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CYCASIN FORMATION IN TISSUE CULTURES OF JAPANESE CYCAD

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Abstract—Calluses were produced from plant tissues of Japanese cycad (*Cycas revoluta* Thunb.). Its toxic component named cycasin, β-glucoside of methylazoxymethanol, was formed by the calluses only in the presence of exogenous methylazoxymethanol. A glucosyltransferase catalysing the formation of cycasin from UDP-glucose and methylazoxymethanol occurred in the calluses. The properties of this enzyme in embryo-derived callus and in leaf tissue were similar.

INTRODUCTION

Cycasin, a toxic component of Japanese cycad, *Cycas revoluta* Thunb., is a β-glucoside of methylazoxymethanol (MAM) [1]. The biosynthetic pathway of cycasin has not been elucidated except that a glucosyltransferase, which catalyses the formation of cycasin from UDP-glucose (UDPG) and MAM, occurs in cycad leaves [2]. There is no earlier report on cycasin formation in plant tissue culture. The purpose of our work was to produce calluses from plant tissues of Japanese cycad and to investigate cycasin formation in these calluses.

RESULTS AND DISCUSSION

Callus production

Since medium compositions used for cycad species other than Japanese cycad [3] were inefficient for callus induction from plant tissues of Japanese cycad, medium compositions were first examined. Murashige and Skoog's [4], Linsmaier and Skoog's [5], and White's [6] media as basal media gave poor callus induction in the presence of 3% sucrose and various concentrations of auxin 2,4-dichlorophenoxyacetic acid (2,4-D) or indole-3-acetic acid. On the other hand, the agar medium of Murashige and Skoog at one-half the concentration, which contains 3% sucrose, 10 ppm 2,4-D and 0.1% activated charcoal, induced calluses from the above plant tissues in 2 weeks. The concentration of 2,4-D was good at 10 ppm. The addition of activated charcoal to the medium effectively suppressed browning and necrosis of calluses.

After calluses induced from embryos were subcultured for 1 month on subculture medium, they were divided into two or three pieces (*ca* 1 g fr. wt), transplanted separately on Murashige and Skoog's agar medium containing 3% sucrose, 0.1% activated charcoal and various concentrations of naphthaleneacetic acid (NAA) and kinetin, as shown in Table 1, and subcultured for one month. Growth of calluses mainly depended on the kinetin concentration (Table 1). In all combinations of NAA and kinetin concentrations, no plantlets were regenerated. Calluses subcultured on the subculture medium showed good growth similar to those on the medium with 10 mM NAA and 1 mM kinetin, which is shown in Table 1, and gave no plantlets.

Cycasin formation and glucosyltransferase activity in callus

Cycad plant tissues and the calluses, which were induced from embryos, epicotyls and roots and subcultured twice on the subculture medium, were analysed for cycasin (Table 2). Neither cycasin nor MAM were detected in these calluses. Both compounds were also undetectable in the embryo-derived calluses subcultured on all the media shown in Table 1, which contained various amounts of NAA and kinetin. These results suggested that cycasin was not formed *de novo* in calluses.

When the calluses (*ca* 1 g fr. wt) derived from embryos, epicotyls and roots were separately incubated for 1 week on the subculture medium spread with 0.8 ml of 10 mM MAM, *ca* 1 μmol of cycasin was found in the calluses, but MAM was not. Glucosyltransferase activity, which forms cycasin from MAM and UDPG, was detected in all the calluses (Table 2). The calluses seemed to contain sufficient enzyme and UDPG to form cycasin from exogenous MAM. The enzyme from the embryo-derived callus was

Table 1. Effect of various concentrations of naphthaleneacetic acid and kinetin on growth of embryo-derived callus*

Kinetin (ppm)	Naphthaleneacetic acid (ppm)				
	0	0.01	0.1	1	10
0	1.2	1.1	1.3	1.2	1.3
0.01	1.3	1.2	1.3	1.2	1.3
0.1	1.2	1.6	1.4	1.5	1.6
1	1.6	1.5	1.6	1.4	1.5
10	3.3	3.0	2.5	4.2	4.0

*Embryo-derived calluses (*ca* 1 g fr. wt) subcultured for about 1 month on the subculture medium were transplanted on agar Murashige and Skoog's medium containing 3% sucrose, 0.1% activated charcoal and various concentrations of plant hormones. The values represent the fr. wt of the calluses after 1 month cultivation.

Table 2. Cycasin content and glucosyltransferase activity in cycad plant tissue and callus

	Cycasin (μmol g^{-1} fr. wt)	Glucosyltransferase activity (units* g^{-1} fr. wt)
Seed		
Albumen	35.5	nd†
Embryo	6.2	6.3
Plant		
Leaf, old	14.0	28.0
Epicotyl from seedlings	35.6	7.0
Embryo-derived callus‡	0	2.8
Epicotyl-derived callus‡	0	0.9
Root-derived callus‡	0	0.9

Results represent the mean of two determinations.

*One unit of enzyme activity was defined as the amount of enzyme catalysing the formation of 1 nmol cycasin per min under the conditions described in the text.

