The molecular species composition of plastid lipids (namely, MGDG, DGDG, and PG) was compared in control and norflurazon-treated plants. In MGDG, the major molecular species was of the 18:3/18:3 eukaryotic type, with all the other molecular species accounting for less than 5 mol % (**Table 1**). After norflurazon treatment, the 18:3/18:3 molecular species decreased from 92 to 71.9 mol %, while 18:2/18:3 increased from 0.3 to 2.7 mol % and 18:2/18:2 from 3.2 to 18.5 mol % (**Table 1**). By contrast, in control plants the DGDG was more saturated than MGDG, and the effect of norflurazon on the molecular species distribution was less marked (**Table 1**).

Table 2. Changes in Molecular Species Distribution of Extrachloroplast Lipids in Soybean Leaves of Plants Treated with Norflurazon[a,b]

molecular species	PC			PE		
(mol %)	control	treated	*P*	control	treated	*P*
18:3/18:3	0.6 ± 0.1	5.3 ± 1.8	**	13.6 ± 1.4	15.8 ± 0.7	NS
18:2/18:3	91.2 ± 1.6	83.6 ± 1.4	*	05.8 ± 0.8	20.6 ± 1.1	**
18:2/18:2	3.0 ± 1.4	2.1 ± 0.8	NS	29.0 ± 2.9	10.9 ± 1.5	**
16:0/18:3	1.9 ± 0.3	3.5 ± 1.3	*	33.5 ± 2.1	31.3 ± 1.1	NS
16:0/18:2	1.9 ± 0.3	3.6 ± 0.8	*	14.5 ± 2.3	20.5 ± 2.0	**

[a] Polar lipids and molecular species were separated by HPLC as explained in the Material and Methods. Fatty acids from each molecular species were analyzed by gas chromatography. [b] Results are the means (±SD) of three independent experiments. *P* value is calculated by ANOVA. *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

In PG, all molecular species were of the prokaryotic type, containing either palmitic acid (C16:0) or Δ^3 trans hexadecenoic acid (Δ^3 trans-C16:1) at the sn-2 position of the glycerol backbone. The most abundant molecular species were 18:1/16:0 and 18:1/16:1t (30.4 mol % and 29.1 mol %, respectively). The molecular species 18:3/16:0 and 18:3/16:1t were each present at about 15 mol %, while 18:2/16:0 (7.2 mol %) and 18:2/16:1t (3.5 mol %) were the least abundant (**Table 1**). The norflurazon treatment decreased the proportion of molecular species containing 16:1t, with 18:3/16:1t and 18:2/16:1t going, respectively, from 15.2 mol % and 3.5 mol % in controls to 9.2 mol % and 0.3 mol % in treated plants. In contrast, treated plants had relatively more molecular species containing 16:0, namely, 18:3/16:0 (a 10 mol % increase) and 18:2/16:0 (a 2.2 mol % increase). The total proportion of 18/16:1t molecular species decreased by about 23%, while the total of 18/16:0 molecular species rose to the same extent.

Analysis of the molecular species composition of extraplastid lipids revealed marked changes in response to norflurazon (**Table 2**). In PC, the proportion of the predominant molecular species 18:2/18:3 was higher in the control (91.2 mol %) than in treated plants (83.6 mol %), while that of 18:3/18:3 molecular species was lower in control (0.6 mol %) than in treated plants (5.3 mol %). In PE, the proportion of the predominant molecular species 16:0/18 (at approximately 50 mol %) fell by less than 10% in the treated plant, while the 18:3/18:3 species increased about 16%; 18:2/18:3 was enhanced about 4-fold, and the direct precursor 18:2/18:2 decreased by about 62%. These changes in

