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Effects of phytohormones on thermal denaturation profiles of Cymbidium DNA: Indication of differential DNA replication

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ABSTRACT

Protocorm pieces of the orchid Cymbidium were aseptically cultured either without phytohormones, or with one of the growth promoting substances, auxin, cytokinin, and gibberellin. The derivative melting profiles of the extracted DNA's differ from each other with respect to the size of various AT- and GC-rich fractions. Evidence has been obtained for the increase of the more AT-rich fractions in auxin-treated cultures, while gibberellin stimulated the expansion of the GC-rich fractions. These results are consistent with earlier cytological and cytochemical findings and indicate the involvement of hormone-controlled differential DNA replication in the development of Cymbidium protocorms in vitro.

INTRODUCTION

Cytophotometrical studies of in vitro differentiating orchid protocorms have shown that the heterochromatin content of certain cells increased disproportionately during the endopolyploidization process (1,2). Analysis of the DNA by analytical ultracentrifugation revealed the existence of an AT-rich satellite DNA, which did, however, not vary at the same extent as did the heterochromatin (3). Further investigations employing fluorescence and electron microscopy indicated that it might be a less AT-rich fraction which is extra replicated during cytodifferentiation (4). In this paper we describe the attempt to characterize this fraction by means of derivative melting profiles, which are known to provide detailed information on DNA heterogeneity (5).

Phytohormones have been found that affect total DNA replication in the endomitotic cycle and differential DNA replication in the amplification cycle in a different extent (6). Here we used these phytohormones to enrich certain types

of nuclei in the cultures, as it is not yet possible to separate heterochromatin-rich and heterochromatin-poor nuclei by an isolation procedure. The results obtained are consistent with the suggestions that (i) phytohormones stimulate the replication of certain DNA fractions differentially, and that (ii) the DNA which is amplified in the heterochromatin-rich nuclei is relatively AT-rich (Tm 78°C).

MATERIAL AND METHODS

Fragments of protocorms of the Cymbidium hybrid "In memoriam Cyrill Strauss" were aseptically cultured on an agar medium (7). Under the culture conditions applied (2) protocorms regenerate within a few weeks. For the present experiments some cultures were grown on the medium without any growth substances, while other protocorm cultures were supplemented with either an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), or a cytokinin, 6-furfurylaminopurine (kinetin), or giberellin, gibberellic acid (GA_3). The protocorms were harvested two months after excision.

For DNA isolation, 2-5g of tissue were disrupted with an ultra-turrax homogenizer for 2 min at 0°C in Tris buffer,pH 9, containing 0.5% sodium dodecylsulfate. DNA was prepared using a modification of the method derived for bacteria by Marmur (8). This method avoids the use of phenol which has been reported to remove satellite DNA's of high AT-content selectively (9).

Purified DNA in 0.12M phosphate buffer, pH 7, was monitored during continuous temperature change (1°C/min) in a closed thermostatically controlled quartz cell of a Gilford 240 UV spectrophotometer. All curves have been corrected for thermal expansion. The DNA samples displayed a 260/280 nm absorption ratio of 1.77 to 1.85, and a 230/260 nm ratio of 0.43 to 0.46. Aliquots of DNA were tested for protein content by the Lowry method (10); the protein content was less than 1%. Different samples showed hyperchromicities between 38 and 40.5%. These values were normalized to 100% to permit a comparison of the relative amounts of the individual fractions.

Derivative melting profiles (per cent hyperchromicity per degree Celsius) were calculated from the melting curves as

shown earlier (4). These profiles revealed heterogeneity of the Cymbidium DNA, so that the amount of several fractions could be estimated. The average base composition of each fraction was determined with the equation given by Mandel and Marmur (11).

Unpublished experiments have shown that the amounts of the individual fractions change with the age of the cultures. Several repeats were, therefore, necessary to obtain reliable values, as the single protocorms to not develop completely synchronously. At least three independent DNA preparations and six Tm measurements have been made from each treatment.

RESULTS

The thermal denaturation profile of Cymbidium DNA which was isolated from protocorms cultured without phytohormones showed a melting temperature of ca. 84°C and, in addition, an early melting fraction with a Tm of ca. 75°C. The derivative curves revealed the existence of six peaks which correspond to distinct fractions of differing average GC content (Fig.1, Table 1). The bulk of the DNA is found in fractions melting at 80 - 86°C, corresponding to 28 - 42% GC (uncorrected for 5-methyl cytosine). The most prominent satellite peak is formed by the early melting fraction with an average AT content of 86%.

When the excised protocorm tissue was cultured in the presence of 10^{-7} to 10^{-4}M kinetin, the thermal denaturation profiles exhibited, essentially, the same peaks. The rather small second peak, however, was absent in most diagrams.

When the protocorms were cultured on a medium containing 4.5×10^{-7} to 4.5×10^{-6}M 2,4-D, a synthetic auxin, the derivative thermal denaturation profiles were clearly different from those of the control cultures (Fig. 1). The slightly AT-rich fraction no.2 was drastically enlarged, the very AT-rich fraction no.1 a little less, and the more GC-rich fractions displayed reduced peaks. The proportion of each fraction is given in Table 1.

