Biotechnology and Bioprocess Engineering **2006**, 11: 38-42

Identification of Marker Compounds for Discriminating between Embryogenic and Nonembryogenic Calluses of Higher Plants Using Pyrolysis Gas Chromatography Mass Spectrometry and Genetic Programming

Suk Weon Kim[1], Sung Hee Ban[1], Ook Joon Yoo[2], and Jang Ryol Liu[1]*

[1] Laboratory of Plant Cell Biotechnology and Laboratory of Plant Genomics Services, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-333, Korea
[2] Department of Biological Science, Korea Advanced Institute of Science and Technology, Daejeon 305-701, Korea

Abstract When whole cells are subjected to pyrolysis gas chromatography/mass spectrometry (Py-GC/MS) analysis, it provides biochemical profiles containing overlapping signals of the majority of compounds. To determine marker compounds that discriminate embryogenic calluses from nonembryogenic calluses, samples of embryogenic and nonembryogenic calluses of five higher plant species were subjected to Py-GC/MS. Genetic programming of Py-GC/MS data was able to discriminate embryogenic calluses from nonembryogenic calluses. The content ratio of 5-meyhyl-2-furancarboxaldehyde and 5-(hydroxymethyl)-2-furancarboxaldehyde was greater in nonembryogenic calluses than in embryogenic calluses. However, the content ratio of phenol, p-cresol, and ^{1}H-indole in embryogenic calluses was 1.2 to 2.4 times greater than the ratio in nonembryogenic calluses. These pyrolysates seem to be derived from the components of the cell walls, which suggests that differences in cell wall components or changes in the architecture of the cell wall play a crucial role in determining the embryogenic competence of calluses.

Keywords: genetic programming, marker, plant calluses, pyrolysis gas chromatography mass spectrometry (Py-GC/MS)

INTRODUCTION

Somatic cells can be stimulated to undergo embryogenesis in higher plants. This phenomenon has been studied intensively throughout the last few decades. When cultured on medium containing auxin (2,4-dichlorophenoxyacetic acid (2,4-D) is most often used), explants produce two types of morphologically distinct calluses, embryogenic and nonembryogenic calluses. Embryogenic calluses are capable of developing into somatic embryos without additional external stimuli, whereas nonembryogenic calluses do not have the potential to do so. To understand the mechanisms for acquiring embryogenic competence of somatic cells, histological, morphological, and biochemical comparisons of the two types of calluses have been conducted, and have provided a considerable amount of descriptive information, including early biochemical changes associated with embryogenic induction by auxins [1], accumulation of polyamine [2] and arabinogalactans [3], and more extensive accumulation of proteins in embryogenic or nonembryogenic calluses [4]. However, comparison of the two types of calluses at the metabolome level is lacking.

Pyrolysis-gas chromatography in combination with mass spectrometry (Py-GC/MS) thermally destroys samples, breaking them down into volatile fragments (pyrolysates) in a pyrolyzer in a helium atmosphere. The pyrolysates are separated in a gas chromatograph, which are subsequently detected in a mass spectrometer. Some of the advantages of Py-GC/MS are that the method requires simple sample preparation, small samples can be used, and any organic material that fragments into volatile pyrolysates at up to 800°C without fragmentation can be analyzed by mass spectrometry, thereby aiding in the identification of products.

In this study, to understand somatic embryogenesis at the metabolome level, embryogenic and nonembyogenic calluses of five higher plant species were compared by Py-GC/MS data, and chemical compounds that contributed the most to the discrimination of embryogenic calluses from nonembryogenic calluses were deduced from Py-GC/MS data.

Corresponding author
Tel: +82-42-860-4430 Fax: +82-42-860-4608
e-mail: jrliu@kribb.re.kr

Table 1. Plant materials used for Py-GC/MS

Order	Family	Genus	Species	Status[1]
Campanulales	Campanulaceae	*Platycodon*	*grandiflorum*	EC
		Platycodon	*grandiflorum*	NEC
Umbellales	Araliaceae	*Panax*	*ginseng*	EC
		Panax	*ginseng*	NEC
		Acanthopanax	*senticosus*	EC
		Acanthopanax	*senticosus*	NEC
	Umbelliferae	*Coriandrum*	*sativum*	EC
		Coriandrum	*sativum*	NEC
Gentianales	Apocynaceae	*Catharanthus*	*roseus*	EC
		Catharanthus	*roseus*	NEC

[1] Status of calluses, EC: embryogenic calluses; NEC: nonembryogenic calluses.