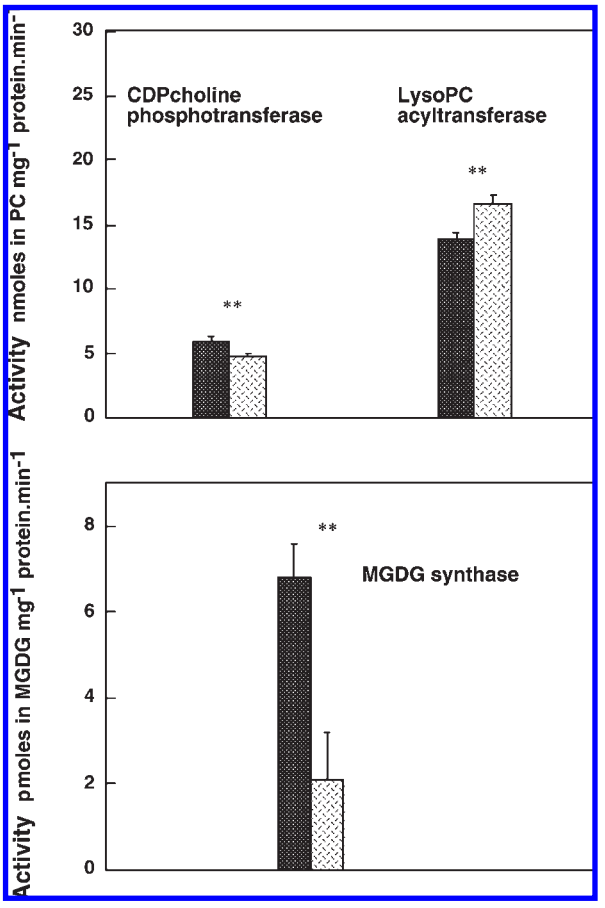

Figure 2. In vitro activities of CDP-choline phosphotransferase, lysoPC-acyltransferase, and MGDG synthase in soybean leaves of control plants (black bars) and plants treated with 100 μM norflurazon (hatched bars). Choline phosphotransferase and lysoPC-acyltransferase activities in isolated microsomes were assayed in a medium containing 265 pmol of CDP-[^{14}C] choline (1.92 GBq mmol^{-1}) for 20 min at 30 °C or 650 pmol of oleoyl [1-^{14}C] CoA (2.04 GBq mmol^{-1}) for 6 min at 30 °C. MGDG synthase activity in chloroplastic membranes was measured in an incubation mixture containing 82 μM UDP [^{14}C]galactose (12 GBq mmol^{-1}) for 30 min at 30 °C. Enzyme activities were determined as the radioactivity incorporated into PC or MGDG. The results are the means (±SD) of three independent experiments. *P* value is calculated by ANOVA. *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

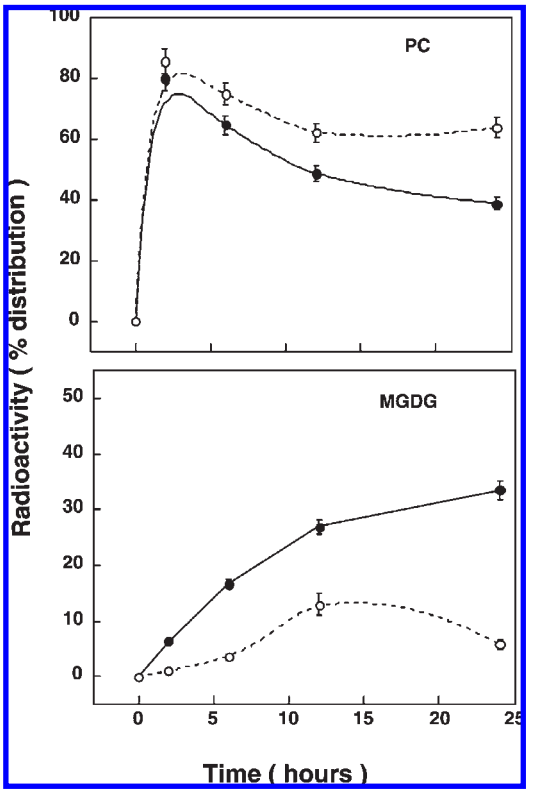

Figure 3. Distribution of radioactivity in PC and MGDG in leaves of control soybean plants (black circles) and plants treated with 100 μM norflurazon (white circles). Microdroplets of ammonium [1-^{14}C] oleate were deposited onto leaves. Lipids were extracted with methanol:chloroform:water (1:1:1, v/v/v) and separated by TLC, and the incorporated radioactivity was counted by liquid scintillation spectrometry. The results are the means (±SD) of three independent experiments.

the molecular species composition of PC and PE suggest that ω^3 FAD3 desaturase acting in the ER compartment is stimulated.

