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DNA METHYLATION AND GENE EXPRESSION DIFFERENCES DURING ALTERNATIVE *IN VITRO* MORPHOGENETIC PROCESSES IN EGGPLANT (*SOLANUM MELONGENA* L.)

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Summary

In vitro-cultured cotyledon explants of eggplant (*Solanum melongena* L.) are capable of producing somatic embryos or shoots, depending on which growth regulators are exogenously applied (auxins versus cytokinins). Rapidly growing suspension cultures were established to model these alternative morphogenetic processes. Clear differences in DNA methylation and gene expression patterns could be detected when suspension cultures grown under different growth regulator regimes were compared by restriction fragment length polymorphism (RFLP) or Differential Display analyses. Some of the random genomic clones showing methylation pattern differences, as well as some differentially expressed transcripts were analyzed in detail. One of the genomic clones, SMG56, proved to be the first identified 1-aminocyclopropane-1-carboxylate (ACC) synthase gene in eggplant. From 15 cloned Differential Display fragments two somatic embryogenesis (SME39, SME50) and one organogenesis (SME88) related transcripts were identified.

Key words: somatic embryogenesis; organogenesis; Differential Display; ACC synthase.

Introduction

The two major types of plant growth regulators, auxins and cytokinins, interact in a complex manner to control many aspects of growth and differentiation. Under *in vitro* conditions explants can be induced to form organs (mainly shoots or roots) or bipolar embryos. Plant growth regulators play a principal role in these processes. A strong shock by the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), for example, is capable of restarting the ontogenetic program in cultured plant cells (Dudits et al., 1995). The primary action of growth regulators during *in vitro* differentiation occurs through the influencing of gene expression at the transcription level, but epigenetic processes such as changes in the level and state of DNA methylation and transient changes in repetitive DNA content have also been shown to be substantial indicators of the activities of auxins and cytokinins (LoSchiavo et al., 1989; Arnholdt-Schmitt et al., 1991; Munksgaard et al., 1995).

Eggplant (*Solanum melongena* L.) is a suitable object for studying alternative morphogenetic processes, because *in vitro* plant regeneration can be obtained through direct organogenesis, as well as through somatic embryogenesis from cotyledon explants, depending on which growth regulators are exogenously applied (Matsuoka and Hirata, 1979; Fári et al., 1995). In this paper, we present data of comparative investigations on growth regulator-induced changes during these alternative differentiation processes of eggplant *in vitro* cultures. Special attention was given to DNA methylation and transcriptional differences.

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Materials and Methods

Plant material and tissue culture. Seeds of *Solanum melongena* L. cv. 'Kecskeméti lila' were surface-sterilized and placed onto MS medium (Murashige and Skoog, 1962). Germination occurred at 25°C under 16/8 h photoperiod. Cotyledons were harvested from 7–10-d-old aseptic seedlings, halved along the midvein and placed onto MS medium supplemented with 0.5 mg l^{-1} picloram or 4 mg l^{-1} α-naphthaleneacetic acid (NAA) to induce somatic embryogenesis, or 1 mg l^{-1} 6-benzyladenine (BA) or 1 mg l^{-1} kinetin to induce organogenesis. Suspension cultures were continuously maintained in B5 liquid medium (Gamborg et al., 1968) containing 0.5 mg l^{-1} picloram by regular subcultures (5–10-fold dilutions) at 5–7 d intervals. Cells from pro-embryogenic and organogenic cultures were collected, frozen in liquid nitrogen, and stored at −70°C for further analysis.

DNA isolation and Southern hybridization. Genomic DNA was extracted as described by Dellaporta et al. (1983). Southern analysis was performed according to Bucherna et al. (1999).

Production of eggplant genomic clones. A partial genomic library was established by cloning of random *Pst*I restriction fragments from eggplant leaf nuclear DNA in the plasmid vector pBluescriptSK+ (Stratagene). Recombinant plasmids that contained inserts between 0.4 and 4 kb in length were selected for further analysis, numbered, and designated as SMG clones.