MATERIALS AND METHODS

Plant Materials

Embryogenic and nonembryogenic calluses of five higher plants (*Platycodon grandiflorum* (Jacq.) A. DC [5], *Acanthopanax senticosus* (Rupr. et Maxim.) Harms, *Catharanthus roseus* (L.) G. Don [6], *Coriandrum sativum* L. [7], and *Panax ginseng* C. A. Meyer [8]) maintained on MS medium [9] supplemented with 3% sucrose and 4.52 μM of 2,4-D were used (Table 1). Embryogenic calluses usually maintain their embryogenic competence for a prolonged period during the subculture. To minimize the effect of differences in culture media, all cultures were subcultured using the same culture medium. After two rounds of subculture, four-week-old calluses were harvested and immediately plunged into liquid nitrogen before homogenizing with a pestle. Homogenized samples were then stored at −70°C until use. Samples were run in triplicate using homogenized callus samples.

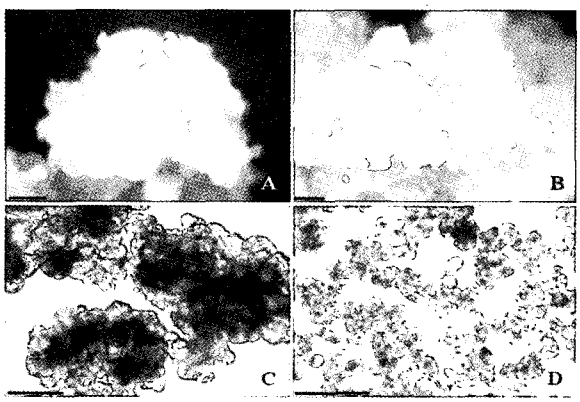

Fig. 1. Embryogenic (A) and nonembryogenic (B) calluses of *Platycodon grandiflorum*. Each callus was cultured on MS medium supplemented with 4.52 μM of 2,4-D. Cell suspension cultures of embryogenic cells (C) and nonembryogenic cells (D) were maintained on MS liquid medium of the same composition, and were subcultured at two-week intervals. Bars represent 200 μm.

Pyrolysis-gas Chromatography Combined with Mass Spectrometry

Freeze-dried calluses (approximately 1 mg) were weighed. Thermal desorption was performed on a Double-Shot Pyrolyzer™ (PY-2020ID, Frontier Laboratories) without any preparation. The thermal desorption temperature was 150~250°C (15 min hold, 20°C/min). Volatile components were trapped at the head of the GC column with the Micro Jet Cryo Trap from Frontier Lab. The Ultra ALLOY™ capillary column (Frontier Lab, PEG 20 M, 30 m, i.d. 0.25 mm, film thickness 0.25 μm) was used for separation. Carrier gas flow rate was 1 mL/min and the split ratio was 50:1. The GC oven temperature was programmed from 40°C (5 min) to 250°C (10°C/min).

Determination of Marker Compounds by Genetic Programming

Genetic programming (GP), an evolutionary technique which uses the concepts of Darwinian selection to generate and optimize a desired computational function or mathematical expression [10,11], was used to determine the marker compounds that distinguish embryogenic and nonembryogenic samples. To determine marker compounds from Py-GC/MS data, the Py-GC/MS chromatogram was first trimmed to 0~20 min. Secondly, to minimize the effect of sample size, Py-GC/MS chromatogram data was normalized according to total ion percentage. Finally, the data point and interval of the Py-GC/MS chromatogram was adjusted using internal standard compounds detected in all Py-GC/MS spectra, including 1-hexene, benzene, and toluene peaks. Genetic programming (Gmax-bio software, Aber Genomic Computing) was used with the following default parameters: population size, 1,000; maximum program length, 44 nodes; fitness, based on tournament selection/Gmax(v); crossover operator used 80% of the time; and of the mutations, terminals were selected 20% of the time. The operators used were the default numeric (0.1, 1, 3, 5, and rand) and arithmetic (1, 2, /, and *) operators.

