JARQ 30, 175-179 (1996)

Identification of mRNAs Differentially Expressed between Embryogenic and Non-Embryogenic Cultivars of Eggplant during Somatic Embryogenesis

John Cliff AFELE*1, Yutaka TABEI*1, Tomohiro YAMADA*2, Takahiro MOMIYAMA*3, Fumio TAKAIWA*1, Toshiaki KAYANO*1, Shigeo NISHIMURA*4, and Takeshi NISHIO*5

*1, 4 Department of Cell Biology, National Institute of Agrobiological Resources (Tsukuba, Ibaraki, 305 Japan)
*2 College of Agriculture, Osaka Prefecture University (Sakai, Osaka, 593 Japan)
*3 Institute of Agriculture and Forestry, University of Tsukuba (Tsukuba, Ibaraki, 305 Japan)
*5 Institute of Radiation Breeding, National Institute of Agrobiological Resources (Ohmiya, Ibaraki, 319-22 Japan)

Abstract

The molecular basis of differential cultivar response to the induction of somatic embryogenesis was investigated by using the digoxigenin (DIG)-differential display with 2 eggplant (*Solanum melongena* L.) cultivars, Wase Shinkuro (WS) and Kumamoto Naga (KN). WS produced embryogenic callus (EC) which was capable of differentiating into mature somatic embryos while KN cultures treated similarly failed to differentiate somatic embryos. DIG-differential display of 0, 1, 2, 4, and 10-day old cultures of both cultivars revealed several PCR products derived from WS, but not from KN, at a crucial stage during embryogenesis. These results indicated that the cultivar difference in EC induction may be attributed, in part, to differences in mRNA expression between the cultivars during the culture process.

Discipline: Biotechnology
Additional key words: DIG-differential display, PCR, Solanum melongena L.

Introduction

Somatic embryogenesis, a phenomenon common to a large number of higher plant species[11], is favorable for the production of an unlimited number of uniform embryos and development of new plant varieties through genetic transformation. However, since not all cultivars within the same species are amenable to the culture process, the contribution of the genetic background to induction of somatic embryogenesis has been investigated in many plant species, including eggplant (*Solanum melongena* L.)[2,9]. Classical genetic models have predicted that only a few genes are responsible for the induction of somatic embryogenesis and that the trait is highly heritable[1,5]. However, the high degree of variability associated with the response of explants to cultural practices *in vitro*[1] restricts precise determination of genetic parameters related to the control of embryogenesis.

We have developed a differential-display technique to identify genes specifically expressed during somatic embryogenesis, using the eggplant cultivar Wase Shinkuro (WS)[8]. This technique is an effective method to isolate and clone individual mRNAs by PCR[6]. Our ultimate goal is to unequivocally identify the molecular basis of embryogenic callus (EC) induction, using eggplant as a model. We have adopted a unique strategy in the pursuit of this objective. The methodology involves 4 sets of conditions: (1) media supplements, for example growth regulator treatments, (2) genotypes, (3) explant tissues, and (4) amino acid supplements, which lead to induction (plus) or suppression (minus) of embryogenesis. This

Present address:
*4 Plant Biotechnology Institute, Ibaraki Agricultural Center (Ago, Nishiibaraki, Ibaraki, 319-02 Japan)

approach would uncover a wide array of cDNA candidate clones involved in various physiological processes culminating in the induction of somatic embryos. cDNA clones differentially expressed within 1 set of plus-minus treatments and which are common to the 3 sets of conditions would be unequivocally implicated in EC induction. The earlier report[8] concerned the first condition above, media supplements, but it involved the use of a radio-isotope, [α-^{32}P]-dCTP, and polyacrylamide denaturing gel, which are difficult to handle. To overcome these shortcomings, we developed an alternative method referred to as DIG-differential display, using digoxigenin (DIG)-dUTP to label PCR products instead of [α-^{32}P]-dCTP[12].

In the present report, we describe the identification of differentially expressed genes between 2 eggplant cultivars, WS and Kumamoto Naga (KN), during the first 10 days of culture, by means of the DIG-differential display technique. Several PCR products which were specifically expressed during the culture process in WS were identified.

Materials and methods

1) Somatic embryogenesis

Somatic embryos were induced from young cotyledon explants in the presence of 45 μM 2,4-dichlorophenoxy-acetic acid (2,4-D), as described previously[8,10], except that the differentiation and maturation medium was modified to contain 10 μM abscisic acid and 10 mM L-glutamine.