RNA isolation and Northern hybridization. Frozen plant material was ground to fine powder in liquid nitrogen after addition of sterile quartz sand. Powdered tissue was homogenized in extraction buffer (4 *M* guanidine thiocyanate, 25 m*M* sodium citrate buffer pH 7.0, 0.5% Sarkosyl, 100 m*M* β-mercaptoethanol). After two phenol/chloroform extractions RNA was precipitated by adding 8 *M* LiCl to a final concentration of 2.7 *M*. After centrifugation at 10 000 rpm for 30 min at 4°C, RNA was washed twice with 3 *M* sodium acetate and once with cold 70% ethanol. Dried RNA pellets were dissolved in formamide and stored at −70°C. For Northern analysis, about 30-μg RNA samples were separated on a formaldehyde agarose gel and blotted onto Hybond-N membranes (Amersham, Germany). cDNA clones were labeled with [α-^{32}P]dCTP using the random priming method of Feinberg and Vogelstein (1982) and used as probes. Hybridizations were carried out under standard conditions at 65°C.

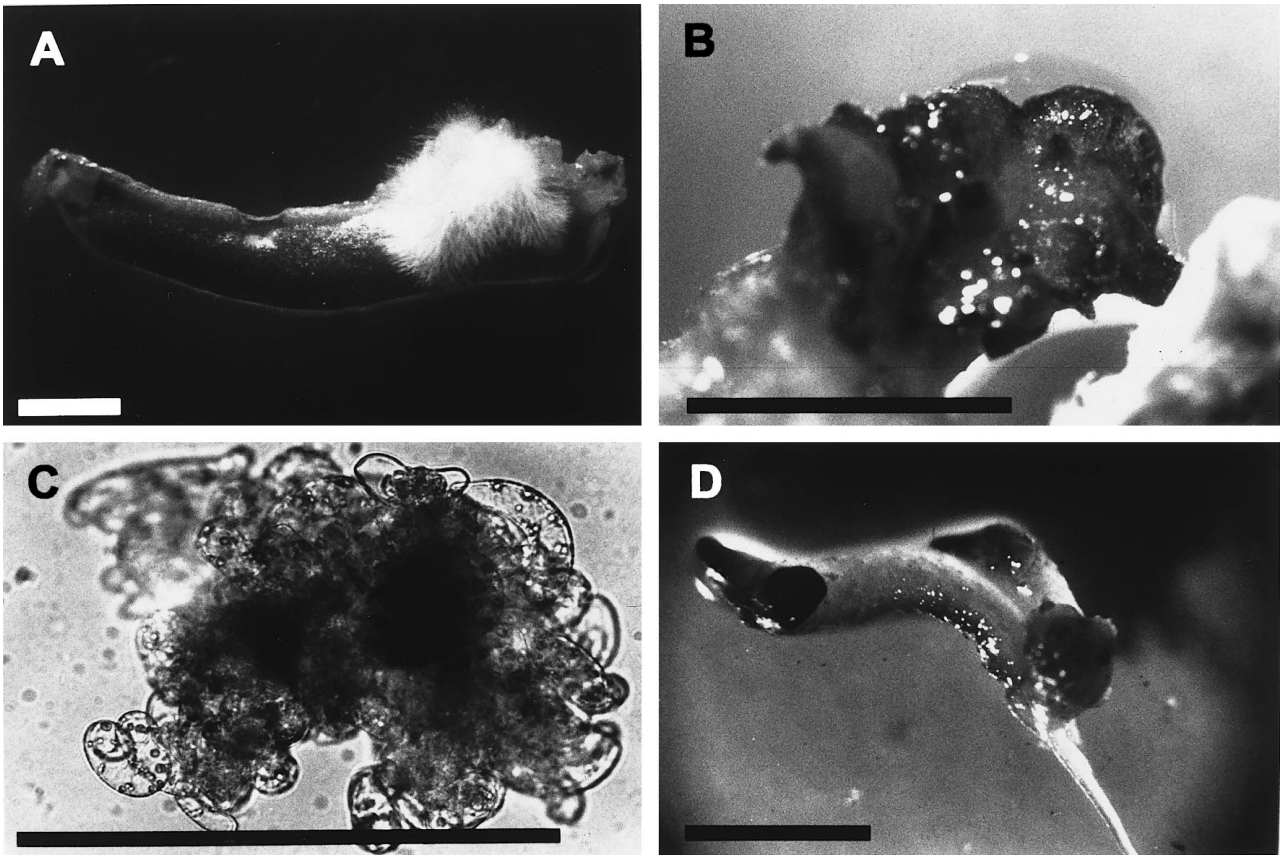

FIG. 1. Somatic embryogenesis (*A*) and organogenesis (*B*) from longitudinally halved cotyledon explants of *Solanum melongena* (*A*, *bar* = 5 mm; *B*, *bar* = 3 mm). *C*, Cell colony from a rapidly growing suspension culture grown in B5 liquid medium containing 0.5 mg l^{-1} picloram (*bar* = 1 mm). *D*, Shoot induction from suspension culture-derived cell colonies in B5 liquid medium containing 1 mg l^{-1} BA (*bar* = 5 mm).

Differential Display and cloning of PCR product. Gene expression differences during somatic embryogenesis and direct organogenesis were compared at the transcription level following the method originally developed by Liang and Pardee (1992). A Differential Display kit from Display Systems Biotech (Vista, CA, USA) was used. Reverse transcription reactions were carried out using 100 ng of total RNA and 25 μ*M* $T_{11}GG$ oligonucleotide primer at 42°C for 60 min with 30 units of M-MuLV reverse transcriptase. Reactions were stopped by an incubation at 95°C for 5 min. First-strand cDNAs were amplified using random primers obtained from Display Systems Biotech. PCR reactions were carried out in 20-μl reaction volumes using two units of display TAQ FLDNA polymerase. After 40 cycles of 94°C for 30 s, 40°C for 90 s, 72°C for 60 s, a final extension step