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Short Communication

Differential gene expression in embryogenic and non-embryogenic clusters from cell suspension cultures of *Coffea arabica*

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Summary

Somatic embryogenesis (SE) is a very useful system for studying the differentiation process in plants and involves gene regulation at several levels. During SE induction in *Coffea arabica* cv. Catura Rojo two types of cell clusters, embryogenic (EC) and non-embryogenic (NEC), were observed. The goal of this work was to compare the most relevant characteristics between EC and NEC for a better understanding of the mechanism driving SE. Morphohistological observations indicated a correlation between the morphological features of clusters and their embryogenic competence. On the other hand, no variation at the DNA level, studied by AFLP, were found to explain the disparity in embryogenic competence of clusters, but gene expression, observed by RNA differential display, and SDS-PAGE showed differences that can explain that disparity. Our results lead us to propose that differential gene expression can modulate the embryogenic capacity of coffee cells and that the number of genes turned off in somatic cells to allow for the change from a somatic to an embryogenic state, is higher than those genes that are turned on.

Key words: *Coffea arabica* – embryogenic cluster – non-embryogenic clusters – somatic embryogenesis

Abbreviations: EC = embryogenic cluster. – NEC = non-embryogenic cluster. – SE = somatic embryogenesis

Introduction

Somatic embryogenesis (SE) is a feasible model for studying the differentiation process in plants. In contrast to its zygotic counterpart, SE can easily be observed, the culture conditions can be controlled and large quantities of embryos can be obtained. SE can be used for the study of early morphogenetic and molecular events occurring during embryogenesis in higher plants. The embryogenic competence of cultures is often a characteristic confined to a very limited fraction of the cell population (Nomura and Komamine 1985) or to cells in discrete zones on clusters (Vasil and Vasil 1981). Although in many cases the physical features may be sufficient for the distinction between embryogenic and non-embryogenic clusters, they do not provide information for studying the mecha-

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nisms involved in plant cell differentiation, especially in the process of SE induction.

Somatic embryogenesis has been reported for the two most important species of *Coffea* genus (Staritsky 1970, Herman and Haas 1975). However, even now, very little is known about the mechanisms of somatic embryogenesis induction in these models. The aim of the present study was to analyze the most relevant characteristics between embryogenic and non-embryogenic clusters of *Coffea arabica* at different levels. These cluster types had the same origin and were cultured under the same conditions. In this work we report some morphological, molecular and biochemical differences in both types of clusters that can explain the disparity in embryogenic competence observed.

Materials and Methods

Plant material and culture conditions

Embryogenic cell suspension cultures of *Coffea arabica* cv. Caturra Rojo were maintained and embryogenesis was initiated as previously described by Quiroz-Figueroa et al. (2002).

AFLP analysis

For AFLP development, a modification of the procedure originally reported by Vos et al. (1995), was used, omitting the first pre-selective PCR. A binary matrix was obtained from scored band patterns by absence (0) or presence (1) of bands. The bootstrap was calculated, employing 500 repetitions and 25 % of the resampled binary matrix, using the Phylogenetic Computer Tools for Windows ver. 1.18. The average bootstrapped matrix was used to calculate the similarity matrix using the formula reported by Nei and Li (1979). The similarity matrix was analyzed using the DICE coefficient and the clusters were analyzed by the Unweighted Pair-Group Method (UPGMA), as reported by Saitou and Nei (1987). Bootstrap data were used for the statistical analysis using STATISTICA for Windows (StatSoft, Inc., I 1998).

Isolation of total RNA and protein

TriPure® Isolation Reagent (Boehringer, Mannheim) was used for isolation of total RNA and protein, as recommended by the supplier, from 100 mg (dry weight) of the different tissues. RNA integrity was checked by electrophoresis in agarose gels and by ethidium bromide staining. Proteins were resuspended in 0.3 mL of 50 mmol L^{-1} sodium phosphate buffer (pH 7.5) containing 250 mmol L^{-1} sucrose and 2.5 mmol L^{-1} EDTA.

