Biological dynamics and distribution of 3-hydroxy fatty acids in the yeast *Dipodascopsis uninucleata* as investigated by immunofluorescence microscopy. Evidence for a putative regulatory role in the sexual reproductive cycle

J.L.F. Kock[1], P. Venter, D. Linke, T. Schewe, S. Nigam*

Eicosanoid Research Division, Department of Gynecology, University Medical Centre Benjamin Franklin, Free University Berlin, D-12200 Berlin, Germany

Received 12 February 1998; revised version received 31 March 1998

Abstract ***Dipodascopsis uninucleata*** **has been recently shown to produce 3-hydroxy polyenoic fatty acids from several exogenous polyenoic fatty acids. In order to examine whether endogenous 3-hydroxy fatty acids (3-OH-FA) may be implicated in the developmental biology of this yeast, we mapped by immunofluorescence microscopy their occurrence in fixed cells with or without cell walls using an antibody raised against 3*R*-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (3*R*-HETE), the biotransformation product from arachidonic acid (AA). This antibody turned out to cross-react with other 3-OH-FA. 3-OH-FA were detected in situ in gametangia, asci, as well as between released ascospores, and proved to be associated with the sexual reproductive stage of the life cycle of the yeast. Acetylsalicylic acid (1 mM), which is known to suppress the formation of 3-OH-FA from exogenous polyenoic fatty acids, inhibited the occurrence of immunoreactive material as well as the sexual phase of the life cycle suggesting a prominent regulatory role of 3-OH-FA for the latter.**

Key words: AA; 3-HETE; 3-Hydroxy fatty acid; Immunofluorescence; *Dipodascopsis uninucleata*

1. Introduction

Long chain fatty acids are widely distributed in plants and animals. These oxylipins exert a myriad of biological actions [1–4]. A number of fungal species, particularly representatives of the Endomycetaceae, Lipomycetaceae and Dipodascaceae, has been reported to produce unsaturated fatty acids, e.g. linoleic and α-linolenic acid [5], which form the basis of oxylipins. Screening of members of the Lipomycetaceae in our laboratory revealed that *Dipodascopsis uninucleata* is capable of transforming the exogenously fed AA to 3-HETE [6,7] and other polyenoic fatty acids to respective 3-OH-FA through a sequence of reactions analogous to β-oxidation, however stereochemically inverse, or through a direct monooxygenase reaction [8]. The prerequisite for the formation of 3-OH-FA from the corresponding polyenoic fatty acid turned out to be the presence of a 5*Z*,8*Z*-diene system either directly or upon preceding partial β-oxidation [8].

As suggested earlier, the intracellular production of 3-OH-FA may be implicated in the sexual reproductive stage of *D. uninucleata* [7]. The life cycle of this yeast is characterized by alternating asexual and sexual reproductive stages [5,9,10]. During the asexual stage, i.e. first 32 h, if grown in synchronous culture from ascospores (eventually present in attached clusters) the latter separate themselves, swell and germinate to produce well separated hyphae. This process is followed by the sexual stage (32 to 45 h) which is characterized by the formation of conjugated gametangia, karyogamy, meiosis and ascus formation. Each ascus contains hundreds of ascospores formed through the process of ascosporogenesis and are liberated in clusters within 45 h. In as much as the cell cycle has been proved to be sensitive to acetylsalicylic acid which at the same time also inhibits the formation of 3-HETE from AA, it was tempting to speculate that the formation and action of certain 3-OH-FA may be involved in ascosporogenesis. If this assumption holds true, 3-OH-FA must be detectable in situ at the corresponding developmental stages of the yeast.

In the present study we investigated the in situ occurrence and localization of 3-OH-FA in *D. uninucleata* during the course of the vegetative and sexual reproductive stages of the cell cycle with the help of immunofluorescence microscopy. For this purpose we used an antibody raised against 3-HETE in rabbits, that turned out to cross-react also with other 3-OH-FA. Despite the apparent impediments to effective immunofluorescence such as encasement of the *D. uninucleata* cells in a tough and impervious cell wall, the method described here has permitted the effective visualization of intracellular structures in yeast cells.

2. Materials and methods

2.1. Strain used

D. uninucleata UOFS-Y128 was used throughout this study and is held at The University of the Orange Free State, Bloemfontein, South Africa.

