Photoautotrophic Cultured Plant Cells: A Novel System to Survey New Photosynthetic Electron Transport Inhibitors

Fumihiko Sato[a], Yasuyuki Yamada[a], Sang Soo Kwak[b]*, Katsunori Ichinose[b], Mitsuhiro Kishida[b], Nobutaka Takahashi[c], and Shigeo Yoshida[b]

[a] Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
[b] Chemical Regulation of Biomechanism Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan
[c] Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo, Bunkyo, Tokyo, Japan

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The responses of photoautotrophic (PA) cultured cells of tobacco (*Nicotiana tabacum cv.* Samsun NN) and liverwort (*Marchantia polymorpha* L.) to thirty-eight cyclohexanedione derivatives were surveyed. Each derivative was also tested for inhibitory activity on photosynthetic electron transport (PET), using isolated thylakoids, and herbicidal activity, using seedlings and mature plants. Comparison of the results from the different assays showed that the responses of PA cells to each compound correlated more closely with the responses of seedlings and mature plants than did the results of the Hill reaction assays. Our findings suggest that PA cultured cells would be a suitable screening material for identifying potential herbicides with PET-inhibiting activity.

Introduction

Testing new compounds for inhibitory activity in Hill reaction assays using isolated thylakoids is an effective method to find new photosynthetic electron transport (PET) inhibitors. However, this method is not well suited to identifying potential herbicides, because the results do not correlate well with the results of assays of herbicidal activity on whole plants. Differences in outcome of the two types of assays can be caused by several factors including the permeability to, and stability of, the compounds at various barriers between the surface of the entity and the site of action in the thylakoid membrane.

Cultured plant cells would be expected to be intermediate in their response to potential herbicides because they retain the chloroplast and cell membranes and cell wall while lacking barriers presented by tissue organization like epidermis. Photoautotrophic (PA) cultured cells should be particularly well suited to assays of potential photosynthesis inhibitors because of their growth mechanism [1]. Photomixotrophic (PM) and heterotrophic cells may also be useful, however, as comparison with PA cells may allow the mode of action of inhibitors to be determined more precisely [2].

In the experiment reported here, we monitored the responses of PA cultured cells of tobacco (*Nicotiana tabacum cv.* Samsun NN) and of liverwort (*Marchantia polymorpha* L.) to various cyclohexanedione derivatives including several potent PET inhibitors. Our results support the use of PA cells for more extensive screening of potential herbicides.

Abbreviations: PA, photoautotrophic; PET, photosynthetic electron transport; PM, photomixotrophic.

* Present address: Plant Cell Biology Lab., Genetic Engineering Research Institute, KIST, P.O. Box 17, Taedok Science Town, Taejon, 305-606, Korea.

Reprint requests to F. Sato.

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Materials and Methods

Herbicides and new PET inhibitors used

Diuron purchased as analytical reagent, was used after the recrystalization. All the new cyclohexanedione derivatives tested were synthesized according to the methods described elsewhere [3, 4].

Cultured cells

Cultured cells of tobacco (*Nicotiana tabacum cv.* Samsun NN, [1]) and liverwort (*Marchantia polymorpha* L., [5]) were used for these experiments.

The PA cell line, which can grow in the light without an organic carbon source, also exhibits considerable photosynthetic activity in liquid medium with a high sucrose concentration [1, 6].

PA cultures of tobacco were maintained in the light (*ca.* 120 μE/m²/sec) in liquid Linsmaier-Skoog basal medium [7] containing twice the original concentration of inositol and thiamine with 10 μM 1-naphthaleneacetic acid, 1 μM kinetin but lacking sucrose. PM cultures of tobacco were maintained in liquid medium as above with 3% sucrose. PA cultures of liverwort were maintained in M 51 medium (NH_4NO_3, 400 mg/l; KNO_3, 2000 mg/l; $CaCl_2$, 300 mg/l; KH_2PO_4, 275 mg/l; $MgSO_4 \cdot 7H_2O$, 370 mg/l; vitamins and micronutrients of B 5 medium [8], pH 5.6) including casamino acids (1 g/l), glutamine (200 mg/l) and 2,4-D (1 mg/l) under the same culture condition as tobacco PA cells. Double tier flasks with 2 M carbonate buffer in the lower part as described by Husemann and Barz [9], were employed to elevate the atmospheric concentration of CO_2 to 1–2% during PA culture. All cells were cultured at 26 ± 2 °C with reciprocal shaking (100 rpm).

