Vol. 165, No. 1, 1989
November 30, 1989

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 125-130

BIOCHEMICAL CHARACTERIZATION OF A STEROL MUTANT PLANT REGENERATED FROM A TOBACCO CALLUS RESISTANT TO A TRIAZOLE CYTOCHROME-P-450-OBTUSIFOLIOL-14-DEMETHYLASE INHIBITOR

Pascale MAILLOT-VERNIER [1], Hubert SCHALLER [1], Pierre BENVENISTE [1] and Geneviève BELLIARD [2]

[1] UA 1182, Laboratoire de Biologie et Biochimie du Développement des Plantes, Institut de Botanique, 28 rue Goethe, 67083 Strasbourg Cedex, France

[2] UA 115 CNRS, Laboratoire d'Amélioration des Plantes, Université d'Orsay, Paris XI, Bâtiment 360, 91405 Orsay, France

Received October 6, 1989

Summary : We report here, for the first time, the biochemical characterization of a plant mutant impaired in sterol biosynthesis. A fertile plant was regenerated from a tobacco callus resistant to LAB170250F, a potent inhibitor of the cytochrome-P450-obtusifoliol-14-demethylase. The resistant callus and the leaves from the regenerated plant are characterized by profound qualitative and quantitative changes in their sterol content. Self-fertilization of this plant yielded seeds with the same biochemical features, indicating that the new phenotype is of mutational origin.

It has been shown in our laboratory that LAB170250F, a triazole compound was a potent inhibitor of the cytochrome-P-450-obtusifoliol-14-demethylase (1) which is a key enzyme in plant sterol biosynthesis (2,3). In addition, treatment of tobacco calli with the triazole was shown to result in the accumulation of 14 α-methyl- Δ8-sterols such as obtusifoliol, the natural substrate of the cytochrome-P450-obtusifoliol-14-demethylase and its C4-demethylated metabolite : 14α-methyl-24(28)-dihydrofecosterol (4). This accumulation occurred at the expense of the usual pathway end-products : campesterol, sitosterol and stigmasterol (fig.1). Furthermore, LAB170250F is toxic to tobacco protoplast cultures and lethal doses have been determined (4). Consequently we hase used LAB170250F as a potential selective agent to screen populations of microcalli originating from mutagenized tobacco protoplasts in the hope that some of the resistant genotypes thus obtained might be mutants modified in sterol biosynthesis. Indeed, among

0006-291X/89 $1.50

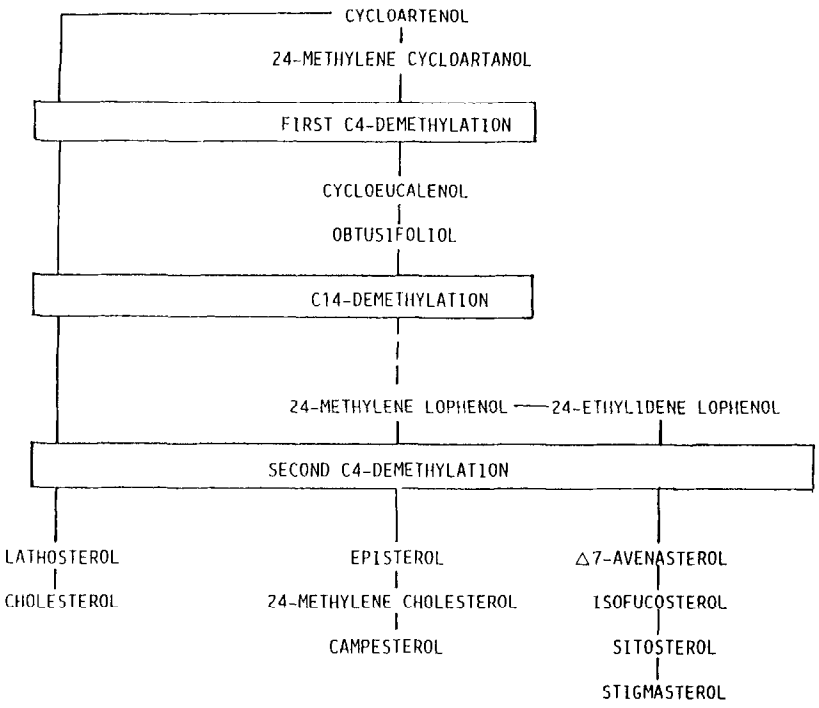

Figure 1 : Sterol biosynthesis in tobacco, downstream to cycloartenol.

forty calli recovered after selection, two showed an unusual sterol profile (4). Only one of the two genotypes LAB 1-4 allowed the regeneration of a fertile plant. Here, we report the first characterization of this genotype through sterol analysis of callus, leaves of the regenerated plant and seedlings resulting from self-fertilization.