Table 2. Marker compounds for discriminating embryogenic calluses from Py-GC/MS

Plan species	Status of callus[1]	5-methyl-2-furancarboxaldehyde		phenol		p-cresol		^{1}H-indole		5-(hydroxymethyl)-2-furancarboxaldehyde	
		Content[2] (%)	EC/ NEC[3]	Content (%)	EC/ NEC	Content (%)	EC/ NEC	Content (%)	EC/ NEC	Content (%)	EC/ NEC
Platycodon grandiflorum	EC	0.00076		0.00129		0.00193		0.00234		0.00074	
	NEC	0.00120	0.64	0.00081	1.59	0.00086	2.24	0.00135	1.73	0.00704	0.11
Acanthopanax senticosus	EC	0.00077		0.00152		0.00214		0.00278		0.00090	
	NEC	0.00112	0.69	0.00063	2.41	0.00109	1.95	0.00163	1.71	0.00144	0.62
Catharanthus roseus	EC	0.00056		0.00116		0.00225		0.00226		0.00125	
	NEC	0.00168	0.33	0.00094	1.22	0.00120	1.87	0.00162	1.4	0.00150	0.84
Coriandrum sativum	EC	0.00041		0.00147		0.00279		0.00285		0.00079	
	NEC	0.00193	0.21	0.00104	1.42	0.00159	1.75	0.00160	1.78	0.00198	0.40
Panax ginseng	EC	0.00173		0.00106		0.00176		0.00250		0.00091	
	NEC	0.00209	0.83	0.00065	1.62	0.00073	2.41	0.00122	2.05	0.00483	0.19

[1] Status of callus represents embryogenic (EC) and nonembryogenic callus (NEC), respectively.
[2] The percentage of each pyrolyzed compound from total metabolites was calculated.
[3] Relative ratio of each compound from embryogenic to nonembryogenic callus was calculated.

RESULTS AND DISCUSSION

Morphological Characteristics of Embryogenic and Nonembryogenic Calluses

The representative embryogenic and nonembryogenic calluses of *P. grandiflorum* are shown in Fig. 1. Numerous somatic embryos were developed from embryogenic calluses (Fig. 1A), whereas nonembryogenic calluses had proliferated well without morphological differentiation (Fig. 1B). The other embryogenic and nonembryogenic calluses exhibited similar morphological characteristics (data not shown). Embryogenic cell suspension cultures consisted of small, round cells with dense cytoplasm (Fig. 1C), whereas nonembryogenic cell suspension cultures mainly consisted of largely vacuolated cells with cytoplasm which was less dense (Fig. 1D).

Determination of Marker Compounds for Embryogenic Calluses

Qualitative Py-GC/MS data for calluses were obtained. GP analysis of Py-GC/MS data ranked the top six variables for discriminating embryogenic calluses from nonembryogenic calluses. These variables included V725 (10.921 min), V784 (11.628 min), V968 (13.816 min), V1254 (17.288 min), and V1349 (18.525 min) (Fig. 2). Interestingly, in embryogenic calluses, the percent ratios of phenol, *p*-cresol, and ^{1}H-indole were increased, whereas the ratios of 5-meyhyl-2-furancarboxaldehyde and 5-(hydroxymeyhyl)-2-furancarboxaldehyde were decreased (Table 2). The exact mechanism of phenolic compounds in somatic embryogenesis has not yet been fully studied. Additionally, we were not able to definitively determine which of the various putative compounds, such as cell wall components or secondary metabolites [12], that the pyrolyzed phenolic compounds were derived from. Therefore, an exact conclusion regarding the functional roles of these compounds in numerous metabolic pools for somatic embryogenesis remains to be determined.