at 72°C for 5 min was performed. From each reaction 3 μl were loaded onto 5% denaturing polyacrylamide gels. Gels were dried and autoradiographed using X-ray film for 2 d. DNA fragments were isolated by cutting out the parts from the dried gels that contained the bands of interest. The fragment-containing gel pieces were soaked in 100 μl of sterile water and boiled at 95°C for 5 min. One microliter from the 1/10 diluted solution was used for reamplification. Reaction conditions were identical to those described above, except that no radioactivity was used and the annealing temperature was increased to 42°C. The amplified DNA fragments were cloned into the pGEM5Zf + T-vector (Promega) under standard conditions. Cloned Differential Display fragments were designated as SME clones.

Sequence analysis and construction of phylogenetic trees. DNA sequencing was carried out under standard conditions using the Taq Dye Deoxy Terminator Cycle Sequencing procedure on ABI model 370 sequencers (Applied Biosystems). Using the individual sequences as query, database searches were carried out through World Wide Web interfaces for the Blast N program (Altschul et al., 1997). Any other sequence analysis programs were run on a local PC under the Linux operating system. In the case of the eggplant genomic clone SMG56 alignments were made using the deduced amino acid sequences of the most related clones by the Clustal W program run under the Clustal X interface (version 1.8, Thompson et al., 1994, 1997). Sequences were trimmed for leaving out terminal regions missing in one or more genes and phylogenetic trees were constructed by the PROTPARS program of the PHYLIP package (version 3.57c, Felsenstein, 1993). Graphical rendering of phylogenetic trees was made by using the DRAWTREE program of the PHYLIP package.

RESULTS

In vitro cultures. Eggplant cotyledon explants produced bipolar somatic embryos within 7 or 8 d on media containing high auxin concentrations (4 mg l^{-1} NAA or 0.5 mg l^{-1} picloram, Fig. 1A). When the medium contained cytokinins (e.g. 0.5–2 mg l^{-1} kinetin or BA) instead of auxins, a greenish callus was produced on the cutting surface that frequently built unipolar shoot primordia, resulting in shoot development after prolonged (2–3 wk) culture on the same medium (Fig. 1B).