MATERIAL AND METHODS

The original haploid genotype of Nicotiana tabacum cv xanthi (2n=2x=24) was provided by the laboratoire de Biologie Cellulaire (C.N.R.A., Versailles, France). The LAB170250F resistant callus : LAB 1-4 was obtained from a selection experiment adapted from an experimental procedure developed by Grandbastien et al. (5) which was previously described (4). This callus resists to a lethal concentration (3.5 μM) of the inhibitor. The control callus (wild-type) was obtained from protoplasts of the same selection experiment which were not submitted to UV-irradiation or to the selective pressure. Both resistant and control calli were subcultured in vitro on a non selective multiplication medium (4) in order to promote undifferentiating growth. About 2 g of fresh material were harvested in order to perform a sterol analysis. A fragment of each two calli was transferred to a regeneration medium (4). Shoots, then roots were induced. Plantlets were finally grown in a greenhouse at 25°C. Leaves were harvested before flowering of the regenerated plant. Seeds were obtained from self-fertilization of wild-type and LAB 1-4 plants, indicating that the plants were fertile (chromosome doubling probably occurred spontaneously during in vitro propagation). Seeds were germinated on an agar solidified medium (4) and

the obtained seedlings were allowed to grow for one month. Finally 50 seedlings of both LAB 1-4 and wild-type genotypes were harvested in order to be analyzed.

RESULTS AND DISCUSSION

The results of the quantitative measure of the sterol content of calli, leaves and seedlings of the wild-type and the LAB 1-4 genotypes are shown in Table I and fig 2. A dramatic increase in the amount of total sterols was found in callus, leaves and seedlings of the LAB 1-4 genotype in comparison with the wild-type genotype. The LAB 1-4 genotype also showed profound qualitative changes. Some intermediate sterols which are only present in trace amounts in the control accumulate : the increase, moderate in the case of 24-methylene cholesterol, Δ7-avenasterol and obtusifoliol, is very important in the case of 24-ethylidene lophenol, 24-methylene cycloartanol and cycloartenol. The

TABLE I : Sterol composition of calli, leaves and seedlings of the wild-type and LAB 1-4 genotypes

	CALLUS		LEAVES		SEEDLINGS	
	wild-type	LAB 1-4	wild-type	LAB 1-4	wild-type	LAB 1-4
cycloartenol	13	1 505	200	10 507	56	730
24 methylene cycloartanol	0	695	0	671	16	315
obtusifoliol	0	216	0	607	0	195
24 ethylidene lophenol	0	426	0	1 279	26	395
Δ7 avenasterol	0	126	0	821	0	130
24 methylene cholesterol	0	256	0	0	0	0
isofucosterol	0	456	85	1 500	96	430
cholesterol	0	0	285	286	216	355
campesterol	122	486	578	1 907	460	740
sitosterol	270	1 874	300	2 907	376	1 445
stigmasterol	83	179	1 500	1 929	1 080	1 515
Total sterol amount (μg/g dry weight)	488	6 219	2 948	22 414	2 326	6 250

Fresh material was frozen then lyophilized. Tissues were analyzed according to a method described previously (6) with only one modification : after the first chromatographic step on the crude extract, the bands corresponding to 4,4-dimethyl sterols, 4α-methyl sterols and 4-desmethyl sterols were pooled, then treated and analyzed as described. Each result was confirmed by a second analysis in the same conditions. The method employed here included a saponification step (6). Consequently the recovered fraction contained the sterol moiety of esterified sterols in addition to free sterols. All sterols were identified by GC-MS analysis and comparison with standard compounds.

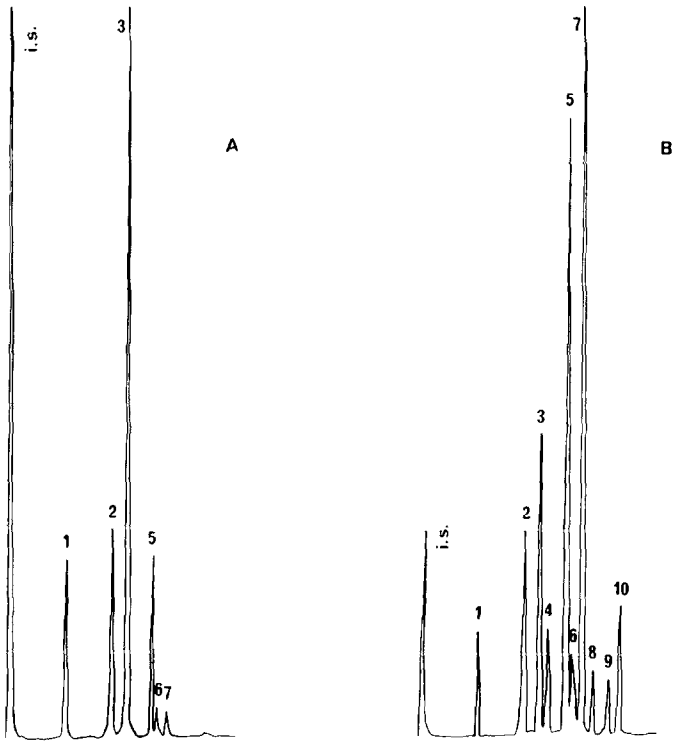

Figure 2 : Gas-chromatography profile of total sterols of leaves from wild-type (A) and mutant (B) plants obtained from 200 mg of lyophilized tissues. Peaks were successively : 100 ng of cholesterol added as internal standard (i.s.), then the steryl acetates of cholesterol (1), campesterol (2), stigmasterol (3), obtusifoliol (4), sitosterol (5), isofucosterol (6), cycloartenol (7), Δ7-avenasterol (8), 24-methylene-cycloartanol (9) and 24-ethylidene lophenol (10).