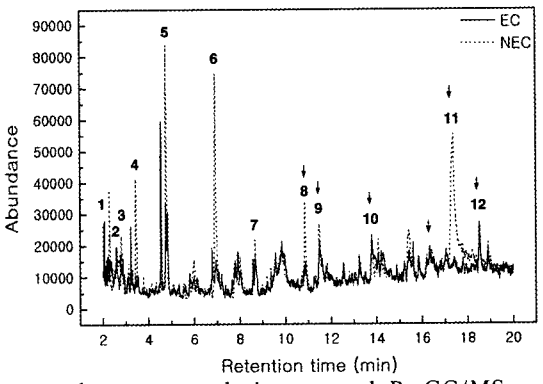

Fig. 2. Marker compounds in averaged Py-GC/MS spectra from embryogenic and nonembryogenic calluses identified by GP analysis. Arrows indicate the six major peaks (marker compounds) of V725 (10.921 min), V784 (11.628 min), V968 (13.816 min), V1195 (16.543 min), V1254 (17.288 min), and V1349 (18.525 min). Numbers represent the compounds identified by GC/MS (1: 1-hexene; 2: 2-methylfuran; 3: benzene; 4: 2,5-dimethylfuran; 5: toluene; 6: 2-furancarboxaldehyde; 7: styrene; 8: 5-meyhyl-2-furancarboxaldehyde; 9: phenol; 10: *p*-cresol; 11: 5-(hydroxymethyl)-2-furancarboxaldehyde; 12: ^{1}H-indole.

In plant systems, peroxidase is likely to play a role in the synthesis of the plant cell wall. The enzyme cross-links phenolic residues of cell wall polysaccharides and glycoproteins, which serve to strengthen the cell wall components and protect against UV illumination [11, 13,14]. Recently, Alemanno *et al.* [15] reported that changes of phenolic compounds occur in embryogenic and nonembryogenic calluses during embryogenic induction. They suggested that embryogenic competence was associated with a balanced concentration of phenolics. In contrast, the total content of phenolic acids was higher in

nonembryogenic cultures compared with embryogenic cultures in *Medicago sativa* [16]. In this study, we examined five different species using Py-GC/MS in combination with GP. The increase of phenol, *p*-cresol, and ^{1}H-indole in embryogenic calluses was a common phenomenon, regardless of the plant species. Taken together with these results, it is suggested that qualitative changes of phenolic compounds in cell wall components from embryogenic calluses play a crucial role in determining the embryogenic competence of calluses.

The Py-GC/MS data were also subjected to principal component analysis (PCA), the most often-used supervised learning method for hyperspectral data analysis. However, PCA was unable to separate the samples into two distinct clusters (data not shown). GP seems more useful when the widely-used statistical modeling methods, such as PCA and partial least square, cannot discriminate samples from different sources [11]. In this study, we demonstrated metabolome level differences between embryogenic calluses and nonembryogenic calluses of higher plants by Py-GC/MS data. Because of their rapidity and simplicity, Py-GC/MS combined with GP can be a powerful method for investigating biological systems at the metabolome level. Furthermore rapid metabolic examination could be applied to high throughput screening of plant cell lines or plant materials for the production of medically important metabolites including decursinol angelate [17] and ginkgolides [18,19].

Acknowledgements This work was supported by a grant (M10104000234-01J000-10710) to JRL from the National Research Laboratory Program, a grant to SWK from the Nano/Bio Technology Development Program through the Genetic Resources and Information Network Center, a grant to JRL from the Korea Science and Engineering Foundation through the Plant Metabolism Research Center of the Kyung Hee University and funded by the Korean Ministry of Science and Technology, and a grant to JRL from the KRIBB Research Initiative Program.

REFERENCES

[1] Komamine, A., R. Kawahara, M. Matsumoto, S. Sunabori, T. Toya, A. Fujiwara, M. Tsukahara, J. Smith, M. Ito, H. Fukuda, K. Nomura, and T. Fujimura (1991) Mechanism of somatic embryogenesis in cell cultures: Physiology, biochemistry and molecular biology. *In Vitro Cell. Dev. Biol.* 28: 11-14.

[2] Bajaj, S. and M. V. Rajam (1996) Polyamine accumulation and near loss of morphogenesis in long-term callus cultures of rice (Restoration of plant regeneration by manipulation of cellular polyamine levels). *Plant Physiol.* 112: 1343-1348.