RNA differential display

This study was performed as described previously by Rojas-Herrera et al. (2002). The first strand was synthesized from 2 µg of the total RNA, using 2.5 µmol L^{-1} of oligo$(dT)_{13}$AA as anchor primer and incubated for 1 hour at 37˚C. PCR reaction was performed using 2.5 µL of first strand reaction as the template. The amplification products were loaded in a polyacrylamide sequencing gel (5 %) and exposed at –80˚C in cassettes with an intensifier screen using Hyperfilms (Kodak).

Protein quantification and SDS-PAGE electrophoresis

The protein content was estimated by Peterson's method (Peterson 1977) using BSA (Sigma, USA) as standard. SDS-PAGE on 10 % gel was performed essentially as described by Laemmli (1970). Fifteen µg of protein was loaded in each lane. Bands were visualized by staining with ammoniacal-silver according to Wray et al. (1981).

Results and Discussion

During SE induction two distinguishable clusters, embryogenic (EC) and non-embryogenic (NEC), growing under the same condition were observed. EC was brown, embryos emerged from day 12, and up to 30 somatic embryos per cluster were scored. NEC emerged from EC around day 16 after induction; they were pale-yellow and did not give rise to somatic embryos.

Browning of tissues, probably caused by the accumulation of phenolic compounds, seems to be necessary for the somatic embryogenesis process in coffee and may be a very useful visual marker to distinguish between embryogenic and non-embryogenic cultures. Both embryogenic and non-embryogenic cells are quite similar at the histological level but they differ in cell size and in the presence of a larger quantity of inclusion bodies in non-embryogenic cells (Fig. 1).

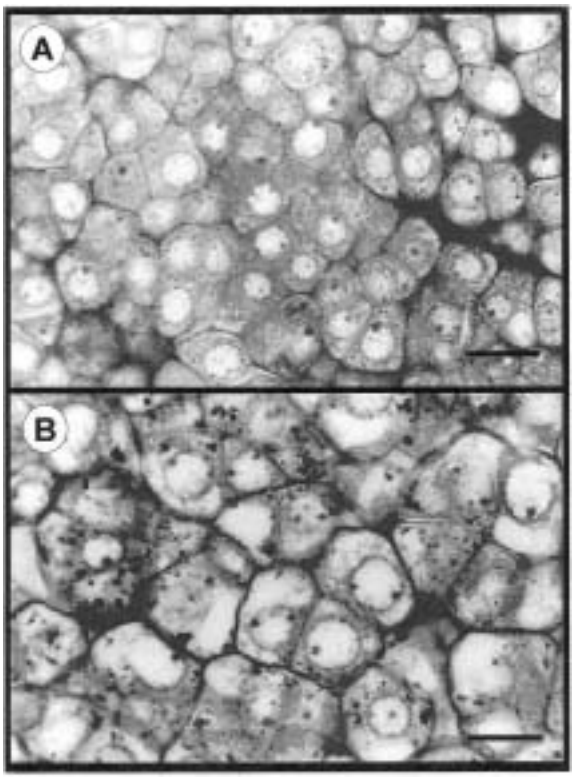

Figure 1. Histological aspect of embryogenic and non-embryogenic clusters. **A** Cross-section of embryogenic cluster. **B** Cross-section of non-embryogenic cluster. All sections were stained with toluidine blue. Bars = 20 µm.

Our histological observations indicated a correlation between cell types and embryogenic capacity of clusters, agreeing with the study by Nomura and Komamine (1985) in *Daucus carota* suspension cultures. However, Menéndez et al. (1994) and Tahara et al. (1995) observed the presence of elongated and highly vacuolated cells in the non-embryogenic callus in contrast to embryogenic cells, which resemble meristematic cells. A third set of observations shows that the capability of individual single cells to form somatic embryos is not restricted to a particular distinguishable cell type based on its morphology (Toonen et al. 1994). It is possible that the observed differences are the consequence of the systems used. The presence of two cell clusters coming from the same origin and growing in the same medium may represent an advantage over other systems where embryogenic and non-embryogenic lines are usually obtained from different sources (Jiménez and Bangerth 2001) or culture conditions (Grotkass et al. 1995). The differences found could therefore be attributed to the presence or absence of a particular substance in the medium as well as other factors caused by the explant source.