2) mRNA isolation, cDNA synthesis, digoxigenin-labeled PCR, and electrophoresis

Explants were sampled from cultures 1, 2, 4, and 10 days following culture initiation and stored at −80°C until they were ready to be used for molecular analyses. Samples were ground in 1 ml ISOGEN™ (Nippon Gene) and total RNA precipitated from the aqueous phase as described by Chomczynski[3]. RNA was also prepared from uncultured cotyledon pieces to establish the background gene expression. Five μg of total RNA served as template in the first-strand cDNA synthesis using an anchored primer of sequence $(T)_{12}GC$[6]. The cDNA, in turn, was used as template for PCR. Two primers, the $(T)_{12}GC$ sequence-anchored primer which was used in the first-strand cDNA synthesis, and a random decamer primer, designated as RA 05[7], whose sequence was 5'-AAGCAGCAAG-3', were used in the PCR.

The PCR method involved the incorporation of digoxigenin (DIG)-labeled dUTP (Boehringer, Mannheim) during the reaction and PCR products were subsequently visualized by the DIG-immunological detection method. The DIG-differential display procedure was essentially the same as that described by Tabei et al.[12], except that the template was cDNA, and also that 2 primers were used. The PCR reaction mixture (20 μl) contained 1 μl template, 1 μl of each primer (20 μM), 0.5 μl dNTP (1 mM each), 0.5 μl PCR DIG-labeling mix (1 mM each), and 1 unit of *Tth* polymerase (Toyobo) in the manufacturer's recommended buffer. The reaction mixture was preheated to 92°C for 1 min prior to 40 cycles of PCR (Iwaki Thermal Sequencer) under the following conditions: denaturation at 92°C for 1 min, annealing at 45°C for 1 min, and extension at 74°C for 2 min, followed by an additional extension at 74°C for 5 min.

PCR products were separated by electrophoresis on 5% polyacrylamide gel, blotted onto nylon membrane (Hybond N$^+$, Amersham) and subsequently stained with the DIG-DNA labeling and detection kit, as recommended by the supplier.

3) DNA isolation and DIG-dUTP-labeled arbitrarily-primed PCR (AP-PCR)

Samples of uncultured cotyledons of both eggplant cultivars were ground in ISOGEN™, and genomic DNA precipitated from the interphase and organic phases, as described by Chomczynski[3]. Two μl of the final DNA solution was used as template for the PCR. The reaction mixture and conditions were similar to those of the DIG-differential display. The primers used were RA 05 and RA 01 (5'-GTCTGACGGT-3')[7].

Results and discussion

Callus formation was visible from cultured tissues by the 5th day after culture initiation and calli proliferated rapidly. Ten days after culture initiation, almost the entire surface of explants became a mass of proliferating calli (Plate 1). WS cultures were slightly more advanced in callus proliferation, and more yellowish in color compared to KN cultures which were slightly pale. Also, WS cell lines produced a higher PCV compared to KN (Table 1) by the end of the cell suspension culture period. On average, about 42 somatic embryos were differentiated from a 100-fold dilution of each 20 μl PCV of WS cultures compared to only 2 somatic embryos

Plate 1. Progression of callus proliferation in cultured cotyledon explants of 2 eggplant cultivars, Wase Shinkuro (top row) and Kumamoto Naga (bottom row), on 2, 3, 4, 7, 9, 10, and 11 days, following culture initiation

Table 1. Frequency of somatic embryogenesis in 2 cultivars of eggplant (*Solanum melongena* L.)

Cultivar	Packed cell volume[a)] (ml)	Frequency of somatic embryos/ 20 μl PCV[b)]
Wase Shinkuro	1.9	41.6
Kumamoto Naga	0.5	2.2

a): Cell suspensions were filtered through 1.0 mm wire mesh and centrifuged. The packed cell volume (PCV) was determined after centrifugation and diluted 100-fold with basal medium.

b): Two ml of the dilution was cultured on differentiation and maturation medium and observed for somatic embryo differentiation 3 to 4 weeks subsequently.

from similar PCV of KN cultures. Thus while WS produced only about 4-fold more PCV than KN, somatic embryo differentiation was about 20-fold higher in the former compared to the latter. The low frequency of embryogenesis in KN could therefore not be attributed to the lower PCV alone. Since the culture procedure was similar for both cultivars, the difference in frequency of embryogenesis was probably due to differential gene expression between the cultivars. Thus, in this report, WS and KN were designated as "embryogenic" and "non-embryogenic" cultivars, respectively.