The data show that in the presence of norflurazon there is a decrease in unsaturated molecular species in chloroplast membrane lipids and an increase in these species in extraplastid lipids.

Norflurazon Modifies in Vitro Activity of Enzymes of PC and MGDG Syntheses. To study the effect of norflurazon on major lipids of plastidial and extraplastidial compartments, we measured the in vitro activity of enzymes involved in PC and MGDG biosynthesis: CDP-choline phosphotransferase, lysoPC-acyltransferase, and MGDG synthase.

Norflurazon affected different enzymes in different ways. It induced a 20% inhibition of CDP-choline phosphotransferase activity, a decrease from 6.0 nmol mg^{-1} protein min^{-1} in controls to 4.8 nmol mg^{-1} protein min^{-1} in treated plants (**Figure 2**). In contrast, the activity of lysoPC-acyltransferase was activated by 20% going from 13.9 nmol mg^{-1} protein min^{-1} in controls to 16.6 nmol mg^{-1} protein min^{-1} in treated plants (**Figure 2**). These results suggest that the biosynthesis of PC is reduced, but the acylation of oleate by lysoPC-acyltransferase is favored as a mechanism in PC biosynthesis induced by norflurazon treatment.

Norflurazon caused a 69% inhibition of MGDG synthase activity (**Figure 2**) which decreased from 6.8 pmol mg^{-1} protein min^{-1} in control plants to 2.1 pmol mg^{-1} protein min^{-1} in treated plants.

Norflurazon Modulates Chloroplast and Extraplastid Desaturation Levels. To clarify the effect of norflurazon on fatty acid desaturation, we determined the sequence in which [1-^{14}C] oleate, the direct precursor for linoleic and linolenic acid synthesis, is desaturated.

Norflurazon Modulates the Oleate Acylation in PC and MGDG. In control plants, the incorporation of radioactivity in PC was an early event with about 80% being incorporated after 2 h of labeling, falling to 38.8% after 24 h. Only 5% of the radioactivity was incorporated in MGDG after 2 h, but this rose to 35% after 24 h of labeling. The decrease in labeled PC was concomitant with an increase in labeled MGDG (**Figure 3**). The other lipids, DGDG, PG, PE, and PI, were only weakly labeled in both control and treated plants (data not shown).

In treated plants, the amount of PC labeled was higher than in control plants, especially after 24 h when about 64% was labeled. In contrast, the incorporation of [1-^{14}C] oleate in MGDG remained low in treated plants (**Figure 3**). The weak MGDG labeling and the parallel increase in PC labeling in treated plants was probably due to a diminution in PC transfer to chloroplasts for galactolipids synthesis.

Norflurazon Affects the Sequence of Oleate Desaturation in PC and MGDG. The in vivo rate of desaturation was assessed by