According to Lo Schiavo et al. (1989) culture conditions could induce changes in genetic information at the DNA level. To test this hypothesis, AFLP markers were obtained employing 11 primer combinations. A total of 987 bands were scored, resulting in 85 (8.61%) polymorphic bands. The average number of bands per combination of primers was 89; moreover, both the total and the polymorphic number of bands varied according to the primer combination analyzed. The similarity index between EC and NEC was 0.994. Several factors concerning tissue culture-induced variation, such as genotype (Söndahl and Bragin 1991), explant source (Van den Bulk et al. 1990), growth regulators employed (Van den Bulk et al. 1990) and age of culture (Müller et al. 1990) have been reported. Nevertheless, our results suggest that the difference in embryogenic capacity between EC and NEC might not be due to DNA variation. However, DNA methylation status could be involved in driving the genetic program toward the embryogenic pathway. To our knowledge this is the first evidence that embryogenic and non-embryogenic clusters are equal at the DNA level, as determined by AFLPs.

To assess whether there were differences in the gene expression pattern between EC and NEC, samples of RNA from day 16 and day 20 after embryogenesis induction were compared by differential display analysis (Liang and Pardee 1992). Nine combinations of primers were employed and a total of 295 bands in EC and 280 bands in NEC were scored. It is important to note that down-regulation is more abundant in both clusters (3.3% of total scored bands) and the number of regulated genes in EC is much higher (6.8%) than in NEC (3.9%). On the other hand, 17 specific bands were observed in the EC but there were only 3 in the NEC (Table 1). Our results lead us to propose that differential gene expression can modulate the embryogenetic capacity of coffee cells and that the number of genes turned off in somatic cells, to allow for the change from a somatic to an embryogenic state, is higher than those genes that are turned on.

Table 1. Comparison by differential display analysis of the gene expression patterns from embryogenic and non-embryogenic clusters.

	Embryogenic clusters	Non-embryogenic clusters
Total Bands	295	280
Invariant	275 (93.22%)	269 (96.07%)
Up regulated	7 (2.37%)	5 (1.78%)
Down regulated	13 (4.40%)	6 (2.14%)
Specific	17	3

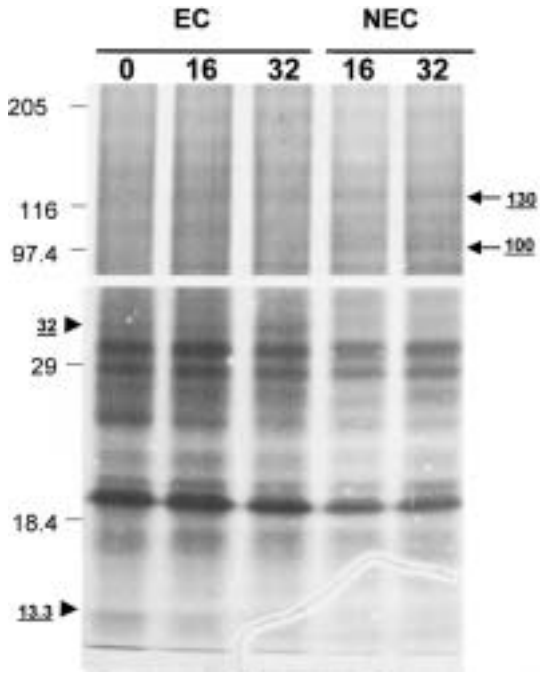

Figure 2. Sodium dodecyl sulfate-polyacrilamide gel electrophoresis profile of intracellular proteins from *Coffea arabica* embryogenic and non-embryogenic clusters. Numbers at the top are days after embryogenesis induction. Numbers in the left margin refer to the kiloDalton values of marker proteins. Underlined number in both margins refer to the molecular weight of the specific proteins in embryogenic (headarrows) and non-embryogenic (arrows) clusters. Analysis was done using 10% SDS-PAGE.

