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CAFFEIC ACID-*O*-METHYLTRANSFERASE IN A SUSPENSION OF CELL AGGREGATES OF TOBACCO

TORU KUBOI and YASUYUKI YAMADA
Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; lignification; *O*-methyltransferase; cell aggregates.

Abstract—Aggregates of tobacco cells in suspension in 2,4-D (10^{-6} M) and kinetin (10^{-5} M) cultures were fractionated by size, then their *O*-methyltransferase (OMT) activities were assayed. Only the kinetin culture showed high OMT activity, which was higher in the larger than the smaller aggregates at all stages of cell growth. The contents of phenolic acids were also greater in the larger cell aggregates in the kinetin culture. However, when the kinetin cultured cells were transferred to a medium containing 10^{-6} M of 2,4-D, the relationships between the cell size of the aggregates and OMT, lignin and the phenolic acids disappeared. The importance of kinetin and cell association for OMT and the subsequent lignification of the cells is discussed.

INTRODUCTION

In almost all cases, suspension cultured cells of higher plants proliferate to some degree as cell aggregates. Cells in aggregate may be considered to be in quite a different environment from free floating cells. Different sizes of cell clusters from a suspension culture possess different embryogenetic potentials [1] and mitotic activities [2]. Moreover, chloroplast differentiation [3] and peroxidase activity [4] are stimulated in the large aggregates. These phenomena suggest that the inherited characters of plant cells in suspension are shown by different levels of aggregation. Thus, further studies on the physiological and biochemical differences associated with the aggregation of cells are necessary.

In a previous paper, we reported changes in OMT activity and the subsequent lignification of cultured cells of tobacco [5]. We here present some differences in OMT activity and in the lignification rate which correlate with the degree of cell aggregation, as studied in tobacco cells in suspension.

RESULTS AND DISCUSSION

During growth of T5S cells in a 2,4-D culture, most cells were in aggregates. At the early log stage of growth (2–5 days in Fig. 1), the S fraction (cell mass less than 0·5 mm in diameter; usually 8 cells per aggregate) increased and the M fraction (cell mass 0·5–1 mm, usually 30 cells per aggregate) decreased. The L fraction (cell mass more than 1 mm, usually 600 cells per aggregate) also decreased (Fig. 1). This indicates that in the early stage of growth, the cell aggregates of the M and L fractions dissociate to form the S fraction, and that in the later stage (5–10 days in Fig. 1), cells in the S fraction expand and divide in aggregate to form the M fraction. When these 2,4-D cultured cells were transferred to a kinetin medium, different patterns of fluctuation were observed. Kinetin was not very effective for aggregate formation, at least initially. But, during successive kinetin

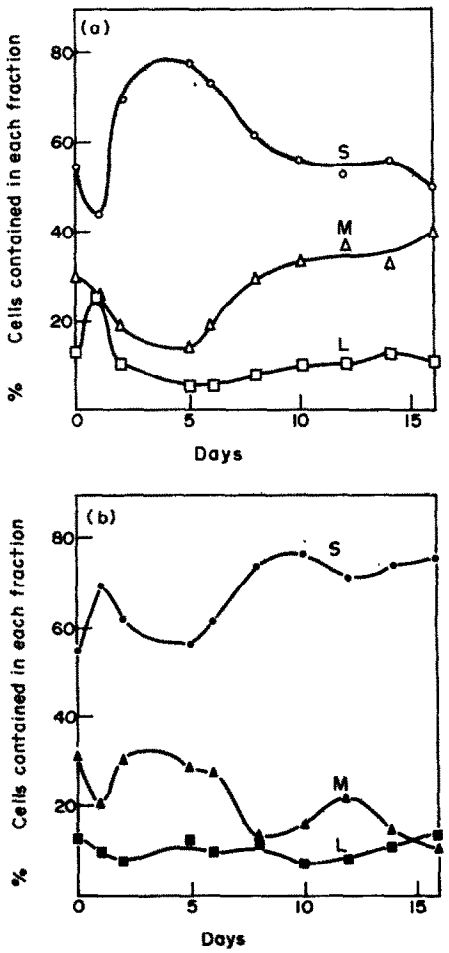

Fig. 1. Changes in % cells contained in fractions of varying cell cluster size during 2,4-D culture (a), and kinetin culture (b). Cell cluster sizes: S, cell mass less than 0·5 mm (circles); M, 0·5–1·0 mm (triangles); L, more than 1·0 mm (squares).

cultures, the large aggregates increased and the cell size of the L fraction decreased.

