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Chromatin reorganization and endogenous auxin/cytokinin dynamic activity during somatic embryogenesis of cultured cotton cell

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Abstract We conducted a systematic assessment and comparative study on the biochemical and cellular characteristics of cultured cotton cells during the entire process of somatic embryogenesis (SE). All staged cultures were widely investigated in this assay. Cell and tissue ectogenesis manipulation combined with flow cytometry (FCM) was employed to cellular study during the whole totipotency process of dedifferentiation and redifferentiation. We identified two phases of chromatin decondensation during the dedifferentiation and redifferentiation. At the same time, sharp increase in the ratio of indoleacetic acid (IAA), isopentenyladenosine group (iPAs) at the same stage of cell dedifferentiation and redifferentiation process serve as distinct biochemical maker of dedifferentiation and SE initiation with the unique feature. Our results suggest the two phases of chromatin reorganization associated with endogenous auxin/cytokinin dynamic activity may underlie dedifferentiation and redifferentiation during the entire SE process in cotton.

Keywords Chromatin reorganization · Dynamic activity · Endogenous auxin/cytokinin · Flow cytometry · *Gossypium hirsutum* · Somatic embryogenesis

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
EC	Embryogenic calli
FCM	Flow cytometry
MS	Murashige and Skoog medium
NEC	Nonembryogenic calli
PEM	Proembryogenic masses
SE	Somatic embryogenesis
IAA	Indoleacetic acid
iPAs	Isopentenyladenosine group

Introduction

Cotton is globally one of the most important commercial crops. To meet persistent demand for developing new cotton varieties for cotton production, biotechnological approaches, such as tissue culture and genetic engineering, have been applied widely to cotton breeding. The transgenic cotton production normally requires an effective plant regeneration procedure from the cotton somatic cells (Wilkins et al. 2000, 2004).

Somatic embryogenesis (SE) undergo the well-known process of dedifferentiation and redifferentiation. As an in vitro tissue culture method, SE has been viewed as a potential model system for the study

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of the basic mechanisms of development reprogramming such as embryogenesis, morphogenesis in the life of higher plants (Zimmerman 1993). In addition, as the initial basis of cellular and genetic engineering, SE plays an important role in genetic engineering, cell fusion, and somaclonal variation. By now, however, most cultivars have been recalcitrant and few reports of high-frequency regeneration in cotton via SE have come forth due to a genotype-dependent response (Zhang et al. 1991; Mishra et al. 2003; Ganesan and Jayabalan 2004).

The cotton cell culture system provides an outstanding experimental tool for studying the biochemical and molecular bases of cellular SE. The identification and isolation of the vital genes of SE in our laboratory (Zeng et al. 2006) will surely provide novel target genes for improving the embryogenic competence and regenerability of a wider range of cotton cultivars. In addition to differential gene expression in somatic cells, cytological and biochemical dynamic activities are crucial factors in conferring on somatic cells the ability to manifest embryogenic potential during SE. In addition, auxin/cytokinin are the most likely candidates as regulators of developmental switches. Endogenous auxin/cytokinin are more important than exogenous growth regulators, however, because they directly determine cell division and differentiation after induction by exogenous growth regulators. Thus far, little is known about endogenous auxin/cytokinin during SE progression, especially during primary dedifferentiation and redifferentiation during the initiation and progression of SE. Thus, an integrated investigation on the biochemical and cellular characteristics during the entire SE process in cotton is another key task for a better understanding of totipotency of cells in higher plants and the improvement of SE ability, as well as other aspects of genetic engineering, which will accelerate the production of transgenic plants. Thus, monitoring cell behavior and comparative cellular and biochemical analysis are needed to understand the relationship between SE and the state of cells in vitro.

Because flow cytometry (FCM) allows rapid analysis of cultures on a cell-by-cell basis, it allows the collection of cell cycle data that distinguish between active and nonactive subpopulations. Cell sorting also allows selection of cells with particular properties. In plants, FCM has mostly been used to analyze nuclear DNA content, ploidy, and the cell cycle (El Maataoui and Pichot 1999; Moscone et al. 2003; Pinto et al. 2004). In particular, chromatin decondensation can be monitored by FCM (Zhao et al. 2001). This study provides an integrated assessment of the cytological, and biochemical dynamic activity during the entire SE process in cotton.