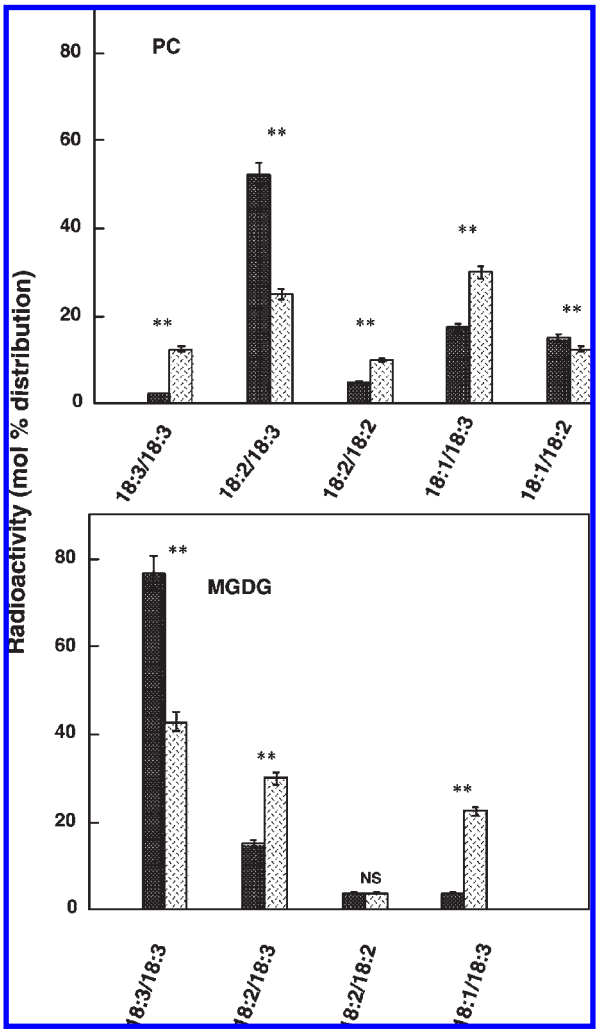

Figure 4. Distribution of radioactivity in PC and MGDG molecular species in leaves of control soybean plants (black bars) and plants treated with 100 μM of norflurazon (hatched bars). Microdroplets of ammonium [1-^{14}C] oleate were deposited onto leaves. Lipids were extracted after 24 h with methanol:chloroform:water (1:1:1, v/v/v). Polar lipids and molecular species were separated by HPLC. Fatty acids from each molecular species were analyzed by radio gas chromatography. The results are the means ($\pm$SD) of three independent experiments. *P* value is calculated by ANOVA. *, $P \leq 0.05$; **, $P \leq 0.01$; NS, not significant.

following the distribution of ^{14}C molecular species in PC and MGDG after [1-^{14}C] oleate incorporation. In PC, after 24 h of labeling, the proportion of 18:3/18:3 molecular species was four times higher in treated than in control plants, whereas the labeling of 18:2/18:3 was lower. An enhancement of labeling in 18:2/18:2 and 18:1/18:3 was also observed, whereas the labeling of 18:1/18:2 was decreased by the herbicide treatment (**Figure 4**).

In MGDG, after 24 h the level of the most unsaturated molecular species 18:3/18:3 decreased from 77 mol % in controls to 43 mol % in treated plants. In contrast, the precursor molecular species 18:2/18:3 and 18:1/18:3 increased from 15 mol % to 30 mol % and from 4.3 mol % to 22.3 mol %, respectively (**Figure 4**).

The desaturase index for linoleoyl desaturase is estimated from the ratio (18:3/18:2) of radioactivity in C18:3 compared to that in C18:2 (*37*). In control plants, the ω^3 desaturase index (linoleoyl desaturase) of the plastid compartment is higher than for the extraplastid compartment. After 24 h of labeling, the ω^3FAD7 desaturase index was 4.7, and the ω^3 FAD3 desaturase index was 0.2. In the presence of norflurazon, PC linoleoyl desaturase ω^3 FAD3 desaturase indexes was higher in treated plants (0.4 after 24 h of labeling). In contrast, in MGDG, the ω^3 FAD7 index fell by about 50% (**Table 3**). These results show that norflurazon inhibits ω^3 FAD7 desaturase activity in the plastid compartment. On the contrary, this herbicide appears to enhance endoplasmic reticulum ω^3 FAD3 desaturase activity in the extraplastid compartment.

Table 3. Effect of Norflurazon on Plastid and Extraplastid Desaturase Indexes[a]

	desaturase index	
	ω^3 FAD 7 desaturase	ω^3 FAD 3 desaturase
control	4.7	0.2
treated	2.4	0.4

[a] Leaves were labeled with [1-^{14}C] oleate as described in the Materials and Methods. ω^3 desaturase indexes were calculated as the ratio of ^{14}C18:3/^{14}C18:2 found in MGDG (ω^3 FAD 7 desaturase) or PC (ω^3 FAD 3 desaturase).