2.2. Cultivation and harvesting of cells

D. uninucleata was cultivated for 72 h in three 250 ml conical flasks each containing 50 ml synthetic medium (4 g/l glucose; 6.7 g/l Yeast Nitrogen Base from Difco Laboratories, Germany). The cultures were incubated on a rotary shaker (160 r.p.m., throw = 50 mm) at 30°C. To one of the flasks, 1.0 mg AA in ethanol (Sigma, Germany) was added after 72 h followed by further cultivation for 3 h. To another flask,

*Corresponding author. Fax: +49 (30) 815 5510.
E-mail: nigam@zedat.fu-berlin.de

[1]On sabbatical leave from Dept. of Microbiology and Biochemistry, Lipid Biotechnology Group, University of the Orange Free State, Bloemfontein, South Africa.

This paper is dedicated to Professor Dr. Dr. h.c. Johannes P. Van Der Walt, Pretoria, South Africa, who devoted his life time work to the taxonomy of yeasts.

PII S0014-5793(98)00406-2

acetylsalicylic acid was added to a final concentration of 1 mM prior to cultivation [5] followed by the addition of AA as described previously. No extra additions were made to the third flask. This experiment was performed at least in duplicate.

2.3. Detection of 3-HETE and other 3-OH-FA by immunofluorescence microscopy

2.3.1. Preparation of antibody. 3*R*-HETE was synthesized by us as described previously [11]. The antibodies against 3-HETE were raised in rabbits as follows: the carboxyl group of 3*R*-HETE was conjugated to amino groups of bovine serum albumin (BSA) by the *N*-succinimidyl ester method [12]. The conjugate (1 mg protein) was emulsified in an equal volume of Freund's complete adjuvant (for the first injection) or incomplete adjuvant (for later injections). The emulsion was injected subcutaneously into several sites on the back of a female New Zealand white rabbit every second week for a total of 7 times for about 3 months. Finally the whole blood was collected from the carotid artery, left at room temperature for 2 h and centrifuged at $1200\times g$ for 20 min at 4°C. The sera were affinity purified by Biogenes, Berlin.

2.3.2. Characterization of antibody. Characterization of the antibody was accomplished by the determination of its titer, sensitivity and specificity. Since the [^{14}C]-3-HETE is not commercially available, a little amount of the tracer was prepared by the transformation of [^{14}C]-AA (sp. act. 52 mCi/mmol) by *D. uninucleata* to [^{14}C]-3-HETE and purified with the help of radio-HPLC. The titer of the antibody gave a binding of approximately 30% labeled 3-HETE at a dilution of 1:100 in the absence of unlabeled 3-HETE. The sensitivity (minimum detectable amount) of 3*R*-HETE was 30 pmol as determined by 10% displacement of the radioactivity by unlabeled 3-HETE from the zero point (maximum binding of labeled 3-HETE). The specificity of the antibody was expressed in terms of cross-reactions with structurally related compounds. Cross-reactivity of the antibody with 5-, 12- or 15-HETE was $<0.5\%$. While investigating the distribution of 3-HETE during the life cycle of *D. uninucleata* we observed substantial cross-reactivity of the antibody against other 3-OH-FA produced in the yeast. Therefore, we checked the immunoreactivity of several commercially available hydroxylated and free fatty acids against our antibody with the help of immunofluorescence microscopy. The cross-reactivities expressed in terms of fluorescence intensities (maximum, +++; medium, ++; minimum, +; none, −) were for the FA as follows: 3*R*-HETE, +++; 3*S*-HETE, ++; 3*R*-OH-5*Z*,8*Z*-tetradecadienoic, acid +++; 3-OH-palmitic acid, ++; 3-OH-myristic acid, ++; 3-OH-lauric acid, +; 3-OH-butyric acid, +; arachidonic acid, −; linoleic acid, −; palmitoleic acid, −; oleic acid, −; stearic acid, −; palmitic acid, −; myristic acid, −; lauric acid, −. Hence, in our study the immunoreactivity indicates solely the presence of 3-OH-FA.

2.3.3. Immunofluorescence microscopy. Fixation and immunofluorescence of yeast cells were performed as described [13,14]. Briefly, yeast cells were centrifuged at 900 r.p.m. for 5 min onto glass microscope slides using a Cytospin (Shandon, Germany) and fixed in acetone at room temperature. The slides were then treated with the antibody against 3-HETE (30 μl; 1:10 dilution v/v) and left for 30 min. After washing with BSA-PBS, the affinity purified FITC anti-rabbit IgG (Sigma, Germany) was added (30 μl; 1:10 dilution v/v). Following adequate washing, the slides were photographed using Kodak Gold Ultra 400 ASA film on an Olympus BX40 standard microscope equipped for epifluorescence with a 500 W high pressure mercury lamp. The stained cells for immunofluorescence detection were compared with appropriate controls including preimmune sera from the rabbit.