accumulation of cycloartenol is noteworthy in leaves. As 24-ethylidene lophenol and 24-methylene cycloartanol are generally considered to be natural substrates of the two C4-desmethylation reactions in plant sterol biosynthesis (fig 1) (3), it may be suggested that a defect in these reactions occurred in the LAB 1-4 genotype. Likewise, as cycloartenol is the natural substrate of the first methylation at C-24 occurring during the biosynthesis (7), this reaction might also be impaired in the LAB 1-4 genotype. However it is more probable that the accumulation of biosynthetic intermediates would reflect an increase of the biosynthetic flow which would lead to a higher amount of total sterols. The increase of the flow could unmask some limiting steps, such as the C-4-desmethylase or the C-24-methyltransferase reactions. Anyway, since the biochemical features described above were conserved at the seedling level, we may conclude that this phenotype is of mutational origin. The increase of the sterol biosynthetic flow could result from any mutation leading to a faster rate of an enzyme located upstream to the C24-methyltransferase.

It is interesting to notice that the total sterol amount of LAB 1-4 seedlings is only three times higher than those of wild-type seedlings, whereas the sterol amount of LAB 1-4 callus and leaves is about ten times higher than the sterol amount of wild-type tissues. To allow the determination of the sterol profile at the seedling level, 50 individuals were pooled. Since these seedlings were obtained from the self-fertilization of the homozygous genotype, no segregation of the mutant phenotype would be expected within the progeny. Thus the sterol profile of such a seedling population should be identical to individual sterol profiles. The lower increase of the sterol content observed for LAB 1-4 genotype at the seedling level might be explained by a different level of expression of the mutation depending on the developmental stage of the tissue.

The mutant described in the present study is of great interest in the elucidation of regulatory and functional aspects of phytosterol biosynthesis. Indeed the main biological functions seem not to be disturbed in the LAB 1-4 genotype. Particularly, the dramatic increase of the total sterol amount has no effect on callus growth, on the plant regeneration process and on seed production and germination. It is well admitted that some special structural features are required for sterols to assume their regulatory role in membrane structure and functions (8). In particular, only Δ5-sterols with a free β-hydroxyl at C-3 are supposed to be efficiently associated with the plasma membrane to play this role (8,9). Moreover, the concentration of sterols in membranes seems to be tightly regulated (10). Taking into account our results and the preceding considerations, one can address the following questions : What is the proportion of esterified and free sterols in the mutant genotype ? Which sterols are integrated into the plasma membrane ? Which role are the biosynthetic intermediates playing in the mutant genotype, especially cycloartenol in the leaves ? Finally, which mechanism is involved in the resistance of the LAB 1-4 callus to LAB170250F ? Further biochemical analysis, in progress in our laboratory, should allow to answer these questions. In parallel, the study of the inheritance pattern of the mutation will be performed.

ACKNOWLEDGMENTS : We thank Dr. M. Caboche (Centre National de la Recherche Agronomique, Versailles, France) for having provided us with the haploid tobacco plants. We are also very much indebted to the B.A.S.F. (Limburgerhof, F.R.G.) for their gift of LAB170250F.

REFERENCES

1. Taton, M., Ullmann, P., Benveniste, P. & Rahier, A. (1988) Pestic. Biochem. Physiol. 30, 178-189.

2. Rahier, A. & Taton, M. (1986) Biochem. Biophys. Res. Commun. 140, 1064-1069.
3. Benveniste, P. (1986) Ann. Rev. Plant Physiol. 37, 275-308.
4. Maillot-Vernier, P., Schaller, H. Benveniste, P. & Belliard G. (1989) Plant Physiol. (in press).
5. Grandbastien, M.A., Bourgin, J.P. & Caboche, M. (1985) Genetics 109, 409-425.
6. Costet-Corio, M.F. & Benveniste, P. (1988) Pestic. Sci. 22, 343-357.
7. Wojciechowski, Z.A., Goad, L.J. & Goodwin, T.W. (1973) Biochem. J. 136, 405-412.
8. Bloch, K.E. (1983) Crit. Rev. Biochem. 14, 47-91.
9. Demel, R.A. & de Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109-132.
10. Hartmann-Bouillon, M.A. & Benveniste, P. (1978) Phytochem. 17, 1037-1042.