Cell Growth and Organ Differentiation in Cultured Tobacco Cells under Spaceflight Condition

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Abstract The responses of cultured tobacco cells to microgravity were examined. Cultured tobacco cells grew and regenerated shoots under microgravity conditions. However, such growth, especially of stems, was much more heterogeneous than that in the ground control, and the increase in fresh weight of the flight samples was less than that of ground control. In addition, multiple shoot formation was not observed in flight samples. Microscopic observation showed that the meristem of regenerating shoots under microgravity was smaller than that in the ground control. Electron microscopy showed that chloroplasts in the ground control were slightly more developed than those in flight samples and extensive arrays of microtubules were more evident in ground control than in flight samples. Analyses of enzymes involved in primary and secondary metabolism indicated that callus grown on shoot regeneration medium under microgravity conditions had a much lower activity of caffeic acid O-methyltransferase, which is involved in lignin biosynthesis, than those grown on Earth. In addition, two-dimensional polyacrylamide-gel electrophoresis analysis of ³⁵S-labeled proteins suggested that gene expression in the flight samples may be similar to that in the ground control.

Keywords: caffeic acid O-methyltransferase, cell growth, cultured tobacco cells, microgravity, shoot formation

Introduction

Plant shoots grow against the Earth’s gravity, whereas roots develop toward the center of the Earth. Together, these phenomena are known as geotropism. Although several studies have been conducted on these topics using spaceflight, clinostat and mutant plants (Cowles et al., 1984; Halstead and Dutcher, 1984; Slocum et al., 1984; Volkmann et al., 1986; Levine and Krikorian, 1992; Hoson et al., 1997; Fukaki et al., 1998), little is known about the long-term effects of microgravity on the growth and development of plant cells. This is primarily due to the relatively slow responses of plant cells to environmental changes, as well as to the difficulties inherent in developing systems to grow intact plants under microgravity conditions.

An experiment by Krikorian and Steward (1978) aboard the Cosmos 782 biosatellite investigated the impact of microgravity on the developmental stages that proceed the establishment of organs and organ polarity. When totipotent carrot cells, which had been manipulated nutritionally to induce somatic embryogenesis, were grown for about 20 days under microgravity conditions, the proportion of embryos at each of several stages of development was the same in both the flight and flight centrifuge-stimulated 1 g control growth.

Plants have also been grown in space in the form of bulbs, tubers, roots and cuttings to study the effects of spaceflight on vegetative and reproductive development (Halstead and Dutcher, 1984; Levine and Krikorian, 1992; Kordyum et al., 1997). However, experimental plants have usually died during extended vegetative growth. One of the obstacles to completing the life cycle under microgravity conditions is the difficulty in maintaining growth conditions for intact plants through an entire life cycle. Our research objective is to investigate the effect of microgravity on the organ differentiation and cell growth of tobacco cells using in vitro cell culture systems. One of the advantages of using an in-vitro culture system is that we can distinguish between the differentiation/developmental processes during the life cycle and evaluate the effects of microgravity on each process.

For this purpose, tobacco stem tissues or calluses (dedifferentiated cells) were inoculated on agar medium in specially designed culture vessels. The culture medium contained nutrients and plant hormones, i.e., auxin and cytokinin, to control the differentiation/dedifferentiation (cell proliferation) of plant cells. These inoculated tissues and cells are launched to orbit, and cultivated for 8 days under microgravity conditions (Speacelab-J, Sep., 12-20, 1992). After their return to Earth, the differentiation and growth responses of flight samples were compared to those
of a ground control. Due to the limited period of growth under microgravity conditions, enzyme activities, particularly those in lignin biosynthesis, were measured to evaluate the level of differentiation and dedifferentiation (growth) of cells, in addition to the electron microscopic observation of ultrastructural changes. Furthermore, general gene expression patterns in flight samples and the ground control were compared using \(^{35}\text{S}\)-labeling of proteins and subsequent 2D-PAGE analysis.

