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LIPID CHANGES DUE TO GROWTH-FACTOR SUPPLEMENTS IN CALLUS AND PLASMA MEMBRANE-ENRICHED FRACTION OF RICE CULTURES

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Key Word Index—*Oryza sativa*; Poaceae; rice; callus cultures; membrane lipids; choline; plasma membrane.

Abstract—Membrane-lipid composition of total callus and its plasma membrane enriched fraction of cultures of *Oryza sativa* grown on media supplemented with various growth factors, such as choline, putrescine and pectin, was determined. Whereas choline was growth stimulatory, putrescine and pectin inhibited growth in these cultures. Growth response due to the growth-factor supplements was associated with significant changes in the total content, as well as the relative amounts of phospholipids and galactolipids. Whereas choline supplementation led to an increase in the relative amounts of phosphatidylcholine and phosphatidylinositol, putrescine/pectin supplemented cultures showed higher level of phosphatidic acid. A plasma membrane-enriched fraction of choline-supplemented cultures had higher content of phospholipids and galactolipids, together with enriched contents of phosphatidylcholine. Results are discussed in relation to the role of membrane lipids in *in vitro* growth. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Establishment of fast proliferating callus and cell cultures and induction of desired organogenetic responses in rice has been difficult due to its recalcitrance *in vitro* [1, 2]. Studies on genetic transformations of this important crop-species depend on the availability of defined *in vitro* protocols for inducing desirable growth and developmental responses *in vitro* [2]. Because the membrane lipids are known to be functionally important [3–6], biochemical characterization of *in vitro* responses on the basis of membrane-lipid changes would be useful in the formulation of appropriate *in vitro* protocols, such as defined culture media and hormonal/growth-factor conditions [7]. Several studies have highlighted the specific role of polar-lipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE), in the regulation of membrane-bound enzymes [3] and the phosphoinositides (phosphatidylinositol) (PI) and its phosphorylated derivatives) as 'second messengers' in a well-defined signal-transduction pathway [4]. Dorman *et al.* [5], by characterizing a mutant of *Arabidopsis thalliana* which was defective in galactolipid biosynthesis, reported the role of galactolipids in organizing the active photosystem of chloroplasts. The role of plasma membrane lipids in conferring stress-tolerance to factors such as dehydration, salinity and freezing, also highlighted their involvement in stress-adaptive responses [8-10].

In the present study, growth rate and changes in lipid composition of callus cultures of rice due to various growth-factor supplements, such as choline, putrescine and pectin [11], is presented. Choline supplementation resulting in the enrichment of PC and its growth stimulation is highlighted. Also, the changes in polar-lipid composition of a plasma membrane enriched fraction (PMEF) due to choline supplementation has been examined.

RESULTS AND DISCUSSION

In order to optimize a growth-medium for the callus cultures of rice, various growth factors, viz., choline, putrescine and pectin, were individually supplemented to Murashige and Skoog's (MS) medium + 2,4-D (control). It was found that choline supplementation stimulated the growth of cultures by *ca* 2.4-fold, as compared to the control (Table 1). However, putrescine and pectin were found to be growth inhibitory by *ca* 30%. Gawer *et al.* [12, 13] reported growth inhibition and subsequent cell-death due to choline supplementation in a cell line of tobacco cultures which was sensitive to choline supplementation. In their study, when comparisions were made between choline sensitive-and choline resistant-cell lines, on the basis of the activity profile of microsomal CTP: phosphocholine

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Table 1. Growth of callus cultures of rice due to growth-factor supplements

Growth factor	Dry matter accumulation* (mg/culture tube)
MS + 2,4-D(Control)	30
+ Choline	71
+ Putrescine	22
+ Pectin	21

*Cultures were initiated with an inoculum of *ca* 150 mg callus and growth was determined after 4 weeks.