When the protocorms were cultured in the presence of gibberellic acid (GA_3), only a few heterochromatin-rich nuclei could be found, and the derivative melting profiles deviated

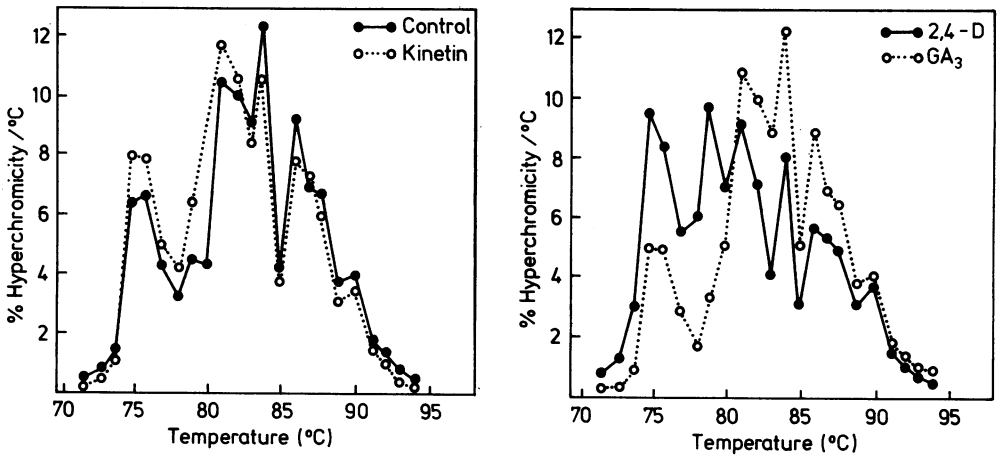

Figure 1. Derivative thermal denaturation profiles characteristic for DNA which has been isolated from Cymbidium protocorms cultured in vitro either without any phytohormone, or with the cytokinin, kinetin (KIN), or the auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), or the gibberellin, gibberellic acid (GA_3).

TABLE I

Fraction (peak) no.	Tm (°C)	% GC	% GC (corr.)	% of total DNA			
				Contr.	2,4-D	GA_3	KIN.
1	75.0	14.30	10.30	12.8	17.8	9.9	15.7
2	78.0	21.62	17.62	4.4	9.6	3.2	6.3
3	80.8	28.45	24.45	20.3	16.0	21.9	22.2
4	84.0	36.26	32.26	12.3	7.9	12.2	10.5
5	86.5	42.36	38.36	24.5	15.5	24.5	20.8
6	90.0	50.90	46.90	3.8	3.6	4.0	3.4
"valleys"				21.9	29.6	24.3	21.1

Individual peaks of the derivative melting profiles were equaled to fractions no. 1 - 6. The average GC content of each fraction was estimated (11) and corrected for its 5-methyl cytosine content (5). The proportion of each fraction was calculated from the size of the individual peaks.

from the control curves inasmuch as the AT-rich fractions displayed reduced peaks (Fig.1). Thus, the size of the AT-rich fractions in the thermal denaturation profiles clearly resembles the size of the population of heterochromatin-rich nuclei. - The statistical data of the experiments are given in Table 2.

TABLE II

Culture	% of total DNA, S.D., and significance of difference from control					
	Fractions					
	1	2	3	4	5	6
Control	12.8±3.3	4.4±0.7	20.3±2.9	12.3±0.9	24.5±1.1	3.8±0.5
2,4-D	17.8±2.8 ++	9.6±0.8 ++	16.0±1.4 ++	7.9±0.8 ++	15.5±1.7 ++	3.6±0.4 -
GA_3	9.9±1.0 ++	3.2±0.1 ++	21.9±1.4 -	12.2±0.8 -	24.5±1.9 -	4.0±0.3 +
KIN	15.7±2.0 +	6.3±0.8 -	22.2±2.6 -	10.5±1.1 -	20.8±2.1 +	3.4±0.3 -

The number of profiles evaluated, and the number of independent DNA preparations used for them (in parantheses) are the following: Control: 9(4), 2,4-D: 12(5), GA_3: 6(3), KIN: 6(3). The t-test has been made for determination of the significance of the differences; ++ indicates significance at the 1% level, + at the 5% level, - indicates that differences are not significant.

DISCUSSION

We have described here the variation of thermal denaturation profiles of the DNA that has been extracted from *in vitro* cultured protocorms of the orchid *Cymbidium* after treatment with various phytohormones. We like to interpret the changes in the proportion of AT- and GC-rich fractions as a consequence of differential replication of these fractions in hormone-treated tissues. This interpretation is consistent with other findings made in this system. In an earlier cytological study it has been observed that phytohormones affect the pattern of ^{3}H-thymidine incorporation into, and the DNA content of, the protocorm nuclei (2,6). Evidence has been obtained for DNA extra replication in heterochromatin after treatment with 2,4-D (1). CsCl equilibrium ultracentrifugation yielded results which are in agreement with our present findings: The AT-rich satellite DNA is sensible to hormonal treatments (3), although its variation is much less distinct than expected from the cytological data underlaying the assumption that the satellite DNA is located within heterochromatic portions of the nuclei. Recent fluorescence and electron microscopic studies indicated

that it is mainly a slightly AT-rich fraction which is extra replicated in developing Cymbidium protocorms (4).

Our findings on differential DNA replication in a higher plant is not unique. Underreplication of repetitive DNA has been observed in pea epicotyls (12) and Phaseolus suspensors (13), extra replication (DNA amplification) has been envisaged to occur in hypocotyls of Sinapis alba (14,15), and the metaxylem of Allium cepa (16); many other cases are discussed in a recent review (17). The influence of phytohormones on the differential replication of certain DNA sequences has been reported for cucumber (18) and artichoke (19). The manifold examples for differential DNA replication in animals cannot be discussed here. All these findings strongly indicate that variation in the proportions of certain DNA sequences may be a common mechanism of eukaryotic cytodifferentiation.

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