6-1-1971

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Published version. *Proceedings of the National Academy of Sciences*, Vol. 68, No. 6 (June 1971): 1178-1179. 

*Proc. Nat. Acad. Sci. USA*
Vol. 68, No. 6, pp. 1178–1179, June 1971

# Induction of Cytokinin-Independent Tobacco Tissues by Substituted Fluorenes

**(aminofluorenes/fluorene-9-carboxylates/indole-3-acetic acid/kinetin/tumors)**

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*Communicated by Armin C. Braun, April 6, 1971*

**ABSTRACT Two morphactins and three aminofluorenes initiated the formation of compact tissue nodules in hormone-dependent tobacco callus cultures. These nodules, upon subculture, behaved like partially transformed plant tumors. They grew on cytokinin-free media, while control callus and nonnodule tissue still required an exogenous cytokinin source. The data indicate that substituted fluorenes, including carcinogenic aminofluorenes, can cause a neoplastic growth response in cultured tobacco tissues. Of particular interest in this study is the finding that a directed and heritable cellular change is induced in tobacco tissue in which a specific (the endogenous cytokinin) biosynthetic system is regularly and persistently activated.**

The morphactin regulators of plant growth, methyl-2-chloro-9-hydroxyfluorene-9-carboxylate and *n*-butyl-9-hydroxy-fluorene-9-carboxylate, generally inhibit and stunt intact plants, but do not act directly as herbicides at low doses (1). They inhibit seed germination and prevent expression of apical dominance (2). Furthermore, the phototropic and geotropic responses by dicotyledonous and monocotyledonous shoots and roots are lost upon morphactin treatment (3). These responses to physical stimuli are mediated by the native auxin, indole-3-acetic acid, according to the Cholodny–Went hypothesis.

Another group of substituted fluorenes, the aminofluorenes, are carcinogens (4); they require the amino group for tumorigenic activity in animals (5). This property is enhanced when the amino substituent is hydroxylated to produce a potent, more proximate carcinogen (6).

We have examined the growth regulating effect of morphactins and three aminofluorenes, *N*-acetylaminofluorene, 2-aminofluorene, and 2,7-diaminofluorene, by plant tissue-culture techniques.

## MATERIALS AND METHODS

Tobacco pith tissue from *Nicotiana tabacum* var. Wisc. 38 in culture has absolute requirements for an exogenous auxin and for a cytokinin, such as kinetin; these hormones control cell enlargement and cell division. The tobacco bioassay permits study of the interaction of substituted fluorenes with both plant-growth regulators. The media employed and cultural methods have been described (7).

The fluorenes were filter-sterilized and added to the autoclaved medium just before gelation of agar.

## RESULTS

In addition to other growth-modifying effects to be discussed in detail elsewhere, all substituted fluorenes tested induced the formation of morphologically identical nodules in the cultured tobacco callus. Nodules appeared in cultures that contained substituted fluorenes at concentrations between 0.5 and 12.5 $\mu$M. The concentration of Ind Ac was 10 $\mu$M for all assays; kinetin concentration was 0.1–2.5 $\mu$M. Nodules formed in the presence of methyl-2-chloro-9-hydroxyfluorene-9-carboxylate and *N*-acetylaminofluorene are illustrated in Fig. 1. No nodules were observed in fluorene-free cultures.

Subcultures of tissues from the nodules grew on cytokinin-free media, while nonnodule tissue and callus from fluorene-free cultures were still dependent upon an exogenous source of cytokinin. Routine subculture of stock callus on hormone-free media has not produced any spontaneous auxin- or cytokinin-autonomous tissues during the past 4 years. Data from a typical subculture on four hormonal programs are given in Table 1. Callus tissue from fluorene-free treatments grew little and died unless both Ind Ac and kinetin were present. Kinetin in the absence of Ind Ac had no effect on the growth of cells isolated from morphactin-induced nodules; however, Ind Ac alone produced significant growth. Kinetin plus Ind Ac increased the yield, suggesting that endogenous cytokinin production or the degree of circumvention of the cytokinin requirement is a growth-limiting factor in the morphactin-induced nodule tissues. The subcultures have maintained their vigor during successive fluorene/cytokinin free passages for the past 2 years.