The detection of 3-HETE and other 3-OH-FA in protoplasts was accomplished by fixing the yeast cells (100 μl) from each culture in paraformaldehyde for 90 min at room temperature followed by stripping of the cell walls with β-glucuronidase and lyticase (Sigma, Germany) as described [14]. Protoplasts were then attached to ChemMate Capillary Gap Plus Slides (DAKO Biotek, USA) and fixed by methanol for 6 min and acetone for 30 s at −20°C. The treatment with antibodies and detection was performed as described above.

3. Results and discussion

In earlier communications we described a sizeable formation of 3-HETE in *D. uninucleata* when fed with AA which is not present in this yeast [7]. Since this 3-HETE is now available in sufficient amounts, we raised an antibody against 3-HETE in a rabbit and checked it for its specificity. Fortunately, the antibody exhibited substantial cross-reactivity against authentic 3-hydroxy-tetradecanoic acid. Owing to this observation it is reasonable to presume that it has also cross-reacted with those 3-OH-FA which are derived from endogenously occurring polyenoic fatty acids in *D. uninucleata*, in particular 3*R*-hydroxy-5*Z*,8*Z*-tetradecadienoic acid that has been shown to be formed from linoleic acid [8]. The endogenous distribution of these 3-OH-FA should be in relation to the composition of free polyenoic fatty acids in *D. uninucleata* in the order linoleic acid $\gg$ oleic acid $\approx$ α-linolenic acid [7]. However, when 5*Z*,8*Z*,11*Z*-eicosatrienoic, 5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic (AA), 5*Z*,8*Z*,11*Z*,14*Z*,17*Z*-eicosapentaenoic (EPA), 11*Z*,14*Z*,17*Z*-eicosatrienoic or 9*Z*,12*Z*-octadecadienoic (linoleic) acids were exogenously fed, *D. uninucleata* transformed the first 3 FA to their 3*R*-hydroxylated form without alteration of the chain length, whereas the last 2 FA were shortened by 6 and 4 carbon atoms to 3*R*-OH-5*Z*,8*Z*,11*Z*-tetradecatrienoic and 3*R*-OH-5*Z*,8*Z*-tetradecadienoic acids, respectively [8].

By using this antibody we mapped by immunofluorescence microscopy the occurrence and localization of 3-OH-FA in cells representing different stages of the life cycle of *D. uninucleata*. For these experiments we used fixed cells with cell walls from AA-fed non-synchronous cultures of the yeast (Fig. 1A–E). No significant fluorescence could be visualized in the vegetative hyphae (Fig. 1B). Marked fluorescence was however observed at the stage of initiation of the sexual phase, i.e. during gametangiogamy (Fig. 1C). Immunoreactive compounds were also visualized in immature asci (Fig. 1D) and were eventually released together with the ascospores (Fig. 1E, A). With protoplasts of the cells positive immunoreaction was also observed only in asci before (Fig. 1G) and after ascospore release (Fig. 1F) as well as between ascospores. This immunoreaction was however abrogated when *D. uninucleata* was grown in the presence of 1 mM acetylsalicylic acid (data not shown).

Identical results were obtained with cells grown in the absence of AA with the exception that the fluorescence intensities in the micrographs were diminished (data not shown). It must be, however, emphasized that this observation must not reflect a lower concentration of 3-OH-FA under these conditions, but rather a relative low immunoaffinity of the antibody against the 3-OH-FA formed from endogenous polyenoic fatty acids.