To investigate the molecular basis of the difference in embryogenic potential between the 2 cultivars, we applied the modified differential-display technique as described in Materials and methods above. The DIG-differential display revealed several fragments that were differently expressed between the "embryogenic" and "non-embryogenic" cultivars during the culture phase (Plate 2). The mRNA species of both cultivars were similar until the 4th day of culture after which the pattern of gene expression changed. Differences in mRNA species and abundance between the 2 cultivars were related to

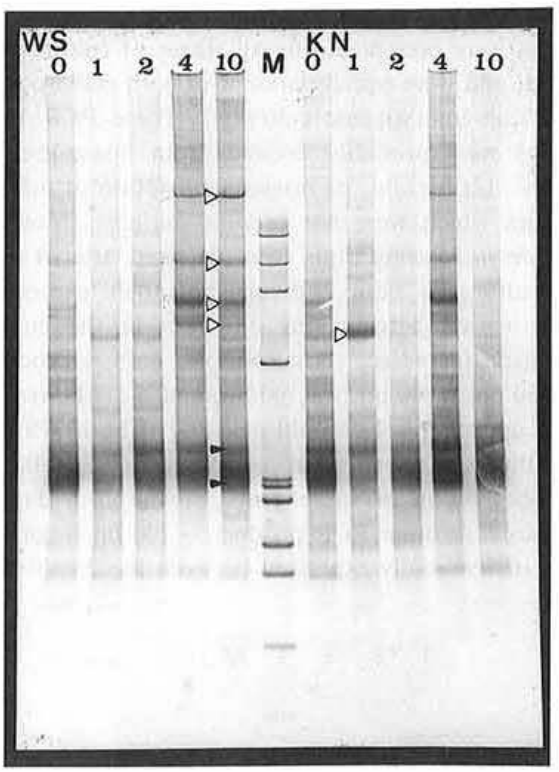

Plate 2. DIG-dUTP-labeled reverse-transcribed PCR products of embryogenic (Wase Shinkuro (WS); left panel), and non-embryogenic (Kumamoto Naga (KN); right panel) eggplant cultivars

Lanes 0, 1, 2, 4, and 10 refer to samples of uncultured cotyledons, and 1, 2, 4, and 10-day old cultures, respectively; lane M indicates ϕX174/*Hae* III digest labeled by DIG-Oligonucleotide 3'-End Labeling Kit. PCR products were separated by electrophoresis on 5% polyacrylamide gel. Putative embryogenesis-related fragments differentially expressed between the 2 cultivars during the culture process are indicated by open arrowheads (▷); PCR fragments constitutively expressed, and common to both cultivars are indicated by solid arrowheads (▶).

EC induction. Callus was observed to be initiated from the cut ends and along the mid-veins of explants by the 5th day of culture but prior to that some significant changes in PCR product patterns were observed, between the 2nd and 4th days of culture (Plate 2). Thus modifications at the gene level preceded visible changes in explant development, particularly callus proliferation.

Generally, 3 categories of genes were expressed, based on the DIG-differential display results (Plate 2)

as follows: (1) First, constitutively expressed genes which were present during all stages of culture analyzed, and were equally present in both embryogenic and non-embryogenic cultivars. These PCR fragments were probably derived from house-keeping genes. (2) Second, *de novo* transcription of mRNA species which were not present initially. Most of the *de novo* transcripts were detected after 4 days of culture in both cultivars but their expression later varied between the cultivars as the culture duration increased. For example, 4 PCR products (−750 bp, −800 bp, −1,100 bp, and >1.4 kbp) were first detected in 4-day old cultures of both WS and KN but only the embryogenic cultivar (WS) retained the expression of these genes by the 10th day of culture. Another PCR product, −700 bp fragment, was differentially expressed between the 2 cultivars.

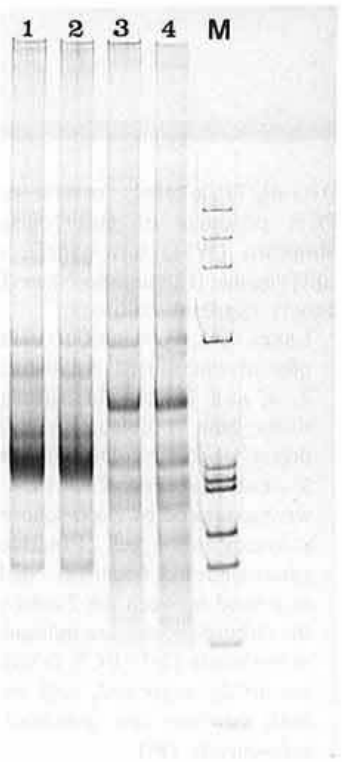

Plate 3. DIG-dUTP-labeled arbitrarily primed-PCR of genomic DNA isolated from uncultured cotyledons of 2 eggplant cultivars, Kumamoto Naga (lanes 1 and 3), and Wase Shinkuro (lanes 2 and 4)

Two μl of the DNA solution was used in the PCR with one random primer, RA 05 (lanes 1 and 2), or RA 01 (lanes 3 and 4), and the PCR products separated by electrophoresis on 5% polyacrylamide gel. M is as indicated in Plate 2.

While this fragment was present in either cultivar from explant until day 2, its expression was significantly enhanced in KN by day 1, compared to WS, and was barely noticeable in the succeeding days. (3) Third, seedling-specific genes, whose expression declined during the culture period, for example, −110 bp fragment, were present in uncultured cotyledons and 1-day old cultures of WS but disappeared by the 2nd day of culture.