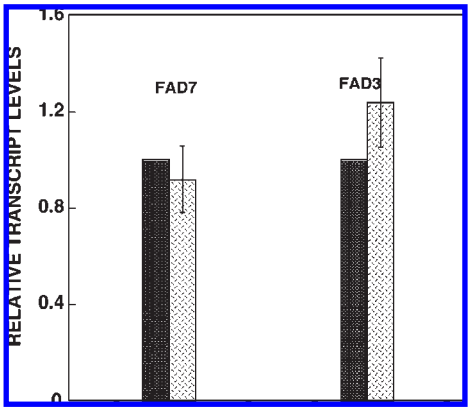

Figure 5. Relative levels of transcripts in leaves of control soybean plants (black bars) and plants treated with 100 μM of norflurazon (hatched bars). Quantification of the relative abundance of transcripts on autoradiograms was done using scanning densitometry and Scion Image software. Results were expressed as the ratio of the integrated density of the signal/the integrated density of the 18S rRNA signal. Results are normalized to control. The differences between treated and control plants were not significant.

Norflurazon Does Not Significantly Affect the Levels of *FAD7* and *FAD3* Transcripts. We examined whether changes in desaturase activities could be a result of changes in the expression of the corresponding *FAD* genes. Transcript levels of *FAD7* and *FAD3*, which encode ω^3 desaturase activities, of untreated and treated plants were compared by Northern blot analysis. After herbicide treatment, *FAD7* transcript levels were not significantly altered in treated seedlings (**Figure 5**). In addition, most of the white norflurazon-treated seedlings lacked or had less plastid rRNA, indicating that the plastid translational machinery was impaired.

FAD3 transcripts were not affected by herbicide treatment due to the fact that there was no significant change in transcript abundance (**Figure 5**).

These results suggest that norflurazon regulates the chloroplast and endoplasmic reticulum of ω_3 desaturases at a posttranslational level.

DISCUSSION

Developing soybean seedlings treated with norflurazon showed characteristic interveinal whitening of leaf and stem tissue, giving partially or totally white plants for the majority of them. Bleached seedlings grew more slowly, a phenotype that can

be explained by the curtailed availability of organic nutrients due to the lack of photosynthetic activity. Such a symptom can also be induced by other herbicides (*38*). The variability in individual responses to herbicide treatment likely results from differences in either the absorption/transport of the herbicide or in metabolizing/detoxification mechanisms, such as those involving Cyt P_{450} and glutathione S transferase (*39*).

Phytoene desaturase present in plastid membranes (*40*) is widely accepted to be the primary target of norflurazon (*2*). Inhibition of this activity interrupts the carotenoid biosynthesis pathway resulting in phytoene accumulation. Colorless carotenoids, such as phytoene, with short chromophores cannot protect the plants against photooxidation which leads to chlorophyll degradation and eventually the dismantling of thylakoids and 70S ribosomes (*38*).

Our results show that norflurazon specifically affects lipid biosynthesis and molecular species composition of the plastid compartment as norflurazon caused a strong inhibition (69%) of in vitro MGDG synthase activity. On the basis of the different changes in molecular species composition and desaturation after labeling with oleate, the results demonstrate that dilinolenoyl MGDG (18:3/18:3) is the lipid most affected by the herbicide since its level markedly decreased in response to treatment, while the precursors 18:2/18:3 and 18:2/18:2 accumulated (**Figure 4**). This indicates that norflurazon inhibits linoleate desaturase (ω^3 FAD7 desaturase) activity that acts on MGDG in plastid membranes. The effect of norflurazon on plastid desaturase activity was confirmed by calculating the desaturase index (**Table 3**).

The decrease in 18:3/18:3 MGDG could also be caused by peroxidation of polyunsaturated MGDG fatty acids due to oxidative stress generated by norflurazon treatment and the absence of lipid protection resulting from a lack of carotenoids (*8*). We have observed an increase in lipid peroxides in norflurazon-treated *Phaseolus* and *Brassica* seedlings (data not shown). The decrease in MGDG biosynthesis and unsaturation induced by norflurazon may also be compared to the process of senescence; atMGD1 expression and levels of 18:3/18:3 MGDG are reduced in senescing leaves of *Arabidopsis* (*41*).