Materials and methods

Induction of somatic embryogenesis on solid medium

Callus initiation was done from 5- to 10-mm-long hypocotyl sections of *Gossypium hirsutum* Coker 201, as described by Wu et al. (2004), in MSB medium (MS medium plus B_5 vitamins) containing 0.045 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.46 μM kinetin. Following callus initiation, the calli were subsequently isolated and subcultured in hormone-free MSB medium to induce embryogenic calli (EC) as described by Zeng et al. (2006). The EC and nonembryogenic calli (NEC) were identified as described by Wu et al. (2004). Primary dedifferentiated callus and late nonembryogenic callus were respectively sampled 2 days and 10 days after callus initiation. Proembryogenic masses (PEM) were sampled before primary embryogenic clumps were first identified. Callus induction was performed at 30°C under illumination (35 μmol m^{-2} s^{-1}) with cool-white fluorescent lamps (14/10 h light/dark photoperiod).

Preparation of material for flow cytometry analysis

Approximately 0.1 g fresh weight of each sample was collected and chopped with a razor blade in 500 μl nuclei extraction buffer (1.0% [v/v] Triton X–100, 140 mM PVP, 50 mM Na_2HSO_3, 50 mM Tris–HCl), and the homogenate was incubated for 5 min. After filtration through a 50 μm nylon mesh to remove cell fragments, 50 μg ml^{-1} of propidium iodide was added to the samples to stain the DNA. After 1 min staining, FCM analyses were performed with an automatic flow cytometer (Partec, Münster, Germany) within 15 min. Each histogram was

generated by the analysis of at least 10,000 cells and repeated four times (for detail, also see Sun et al. 2004).

Hormone measurements

We sampled the following representative staged cultures: hypocotyls as explant, primary dedifferentiated calli, later NEC, primary redifferentiated EC, and long-term subcultured EC. To prepare samples for hormone measurements, 0.5 g of tissue from each sample was taken from the flask and put into a bottle with 3 ml of precooled 80% methanol. The bottle was put into a refrigerator, and then stored at −80°C for later use.

For endogenous auxin/cytokinin measurement, a sample was placed in an ice bath under weak light. After 3 ml of precooled 80% methanol was added, it was centrifuged at 4000 rpm for 15 min at 4°C. The content of two pivotal SE-related endogenous hormones, IAA and iPAs, were determined by enzyme-linked immunosorbent assay using the procedure described by Hu et al. (2003), which was modified from that described by Zhang et al. (1992) and Smart et al. (1995). The averages taken from eight replicates were used for statistic analysis.

Results and discussion

Morphological characterization

Primary dedifferentiated calli initiated from the two ends of hypocotyl sections were loose and soft, with active cell growth and the ability to divide. Embryogenic calli were compact and showed somatic embryogenic structures, mostly during the preglobular stage (Fig. 1). Long-term subcultured calli maintained on solid MSB medium for 3 years were homogeneous in color, structure, and composition (Fig. 1). Long-term subcultured calli gradually showed homogeneous pale yellow-fine morphological characteristics during repeated subcultures. However, they also gradually lost somatic embryogenic structures, which may have resulted in the ultimate low or nonexistent morphogenic and regeneration ability.

The compactness of embryogenic tissues in cotton (*G. hirsutum* Coker 201) is consistent with the description of such tissues in other species (e.g., *Eucalyptus globulus*; Pinto et al. 2004), but it differs from undifferentiated calli, which are in general much more friable and have large and vacuolated cells. Primary dedifferentiated grayish calli with