Results of AP-PCR of genomic DNA of the cultivars used in the present study did not reveal any major difference between the genomic DNA of the cultivars (Plate 3) and the gene expression pattern was largely similar between the 2 cultivars prior to day 10 of culture. This observation suggested that EC induction could have been initiated in both cultivars, e.g. the first few cell divisions, but subsequent events leading to completion of the process were inhibited in the non-embryogenic cultivar. The present report, and evidence presented earlier[8] from our group suggest that significant changes in PCR products of embryogenic tissues can be detected after 4 days of culture and the expression of these mRNA species remains stable until 10 days after culture initiation. We consider that continuous expression of these transcripts, at least for the first 10 days following culture initiation, reflects the embryogenic condition.

Although the differential-display technique is very sensitive and enables the detection of genes with very low abundance or expressivity, only a few noticeable changes in mRNA species were detected in the present studies. This observation supports previous findings that morphological events related to embryogenesis are controlled by very few modifications of the pre-existing gene program[4] and that the regulation of plant embryogenesis may take place largely via post-transcriptional control, e.g. transcript processing and stability[13].

Investigations of genes involved in somatic embryogenesis have been centered largely on later phases of the process, for example maturation, possibly because genes influencing the early stage of embryogenesis are expressed at very low levels and molecular techniques such as, subtractive hybridization, are not efficient in the detection of these genes. The present study demonstrated that the differential display technique is sensitive to discern the molecular changes associated with successive days of culture, as well as discriminate between embryogenic and non-embryogenic genotypes, very early during the culture process.

Several studies have demonstrated that somatic embryogenesis is partially controlled by genetic factors, some of which are simply inherited[1,5]. In this report we presented evidence that cultivar differences in embryogenesis could be related to differences in mRNA species at the very initial stages of the culture process. Although Northern blot analysis or reverse-transcription-PCR have yet to be performed on the PCR fragments, the pattern of differential display observed using the cultivar difference is supported by a similar pattern observed in our earlier studies using media supplements[8]. Characterization of the putative somatic embryogenesis-related genes which were differentially expressed between the 2 cultivars may provide a more detailed analysis of the influence of genotype on somatic embryogenesis.

Such studies are in progress in our laboratory to fully determine the molecular basis of early events during eggplant somatic embryogenesis, as well as differential cultivar response.

References

1) Afele, J. C. & Kannenberg, L. W. (1990): Genetic studies of corn (*Zea mays* L.) anther culture response. *Theor. Appl. Genet.*, **80**, 459-464.
2) Ali, M., Okubo, H. & Fujieda, K. (1991): In vitro multiplication of intra- and interspecific *Solanum* hybrids through somatic embryogenesis and adventitious organogenesis. *J. Jpn. Soc. Hort. Sci.*, **60**, 601-612.
3) Chomczynski, P. (1993): A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques*, **15**, 532-536.
4) Goldberg, R. B., Barker, S. J. & Perez-Grau, L. (1993): Regulation of gene expression during plant embryogenesis. *Cell*, **56**, 149-160.
5) Hernandez-Fernandez, M. M. & Christie, B. R. (1989): Inheritance of somatic embryogenesis in alfalfa (*Medicago sativa* L.). *Genome*, **32**, 318-321.
6) Liang, P. & Pardee, A. B. (1992): Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, **257**, 967-971.
7) Monna, L. et al. (1994): Determination of RAPD markers in rice and their conversion into sequence tagged sites (STSs) and STS-specific primers. *DNA Res.*, **1**, 139-148.
8) Momiyama, T. et al. (1995): Differential display identifies developmentally regulated genes during somatic embryogenesis in eggplant (*Solanum melongena* L.). *Biochem. Biophys. Res. Comm.*, **213**, 376-382.
9) Rao, P. V. L. (1992): Differences in somatic embryogenic ability of cultured leaf explants of four genotypes of *Solanum melongena* L. *Agronomie*, **12**, 469-475.
10) Saito, T. & Nishimura, S. (1994): Improved culture conditions for somatic embryogenesis using an aseptic ventilative filter in eggplant (*Solanum melongena* L.). *Plant Sci.*, **102**, 205-211.
11) Sterk, P. & de Vries, S. C. (1993): Molecular markers for plant embryos. *In* Synseeds. Application of synthetic seeds to crop improvement. ed. Redenbaugh, K., CRC Press, Boca Raton, 115-132.
12) Tabei, Y. et al. (1996): Development of highly sensitive RAPD method by digoxigenin incorporated PCR: Application for the identification of cultivars. *Breed. Sci.* (In press).
13) Zimmerman, J. L. (1993): Somatic embryogenesis; A model for early development in higher plants. *Plant Cell*, **5**, 1411-1423.

(Received for publication, Nov. 8 1995)