[3] Saare-Surminski, K., W. Preil, J. P. Knox, and R. Lieberei (2000) Arabinogalactan proteins in embryogenic and non-embryogenic callus cultures of *Euphorbia pulcherrima*. *Physiol. Plant.* 108: 180-187.

[4] Hahne, G. and H. Lörz (1988) Embryogenic and callus specific proteins in somatic embryogenesis of the grass, *Dactylis glomerata*. *Plant Sci.* 55: 267-279.

[5] Kim, S. W. and J. R. Liu (1999) Somatic embryogenesis and plant regeneration in zygotic embryo cultures of balloon flower. *Plant Cell Tissue Organ Cult.* 58: 227-230.

[6] Kim, S. W., D. S. In, P. S. Choi, and J. R. Liu (2004) Plant regeneration from immature zygotic embryo-derived embryogenic calluses and cell suspension cultures of *Catharanthus roseus*. *Plant Cell Tissue Organ Cult.* 76: 131-135.

[7] Kim, S. W., M. K. Park, and J. R. Liu (1996) High frequency plant regeneration *via* somatic embryogenesis in cell suspension cultures of coriander (*Coriandrum sativum* L.). *Plant Cell Rep.* 15: 751-753.

[8] Lee, H. S., S. W. Kim, K. W. Lee, and J. R. Liu (1993) Plant regeneration from transformed somatic embryo-derived protoplasts in Korean sinseng (*Panax ginseng*) and the mitotic stability of the transgene through ontogeny. *Kor. J. Plant Tiss. Cult.* 20: 345-350.

[9] Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.

[10] Banzhaf, W., P. Nordin, R. Keller, and F. Francone (1998) *Genetic Programming - An Introduction.* Academic Press, San Francisco, CA, USA.

[11] Jansen, M. A. K., R. E. van den Noort, M. Y. A. Tan, E. Prinsen, L. M. Lagrimini, and R. N. F. Thorneley (2001) Phenol-oxidizing peroxidases contribute to the protection of plants from ultraviolet radiation stress. *Plant Physiol.* 126: 1012-1023.

[12] Morrison, W. H. and D. D. Archibald (1998) Analysis of graded flax fiber and yarn by pyrolysis mass spectrometry and pyrolysis gas chromatography mass spectrometry. *J. Agric. Food Chem.* 46: 1870-1876.

[13] Fry, S. C. (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu. Rev. Plant Physiol.* 37: 165-186.

[14] Kawaoka, A., E. Matsunaga, S. Endo, S. Kondo, K. Yoshida, A. Shinmyo, and H. Ebinuma (2003) Ectopic expression of a horseradish peroxidase enhances growth rate and increases oxidative stress resistance in hybrid aspen. *Plant Physiol.* 132: 1177-1185.

[15] Alemanno, L., T. Ramos, A. Gargadenec, C. Andary, and N. Ferriere (2003) Localization and identification of phenolic compounds in *Theobroma cacao* L. somatic embryogenesis. *Ann. Bot.* 92: 613-623.

[16] Hrubcova, M., M. Cvikrova, and J. Eder (1994) Peroxidase activities and contents of phenolic acids in embryogenic and nonembryogenic alfalfa cell suspension cultures. *Biol. Plant.* 36: 175-182.

[17] Cho, J. S., J. Y. Kim, I. H. Kim, and D. I. Kim (2003) Effects of polysaccharide elicitors on the production of decursinol angelate in *Angelica gigas* Nakai root cultures. *Biotechnol. Bioprocess Eng.* 8: 158-161.

[18] Park, Y. G., S. J. Kim, H. Y. Jung, Y. M. Kang, S. M. Kang, D. T. Prasad, S. W. Kim, and M. S. Choi (2004) Variation of ginkgolides and bilobalide contents in leaves and cell cultures of *Ginkgo biloba* L. *Biotechnol. Bioprocess Eng.* 9: 35-40.

[19] Park, Y. G., S. J. Kim, Y. M. Kang, H. Y. Jung, D. T.

Prasad, S. W. Kim, Y. G. Chung, and M. S. Choi (2004) Production of ginkgolides and bilobalide from optimized the *Ginkgo biloba* cell culture. *Biotechnol. Bioprocess Eng.* 9: 41-46.

[Received July 22, 2005; accepted September 24, 2005]