cytidyltransferase, it was found that this enzyme was regulating PC synthesis. Accordingly, choline-sensitive cells did not show continued activity of CTP: phosphocholine cytidyltransferase on prolonged culture on +choline medium. Also, the enzyme activity was lower as compared to choline sensitive cells grown on choline-free medium. Thus, choline which did not get metabolised through CTP: phosphocholine cytidyltransferase of the CDP-choline pathway was shown to be growth inhibitory and cytotoxic in tobacco cell cultures. However, the results of the present study indicated that choline supplementation was neither growth inhibitory nor cytotoxic, possibly due to its rapid metabolism into PC, which resulted in stimulated growth of rice cultures. It is also known that the metabolism of choline into PC biosynthesis and its growth-supportive/inhibitory nature depended on the *in vitro* culture conditions [14]. It has been shown that polyamines regulate various growth and developmental responses and the efficacy of different polyamines in inducing specific responses varied among the plants [15, 16]. Martin-Tanguy [17] reported similar effects of putrescine in tobacco cultures, which depended on its metabolic conversions.

Qualitatively, the membrane lipids of rice callus consisted of phospholipids, such as PC, PE, PI, phosphatidylserine (PS) and phosphatidic acid (PA) (Table 2). Besides phospholipids, the galactolipid fraction consisted of sulpholipids, monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG). Whereas the above-mentioned membrane-lipids were present in all the different growth-factor supplemented cultures, LPC was also present in + putrescine cultures which represented degradation of PC [18, 19]. Even though phosphatidylglycerol (PG) was present at *ca* 3% in the shoot/root-forming callus cultures of rice (data not presented), it was not detected in either the control or growth-factor supplemented cultures of the present study. PG has been shown to be specifically enriched in chloroplast membrane of green tissues [20]. The rice cultures in the present study were totally non-green and thus lacked detectable amounts of PG.

The content of total phospholipids and galactolipids of the cultures grown with various growth factors was significantly different, compared with the controls (Table 2). +Choline cultures showed an increase of *ca* 18% on a gram dry weight basis and +putrescine/pectin cultures had a lower content (*ca* 33 and 26%, respectively). When the relative amounts of membrane lipids of different cultures was compared, it was found that choline supplementation led to an increase in PC and PI of *ca* 33 and 60%, respectively. There was also decrease in the relative contents of PE and PS in these cultures (*ca* 30 and 25%, respectively). Choline supplementation led to an increase in the PC/PE ratio from 1.1 of control cultures to 2.1 in +choline cultures. Choline is the specific substrate for PC biosynthesis and its supplementation has been shown to induce higher PC/PE ratios in membranes of all plants [12]. From the results of the present study, it is apparent that the observed increase in PC/PE ratio was growth stimulatory in rice cultures, whereas the same change was growth inhibitory and lethal in tobacco cultures [12–14]. Contrastingly, +putrescine/pectin cultures showed significant decrease in the relative amounts of PC and PE, together with an increase in the relative content of PA by *ca* 3–3.6 fold, as compared to the controls. Decreases in the content of phospholipids and galactolipids, together with high concentration of PA in +putrescine/pectin cultures were found to be characteristic of senesced tissues [19]. Whereas the galactolipid fraction was insensitive to choline supplementation, + putrescine/pectin cultures showed a decrease in the relative amount of galactolipids.

Qualitatively, the PMEF of the control, and also the +choline cultures, had similar lipid-profiles to that of the total callus, which consisted of PC, PE, PI, PS and PA (Table 3). Galactolipids represented minor constituent of PMEF, which was <4% of the PMEF phospholipids and galactolipids. PC, PE and PI were the major phospholipids of the PMEF, which together constituted ca 86% and 88% of the phospholipids and

Table 2. Changes in phospholipid and galactolipid composition of callus cultures of rice due to growth-factor supplements

Growth factor	Phospholipids + galactolipids (μmol g^{-1} dry wt)	Phospholipids + galactolipids (% total)								
		PC	LPC	PE	PI	PS	PA	SL	DGDG	MGDG
MS + 2,4-D (control)	76	33	–	30	10	12	5	2	5	3
+ Choline	90	42	–	20	16	9	4	3	4	2
+ Putrescine	51	14	12	24	8	18	20	2	1	1
+ Pectin	56	20	–	30	7	14	25	2	1	1

See text for abbreviations.