We analyzed the total protein patterns between EC and NEC by SDS-PAGE. In both clusters, almost the same pattern was found (Fig. 2) except for minor qualitative differences. Two proteins of 13.3 and 32 kDa (arrowheads) were specific in EC and others of 100 and 130 kDa (arrows) in NEC. Other proteins, such as those of 19, 27 and 29 kDa, were consistently expressed in both clusters. In several species, some proteins are expressed specifically during the development of somatic embryos (Sung and Okimoto 1981, Stirn and Jacobsen 1987). The close similarity observed in the total protein

patterns may be due to the fact that clusters have a common origin, but the presence of specific proteins in EC and NEC could indicate that these may be involved in the determination process of embryo formation. These specific proteins could thus serve as biochemical markers for assessing the embryogenic capacity of *in vitro* cultures. Nevertheless, further biochemical studies will be necessary to understand the nature and role of these proteins.

In conclusion, *in vitro* cultures having EC and NEC from the same origin and cultured under the same conditions, offer the possibility of carrying out comparative studies and searches for pathways involved in the complex process of somatic embryogenesis that will allow for a better understanding of the mechanism that drive SE in higher plants.

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References

Grotkass C, Lieberei R, Preil W (1995) Plant Cell Rep 14: 428–431
Herman EB, Haas GJ (1975) HortScience 10: 588–589
Jiménez VM, Bangerth F (2001) Plant Sci 160: 247–257
Laemmli UK (1970) Nature 227: 680–685
Liang P, Pardee AB (1992) Science 257: 967–971
Lo Schiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V, Marazziti D, Vergara R, Orselli S, Terzi M (1989) Theor Appl Genet 77: 325–331
Menéndez A, de Garcia EG, Nieto MS (1994) Plant Cell Rep 13: 197–202
Müller E, Brownn PTH, Hartke S, Lörz H (1990) Theor Appl Genet 80: 673–679
Nei M, Li WH (1979) Proc Natl Acad Sci USA 76: 5269–5273
Nomura K, Komamine A (1985) Plant Physiol 79: 988–991
Peterson GL (1977) Anal Biochem 83: 346–356
Quiroz-Figueroa FR, Fuentes-Cerda CFJ, Rojas-Herrera R, Loyola-Vargas VM (2002) Plant Cell Rep 20: 1141–1149
Rojas-Herrera R, Quiroz-Figueroa F, Monforte-González M, Sánchez-Teyer F, Loyola-Vargas VM (2002) Mol Biotech 20: 43–50
Saitou N, Nei M (1987) Mol Biol Evol 4: 406–425
Söndahl MR, Bragin A (1991) 14^{e} Colloque de la Association Scientifique Internationale du café. San Francisco, ASIC, pp 701–710
Staritsky G (1970) Acta Bot Need 19: 509–514
Stirn S, Jacobsen H-J (1987) Plant Cell Rep 6: 50–54
Sung ZR, Okimoto R (1981) Proc Natl Acad Sci USA 78: 3683–3687
Tahara M, Nakanishi T, Yasuda T, Yamaguchi T (1995) 16^{e} Colloque Scientifique International su le café. Kyoto, ASIC, pp 860–867
Toonen MAJ, Hendriks T, Schmidt EDL, Verhoeven HA, Van Kammen A, De Vries SC (1994) Planta 194: 565–572
Van den Bulk RW, Lofer HJM, Lindhout WH, Koornneef M (1990) Theor Appl Genet 80: 817–825
Vasil V, Vasil IK (1981) Amer J Bot 68: 864–872
Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) Nucleic Acids Res 23: 4407–4414
Wray W, Boulikas T, Wray VP, Hancock R (1981) Anal Biochem 118: 197–203