PROTEIN CHANGES ASSOCIATED WITH SOMATIC EMBRYOGENESIS IN COTTON (*Gossypium hirsutum* L.)

H. Vandenhout
Laboratory for Tropical Crop Improvement, KU Leuven
Leuven, Belgium

Abstract

Protein samples were extracted from callus and cell suspensions at different developmental stages of the non-commercial Cotton varieties Coker 312, Coker 100 and Delta Pine 15 (*Gossypium hirsutum* L.). Proteins were separated by two-dimensional gel electrophoresis (IEF- PAGE-SDS) and silver stained. Gels were analysed with the ImageMaster software (Pharmacia-Biotech). Protein patterns differed with the level of tissue development. In the case of Coker 312, suspension cultures are characterised by a peek in total spot amount, while callus cultures showed abundant proteins in a low range of molecular weight during early embryogenesis.

The aim of the study is to provide a tool for the identification of proteins specific for somatic embryogenesis and regeneration.

Introduction

Most cotton varieties show a low regeneration capacity and produce few or no somatic embryos in vitro, what results in very slow proceeding of genetic engineering in cotton.

Some differences in protein patterns are indicative for the specific regulation of proteins biosynthesis in zygotic embryos, as shown by one-dimensional analysis (Dure and Galau, 1981). Comparison of total protein extract of embryogenic versus non-embryogenic explants (Pedroso *et al.*, 1995) led to the identification of 2 polypeptides in *Camellia japonica* with a well-described function in the process of somatic embryogenesis. Puüponen-Pimiä (1993) reported of an embryogenic gene found in birch by protein analysis.

Analysis of intracellular proteins of tissue culture of Coker 312, and its parent lines Coker 100 and Delta Pine 15, enables us to search for protein pattern relationships between embryogenic and non-embryogenic cultures. The outcome of this study can provide a tool for detecting proteins involved in embryogenesis in cotton.

Materials and Methods

Tissue Culture and Medium

Sterilised seeds of 3 varieties, Coker 312, Coker 100 and Delta Pine 15, were germinated in tubes on medium with 2.3g/l Murashige and Skoog salts, 30g/l glucose, 2.5g/l fytagel and 0.75g/l magnesium chloride (pH 5.8). 50% of the seeds were placed on medium with additional 50µM IAA, to check the embryogenic response (Ivanova *et al*. 1994).

Hypocotyl segments of the 3-5 days old plantlets were placed on an analogue medium with 4.6g/l Murashige and Skoog salts, and additional 2.5µM kinetine and 0.5µM 2.4D (Trolinder and Goodin, 1987). After 8 weeks, part of the obtained callus was subcultured in a medium without hormones, which was repeated every 6 weeks to obtain embryogenic cultures. Another part of the callus was subcultured in an analogue liquid medium, called indirect suspension culture, and was subcultured every 3-4 weeks.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1439-1441 (2001)
National Cotton Council, Memphis TN

Direct suspension culture was derived from chopped cotyledons of the original plantlets and grown in MS medium with 2.5µM kinetine and 0.5µM 2.4D for 10 weeks with 2 subcultures in between. The same treatment was used as described for indirect suspension culture. Cultures were kept in acclimatised rooms with 16h-photo period (50-70µE/m^2s and 28±2°C).

Prior to each treatment, morphological and physiological observations of the cultures were done.

Protein Extraction

Protein extracts of each culture were taken at different stages of development. Protein extraction was carried out according to Mayer *et al.* (1987). Samples were stored at -75°C immediately after extraction. Samples were thawed at 37°C and kept on ice prior to use (Copeland, 1994).

Two-Dimensional Gel Electrophoresis

According to O'Farell (1975) and O'Farell *et al* (1977), first dimension IEF was carried out. Running-voltages were: 10'-500V; 60'-750V and 150'-850V. 2D –SDS-PAGE standards (Biorad) and carbamylyte carbonic anhydrase (Pharmacia-Biotech) were used. Second dimension SDS-PAGE had a running-voltage of 37'-200V. Silver stain SDS-PAGE standards in low range (Bio-Rad) were used. Gels were silver stained with silver staining kit (Amresco) and air-dried on individual vertical frames (Novex-10P's).

Protein Analysis

Analysis was done on stained and dried gels, with ImageMaster software package (Pharmacia-Biotech). Gel series were formed by a set of gels from each cell culture with protein extracts of different development stages. Gels were scanned and individually treated for spot detection, background subtraction and spot measurement. In each series, a reference gel was prepared to match the other gels in the series. Spots were calibrated bases on the markers of 2D-gel electrophoresis. Gels were characterised by individual spot data on volume, difference, matching and calibration results. Gels were divided in 12 areas with molecular weight between 97.8-45.0-31.0-21.5-14.0 kD and pI from 3-6, 6-7 and 7-10. Series of gels were evaluated and compared with each other.