Table 3. Changes in phospholipid and galactolipid composition of plasma membrane-enriched fraction of callus cultures of rice due to choline supplementation

Growth factor	PMEF phospholipids + galactolipids (μmol g^{-1} dry wt)	Phospholipids + galactolipids (% total)					
		PC	PE	PI	PS	PA	Galactolipids
MS + 2,4-D (control)	552	30	27	29	7	3	4
+ Choline	673	40	23	25	8	2	2

See text for abbreviations.

galactolipids in the control and +choline cultures, respectively. The relative amount of PA was found to be <3% in the PMEF. PA, a hydrolytic product of phospholipase D action on membrane lipids, was found to accumulate in membrane preparations due to degradative changes during the isolation of membrane [21, 22]. Low levels of PA in the PMEF of the present study, thus indicated that this membrane preparation was free of any hydrolytic change. Compared with the PMEF of the control, +choline cultures had a higher content of total phospholipids and galactolipids (*ca* 22%) on a gram basis of PMEF protein. There was significant change in the PC/PE ratio of PMEF of +choline cultures as compared with controls. PC/PE ratios of control and + choline cultures were *ca* 1.1 and 1.7, respectively. The increase in PC/PE ratio of PMEF of + choline cultures reflected a comparable change that occurred in the total callus due to choline supplementation (Table 2). Such an increase in PC/PE ratio may be related to the growth-stimulatory role of choline in rice cultures. Whereas the relative amount of PI was only *ca* 10 and 16% in the total callus of control and +choline cultures, respectively (Table 2), it was *ca* 29 and 25%, respectively, in the PMEF of those cultures (Table 3). It is pertinent to mention that the increase observed in the relative amount of PI of total callus due to choline-supplementation was not found in the PMEF (Tables 2 and 3). In order to explain the increase in the amount of membrane, Somerville [23] proposed that the synthesis of lipids and proteins of biomembrane are regulated coordinately so that an increase in the amount of lipids was always associated with parallel increases in the amount of proteins. Thus, the observed increase in the content of phospholipids and galactolipids and the increase in the relative amount of PC of PMEF of + choline cultures, revealed coordinated accumulation of lipids and proteins in the plasmamembrane, although PC accumulated at a relatively higher concentration by *ca* 33%, compared with other phospholipids. Thus, the present study showed that choline was an ideal growth-supplement for inducing fast proliferation of rice cultures which induced characteristic changes in lipid composition.

EXPERIMENTAL

Chemicals. Lipid standards, phytohormones, growth factors and organic components of nutrient media were obtained from Sigma.

Callus cultures and growth conditions. Callus cultures of rice (*O. sativa* L. cv. Rasi) were initiated from mature seed embryos on MS + 2,4-D (43 μM; [16]). Three-month-old callus was used as the inoculum to raise cultures on various growth factor-supplemented media. The inoculum per culture tube was *ca* 150 mg of callus whose % dry matter content was *ca* 3.5. Choline chloride (5 mM), putrescine (5 mM) and pectin (100 ppm) were supplemented individually to MS + 2,4-D. After 4 weeks of culture on growth factor-supplemented media, callus growth on the basis of dry wt accumulation and associated lipid changes in total callus and also its PMEF were monitored. Cultures were incubated under continuous white light (900 μW cm^{-2}) at 26°.

Preparation of PMEF. This was accomplished essentially according to the procedure of ref. [25], with a modification to inhibit phospholipase D and PA phosphatase activity [21]. Accordingly, choline chloride (4% w/v), glycerophosphate (4% w/v) and ethanolamine (4% v/v) were added in all solns used in the prepn of PMEF [22].

Lipid analysis. A two-step chromatographic procedure involving silica gel CC and TLC was used [7, 22]. Determination of dry matter content was done gravimetrically and that of phospholipids and galactolipids was accomplished through lipid P [26] and lipid galactose [27], respectively. Protein estimation was done according to the procedure of ref. [28].

Data presentation. Results are the means of three expts. The SD for various parameters was as follows: dry matter content, *ca* 6; total and also individual phospholipids and galactolipids, *ca* 3; protein content *ca* 2.

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