Our data show clearly that 3-OH-FA are formed in *D. uninucleata* during the sexual phase of the reproductive cycle but not during the vegetative phase. The changing patterns of 3-OH-FA localization during the yeast cell cycle provide some clues as to the regulatory function of this family of oxylipins which is further supported by the specific suppression of the sexual phase by acetylsalicylic acid. A crucial role in the reproduction biology of fungi has also been proposed for some other oxylipins, so-called psi (precocious sexual inducers) factors. They include 5*S*,8*R*-dihydroxy-octadeca-9*Z*,12*Z*-dienoic acid (5,8-DiHODE), 5*S*,8*R*-dihydroxy-octadeca-9*Z*-(mono)-enoic acid (5,8-DiHOME), 8*R*-hydroxy-octadeca-9*Z*,12*Z*-dienoic acid (8-HODE), 8*R*-hydroxy-octadeca-9*Z*-(mono)enoic acid (8-HOME), etc. [15–18]. Since the yeast contains linoleic acid in abundance, its corresponding conversion product 3*R*-

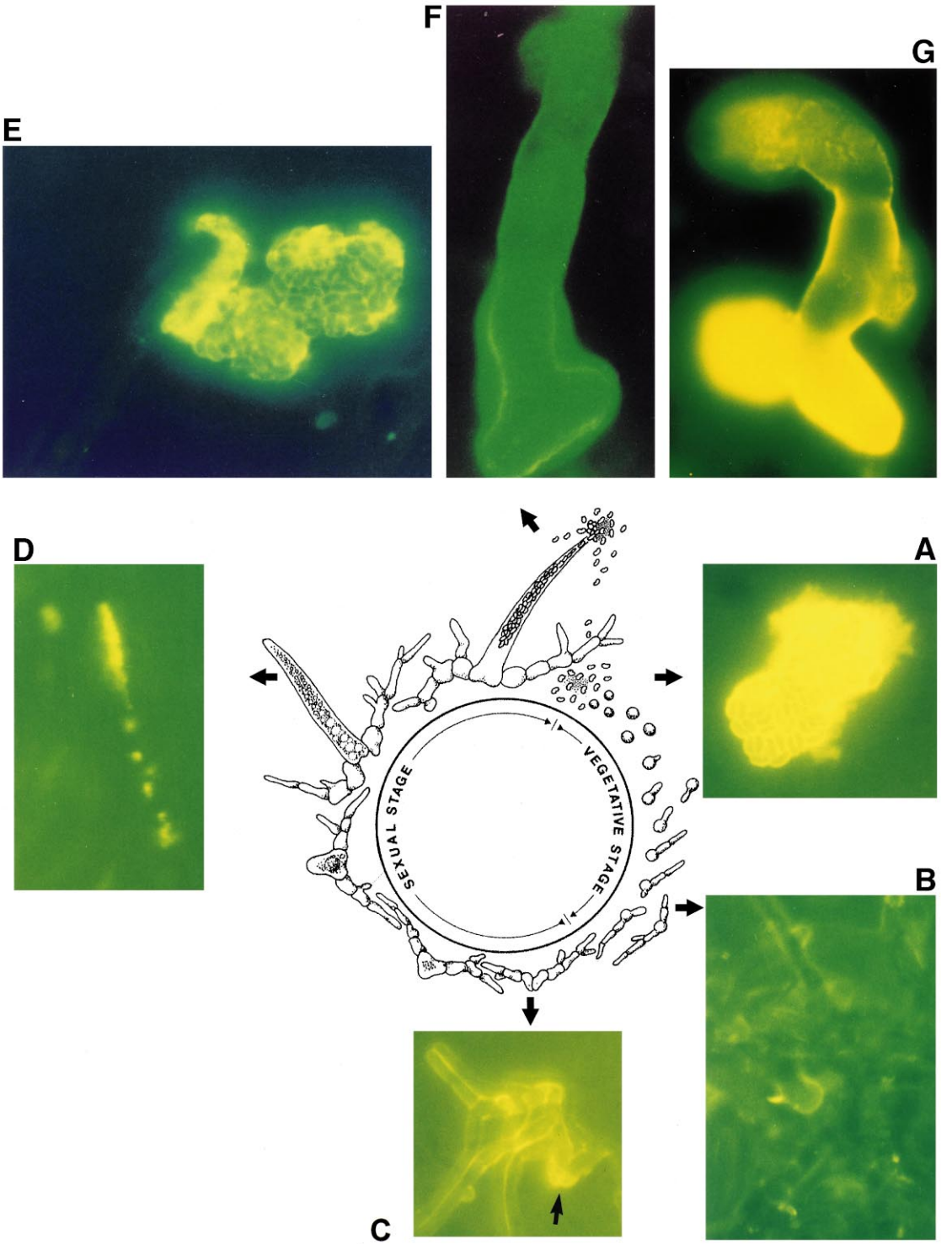

Fig. 1. The life cycle of *Dipodascopsis uninucleata* and distribution of 3-HETE visualized through immunofluorescence mapping. A: liberated ascospores; B: hyphae with cell wall; C: gametangiogamy; D: young ascus with cell wall; E: liberated ascospores from ascus with cell wall; F: empty ascus protoplast: still with characteristic morphology i.e. base (bottom) and shaft; G: deformed mature ascus protoplast containing ascospores mainly at base. (A, E, F, G: 10 mm on photo = 10 μm cell size; B, C, D: 10 mm on photo = 25 μm cell size).

