Induction of Cytokinin-Independent Tobacco Tissues by Substituted Fluorenes

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

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ABSTRACT Two morphactins and three amino-
fluorenes initiated the formation of compact tissue nod-
ules in hormone-dependent tobacco callus cultures. These
nodules, upon subculture, behaved like partially trans-
formed 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 amino-
fluorenes, can cause a neoplastic growth response in cul-
tured 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
dogenous 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-hydroxyflu-
orene-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 geotropnic
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 tumori-
genic 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 morph-
actins 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 auto-
claved 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
μM. The concentration of Ind Ac was 10 μM for all assays;
kinetin concentration was 0.1–2.5 μM. 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 typi-
ical 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/kinetin 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 endoge-
nous cytokinin system in certain cells of the cultured tobacco
callus producing the cytokinin-autonomous nodules. Most
significant is the finding that the morphactins and amino-
fluorenes 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 bio-
synthetic 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). Forma-
tion of a naturally occurring cytokinin, N6-isopentenyl-
adenosine in tobacco by Δ2-isopentenyl-tRNA transferase has
been described (15). This enzyme, also found in yeast and rat liver
(16), couples the isopentenyl group to the N6 position on an
adenine moiety that is adjacent to the 3' end of the anticodon
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

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

<table>
<thead>
<tr>
<th>Fluorene</th>
<th>-K</th>
<th>+K</th>
<th>-Ind Ac</th>
<th>+Ind Ac</th>
</tr>
</thead>
<tbody>
<tr>
<td>None*</td>
<td>0.16 ‡</td>
<td>0.08 ‡</td>
<td>0.19 ‡</td>
<td>18.66</td>
</tr>
<tr>
<td>MeCl-F*§</td>
<td>0.14 ‡</td>
<td>0.19 ‡</td>
<td>10.24</td>
<td>18.30</td>
</tr>
<tr>
<td>But-F*§</td>
<td>0.15 ‡</td>
<td>0.21 ‡</td>
<td>9.54</td>
<td>17.95</td>
</tr>
<tr>
<td>2-AF†</td>
<td>0.19 ‡</td>
<td>—</td>
<td>14.64</td>
<td>—</td>
</tr>
<tr>
<td>AAF†</td>
<td>0.26 ‡</td>
<td>—</td>
<td>7.99</td>
<td>—</td>
</tr>
<tr>
<td>2,7-A2F†</td>
<td>0.26 ‡</td>
<td>—</td>
<td>15.06</td>
<td>—</td>
</tr>
</tbody>
</table>

* 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-acetyl-2-amino; 2,7-A2F 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 mean of four replicate cultures. The standard error of the mean was less than ±0.5 g/flask for all values cited.

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**Fluorene-Induction of Cytokinin Independence**

essential for the neoplastic growth response in tobacco pith callus.

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