In sycamore [2] and in rose cells [6], cells in aggregate increase during the logarithmic phase and free floating cells increase during the stationary stage. Our results indicate, however, that large aggregates vary in a different way. Several factors (the sugar source, auxins, cytokinins, organic nitrogen [2], and the shaking rate [7]) contribute to aggregate formation. Auxins, cytokinins, and the sugar sources also affect tracheid differentiation [8–11]. In this study, kinetin was effective for cell aggregation and organization after several passages in a kinetin (10^{-5} M) culture.

In both the 2,4-D and kinetin cultures, the cells in large aggregates contained more DNA, RNA, and protein than did cells in the smaller aggregates, on a per cell basis (Fig. 2A, B, C). In the 2,4-D culture, however, the contents of phenolic acids (Fig. 2D) and the number of lignified cells (Fig. 3) did not increase with an increase in cell aggregate size. These two factors were related with the size of the cell aggregates only in the kinetin culture. The percentage of lignified cells to the total cells contained in each cluster fraction increased during successive kinetin cultures while retaining a correlation with the size of the cell aggregate (Fig. 3). Strikingly, lignification occurred even in the first transfer to a kinetin medium when cells were less organized. This was confirmed by staining the cells with phloroglucinol–HCl and safranin-*O*. On the 12th day of the kinetin culture, cells in aggregates were stained with phloroglucinol–HCl and

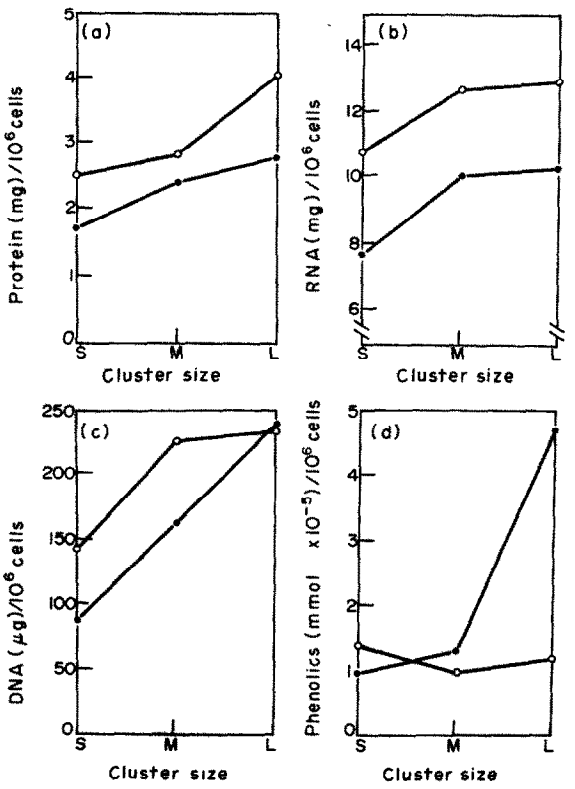

Fig. 2. Changes in (a); buffer soluble protein, (b); RNA, (c); DNA, and (d); phenolic acids contained in a million cells of each cluster fraction on the 12th day of culture of T5S cells. —●— 10^{-5} M kinetin culture; —○— 10^{-6} M 2,4-D culture.

