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Variation of Transhexadecenoic Acid Content in Two Triazine Resistant Mutants of *Chenopodium album* and Their Susceptible Progenitor

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ABSTRACT

Two atrazine resistant nutants of *Chenopodium album* L. and their susceptible progenitor were analyzed for lipid composition. In the phosphatidyldiacylglycerol the Δ3-*trans*-hexadecenoic acid (C16:1 *trans*) percentage was higher in the two resistant phenotypes. However, this difference appears later in the development of the leaves and is not clearly observed in young leaves and seedlings. Thus, the increase of the C16:1 *trans* during the leaf development of the resistant phenotypes is probably a secondary effect of the *psbA* mutation that arises in compensation for some photosynthesis deficiency. The significance of the lipid differences shown between the two resistant mutants is discussed in terms of whether they are responsible of the two different levels of herbicide resistance observed in the field.

Resistance to triazine herbicides at the chloroplast level in higher plants is known to occur in more than 30 species (9). This resistance is related to the alteration of the herbicide binding site, the protein of the secondary electron carrier on the reducing side of PSII called the 32 kD protein (21). It has been shown that a single base substitution on the chloroplast *psbA* gene resulted in a single amino acid change in the 32 kD protein (11). The same substitution at amino acid 264 occurs in all higher plants tested to date (19). This change results in a specific loss of herbicide binding capacity and a reduced rate of electron transport in triazine resistant chloroplasts resulting in a lower growth rate of the resistant plants (10). Ultrastructural studies have shown that the chloroplasts of the resistant plants have more large grana stacks and lower Chl *a*/*b* ratio than susceptible plants (23). This would suggest that they have larger PSII antennae.

Several lipid differences also have been reported repeatedly (5, 20), especially an increase in the C16:1 *trans*[1] fatty acid proportion in the PG of the resistant plants (4, 14, 15). Since the fatty acid composition of the PG has been previously suggested to be implicated in the PSII and antennae organizations (6, 12), and PG occurs only in chloroplast membranes (6), it could be a good marker of a secondary effect of the mutation. Indeed, an increase of the PSII antennae could correspond to the need for a better light energy collection in order to compensate for the deficiency in electron transfer in the resistant plants. Alternately, PG changes could be directly involved in the expression of the resistance as PSII centers are partially embedded in the lipid matrix of the thylakoid membrane:conformational changes due to lipid variations may well affect the atrazine binding.

The aims of this paper are therefore to clearly establish the PG difference between atrazine resistant and susceptible plants and to determine whether this lipid change appears very early or in a later stage of the plant life, as a secondary effect. Moreover, we intended to make a correlation between the lipid composition and low and high levels of resistance in fields. However, such a study needs the use of genetically related plants to provide valuable results free of uncontrolled genetic variations. In the present study, we used a unique plant model showing three levels of atrazine resistance (susceptible, intermediate, and resistant) within the descendance of a single plant line (2, 7). A spontaneous intermediary resistant and an induced highly resistant mutant of *Chenopodium album* L. were both issued from the same susceptible parent plant. As far as these three phenotypes have been studied (morphology, phenology, physiology, isozymes, and DNA), they do not differ from one another except for what concerns the consequence of the 32 kD protein mutation. Since the two resistant plants have the same inbred mother plant, and since the *psbA* mutation was demonstrated to be punctual at the same amino acid in both cases (2), these two resistant materials certainly display nearly the same mitochondrial, muclear, and chloroplastic genomes as their susceptible mother plant.