## DISCUSSION

These data indicate that the nodules behave like the hormone-dependent plant tumors described by Braun (8). Fully autonomous plant tumors do not require an exogenous source of either an auxin or a cytokinin for growth *in vitro*. The substituted fluorenes appear to activate or regulate the endogenous cytokinin system in certain cells of the cultured tobacco callus producing the cytokinin-autonomous nodules. Most significant is the finding that the morphactins and aminofluorenes induce a directed and heritable cellular change in the tobacco tissues in which a specific biosynthetic system is regularly and persistently activated. The product of this biosynthetic system has been found to play a central role in the development of a capacity for autonomous growth of the plant tumor cell.

Cytokinins occur in animal, plant, and bacterial tRNAs (9–11). In addition to their activity in plants, they have been reported to induce cytokinesis in animal cells (12–14). Formation of a naturally occurring cytokinin, $N^6$-isopentenyladenosine in tobacco by $\Delta^2$-isopentenyl-tRNA transferase has been described (15). This enzyme, also found in yeast and rat liver (16), couples the isopentenyl group to the $N^6$ position on an adenine moiety that is adjacent to the 3′ end of the anticodon

Abbreviation: Ind Ac, indole-3-acetic acid.

TABLE 1. *Growth and hormonal requirements of subcultures from control callus and fluorene-induced nodule tissues*

| Fluorene | Fresh weight (g/flask) −K −Ind Ac | +K −Ind Ac | −K +Ind Ac | +K +Ind Ac |
|---|---|---|---|---|
| None* | 0.16‡ | 0.08‡ | 0.19‡ | 18.66 |
| MeCl-F*¶ | 0.14‡ | 0.19‡ | 10.24 | 18.30 |
| But-F*¶ | 0.15‡ | 0.21‡ | 9.54 | 17.95 |
| 2-AF†‖ | 0.19‡ | — | 14.64 | — |
| AAF†‖ | 0.26‡ | — | 7.69 | — |
| 2,7-$A_2$F†‖ | 0.26‡ | — | 15.06 | — |

* 69 days' growth. † 61 days' growth. ‡ Dead.

¶ Fluorene-9-carboxylate derivatives: MeCl-F is methyl-2-chloro-9-hydroxy; But-F is *n*-butyl-9-hydroxy.

‖ Fluorene derivatives: 2-AF is 2-amino; AAF is *N*-acetylamino; 2,7-$A_2$F is 2,7-diamino.

The tissues were grown in the dark at 28°C on basic medium with the hormones indicated. Kinetin (furfurylaminopurine, K) and Ind Ac, when present, were 0.1 and 10 μM, respectively. The data are expressed as the means of four replicate cultures. The standard error of the mean was less than ±0.5 g/flask for all values cited.

in several species of tRNA. We do not know whether the substituted fluorenes activate this specific mechanism of cytokinin biosynthesis *in vivo*. A second mechanism may be involved, these compounds may also persistently activate the biosynthetic system that is responsible for the production of a new type of cell-division factor that was first isolated from plant-tumor tissue and was subsequently shown to be produced by normal plant cells grown in the presence of a 6-substituted purine, such as kinetin (17, 18). Recent work on the chemical structure of that compound clearly indicates that it does not arise directly from an RNA polymer (19).

Information on the biochemical fate of substituted fluorenes in plants is lacking; however, it is known that aminofluorenes can be enzymatically altered and bound to proteins, RNA (including tRNA), and DNA in animal tissues (6, 20, 21).

These observations, in addition to published data, show that substituted fluorenes have the common biological property of inducing neoplastic growth in at least two diverse eucaryotic systems. Insufficient evidence is available to decide whether or not aminofluorene-induced animal tumors are cytokinin autonomous, or even if cytokinins play an analogous role in animal growth and development to their role in plants.