To model these alternative morphogenetic processes on a larger scale, rapidly growing suspension cultures were established from cotyledon-derived callus. Primary cultures grown in liquid medium containing 0.5 mg l^{-1} picloram produced compact, yellowish white cell colonies that often showed asymmetric cell divisions, indicating

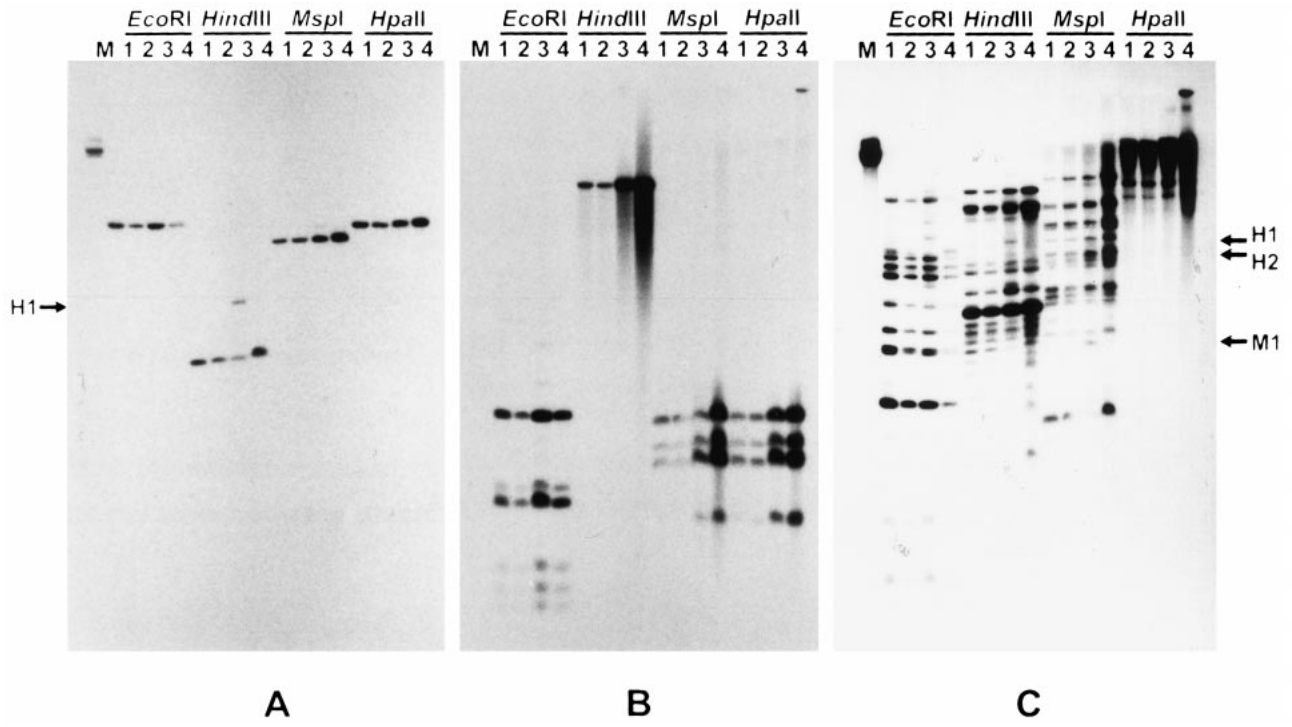

FIG. 2. Genomic Southern hybridization of eggplant DNA samples. DNA was isolated from suspension cultures grown in media containing 4 mg l^{-1} NAA (1); 0.5 mg l^{-1} picloram (2); 1 mg l^{-1} BA (3) and from leaves (4) (M=*Eco*RI and *Hin*dIII digested λ DNA). The following eggplant genomic clones were used a probes: SMG14 (*panel A*), SMG74 (*panel B*), and SMG56 (*panel C*). *Arrows* indicate extra bands after digestions with *Hin*dIII (*panel A*: H1, *panel C*: H1, H2) and *Msp*I (*panel C*: M1).