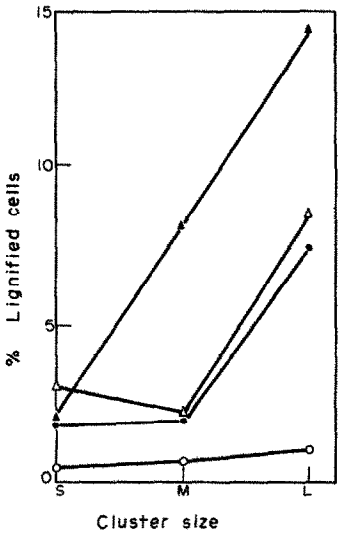

Fig. 3. Relation of the lignification rate of each cell fraction to the sizes of the cell cluster on the 15th day of culture of T5S cells. —○— 10^{-6} M 2,4-D culture; —●— 1st passage; —△— 2nd passage; —▲— 3rd passage in a 10^{-5} M kinetin medium.

safranin-*O* at the point of cell contact with neighbouring cells, but cells in aggregates in the 2,4-D culture did not take the stain. Results shown in Figs. 2D and 3 indicate that the activity of *O*-methyltransferase (OMT) (E.C. 2.1.1.6), a key enzyme in lignin biosynthesis, might be related to the size of the cell aggregates. Cells in the 2,4-D and kinetin cultures were fractionated using sieves at the 2nd, 9th and 14–15th days of culture, which corresponded to the first rise in OMT, the early stage and the maximum of the second rise in OMT, respectively [5]; then crude enzyme for the assay of OMT activity was extracted. At all stages tested, OMT activity was higher in the large cell aggregates in the kinetin culture. But in the 2,4-D culture this relationship was not observed (Fig. 4). Even in the 4th passage of cells from the kinetin culture, the relationship of OMT and lignification to the size of the cell aggregate was retained.

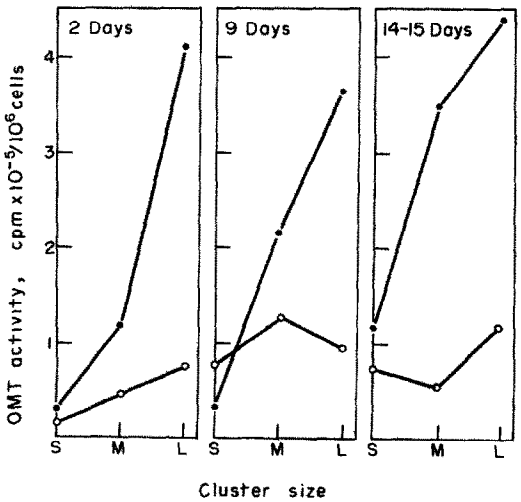

Fig. 4. Relation of OMT activity to the size of the cell cluster in a 10^{-6} M 2,4-D culture (—○—) and in a 10^{-5} M kinetin culture (—●—).

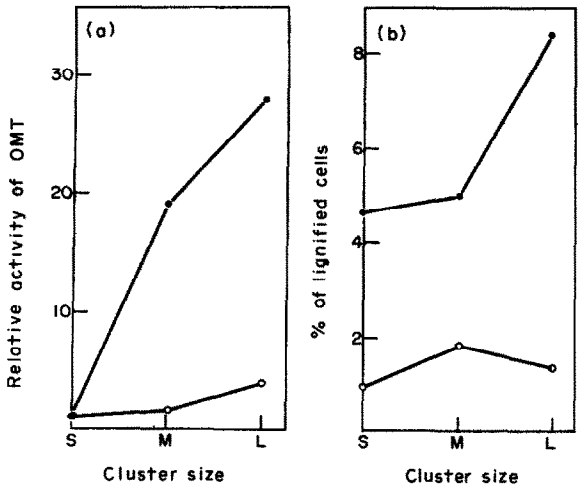

Fig. 5. (a), relation of OMT activity; and (b), the percentage of lignified cells to cells contained in each cluster fraction in the 10^{-6} M 2,4-D culture, to which cells were retransferred from a 3rd passage kinetin culture (—○—), and in the 4th passage kinetin (10^{-5} M), culture (—●—) at the 15th day.

When 3rd passage cells from the kinetin medium were transferred to the 2,4-D culture the relationship of OMT and lignification to the size of the cell aggregate was lost (Fig. 5). This reversal confirms that cell aggregate dependent OMT activity and lignification are inhibited by 2,4-D and/or promoted by kinetin.