**Materials and methods**

**Plant materials**

Both cultured cells and stem tissues of tobacco (*Nicotiana tabacum* cv. Samsun NN) were used as plant materials. Stems (ca 5 mm thick) were cut from sterile seedlings that had been grown on half-strength of Linsmaier-Skoog agar medium (1965) containing 1.5% sucrose for 3-4 weeks. Cultured tobacco cells were maintained on Linsmaier-Skoog medium containing 10 µM naphthaleneacetic acid (NAA), 1 µM benzyladenine (BA) and 3% sucrose until the flight experiments. Both seedlings and cells were maintained at 25 ± 1 °C under 3,500-5,500 lux.

**Inoculation**

Stem tissues and cultured cells were inoculated on shoot-forming medium (regeneration medium; Linsmaier-Skoog basal medium containing 0.1 µM NAA, 10 µM BA and 3% sucrose) and callus-forming medium (dedifferentiation medium; Linsmaier-Skoog basal medium containing 10 µM NAA, 1 µM BA and 3% sucrose) about 22 hr before the launch. Inoculated samples were maintained at room temperature at 1 g condition until launch. Furthermore, flight samples were exposed to ca 2 g during the launch.

**Progress of flight experiment**

Culture vessels were placed into Space lab from mid-deck at the scheduled time (MET; Mean Elapsed Time, 7:09) and cultivated for about 8 days at 22-24.5 °C. The temperature of the ground control was adjusted to match that in the flight experiment. Flight samples were moved to a refrigerator within 3 hrs after the Shuttle returned to Kennedy Space Center at MET 7 day 22:30. Samples were carefully inspected with the naked eye and under a microscope. Some of the samples were transferred to fresh medium and some were fixed for electron microscopic observation. The remaining samples were frozen in liquid nitrogen for enzyme measurements.

**Measurement of fresh weight and electron microscopic observation**

Changes in the fresh weight of tissues were measured using the remaining samples after blotting away excess water with filter papers. The amounts fixed for electron microscopy with glutaraldehyde were calculated from the average weight of tissues.

Some of the samples were fixed in 2% glutaraldehyde-50 mM phosphate buffer (pH 7.0), and shipped to Japan for the electron microscopic observation. Fixed samples were post-fixed with 2% OsO4, dehydrated and embedded. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H400H transmission electron microscope.

**Measurement of enzyme activities and \(^{35}\text{S}\)-amino acid incorporation**

The effects of microgravity on the gene expression in plant tissues were evaluated using \(^{35}\text{S}\)-amino acid incorporation. \(^{35}\text{S}\)-Protein labeling mix (20 µCi, 1000 Ci/ mmol; NEN) was added to culture medium, and cells were cultured as in other experiments. Proteins were extracted and analyzed by 2D-PAGE, as described previously (Takeda et al., 1990). After SDS-PAGE (2nd dimensional), gels were stained with Coomassie brilliant blue, treated with ENHANCE (Du Pont), dried and exposed to X-ray films.

The activities of enzymes involved in lignin biosynthesis, as well as primary metabolism, were measured according to the procedures described by Kuboi and Yamada (1978).

To evaluate the data obtained in the flight experiment, a reference ground experiment was conducted using a rotator as a clinostat. Cultured tubes were vertically fixed on a board and rotated at about 5 rpm to counteract the effects of gravity. Morphological changes, growth, and differentiation of tracheid elements were examined.

**Results**

**Growth and differentiation of plant cells**

Both tobacco tissues (stem tissues) and callus (dedifferentiated cells) grew well on either the dedifferentiation medium or the regeneration medium, while flight samples on callus dedifferentiation medium showed more heterogeneous growth (Fig. 1). Measurement of the increase in fresh weight indicated that the cells in the flight sample grew less than those in the ground control (Table 1). While literature on plant growth in space is both sparse and contradictory, in most case, space flight...