Hill reaction assay

Spinach (*Spinacia oleracea*) thylakoids were obtained as described (10) and were stored in liquid nitrogen. PET activity was measured as an increase in photoabsorption at 600 nm due to the photoreduction of dichloroindophenol (DCIP) under neutral conditions (HEPES pH 7.0, 50 mM; NaCl, 10 mM; methylamine, 20 mM; DCIP, 50 mM). Assay volume for each compound tested was 2 ml with a chlorophyll concentration of 0.5 μg/ml.

Test of herbicidal activity on cultured cells

The effects of each compound on tobacco PA cells, and on liverwort PA cells were measured by different assays. PA cells of tobacco were cultured in the presence of the compound (without elevated atmospheric CO_2 levels) for one week. Chlorophyll content (*ca.* 120–170 μg/g fresh weight) was measured at the beginning and end of the week, and was used as the basis for determining the extent of inhibitory activity. PA cells of liverwort (100 μg chlorophyll/assay; average photosynthetic O_2 evolution 15 μmol/mg Chl/h) were incubated in a Clark type oxygen electrode in cultured medium containing 20 mM $NaHCO_3$ at a light intensity of about 400 μE/m²/sec. Photosynthetic O_2 evolution was measured immediately following addition of the compound being tested and the rates before and after addition were compared to determine % inhibition. The biological activity of each compound on tobacco PM cells was measured by the growth in fresh cell weight over two weeks. In each case, the inhibitory activity of the compounds was measured in three replicates and 50% inhibition was determined by regression analysis. Standard deviations were less than 10% of the mean. pI_{50} is the negative logarithm of the molar concentration required for 50% inhibition.

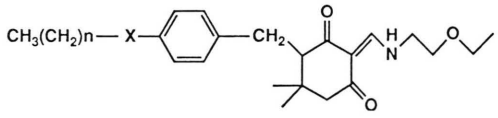
Tests for herbicidal activity on seedlings and mature plants

Tests on seedlings and mature plants were carried out according to standard methods. Uniformly germinating 20 cress seedlings were treated with solutions containing each compound for 1 week. The inhibitory effect of the compounds was evaluated semi-quantitatively using the following scale: Healthy, green seedlings = 0, seedlings showing chlorosis or necrosis = 100. The total score of the 20 seedlings used for each treatment was summed and the relative effect was calculated as follows: relative effect = total score for treated seedlings/20 (%). In each case, the inhibitory activity of the compounds was expressed as the negative logarithm of the molar concentration required for 50% inhibition (pI_{50}) as described above. In pot tests, eight weeds, including *Xanthium strumarium* L., and *Galium spurium* L. were treated with the compounds by foliar spraying at a dosage rate equivalent to 50 g ai/a. The effect of compounds was expressed as % inhibition of growth which was determined semi-quantitatively as described above.

Results and Discussion

The effects of cyclohexanedione derivatives on PA cells of tobacco and liverwort were measured and compared with the effects of the compounds on the Hill reaction in isolated thylakoids and on growth and survival of seedlings and mature plants. Table I shows the results for a series of derivatives in which the length of alkyl chain in the

Table I. Effects of cyclohexanedione derivatives with varying terminal alkyl chain length on the Hill reaction, on the growth of PM and PA cultured cells, and on seedlings and potted plants.



Compound	X	n	Hill reaction (pI_{50})[a]	Tobacco PM cells (pI_{50})	Tobacco PA cells (pI_{50})	Liverwort PA cells (pI_{50})	Cress seedlings (pI_{50})	Pot test[b]
1	–	2	6.7	6.0	4.7	5.1	4.1	40
2	–	4	7.8	5.3	4.2	4.8	3.6	20
3	–	6	8.4	5.3	4.5	3.7	4.3	5
4	–	8	8.2	5.1	4.0	<3	3.7	5
5	–	10	7.3	5.4	<4	<3	<3	5
6	–	12	6.3	5.7	<4	<3	<3	8
7	O	1	6.0	5.4	4.7	4.9	4.5	51
8	O	3	7.1	5.6	4.8	5.1	4.7	33
9	O	5	7.7	5.3	4.4	4.8	3.9	15
10	O	7	8.1	5.0	4.0	<3	3.3	0
11	O	9	7.9	5.0	<4	<3	<3	0
12	O	11	7.2	5.6	<4	<3	<3	0
DCMU			7.3	6.5	6.4	6.4	5.2	100

[a] pI_{50} is the negative logarithm of the molar concentration required for 50% inhibition.
[b] Expressed as % inhibition of growth after application at a level corresponding to 50 g ai/a.

lipophilic part of the molecule was varied. All of these compounds (compounds **1–12**) showed strong PET inhibition. As measured with isolated thylakoids, the maximum PET inhibition activity was observed with heptyl and nonyl substituents ($n = 6, 8$), however, the *in vivo* activity on PA cells, seedlings and mature plants markedly decreased with alkyl chain length more than heptyl ($n = 6$).