MATERIALS AND METHODS

Source of Plant Materials. Seedlings of susceptible plants were collected in a private garden, then transferred in the greenhouse and self-pollinated under individual bags. Their descendance was sown in the greenhouse and the resulting seedlings were analyzed for resistance using the leaf fluorescence test (7). In a few cases, a few intermediary resistant plants (I) appeared together with susceptible ones (Sp) within the same descendance (2, 7). I plants showed to be resistant to 10-fold the lethal dose of the Sp plants and this characteristic was maternally inherited. However, when an I plant was sprayed with a sublethal dose of atrazine, its whole descendance (Ri) resulted to be resistant to at least 200-fold the lethal dose of the Sp plants and to have a fluorescence test different from that of the I plant (7). Therefore, the Sp and I materials used here are sister plants, and the Ri material is the progeny of atrazine treated I plants. It has been demonstrated

[1] Abbreviations: C16:1 *trans*, Δ3-*trans*-hexadecenoic acid; Sp, I, and Ri, susceptible, intermediate and resistant isogenic phenotypes; PG, phosphatidyldiacylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; MGDG and DGDG, mono- and digalactosyldiacylglycerol; C16:0: palmitic acid; C16:3, 7-, 10-, 13-*cis*-hexadecatrienoic acid; C18:0: stearic acid; C18:1, 9-*cis*-octadecenoic acid; C18:2, 9-, 12-all-*cis*-octodecadienoic acid; C18:3, 9-, 12-, 15-all-*cis*-octodecatrienoic acid.

that both I and Ri phenotypes have the same mutation at amino acid position 264 of the 32 kD protein while the Sp phenotype has no mutation (2).

Conditions of Culture and Harvest. Seeds were sown in vermiculite soil, then transplanted when 10 d old in individual pots in the greenhouse (24°C day and 17°C night, at 70% humidity with a 16 h/8 h photoperiod). Three experiments were carried out independently in November, April, and June 1985–1986. For each experiment, leaves of 60 d old plants were harvested (5 repetitions of 10 fully developed leaves for each phenotype), then analyzed for their lipid composition. In one experiment (June), the leaves were harvested at three stages: (a) at 10 d the plants have four small leaves that were all harvested; (b) at 30 d three samples were carried out on three different parts of the plants (the youngest leaves at the very top of the plants, the fully developed leaves at the middle of the plants, and the four primary leaves at the basis of the plants; (c) at 60 d, fully developed leaves were harvested. Leaf surface was measured with an area meter from δ-T devices.

Lipid Analysis. Leaves were boiled in water for lipase inactivation. Total lipids were extracted by grinding the leaves in chloroform methanol 1/1. After addition of water (1 volume), the chloroform phase was separated from the aqueous phase then evaporated to dryness under a N2 stream (3). An aliquot of total lipid extract was transmethylated with boron-trifluoride methanol according to Metcalf *et al.* (17). Fatty acid methyl esters were analyzed by capillary GLC at 180°C using a Girdel 30 equipped with flame ionization detector. A 25 m long, 0.32 mm diameter glass column coated with PEG was used. For quantitative determination of fatty acids, a known amount of heptadecanoate (C17:0) was added as an internal standard during lipid extraction to avoid artefacts in quantitation of fatty acids. Separation of different lipid classes was performed by TLC. Polar lipids were separated as described by Tremolières and Lepage (22). Fatty acid composition and content of lipid classes separated by TLC was determined by scrapping lipid spots vizualized under UV after primuline pulverisation then transesterification and capillary GLC analysis.

RESULTS

Lipid Classes Composition and Fatty Acid Content. Table I presents a typical experiment that shows the relative abundance of several lipid classes in leaves of 60 d old plants. Galactolipids, as in all green leaves, represent the main polar lipid classes with a percentage of about 60%. The PG represents 8% of the polar lipids. No major differences could have been detected between the three phenotypes in either the PG percentage or in any other lipid classes (Student *t* test, 1% level). However, the total amount of fatty acids on a fresh matter basis varied with the phenotype but irrespective to the chloroplast mutation. In contrast, on a leaf area basis, the two mutants clearly showed lower total fatty acid contents. This result was due to the presence of thicker leaves in these two phenotypes.