![Fig. 1 Tobacco cells which grew on dedifferentiation medium under microgravity (Left) and on Earth (Right).](image-url)

In each vessel, the left part was inoculated with cultured cells and the right part was with stem tissues.
Table 1  Summary of the growth and differentiation of cells grown under microgravity (μG) and on Earth (1G)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Materials</th>
<th>Gravity</th>
<th>Inoculum(I) (g FW)</th>
<th>Harvest(H) (g FW)</th>
<th>H/I</th>
<th>Shoot</th>
<th>Root</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus</td>
<td>Stem</td>
<td>μG</td>
<td>0.29</td>
<td>0.97</td>
<td>3.34</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1G</td>
<td>0.32</td>
<td>1.59</td>
<td>2.93</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.23</td>
<td>0.91</td>
<td>3.93</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Callus</td>
<td></td>
<td>μG</td>
<td>1.73</td>
<td>4.24</td>
<td>2.45</td>
<td>2.35</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1G</td>
<td>2.16</td>
<td>5.89</td>
<td>2.73</td>
<td>2.16</td>
<td>2.66</td>
</tr>
<tr>
<td>Shoot</td>
<td>Stem</td>
<td>μG</td>
<td>0.25</td>
<td>0.47</td>
<td>1.88</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1G</td>
<td>0.34</td>
<td>0.96</td>
<td>2.82</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td>0.97</td>
<td>3.46</td>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

Samples were prepared in duplicate.

conditions result in decreased growth rates (Levine and Krikorian, 1992; references therein). Our results provide additional information that growth retardation also occur at cellular level.

While shoot differentiation was observed in both samples, we noticed that direct root differentiation was only observed in the flight sample and multiple shoot formation was only observed in ground control cells (Table 1, Fig. 2). Although further flight experiments are needed to characterize these differences between flight samples and ground controls, other parameters, such as gas composition (Kiss et al., 1998), might affect growth and differentiation.

Some of the flight sample that were brought back to Japan grew well and regenerating shoots (data not shown). These results suggest that short-duration microgravity treatment did not have any severe deleterious effects on differentiation and growth.

Gross anatomy and ultrastructure of cells grown on regeneration medium

The gross anatomy of flight sample and ground control adventitious shoots is represented in Fig. 3. The meristem exhibited the usual apical meristem organization of the shoot. However, the meristem in the flight samples was considerably smaller than that in the ground control and cells in the flight sample were more vacuolated. This smaller meristem may be associated with the absence of multiple shoot-formation in flight samples.

Electron microscopic observations also revealed differences between the ultrastructure of cells in flight samples and ground control. The development of plastid thylakoid membranes in ground control cells was slightly

Fig. 2 Regenerating shoots in flight samples (upper) and ground control (lower)
better than that in flight samples (Fig. 4). When cultured cells were used as inoculum, plastids accumulated more starch than those derived from stem tissues and both flight and ground control cells had multi-granular amyloplasts.

Extensive arrays of microtubules were observed in ground control stem tissues. However, microtubules were not apparent in flight samples (Fig. 5).

**Ultrastructure of cells grown on dedifferentiation medium**

Electron microscopic observation revealed differences between flight samples and ground control, even when they were cultivated on dedifferentiation medium. Plastids of de-differentiating stem shoots in flight samples had less developed thylakoid membranes, while plastids in the ground control retained well-developed thylakoid structure (Fig. 6). This tendency was also observed when callus cells were used as inoculum.

**Measurement of the activities of enzymes involved in primary and secondary metabolism**

While there was little difference in the content of soluble protein, and in the activities of primary metabolism (malate dehydrogenase), and secondary metabolism (peroxidase), caffeic acid O-methyltransferase showed a much lower activity in flight samples of callus on regeneration medium than in those in ground control (Table 2). Since this is the key enzyme in lignin biosynthesis (Kuboi and Yamada, 1978), this result suggests that gravity affects the lignification of plant cells. Ground experiments using a clinostat also showed that offsetting gravity reduced the differentiation of tracheid elements, which are the primary site of lignin biosynthesis (data not shown). The above data and earlier reports (Cowles et al., 1984) support the idea that gravity affects the process of lignification and the differentiation of tracheid elements.