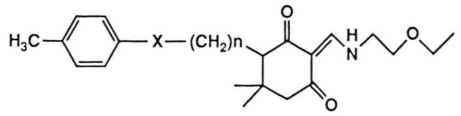
The difference between the pI_{50} measured for the Hill reaction and the pI_{50} measured in the *in vivo* assay increased linearly with the length of the alkyl chain. This suggests that longer alkyl chains prevent the transport of these derivatives to the site of inhibition in the thylakoid membranes. The similarity of this trend among PA cells, seedlings and potted plants suggests that the barriers to penetration for these derivatives are similar in the different systems.

Table II showed the effects on photosynthesis and growth of another series of compounds with varying lengths of alkyl chain between the benzene ring and the cyclohexanedione. These compounds (compounds **13–24**) not only showed strong PET inhibition, but also strong herbicidal activity on potted plants. Effects of length of alkyl chain similar to those noted for compounds **1–12** were also observed for this series of compounds. PET inhibition activity was high in compounds with an alkyl chain longer than three, but *in vivo* activity decreased with alkyl chains longer than seven. Compounds with alkyl chain length between these two limits (compounds **14, 15, 19–21**) showed strong activities on both PA cells and potted plants.

The effects of substitutions on the benzene ring were examined and the results are shown in Table III. All of these compounds except the *meta* substituent (compound **26**) had strong herbicidal activity both on PA cells and on potted plants. The electron-withdrawing substituents (-Cl, -Br) showed higher inhibitory activity than did the electron-donating substituent (-CH3). The activities of these compounds on PA cells correlated well with their activities in the Hill reaction assay and on potted plants. Of the derivatives tested, compounds **14, 19–21** and **29–31** showed nearly the same level of activity as DCMU in all of the assays.

The substitution of furfuryl moieties for ethoxyethylamine moieties significantly decreased the herbicidal activity on potted plants as is shown in

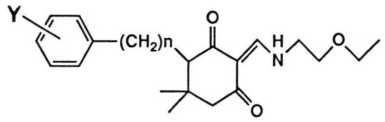
Table II. Effects of cyclohexanedione derivatives with varying internal alkyl chain length on the Hill reaction, on the growth of PM and PA cultured cells, and on seedlings and potted plants.



Compound	X	n	Hill reaction (pI_{50})[a]	Tobacco PM cells (pI_{50})	Tobacco PA cells (pI_{50})	Liverwort PA cells (pI_{50})	Cress seedlings (pI_{50})	Pot test[b]
13	–	2	6.2	5.3	4.5	5.2	3.7	73
14	–	3	7.8	6.3	5.4	6.1	4.8	90
15	–	4	7.2	5.4	4.9	5.0	4.4	88
16	–	5	7.6	5.2	4.0	4.8	4.0	84
17	–	7	7.9	5.3	4.1	3.5	3.5	83
18	–	9	7.4	5.3	<4	<3	<3	43
19	O	2	7.1	6.0	4.9	5.7	4.3	96
20	O	3	7.1	6.5	6.1	5.7	4.4	95
21	O	4	7.3	6.2	5.7	5.3	4.1	93
22	O	6	7.8	6.0	4.6	4.6	3.8	70
23	O	8	7.7	5.8	4.4	<3	3.2	45
24	O	10	7.3	6.0	<4	<3	<3	33

[a, b] pI_{50} and results of the pot test are expressed as described in Table I.

Table III. Effects of cyclohexanedione derivatives with substituted benzene rings on the Hill reaction, on the growth of PM and PA cultured cells, and on seedlings and potted plants.