Fatty Acid Composition of the PG. In order to determine more precisely the fatty acid composition of the PG in the leaves of the three phenotypes and to distinguish between genetic and developmental variations, three distinct cultures of *Chenopodium* were grown and analyzed at three different dates in the year. The results were interpreted through an analysis of variance (Table II). A significant increase of about 20% in the percentage of C16:1 *trans* in the PG of I and Ri phenotypes was observed, but no differences were measured between I and Ri. Correlatively, the C18:1 and the C16:0 percentages decreased in these two phenotypes. In addition, the I phenotype was found to be slightly richer in C18:2 than the two other phenotypes. Other differences were observed in the other lipid classes (Table III). In the PC class, two highly significant differences appeared between the I phenotype on the one hand and the Sp and Ri phenotypes on the other hand for C16:0 and C18:2. Three other highly significant differences were observed in C16:0, C18:1, and C18:3 of the DGDG between the susceptible and the resistant plants.

Evolution of the Fatty Acid Composition of PG According to the State of Development of the Leaves. In order to report variations in the C16:1 *trans* proportion independently of the C18 polyunsaturated chains percentages which change largely during the development (results not presented; and Ref. 13), results are given in terms of the C16:1 *trans*/C16:0 ratio and interpreted through a two-factor analysis of variance (June experiment, Table IV). Both the stage and the phenotype effects were highly significant ($F = 232$ and $F = 36$, respectively, significant at $P \leq 0.001$) as well as the interaction between stages and phenotypes ($F = 6.2$, significant at $P \leq 0.001$). All the phenotypes pooled, the more juvenile stages appeared to have the lowest ratios, while the highest ratios were recorded for the oldest leaves. For example, a mean increase of 50% (from 0.9–1.34) was observed in the four first leaves within 20 d from the seedling stage to the 30 d old plant stage. In average over the five stages, the two resistant I and Ri showed higher C16:1 trans/C16:0 ratio than the Sp material. This is coherent with the analysis carried out at the 60 d old plant stage only. However, no difference was observed between the phenotypes for the juvenile material (four-leaved seedlings and top of 30 d old plants) while differences appeared

Table I. *Polar Lipid Classes Composition, Total Fatty Acid Content, and Specific Leaf Weight of Leaves at the Middle of the Stem of 60 d old Plants of Sp, I, and Ri Phenotypes of Chenopodium*

The data are the mean ± SE.

Component	Sp	I	Ri
		%	
MGDG	41.3 ± 1.9	40.4 ± 2.2	40.8 ± 0.3
PE	5.4 ± 0.3	4.5 ± 0.3	5.1 ± 0.3
PG	8.1 ± 0.4	8.0 ± 0.5	8.5 ± 0.4
DGDG	21.6 ± 0.8	25.9 ± 1.1	24.1 ± 0.4
PC	23.6 ± 1.8	21.2 ± 0.8	21.5 ± 0.9
Total g/cm²	136 ± 5	126 ± 4	112 ± 4
Total g/g fresh wt	6754 ± 208	7430 ± 92	6284 ± 135
Specific leaf weight mg/cm²	20.2 ± 0.6	17.0 ± 0.5	17.8 ± 0.4

Table II. *Fatty Acid Composition of the Phosphatidyldiacylglycerol from Leaves of the Sp, I, and Ri Phenotypes*

Each value is the mean of 15 true repetitions (3 dates, 5 samples each). The analysis of variance indicated the least significant difference (LSD at $P \leq 0.01$); values in the same horizontal line that are followed by the same letter are not different at $P \leq 0.01$.

Fatty Acid	Sp	I	Ri	LSD 1%
		%		
C16:0	30.3 b	25.0 a	26.9 a	2.1
C16:1 *trans*	22.2 a	25.3 b	26.3 b	1.8
C18:0	2.3 a	2.3 a	2.3 a	NS
C18:1	8.2 b	6.8 a	6.1 a	0.7
C18:2	10.5 a	12.5 b	10.9 a	1
C18:3	26.5 a	28.0 a	27.6 a	NS

Table III. *Fatty Acid Composition, of the Main Lipid Classes (except PG, see Table II) in Sp, I, and Ri Phenotypes in Leaves of 60 d old Plants (same leaves as in Table I)*

Mean of 5 repetitions ± SE, in percent of fatty acid content in each lipid class. Means indicated with *, **, and *** are different from that of the other phenotypes at $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$, respectively (Student *t* test).