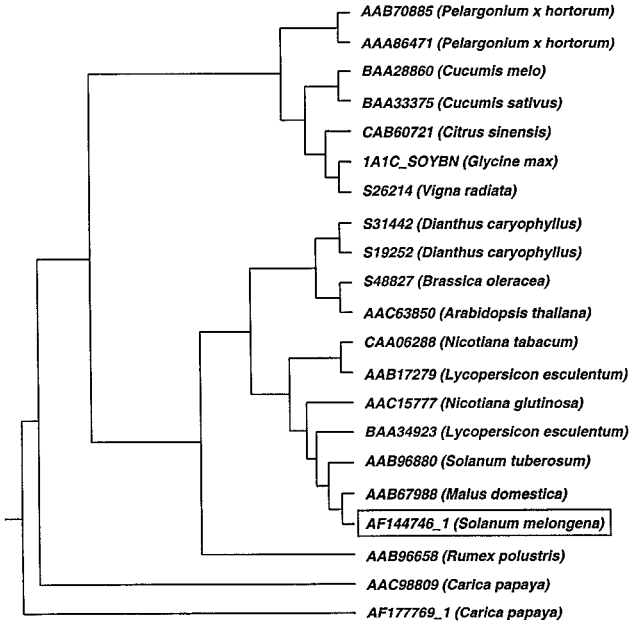

FIG. 3. Phylogenetic tree constructed on the basis of the amino acid sequences of ACC synthase genes.

the pro-embryogenic state of these cultures (Fig. 1C). When auxin was replaced by cytokinin (1 mg l^{-1} BA) direct organogenesis (shoot development) could be obtained after 4–5 wk in liquid media as well as on solid media (Fig. 1B, D).

DNA methylation analysis. Genomic clones were used to detect differences in methylation of DNA preparations from embryogenic and organogenic cultures as well as leaf DNA samples. In most enzyme–probe combinations the restriction pattern of the different DNA preparations were identical. After parallel digestions by the isoschizomers *Msp*I and *Hpa*II, the clones SMG4 or SMG74 produced similar hybridization patterns across all DNA samples in the case of both enzymes (Fig. 2B; note that this picture also indicates that DNA digestion was complete). On the other hand, different restriction patterns were detected when the clones SMG3, SMG14, SMG17, and SMG56 in combination with the methylation-sensitive isoschizomers were used as probes. In the case of SMG14, which represents a single-copy sequence, a weak extra band was visible in the *Msp*I-digested DNA samples from cells cultured in cytokinin-containing medium (Fig. 2A). Another *Msp*I-specific fragment was found in the same DNA samples, when the same membrane was probed with a repetitive clone SMG56. This probe revealed strong methylation differences between DNA samples of different origin (Fig. 2C). No hybridization pattern differences were found between different tissues and cultures when *Bst*NI and its isoschizomer *Eco*RII as well as *Pst*I and *Xho*I methylation-sensitive restriction endonucleases were used to cut genomic DNA samples (data not shown). The most differences were observed when DNA was digested by *Hin*dIII. Seven out of 17 probes tested showed extra bands in the DNA derived from cells cultured in cytokinin medium (Fig. 2A, C).

SMG56 is a 1-aminocyclopropane-1-carboxylate (ACC) synthase homolog. The DNA sequence of the genomic clones showing methylation pattern differences was determined. Database searches