The similarity of biological behavior in the production of cytokinin-independent nodules by all fluorenes tested suggests that biological substitutions, such as amination and hydroxylation of the fluorene nucleus, if such substitutions are required for the production of growths in plant tissue, may also occur with morphactins in tobacco tissue. Alternatively, in contrast to animal systems, the amino group may not be

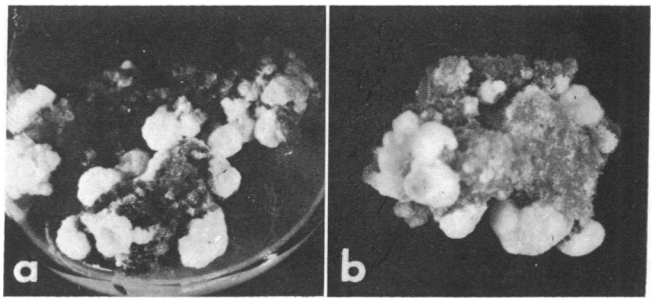


FIG. 1. Tobacco callus, grown in light for 47 days, showing nodules produced in cultures containing (*a*) 2.5 μM methyl-2-chloro-9-hydroxyfluorene-9-carboxylate and (*b*) 2.5 μM *N*-acetylaminofluorene in the presence of 0.5 μM kinetin and 10 μM Ind Ac.

essential for the neoplastic growth response in tobacco pith callus.

We thank Dr. A. C. Braun for helpful discussions, Ruth Sorg, and Ann Hirsch for technical assistance, and E. Merck A.G., Darmstadt, Germany, for samples of morphactins. This research was supported by the American Cancer Society, Milwaukee Division, the Marquette University Committee on Research, and Sigma Delta Epsilon.

1. Schneider, G., D. Erdmann, S. Lust, G. Mohr, and K. Niethammer, *Nature*, **208**, 1013 (1965).
2. E. Merck, A.G., *Morphactins, Technical Data Sheet* (1965).
3. Kahn, A. A., *Physiol. Plant.*, **20**, 306 (1967).
4. Wilson, R. H., F. DeEds, and A. J. Cox, *Cancer Res.*, **1**, 595 (1941).
5. Miller, E. C., and J. A. Miller, *Pharmacol. Rev.*, **18**, 805 (1966).
6. Miller, J. A., *Cancer Res.*, **30**, 559 (1970).
7. Linsmaier, E. M., and F. Skoog, *Physiol. Plant.*, **18**, 100 (1965).
8. Braun, A. C., *The Cancer Problem: A Critical Analysis and Modern Synthesis* (Columbia University Press, New York and London, 1969), p. 28.
9. Robins, M. J., R. H. Hall, and R. Thedford, *Biochemistry*, **6**, 1837 (1967).
10. Peterkofsky, A., and C. Jensensky, *Biochemistry*, **8**, 3798 (1969).
11. Letham, D. S., and C. O. Miller, *Plant Cell Physiol.*, **6**, 355 (1965).
12. Ogawa, Y., A. Yukihide, and K. Jujioka, *Nature*, **180**, 985 (1957).
13. Kevin, P., E. R. Witkus, and C. A. Berger, *Exp. Cell Res.*, **41**, 259 (1966).
14. Hacker, B., and T. L. Feldbush, *Biochem. Pharmacol.*, **18**, 847 (1969).
15. Chen, C-M., and R. H. Hall, *Phytochemistry*, **8**, 1687 (1969).
16. Kline, L. K., F. Fittler, and R. H. Hall, *Biochemistry*, **8**, 4361 (1969).
17. Wood, H. N., and A. C. Braun, *Ann. N.Y. Acad. Sci.*, **144**, 244 (1967).
18. Wood, H. N., A. C. Braun, H. Brandes, and H. Kende, *Proc. Nat. Acad. Sci. USA*, **62**, 349 (1969).
19. Wood, H. N., *Proc. Nat. Acad. Sci. USA*, **67**, 1283 (1970).
20. Epstein, S. M., J. McNary, B. Bartus, and E. Farber, *Science*, **162**, 907 (1968).
21. King, C., and B. Phillips, *Science*, **159**, 1351 (1968).