	Sp	I	Ri
PC			
C16:0	26.7 ± 0.4	21.7 ± 1.1**	26.5 ± 0.9
C18:0	2.0 ± 0.3	1.8 ± 0.1	3.1 ± 0.6
C18:1	20.3 ± 0.9	21.2 ± 0.4	20.4 ± 1.0
C18:2	29.8 ± 0.2	33.4 ± 0.6***	28.9 ± 0.5
C18:3	21.1 ± 0.5	22.1 ± 1.0	21.1 ± 0.5
DGDG			
C16:0	15.2 ± 0.4***	11.2 ± 0.4	10.9 ± 0.4
C16:3	2.2 ± 0.2	2.6 ± 0.1	2.4 ± 0.2
C18:0	1.6 ± 0.3	1.2 ± 0.1	1.7 ± 0.2
C18:1	2.4 ± 0.1***	1.2 ± 0.2	1.2 ± 0.2
C18:2	3.2 ± 0.3	2.6 ± 0.1	2.5 ± 0.6
C18:3	75.5 ± 0.9**	81.3 ± 0.3	81.3 ± 0.8
PE			
C16:0	30.5 ± 2.5	27.8 ± 0.4	32.2 ± 1.9
C18:0	2.1 ± 0.6	2.7 ± 0.4	2.5 ± 0.5
C18:1	11.3 ± 0.5	11.5 ± 0.2	11.3 ± 0.5
C18:2	43.5 ± 2.1	47.1 ± 0.6	41.5 ± 2.2
C18:3	12.7 ± 0.6	10.8 ± 0.6	12.4 ± 1.1
MGDG			
C16:0	2.8 ± 0.3	1.9 ± 0.1*	2.4 ± 0.2
C16:3	5.3 ± 0.5	5.2 ± 0.1	5.8 ± 0.1
C18:0	0.6 ± 0.2	0.2 ± 0.1	0.4 ± 0.1
C18:1	1.2 ± 0.1*	0.8 ± 0.1	0.8 ± 0.1
C18:2	2.2 ± 0.1	3.0 ± 0.2	2.7 ± 0.2
C18:3	88.0 ± 0.9	89.0 ± 0.2	87.9 ± 0.4

clearly at further stages. In two of the three well developed leaf stages, the I value was intermediary between that of Sp and Ri.

DISCUSSION

In this work, both the resistant mutants I and Ri showed a statistically significant enrichment in C16:1 *trans* of the PG fraction of fully developed leaves, as compared to their susceptible progenitor Sp. Over the studies that have dealt with the fatty acid composition of resistant and susceptible plants (4, 5, 14, 15), three showed such a difference, even in higher magnitude (4, 14, 15). Hence, it could be suggested that this is a characteristic of the *psbA* mutation at amino acid 264, at least when genetically related plants are compared. In contrast to the other works, no changes in the MGDG to DGDG ratio nor in the MGDG fatty acid composition were observed here, but a clear-cut difference was shown in the DGDG fatty acid composition. In this lipid class only, the unsaturation indice (total of unsaturated *versus* saturated fatty acids) was higher in the resistant materials (7.1 ± 0.3 and 6.7 ± 0.3 for I and Ri, respectively) than in the susceptible one (5.0 ± 0.2, different at $P \leq 0.01$ by a Student *t* test). This corresponds to a general trend shown in other lipid classes in atrazine resistant plants (16). These slight discrepancies perhaps could be due to the plant materials used. The three materials used in this study are certainly more genetically closely related than in previous works that have used any plant collected in the field. Here, the two mutants are the direct descendants of the same unique self-pollinated mother plant used as susceptible material so that all the plants used have very similar cytoplasmic and nuclear genomes (7).