In addition to its effects on MGDG desaturase, norflurazon caused a 23% reduction in all 16:1t PG molecular species, suggesting that it inhibits PG Δ^3-trans desaturase. Plastid PG is essential for the development of thylakoid membranes (*42*) to maintain the structural integrity of the QB-binding site of PSII reaction center complexes (*43*).

Unlike the plastid compartment, there was an increase in 18:3/18:3 PC in the extraplastid compartment after labeling with oleate which suggests that 18:2 PC desaturation catalyzed by endoplasmic reticulum ω^3 desaturase was activated (**Figure 4**), and this is confirmed by the ω^3 desaturase index (**Table 3**).

The increase in 18:3/18:3 PC could also result from a transfer of 18:3 from the chloroplast to the endoplasmic reticulum as has been suggested (*44*). This could explain the activation of the in vitro lysoPC-acyltransferase activity observed in treated plant samples.

On the other hand, as in 18:3 plants like soybean in which glycolipids are synthesized by the eukaryotic pathway, enhancement of 18:3/18:3 PC could derive from a diminution in PC transfer toward the chloroplast; accordingly, oleate labeled PC accumulated in treated plants, while the amount of labeled MGDG decreased (**Figure 3**). A higher 18:3/18:3 PC content would induce greater fluidity of membranes, hence maintaining integral membrane proteins in an environment compatible with normal biochemical activities.

There is therefore a differential effect of norflurazon on the plastidial and extraplastidial ω^3 desaturases. As norflurazon inhibits the phytoene desaturases by competition with enzyme cofactors (*3*), the differential effect of norflurazon on the activities of the two types of enzymes could result from the cofactors they use: endoplasmic reticulum desaturases use phosphatidylcholine as the lipid substrate and $cytb_5$ as the immediate electron donor (*45*), whereas plastid desaturases use galactolipids as the lipid substrate and reduced ferredoxin as the electron donor (*46*).

We wondered whether the observed variation in desaturase activities was related to transcriptional changes. In treated leaves, *FAD7* and *FAD3* transcript levels did not significantly vary after norflurazon treatment. The lack of correlation between relative levels of *FAD7* and *FAD3* transcripts and MGDG 18:3 or/and PC 18:3 content suggests that the herbicide principally acts at the posttranscriptional level on ω^3 desaturases.

In conclusion, our results demonstrate that phytoene desaturase is not the only enzyme inhibited by norflurazon: MGDG synthase, chloroplast lipid ω^3 desaturase, and PG Δ^3 trans desaturase are also affected. In addition to the decrease in carotenoid levels, decreases in the levels of MGDG (**Figure 2**), 18:3/18:3 MGDG, and 18:3/16:1t PG (**Table 2**) would contribute to the disorganization of photosynthetic membranes and disrupt the primary phase of photosynthesis. It is established that the effects of pyridazinone herbicides on membrane lipids and unsaturation are not secondary effects due to the inhibition of carotenoid biosynthesis. Moreover, the effects on fatty acid desaturation are also induced by substituted pyridazinones which have no significant effects on carotenoid biosynthesis like BASF 13 338 (San 9785) (*47*). On the other hand, it has been reported (*41*) that the mutant *mgd1* is less green than the wild type containing only approximately 50% of the normal amount of chlorophyll. The deficiency in MGDG might contribute to the bleaching of soybean seedlings. Whatever the exact causes of the norflurazon toxicity against the soybean seedlings, the effects were associated with disruption of biosynthesis and desaturation of chloroplast lipids and mainly MGDG.

ABBREVIATIONS USED

Fatty acids: 16:0, palmitic acid; 18:0, stearic acid; 18:1, $\triangle^9$ octadecenoic acid (oleic acid); 18:2, $\triangle^{9,12}$ octadecadienoic acid (linoleic acid); 18:3, $\triangle^{9,12,15}$ octadecatrienoic acid (linolenic acid). FAD, fatty acid desaturase; DAG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FW, fresh weight; GLC, gas−liquid chromatography; HPLC, high-performance liquid chromatography.

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