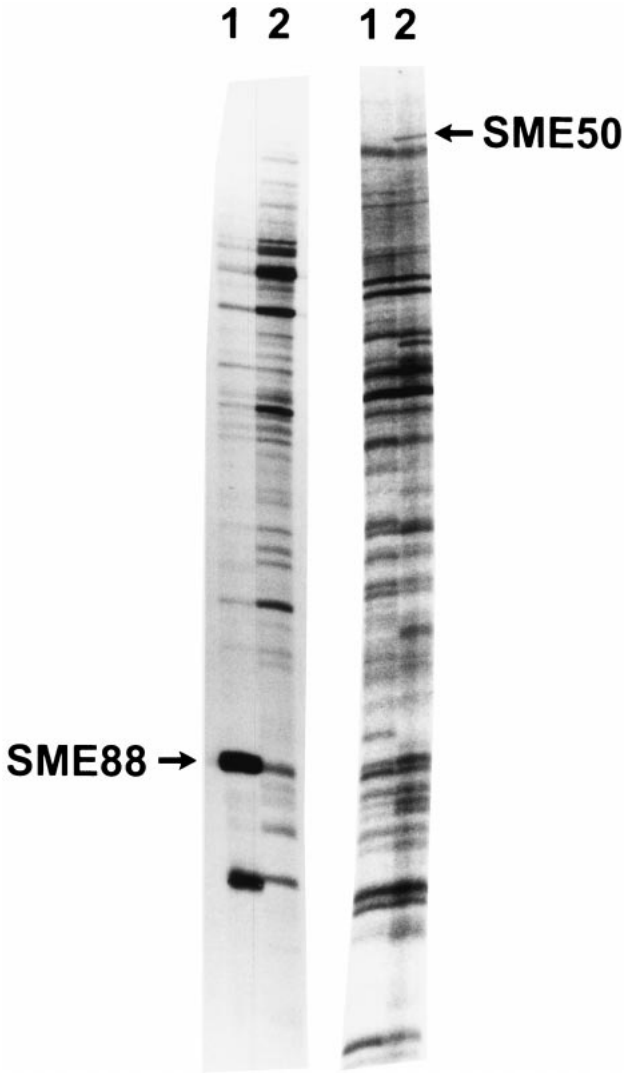

FIG. 4. Portion of a Differential Display autoradiogram. *Lanes 1* and *2* represent samples from suspension cultures grown in the presence 1 mg l^{-1} BA and 0.5 mg l^{-1} picloram, respectively. *Arrows* indicate specific fragments that were isolated and cloned.

using the 1123 bp long sequence representing the clone SMG56 as query, produced 213 hits. All of them were shown to be plant ACC synthase gene homologs. The phylogenetic trees constructed from the sequencing data indicated that the ACC synthase genes are members of a very large and diverse multigene family, containing at least 10 subfamilies. Twenty-one sequences belonging to the most related subfamilies of the group containing the SMG56 sequence were selected and a new phylogenetic tree was constructed using this subset of sequences. This tree produced four to five subfamilies (Fig. 3). Most sequences belonging to the subgroups containing the clone SMG56 were isolated from species of the *Solanaceae* family, but surprisingly the most homologous to SMG56 (99% identity at the amino acid level) is the MdACS-2 gene from *Malus domestica* (Rosenfield et al., 1996). Genomic Southern hybridization results suggested that SMG56 is a member of a multigene family in *Solanum melongena* (Fig. 2C).

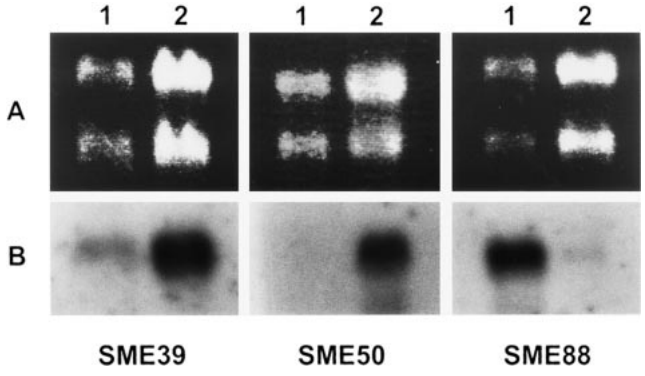

FIG. 5. Northern analysis of three candidates (SME39, SME50, SME88) for differentiation-specific clones. RNA was isolated from suspension cultures grown in media containing 1 mg l^{-1} BA (1) or 0.5 mg l^{-1} picloram (2). In *block A* ethidium bromide-stained gels and in *block B* autoradiograms after hybridization with the indicated clones are presented.

Identification of differentiation-specific transcripts. The Differential Display analysis using RNA from suspension cells cultured in auxin or cytokinin media revealed five gene expression categories: constitutively expressed transcripts; transcripts showing overexpression after auxin *or* cytokinin treatment; transcripts found solely in cultures maintained in media supplemented with auxin *or* cytokinin alone (Fig. 4). Probably the most interesting transcripts were members of the growth regulator-specific categories.

About 50 differentially expressed transcripts were identified and isolated. From these clones 15 could be specifically reamplified and cloned. The expression pattern of these clones were investigated by RNA gel blot analysis using total RNA from cells maintained in auxin- or cytokinin-containing media. The results of Northern analyses showed that three of the clones had growth regulator-dependent, differentiation-specific expression.