With regard to the differences between the two resistant mutants, I and Ri, which show the same *psbA* mutation but different whole plant responses to atrazine treatment (7), no clear differences in the C16:1 *trans* percentage have been detected. However, a higher total fatty acid content per gram of fresh matter and a higher C16:2 percentage in the PC and PG have been observed in the I phenotype. Whether the difference in the levels of field resistance between the I and the Ri resistant phenotypes is directly related to these lipid changes needs further attention.

The proportion of the C16:1 *trans versus* the C16:0 increased with leaf ageing (senescence not involved), as already showed (13). However, this increase varied according to the phenotype: it was slower for the Sp phenotype. From the youngest to the oldest leaves, the C16:1 *trans*/C16:0 ratio changed from 0.26 to 1.12 in the Sp and from 0.18 to 1.6 in the Ri phenotype. Samples of young leaves (the first leaves of the seedling and the leaves at the top of the 30 d old plant) showed no difference between the phenotypes. The I and Ri resistant phenotypes showed higher values at a later stage of the leaf development. Therefore, the increase in C16:1 *trans* percentage of the PG can be clearly considered as a secondary effect of the *psbA* mutation.

How is this secondary effect linked to the mutation affecting the 32 kD protein? The role of the C16:1 *trans* in thylakoids is still not clear. For example, an *Arabidopsis* mutant lacking this fatty acid shows normal growth and chloroplast properties and ultrastructure (18). Moreover, the treatment of *Lemna* with a sublethal dose of atrazine induced a C16:1 increase (8), but also a reduction in the number of lamellae per grana (1), in contrast to what has been observed in triazine resistant weed biotypes (23). Nevertheless, since a correlation between the C16:1 and the light harvesting complex oligomers was observed (6, 12), it can be proposed that the increase in C16:1 *trans* percentage with leaf ageing in the PG of resistant plants corresponds to an increase of the antennae size which may result itself from some compensation for the low efficiency of individual photosynthetic center.

Table IV. *Evolution of Fatty Acid Composition in PG of Leaves during the Development of Sp, I, and Ri Phenotypes*

The data are the C16:1 *trans*/C16:0 ratio of leaves of 10, 30 (3 parts of the plant), and 60 d old plants (June experiment). Each value is the mean of four repetitions. The values followed by the same letter are not different at $P \leq 0.01$ (Newman and Keuls test).

Time	Sp	I	Ri	All phenotypes
At 10 d	0.82 b	0.86 b	1.00 bcd	0.90 x
At 30 d				
Top	0.26 a	0.25 a	0.18 a	0.23 w
Middle	0.95 bc	1.17 cde	1.24 de	1.12 y
Basis	1.12 cde	1.30 e	1.60 f	1.34 z
At 60 d	0.76 b	1.11 cde	1.13 cde	1.00 x
All stages	0.78 α	0.94 β	1.03 β	0.92