hydroxy-5*Z*,8*Z*-tetradecadienoic acid was predominantly present when the yeast was grown in the absence of AA. In the presence of AA, however, 3-HETE may more or less substitute this compound. Hence, we conjecture that the 3*R*-OH-5*Z*,8*Z*-tetradecadienoic acid is the natural growth regulator in *D. uninucleata.* This assumption is firmly supported by the maximal cross-reactivity of the antibody with the 3*R*-OH-5*Z*,8*Z*-tetradecadienoic acid (see Section 2.3).

The 3-OH-FA are presumably formed during gametangiogamy and ascosporogenesis. Their absence in other stages may indicate a relatively high turnover rate. The prospective biological functions in the sexual reproductive phase of the life

cycle of *D. uninucleata* remain to be elucidated in future studies. The availability of an effective immunofluorescence procedure presented here should greatly facilitate the study of this genetically tractable yeast.

Acknowledgements: The authors wish to thank the Association for International Cancer Research, UK (Ni-81025), Free University Berlin, Foundation for Research Development in South Africa for their generous support to conduct this study and Dr. R.K. Bhatt (Dallas, TX) for supplying the synthetic 3-HETE isomers.

References

[1] Schweizer, E. (1989) in: C. Ratledge and S.G. Wilkinson (Eds.), Microbial Lipids, Vol. 2, Academic Press, San Diego, pp. 3–50.
[2] Nomura, T. and Ogata, O. (1976) Biochim. Biophys. Acta 431, 127–131.
[3] Van Dyk, M.S., Kock, J.L.F. and Botha, A. (1994) World J. Microbiol. Biotech. 10, 495–504.
[4] Gardner, H.W. (1991) Biochim. Biophys. Acta 1024, 221–239.
[5] Botha, A. and Kock, J.L.F. (1993) Antonie van Leeuwenhoek 63, 111–123.
[6] Van Dyk, M.S., Kock, J.L.F., Coetzee, D.J., Augustyn, O.P.H. and Nigam, S. (1991) FEBS Lett. 283, 195–198.
[7] Kock, J.L.F., Jansen van Vuuren, D., Botha, A., Van Dyk, M.S., Coetzee, D.J., Botes, P.J., Shaw, N., Friend, J., Ratledge, C., Roberts, A.D. and Nigam, S. (1997) Syst. Appl. Microbiol. 20, 39–49.
[8] Venter, P., Kock, J.L.F., Sravan Kumar, G., Botha, A., Coetzee, D.J., Botes, P.J., Bhatt, R.K., Falck, J.R., Schewe, T. and Nigam, S. (1997) Lipids 32, 1277–1283.
[9] Biggs, R. (1937) Mycologia 29, 34–44.
[10] Zsolt, J. (1963) Acta Bot. Acad. Sci. Hung. 9, 217–226.
[11] Bhatt, R.K., Falck, J.R. and Nigam, S. (1998) Tetrahedron Lett. 39, 249–252.
[12] Hosoda, H., Kawamura, N. and Nambara, T. (1981) Chem. Pharm. Bull. 29, 1969–1974.
[13] Harlow, E. and Lane, D. (1988) in: Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Ch. 10.
[14] Kilmartin, J.V. and Adams, A.E.M. (1984) J. Cell Biol. 98, 922–933.
[15] Champe, S.P., Rao, P. and Chang, A. (1987) J. Gen. Microbiol. 133, 1383–1387.
[16] Champe, S.P. and El-Zayat, A.E. (1989) J. Bacteriol. 17, 3982–3988.
[17] Mazur, P., Meyers, H.V., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1990) Tetrahedron Lett. 31, 3837–3840.
[18] Mazur, P., Nakanishi, K., El-Zayat, A.E. and Champe, S.P. (1991) J. Chem. Soc. Chem. Commun. 20, 1486–1487.