The clone SME39 showed overexpression in cultures maintained in auxin-containing medium, while SME88 seemed to be overexpressed in cultures maintained on cytokinin-containing medium. The clone SME50 was expressed only in the cultures maintained in auxin-containing medium (Fig. 5). Furthermore, Northern analysis results of these clones indicated that the expression patterns of the corresponding endogenous sequences were similar to those of the original Differential Display fragments (Figs. 4 and 5).

Database search results showed that SME88 is a homolog of light-inducible chlorophyll *a/b* binding proteins. No significant homologies were found by scanning the sequence databases for searching sequences related to SME39, whereas the sequence of SME50 showed a high level (91%) of homology with an unidentified carpel mRNA from tomato ovaries (GenBank accession No. AI483923; Fig. 6).

DISCUSSION

In eggplant, highly effective protocols for *in vitro* plant regeneration from cotyledon explants through organogenesis as well as through somatic embryogenesis are available. In this paper we present data on comparative molecular investigations of these two alternative differentiation processes using two different approaches: restriction fragment length polymorphism (RFLP) and Differential Display.

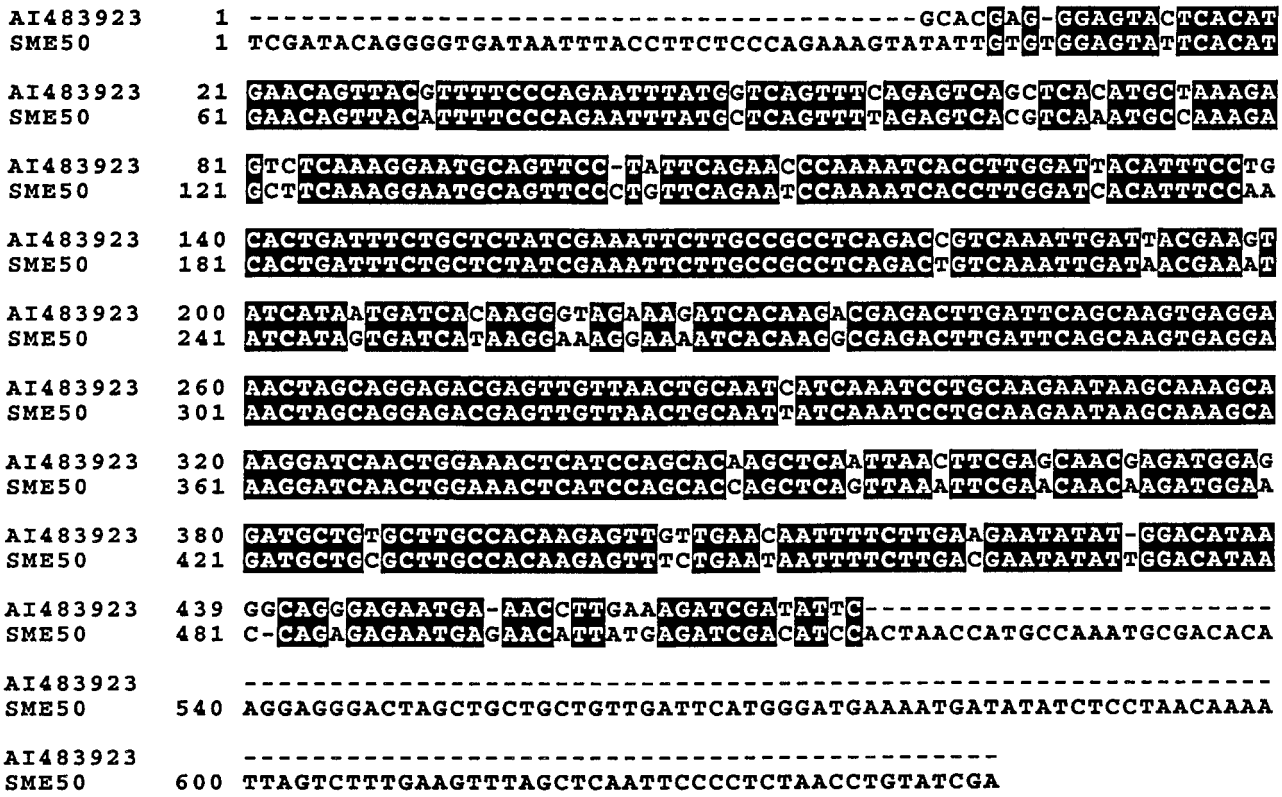

FIG. 6. Alignment of the nucleotide sequences of the SME50 and a carpel mRNA from tomato ovaries (GenBank accession No. AI483923). *Shaded boxes* represent identical nucleotides. Gaps (*as hyphens*) were introduced to maximize the length of matching regions.

Several results suggest that DNA methylation may play a role in the regulation of gene activity and cell differentiation. Our analysis of methylation using the isoschizomer restriction enzymes *Msp*I and *Hpa*II showed that methylation of the internal cytosines in the recognition sequence CCGG varied widely, from no detectable methylation to nearly complete methylation. Only minor differences in methylation were found when DNA samples isolated from auxin and cytokinin cultures were compared (Fig. 2). Similar results were reported by other workers (Vergara et al., 1990; Smulders et al., 1995). A genomic clone showing developmentally regulated expression and different methylation pattern with *Msp*I at different developmental stages was identified in a well-characterized carrot somatic embryogenesis system (Vergara et al., 1990).

We detected more methylation differences when DNA samples were probed after digestion with *Hin*dIII. It is known that *Hin*dIII restriction digestion is inhibited by methylated adenine and cytosine (Huang et al., 1982; McClelland and Nelson, 1985). Some other reports also indicate that *Hin*dIII sites may have methylation differences in plants (LoSchiavo et al., 1989; Brown et al., 1991; Smulders et al., 1995). Extra bands could be found in DNA samples from the early stages of differentiation, when *Hin*dIII digested DNA from callus, *in vitro* regenerants and whole plants were compared (Smulders et al., 1995). We also found extra *Hin*dIII bands in seven cases from 17 genomic hybridization probes in suspension cultures maintained in cytokinin-containing media.