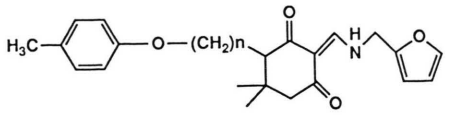
Compound	Y	n	Hill reaction (pI_{50})[a]	Tobacco PM cells (pI_{50})	Tobacco PA cells (pI_{50})	Liverwort PA cells (pI_{50})	Cress seedlings (pI_{50})	Pot test[b]
25	H	1	5.2	5.2	4.5	4.6	4.1	38
26	2-CH_3	1	4.7	5.2	<4	4.1	3.9	0
27	3-CH_3	1	5.2	5.3	4.6	4.6	4.0	68
28	4-CH_3	1	6.0	5.6	4.7	5.0	3.8	74
29	4-Cl	1	6.7	6.2	5.7	5.4	3.9	94
30	3,4-Cl_2	1	7.3	6.8	5.9	5.4	4.4	98
31	4-Br	1	6.9	6.4	5.3	5.5	4.0	99
32	H	2	5.8	5.6	5.7	4.9	3.5	80

[a, b] pI_{50} and results of the pot test are expressed as described in Table I.

Table IV. These compounds, however, retained high PET inhibition activity. The results of the PA cell assay indicate that the furfuryl substitution narrowed the range of alkyl chain lengths compatible with inhibitory activity (Tables II and IV).

For all chemicals tested, pI_{50} values of PA cells showed good correlation with the data of vivo assay on whole plants. When the pI_{50} values for the compounds which data could not determined experimentally were estimated as 3.5, 2.5 and 2.5 for

Table IV. Effects of cyclohexanedione derivatives containing a furfuryl moiety on the Hill reaction, on the growth of PM and PA cultured cells, and on seedlings and potted plants.



Compound	n	Hill reaction (pI_{50})[a]	Tobacco PM cells (pI_{50})	Tobacco PA cells (pI_{50})	Liverwort PA cells (pI_{50})	Cress seedlings (pI_{50})	Pot test[b]
33	2	7.0	5.1	3.8	5.5	4.1	0
34	3	6.9	5.4	5.0	4.3	4.3	0
35	4	6.8	5.1	4.4	4.2	4.2	0
36	6	7.0	5.0	<4	<3	3.9	0
37	8	6.9	5.7	<4	<3	3.1	0
38	10	6.1	4.7	<4	<3	<3	0

[a, b] pI_{50} and results of the pot test are expressed as described in Table I.

tobacco PA cells, liverwort PA cells and cress seedlings, respectively, the following correlations were obtained.

pI_{50}(Cress seedlings) = 1.72 + 0.47 × pI_{50}(Liverwort PA) (r = 0.81)

pI_{50}(Cress seedlings) = 0.72 + 0.67 × pI_{50}(Tobacco PA) (r = 0.74)

pI_{50}(Cress seedlings) = 0.92 + 0.49 × pI_{50}(Tobacco PM) (r = 0.27)

pI_{50}(Cress seedlings) = 4.08 − 0.066 × pI_{50}(Hill reaction) (r = 0.06)

This suggests that PA cells are good materials for the screening for the new PET inhibitors and PM cells with similar chlorophyll contents (70−100 μg/g fresh weight) are less useful for this purpose. Compounds **1−4**, and compounds **13−17** and **19−23** showed similar activity on the PA cells and seedlings, while compounds **13−17** and **19−23** are more active on whole plants in pot than compounds **1−4.** Different sensitivities among PA cells (or seedlings) and whole plants were also observed for compounds **33−35** (Table IV). This result suggests that some differences exist between the assays using cultured PA cells (or seedlings) and pot assay. Several reasons are considered for these differences: 1) Degradation of compound in pot test. 2) Different absorption ability of cultured cells (or seedlings) from whole plants. PA cells would show an intermediate character between thylakoids and whole plant-level because of these differences. But overall, we can conclude that the sensitivities of PA cells and seedlings were highly correlated to make a useful system to screen new PET inhibitors.

Additionally, our results allow a comparison of two different assay methods utilizing PA cells. The cell growth assay (used here with PA tobacco cells) was simple but required a culture period of 1−2 weeks. Direct measurement of O_2 evolution (used here in the case of liverwort PA cells) was rapid but required the high photosynthetic activity and small aggregate size characteristic of the liverwort PA cell cultures but not of the tobacco PA cell cultures. The results obtained by direct oxygen electrode assay and the results for PA tobacco cells were highly correlated.

pI_{50}(Liverwort PA) = −1.35 + 1.24 × pI_{50}(Tobacco PA) (r = 0.81)

This suggests that despite the phylogenetic distance and despite the morphological differences (*e.g.* aggregate size) of the cultured cells, the eventual accessibility of the thylakoids to the compounds was very similar in the two types of cells.

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