LITERATURE CITED

1. BEAUMONT G, A LORD, G GRENIER 1980 Effets physiologiques de l'atrazine à doses sublétales sur *Lemma minor*. V. Influence sur l'ultrastructure des chloroplastes. Can J Bot 58: 1571–1577
2. BETTINI P, S MCNALLY, M SEVIGNAC, H DARMENCY, J GASQUEZ, M DRON 1987 Atrazine resistance in *Chenopodium album*. Low and high levels of resistance to the herbicide are related to the same chloroplast psbA gene mutation. Plant Physiol 84: 1442–1446
3. BLIGH EG, WS DYER 1959 A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–919
4. BURKE JJ, RF WILSON, JR SWAFFORD 1982 Characterization of chloroplasts isolated from triazine-susceptible and triazine-resistant biotypes of *Brassica campestris* L. Plant Physiol 70: 24–29
5. CHAPMAN PJ, J DE FELICE, J BARBER 1985 Characteristics of chloroplast thylakoid lipid composition associated with resistance to triazine herbicide. Planta 166: 280–285
6. DUBACQ JP, A TREMOLIERES 1983 Occurence and function of phosphatidylglycerol containing Trans-hexadecenoic acid in photosynthetic lamella. Physiol Veg 21: 293–312
7. GASQUEZ J, A AL MOUEMAR, H DARMENCY 1985 Triazine herbicide resistance in *Chenopodium album* L. Occurrence and characteristics of an intermediate biotype. Pestic Sci 16: 390–395
8. GRENIER G, JP MARIER, G BEAUMONT 1982 Effects physiologiques de l'atrazine à dose subléthales sur *Lemna minor* VI Influence sur les classes importantes de phospholipides. Physiol Veg 20: 179–185
9. GRESSEL J, HV AMMON, H FOGELFORS, J GASQUEZ, QON KAY, H KEES 1982 Discovery and distribution of herbicide resistant weeds outside North America. *In* H Le Baron, J Gressel, eds, Herbicide Resistance in Plants. Wiley, Inc., New York, pp 31–56
10. GRESSEL J 1984 Biotechnologically confering herbicide resistance in crops. *In* L Van Vloten-Doting, ed, Molecular Form and Function of the Plant Genome. Plenum, New York, pp 489–504
11. HIRSCHBERG, J, L MCINTOSH 1983 Molecular basis of herbicide resistance in *Amaranthus hybridus*. Science 222: 1346–1349
12. HUNER NPA, M KROL, JP WILLIAMS, E MAISSAN PS LOW, D ROBERTS, JE THOMPSON 1987 Low temperature development induces a specific decrease in trans-Δ3. Hexadecenoic acid content which influence LHC II organization. Plant Physiol 84: 12–18
13. LEECH RM, CA WALTON 1983 Modification of fatty acid composition during chloroplast ontogeny and the effects on thylakoid appression and primary photochemistry. *In* WW Thomson, JB Mudd, M Gibbs, eds, Biosynthesis and Function of Plant Lipids. American Society of Plant Physiologists, Rockville, MD, pp 56–80
14. LEHOCZKI E, E POLOS, G LASKAY, T TARKAS 1985 Chemical composition and physical states of chloroplast lipids related to atrazine resistance in *Conyza canadensis* L. Plant Sci 42: 19–24
15. LEMOINE Y, JP DUBACQ, G ZABULON, JM DUCRUET 1986 Organization of the photosynthetic apparatus from triazine-resistant and susceptible biotypes of several plant species. Can J Bot 64: 2999–3007
16. MATTOO AK, JB ST JOHN, WP WERGIN 1984 Adaptative reorganization of protein and lipid components in chloroplast membranes as associated with herbicide binding. J Cell Biochem 24: 163–175
17. METCALFE LB, AA SCHMITZ, JR PELKA 1966 Rapid preparation of fatty acids esters from lipids for gas chromatographic analysis. Anal Chem 38: 514–515
18. MCCOURT P, J BROWSE, J WATSON, CJ ARNTZEN, CR SOMERVILLE 1985 Analysis of photosynthetic antenna function in a mutant of *Arabidopsis thaliana* (L.) lacking trans-hexadecenoic acid. Plant Physiol 78: 853–858
19. MCNALLY S, P BETTINI, M SEVIGNAC, H DARMENCY, J GASQUEZ, M DRON 1987 A rapid method to test for chloroplast DNA involvement in atrazine resistance. Plant Physiol 83: 248–250
20. PILLAI P, JB ST JOHN 1981 Lipid composition of chloroplasts membranes from weed biotypes differentialy sensitive to triazine. Plant Physiol 68: 585–587
21. STEINBACK KE, L MCINTOSH, L BOGORAD, CJ ARNTZEN 1981 Identification of the triazine receptor protein as a chloroplast gene product. Proc Natl Acad Sci USA 78: 7463–7467
22. TREMOLIERES A, M LEPAGE 1971 Changes in lipid composition during greening of etiolated pea seedling. Plant Physiol 47: 329–334
23. VAUGHN CK, SO DUKE 1984 Ultrastructural alterations to chloroplasts in triazine-resistants weed biotypes. Physiol Plant 60: 510–520