Results and Discussion

Morphology

Observation of the tissue cultures of Coker 312 during their growth showed a clear transition from induction over an embryogenic phase, followed by a post-embryogenic phase.

All suspension cultures derived from chopped cotyledons (direct suspension) and from callus (indirect suspension) showed somatic embryogenic cells 23-24 weeks after induction. Growing and formation of somatic embryos stopped 39-40 weeks after induction.

Callus cultures showed the first embryogenic cells 25-31 weeks after induction and continued growing and forming embryos until the end of the experiment, 55 weeks after growth induction.

No influence of IAA in the medium of the seedlings was found on embryogenic response.

Total Number of Protein Spots

The total number of spots in the successive gels of each series of Coker 312 showed a different pattern for suspension and callus. Indirect and direct suspensions show a peek in spot amount during early embryogenesis (figure 1). A first peek with new spots appeared at gel 3 and 4, where embryogenic capacity was first noted in the morphological observations. Direct suspension B showed a re-establishment of spot amount at the end in the series, and indirect suspension IAA showed a second peek, although embryogenic growth was reduced and even ending at that stage.

Callus cultures showed a rather diffuse phase during embryogenesis in gel 3-4, with few spots and a reverse graphic compared to suspension cultures. The phase directly after the first formation of somatic embryogenic cells showed a re-establishment of the number of cells.

No differences were found between cultures derived from seedlings grown on blank medium and these grown on medium with additional IAA in relation to relative spot numbers during early embryogenesis. Although graphics differed between series, no clear connection with embryogenic capacity was found.

Protein Changes during Somatic Embryogenesis

Suspension cultures showed an important increase of new spots in gel areas 4, 7 and part of 5 during early embryogenesis (figure 3). After the embryogenic phase areas 7, 10 and parts of 8 and 11 showed an individual peek of new spots. When matched spots were compared in the gel representing the embryogenic phase with the spots in the gel prior to this phase, areas 4, 5 and 7 showed the most spots increased in volume.

Callus cultures showed an important region of new spots in areas 5 and 6 prior to early embryogenesis, while new spots appeared in areas 7 and 10 immediately after morphological observation of first embryogenic capacity. During early embryogenicity, areas 7, 8 and 10 showed most spots increased in volume compared to the stage prior to the formation of embryogenic cells. In areas 10, 11 and 12 appeared most cells with decreased volume during early embryogenesis.

No influence of IAA in the medium of seedlings was found on protein changes during somatic embryogenesis.

Gel series of the varieties Coker 100 and Delta Pine 15 were analysed (data not shown) and are to be evaluated to compare the results with these presented here.

Conclusions

Suspension cultures of Coker 312 were characterised by a peek in total spot amount, while callus cultures showed abundant proteins in low molecular weight range during early embryogenesis.

In the case of suspension tissue cultures, a peek in total number of spots appeared in the gel where embryogenic capacity first was observed. A reverse picture appeared for callus cultures, where a rather diffuse transition characterised the embryogenic phase. Abundant proteins were found during early embryogenesis in callus cultures. These matched spots were changing in volume. Most increased spots appeared in the area with molecular weight between 31.0 and 14.0 kD and pI between 3 and 6, and the area between 31.0-21.5 kD and pI 6-7. Most decreased spots where found in the area with the lowest molecular weight between 21.5 – 14.0 kD over the whole pI range.

In all gel series of Coker 312, a clear classification of pre-, and post-embryogenic phases occurred, with an embryogenic transition phase in between.

The induction phase, prior to embryogenesis, occurred in the acid region with a low molecular weight (>31 kD and pI<6). The embryogenic phase showed characteristic protein spots in the acid to neutral region, with intermediate molecular weights (45.0-21.5 kD and pI <7) while a post-embryogenic phase showed abundant proteins in the lower molecular weight range (<31 kD).

Tissue cultures were derived either from seedlings grown on blank medium or from medium with additional IAA. No influence of IAA was found on protein changes associated with embryogenic response.

References

Copeland, R.A. 1994. Methods for protein analysis. A practical guide to laboratory protocols. Chapman and Hall, New York.

Dure III, L., G.A Galau. 1981. Developmental biochemistry of cotton seed embryogenesis and germination, XIII. Regulation of the biosynthesis of the principal storage proteins. Plant Physiology 68:187-194.