The increases in OMT activity and in the number of lignified cells with the increasing cell mass at any point during the kinetin culture indicate that kinetin (or a dilution of 2,4-D) and cell aggregation have a decided effect on cytodifferentiation. The reversibility of this relation between cells in the 2,4-D culture and those in the kinetin culture indicates that 10^{-6} M of 2,4-D represses the aggregate dependent OMT activity and subsequent lignification. A diluted 2,4-D media, e.g. a 10^{-8} M 2,4-D medium, and 2,4-D free medium without kinetin slightly induced aggregate dependent OMT activity in T5S cells, but cells were gradually destroyed during the second passage. Even in the presence of 2,4-D (10^{-6} M), 10^{-5} M of kinetin induced aggregate dependent OMT activity.

The gradual increase in the percentage of total cells contained in the L fraction during successive kinetin cultures, and the observation that cells in a large cluster become highly organized after several passages in kinetin cultures suggest that kinetin has an organizing effect. Kinetin may, thus, have secondary effects on the aggregate dependency correlated to OMT, as well as an antagonistic effect to 2,4-D, the maintenance of cell viability and the initiation of organization.

That lignification is initiated at the point where cells are strongly bound to each other, suggests the importance of cell association for the induction of OMT activity. Though Hahlbrock *et al.* [12] reported that phenylalanine ammonia lyase activity was more active in the small aggregates of soybean cells, Verma and Huystee [4] found cell association important for the quantitative and qualitative differences in peroxidase. Using cytochemical enzyme assays De Jong *et al.* [13] also observed that chains of tobacco cells in suspension had individual cells in a single aggregate which differed greatly in their enzyme activities. The heterogeneity of cells in a culture probably is caused by differences in the micro-environment of each cell. But the results of De Jong *et al.* strongly suggest that contact between cells is more important. Cell association is clearly important for several aspects of differentiation; greening [3], vascularization [4,14], alkaloid syntheses [15] and phenolic syntheses [16].

Henshaw *et al.* [2] reported high mitotic activity in large aggregates of sycamore cells. Our results for DNA, RNA, and protein contents also suggest the viability of cells in a large aggregate. To activate phenolic, cytokinin seems to be required along with cell aggregation.

Loewenstein and his co-workers [17,18] reported a deficiency in the intercellular communication between cancer cells, and between that of a normal cell and a cancer cell. Fetal calf serum also effects the formation of communication in some fibroblastic cells [19]. Intercellular communication may be important in cultured cells of higher plants as well; thus an investigation is needed as to whether it is induced by kinetin.

EXPERIMENTAL

Culture conditions. T5S [20] strain of *Nicotiana tabacum* var. Bright Yellow was used. Culture conditions were the same as in a previous paper [5].

Measurement of growth. For fr. wt determination, cells were collected by vacuum filtration for 3–5 min and weighed. For determination of cell number, aliquots were digested with 5% chromic acid–5% HCl, and cell numbers were counted under a microscope [21]. Lignified cells were also counted after this digestion.

Fractionation of cell clusters. Cells free or aggregated were fractionated with metal sieves (0·5 and 1 mm) into three fractions; a cell mass <0·5 mm in diam. (S fraction), a mass of 0·5–1 mm in diam. (M fraction), and one of more than 1 mm in diam. (L fraction).

DNA, RNA, phenolics and protein determinations. DNA and RNA were determined after treatment with 5% PCA at 90° for 15 min. DNA was estimated by the diphenylamine reaction as modified by Burton [22], and RNA was estimated by the orcinol reaction [23]. Total phenolic acids were extracted by the method of Rhodes and Wooltorton [24] and determined by the method of Swain and Hillis [25]. Phenol was used as standard. Buffer soluble protein in cells was extracted with 10 mM Pi buffer (pH 7·5). The subsequent solution, after centrifugation for 30 min at 15000 *g*, was assayed for protein [26]. OMT was extracted and assayed as in a previous paper [5].

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