†Not determined because of very high activity of β-glucosidase.

‡Calluses induced from embryos, epicotyls and roots were successively twice subcultured on the subculture medium.

compared with that from the leaf for some properties by using crude enzyme solutions. The two enzymes were localized in the cytosolic fraction and showed the same substrate specificity for sugar nucleotides (ADP-glucose and GDP-glucose were inactive, but UDP-galactose slightly active for both enzymes) and similar apparent K_m values for MAM (0.11 mM at 25 mM UDPG for both callus and leaf enzymes) and UDPG (0.40 mM at 3 mM MAM for the callus enzyme and 0.46 mM for the leaf one).

Ethanolamine, which is a component of cycad leaves, stimulated cycasin formation in cycad leaves, but not in the embryo-derived callus (unpublished data). This finding and the results described above indicated that MAM was not formed *de novo* in calluses. An unidentified nitrogen compound(s) formed by symbiotic nitrogen-fixing cyanobacteria of the genus *Nostoc*, which are present in coralloid roots of trees [7], probably participates in MAM formation in calluses. The exact form in which the fixed nitrogen substance(s) leaves the cyanobacteria and is assimilated by the plant is not known [8].

Although some papers describe callus production in cycads other than Japanese cycad [3], this is the first report to describe callus production from plant tissues of Japanese cycad, and the occurrence in callus of the glucosyltransferase forming cycasin from UDPG and MAM.

EXPERIMENTAL

Cycad seeds were collected in the campus of Kagoshima University. Cycasin was obtained from our laboratory collection.

Prepn of methylazoxymethanol. A pure specimen of MAM was prepd by the method of ref. [9].

Determination of cycasin. Plant tissues and calluses (*ca* 1 g, fr. wt) were homogenized with cold 80% aq. EtOH (− 50°), and the cycasin content was analysed by HPLC [10].

Prepn of crude enzyme solution and assay of activity. A crude enzyme prepn was made from plant tissues or calluses (*ca* 1 g, fr. wt) as described previously [2]. To the enzyme soln (0.8 ml) were added 0.1 ml of 30 mM MAM and 0.1 ml of 250 mM UDPG, and the mixt. was incubated at 30° for an appropriate time, the rate of glucosylation being linear during the incubation time. After the reaction was stopped by heating at 100° for 10 min, a small amount of Dowex 1 × 4 (AcO^-) was added to the mixt. and the cycasin analysed by HPLC [10].

Prepn of cycad seedlings. Japanese cycad kernels were aseptically germinated on cotton layers moistened with sterile H_2O at 28° in the dark for 1–2 months after complete sterilization with 70% aq. EtOH for 1 min and 10% $Ca(OCl)_2$ for 20 min, and then 3 rinsings in sterile H_2O. The epicotyls and roots were used as source materials for callus induction.

Callusing and subculture. Callus medium: 1% agar medium of ref. [4] in one-half the concn containing 3% sucrose, 10 ppm 2,4-D and 0.1% activated charcoal. Subculture medium: 2 ppm 2,4-D was substituted for 10 ppm 2,4-D in the callus medium.

Embryos with suspensors were obtained from kernels sterilized as described above. Epicotyls and roots were excised from the cycad seedlings. Epicotyls were divided into 4 segments. Roots divided in *ca* 1 cm-length were longitudinally bisected. Embryos, epicotyls and roots were separately put on 20 ml of the callus medium in 100 ml conical culture flasks with polypropylene caps. Calluses induced from these tissues and grown at 30° in the dark for 1 month were divided into 2–3 pieces (*ca* 1 g

fr. wt), transplanted separately on 20 ml of the subculture medium in the culture flasks, and subcultured at 30° in the dark for *ca* 1 month. Grown calluses were divided into 2–3 pieces, subcultured again, and used for analyses of cycasin and glucosyltransferase activity and for a MAM-uptake experiment.

REFERENCES

1. Nishida, K., Kobayashi, A. and Nagahama, T. (1955) *Bull. Agric. Chem. Soc. Jpn* **19**, 77.
2. Tadera, K., Yagi, F., Arima, M. and Kobayashi, A. (1985) *Agric. Biol. Chem.* **49**, 2827.
3. Osborne, R. (1990) *Mem. New York Bot. Garden* **57**, 82.
4. Murashige, T. and Skoog, F. (1962) *Physiol. Pl.* **15**, 473.
5. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Pl.* **18**, 100.
6. White, P. M. (1943) *A Handbook of Plant Tissue Culture*. J. Cattell Press, Lancaster, PA.
7. Lindblad, P. (1990) *Mem. New York Bot. Garden* **57**, 104.
8. Pate, J. S., Lindblad, P. and Atkins, C. A. (1988) *Planta* **176**, 461.
9. Kobayashi, A. and Matsumoto, H. (1965) *Arch. Biochem. Biophys.* **110**, 373.
10. Yagi, F., Tadera, K. and Kobayashi, A. (1983) *Agric. Biol. Chem.* **47**, 137.