Ivanova, A., M. Velcheva, P. Denchev, A. Atanassov, H.A. Van Onckelen. 1994. Endogenous hormone levels during direct somatic embryogenesis in *Medicago falcata*. Physiologia Plantarum 92:85-89.

Mayer, J.E., G. Hahne, K. Palme, J. Schell. 1987. A simple and general plant tissue extraction procedure for two-dimensional gel electrophoresis. Plant Cell Reports 6:77-81.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250:4007-4021.

O'Farrell, P.Z., H.M. Goodman, P.H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12:1133-1142.

Pedroso, M.C., J.L. Hilbert, J. Vasseur, M.S. Pais. 1995. Polypeptides associated with the induction of direct somatic embryogenesis in *Camellia japonica* leaves. I. Identification of embryo-specific polypeptides. Journal of Experimental Botany vol. 46, 291: 1579-1584.

Puupponen-Pimiä, R., M. Saloheimo, T. Vasara, R. Ra, J. Gaugecz, U. Kurtén, J.K.C. Knowles, S. Keränen, V. Kauppinen. 1993. Characterization of a birch (*Betula pendula* Roth.) embryogenic gene, BP8. Plant Molecular Biology 23:423-428.

Trolinder, N.L., J.R. Goodin. 1987. Somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Reports 6:231-234.

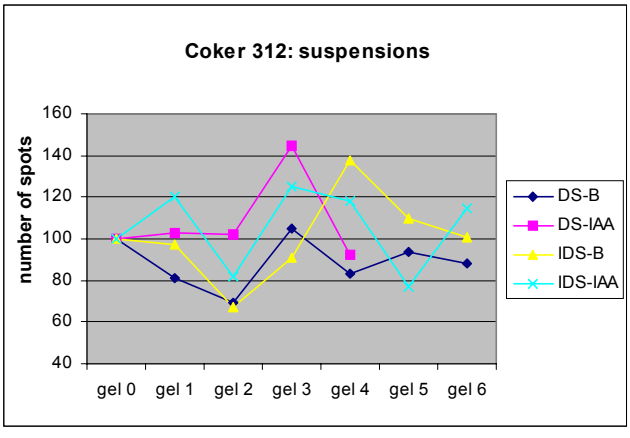

Figure 1: Total number of spots in each gel of suspension series of Coker 312. DS: direct suspension, IDS: indirect suspension, B: blank seed germination medium, IAA: seed germination medium with 50µM IAA.

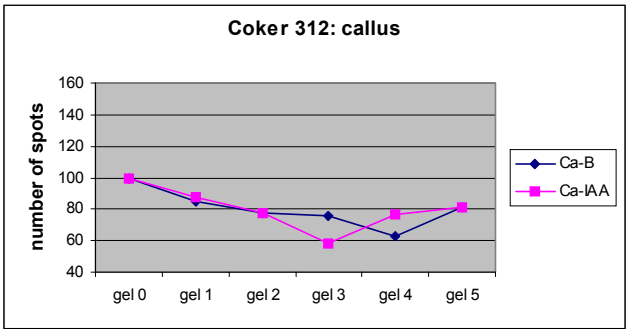

Figure 2: Total number of spots in each gel of callus series of Coker 312. B: blank seed germination medium, IAA: seed germination medium with 50µM IAA.

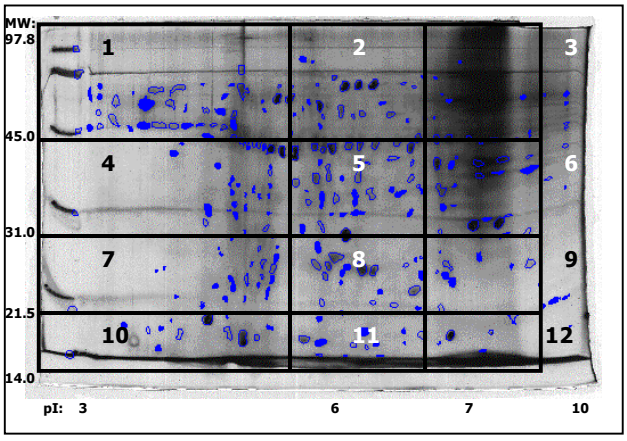

Figure 3: Example of gel building image after analysis. Molecular weight range: 97.5-14.0 kD and pI from 3-10. Gel areas: 1-12 with area 1 most acid and highest molecular weight; area 12 most alkalic with lowest molecular weight.