**Incorporation of 35S- amino acids**

Incorporation of 35S-amino acids by proteins was examined in flight samples and ground control. The type of inoculum cell determined the incorporation of 35S-amino acid: i.e., calluses showed higher incorporation of 35S-amino acids than stem tissues (data not shown). However, the difference between flight samples and ground control was small. Two dimensional polyacrylamide gel electrophoresis of extracted proteins and fluorography showed that the 35S-labeling pattern of proteins in the flight samples was similar to that in the ground control (Fig. 7). This result suggests that general gene expression in the flight samples may be similar to that in the ground control. Hampp et al. (1997) also reported that space flight did not
Fig. 5 Microtubles in flight samples (A) and ground control (B) 
Stem tissues were inoculated on regeneration medium and cultured for 8 days. 
W: cell wall, PD: plasmodesmata, MT: microtubule

Fig. 6 Ultrastructure of mesophyll cells in the flight sample (A) and ground control (B) grown on 
dedifferentiation medium 
Stem tissues were inoculated on dedifferentiation medium and cultured for 8 days. Bar indicates 1 μm. 
N: nucleus, Nu: Nucleolus, P: plastid

Table 2 Summary of the enzyme activities in cells grown under microgravity (μG) and on Earth (1G)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Materials</th>
<th>Gravity Protein (mg/g FW)</th>
<th>MDH* (μmol/mg protein/min)</th>
<th>POD** (μmol/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callus</td>
<td>Stem</td>
<td>μG 0.32</td>
<td>91</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.43</td>
<td>87</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.48</td>
<td>58</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.78</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Callus</td>
<td>μG 0.69</td>
<td>35</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.34</td>
<td>20</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1G 0.41</td>
<td>49</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.43</td>
<td>43</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Shoot</td>
<td>Stem</td>
<td>μG 0.67</td>
<td>49</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0.94</td>
<td>28</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1G 0.82</td>
<td>28</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.85</td>
<td>34</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Callus</td>
<td>μG 0.85</td>
<td>37</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>32</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1G 1.53</td>
<td>34</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.61</td>
<td>25</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

* malate dehydrogenase, ** peroxidase, *** caffeic acid O-methyltransferase
affect the composition of cytoskeleton constituents and the levels of osmotin, a stress-indicating pathogenesis-related protein, and phenylalanine ammonia lyase, a marker enzyme for an initial step into secondary metabolism. However, we should note that plant materials were incubated for almost an entire day at room temperature at 1 g before they were exposed to microgravity because of the lack of space in the refrigerator. Therefore, the present results should be interpreted carefully and additional experiments are needed before a definite conclusion can be reached.

**Discussion**

Gravity is one of the fundamental stimuli that affects the growth and development of plants. However, the growth and development of plants under microgravity conditions is poorly understood, since opportunities to examine flight samples have been limited. Several flight experiments have indicated that root tissue organization is not affected by microgravity, although root cap amyloplast orientation under microgravity conditions is random (Cowles et al., 1984; Halstead and Dutcher, 1984; Slocum et al., 1984; Volkman et al., 1986; Hoson et al., 1997). This agrees with the observed random growth orientation of unsupported roots in space. Somatic embryogenesis under microgravity also indicates that microgravity does not affect the embryogenesis (Krikorian and Steward, 1978; Theimer et al., 1986). However, our flight experiment suggests that microgravity does affect the development of shoot-organization. In particular, multiple shoots were not observed in the flight samples. This may be associated with the reduced development of shoot apical meristem in the flight samples. Several ultrastructural changes were also found in flight samples. These data suggest that plant cells sense gravity at the cellular level and exhibit different growth and development under microgravity and 1 g conditions.

However, we should be careful in concluding that microgravity affected the development of plant cells. Although microgravity is certainly the key environmental factor in space, several other factors may affect the development and the growth of cells, e.g., hyper-gravity, vibration, etc. Additional experiments are required before we can be certain of the effect of microgravity during flight. At a minimum, future experiments should examine callus initiation and regeneration under microgravity and 1 g. A study of long-term culture in un-manned flight would be useful. Furthermore, we note that our flight samples were kept under 1 g conditions at room temperature for almost an entire day before launch. This may have affected the differentiation, growth and incorporation of $^{35}$S-amino acids. Several limitations in Space Shuttle, including refrigerator space, availability of electricity should be addressed for future life science experiments.
Acknowledgment

We greatly appreciate to whom involved in SL-J project for their extensive cooperation, while we do not list. Special acknowledgement is given to Mr. Mutuki Nagase and Mr. Keiichi Araki of Chiyoda Co., and to Dr. Muneki Takaoki and Ms. Atsuko Nishi of Mitsubishi Heavy Industries, for their help in this project.

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