In general, we concluded that DNA is methylated to a higher extent when suspension cultures are maintained by the addition of cytokinins compared to auxin. LoSchiavo et al. (1989), analyzing the volume of total methylated cytosine by HPLC, found that auxin induction resulted in the increase of methylated cytosines.

One of our eggplant genomic clones (SMG56) that revealed methylation differences during auxin- and cytokinin-induced morphogenetic processes was identified as an ACC synthase gene. ACC synthases are encoded by genes of a divergent multigene family. The expression of each member of the ACC synthase family is modulated differentially by developmental, hormonal, and environmental factors in a tissue-specific manner (Kende and Zeevart, 1997). Our results suggest that particular ACC synthase genes can also play an important role in the differentiation processes.

The second characterized clone SME88, isolated by Differential Display, showed an increased expression level in suspension cultures grown in cytokinin-containing media. This sequence showed homology to genes coding for light-inducible chlorophyll *a/b* binding proteins. We suspect that the accumulation of the corresponding transcript in cytokinin cultures could have been a consequence of the advent of leaf differentiation and greening in the cultures.

Another interesting candidate for differentiation-specific genes was represented by the clone SME50. This clone was identified by Differential Display in eggplant suspension cultures maintained in auxin-containing medium. Northern analyses showed that this clone has growth regulator-dependent, differentiation-specific expression (Fig. 5). Momiyama et al. (1995) isolated an early somatic embryogenesis-related clone by Differential Display, but the expression of this clone could not be investigated by Northern

analysis due to its low level of expression. In the case of SME50 a relatively higher expression level was detected in embryogenic suspension cultures. Only one related sequence could be found in the databases. This sequence represented an uncharacterized tomato EST sequence and was isolated from tomato ovaries. The EST sequence revealed high similarity to SME50 (91% identity at the nucleic acid level). The EST sequence's strictly tissue-specific expression in tomato—and the occurrence of its homolog (SME50) in differentiating eggplant *in vitro* cultures on the other hand—indicated that this transcript may have a function in morphogenesis and differentiation.

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