Fig. 1 Various stages of cultured embryogenic calli in cotton **(A)** Hypocotyls as explants incubated on MSB medium; **(B)** primary dedifferentiated calli; **(C)** proembryogenic masses; **(D)** primary redifferentiated embryogenic calli; **(E)** short-term subcultured embryogenic calli; **(F)** long-term subcultured embryogenic calli; **(G)** Late nonembryogenic callus; and **(H)** Cycling suspension cells
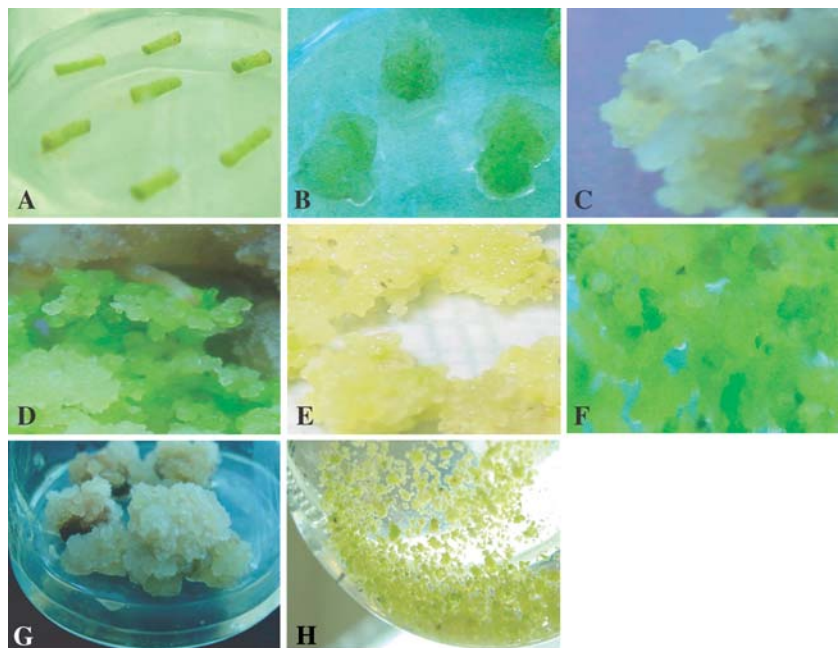
amorphous, soft, and friable tissue divide intensively. Cellular dedifferentiation is the major process underlying totipotency, regeneration, and formation of new stem cell lineages in multicellular organisms.

Because they were all derived from hypocotyls of Coker 201, this morphology may be associated with embryogenic competence. Formation of embryogenic cells can be correlated with characteristic morphological changes in most embryogenic systems (for review, see Yeung 1995). The fate of embryogenic carrot cells was followed by video cell tracking by Toonen et al. (1994); the single-cell fraction of the established embryogenic cell culture contained cells that could be classified into five morphological groups. Thus, cell morphology can be used as an early marker of embryogenic competence.

Two phases of chromatin reorganization during dedifferentiation and redifferentiation

The morphogenesis of plants is based on coordinated cell division and elongation followed by differentiation. Dedifferentiation and redifferentiation are key events in cellular adaptation, causing genetic, metabolic, and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells. Activation of cell division is required to maintain the dedifferentiated cell fate, as well as for embryo differentiation.

We identified two phases of chromatin reorganization during the entire SE process. The first phase took place in the course of primary dedifferentiation after somatic cells were induced with phytohormones to re-enter the cell cycle, and the second occurred when redifferentiation was initiated. We compared the FCM histograms of nuclei isolated from freshly prepared primary dedifferentiated cells 48 h after somatic cells were induced for callus initiation (t-48 h callus) versus nuclei prepared from hypocotyls as explants. Both t-48 h dedifferentiated cell and hypocotyl nuclei displayed a single peak corresponding to the G_0/G_1 content of DNA (Fig. 2). However, nuclei prepared from t-48 h dedifferentiated cells (Fig. 2B, G_1 nuclei) consistently showed an upward shift in fluorescence intensity compared with hypocotyl nuclei (Fig. 2A, G_0 nuclei).

Because propidium iodide intercalates between the strands of DNA without sequence specificity, its fluorescence intensity is directly proportional to the level of accessible DNA, thus reflecting the condensation state of chromatin. However, cycling cotton cells showed G_1 peaks (see Fig. 2C), suggesting that differentiated cells re-enter the cell cycle and undergo cellular reprogramming with the upward shift in the G_1 peak. This pattern suggests that the upward shift represents the stage at which nuclei are competent to re-enter the S phase and, therefore, may be equivalent to G_1 nuclei of cycling cells. To examine this possibility, we established a cell suspension culture of cotton (cycling cells) and compared the FCM histograms of nuclei prepared from this culture with that of cotton dedifferentiated cells. The G_1 nuclei of cycling cells had a higher fluorescence intensity than nuclei of hypocotyls (cf. Fig. 2A and C), coinciding with the position of the t-48 h dedifferentiated cells nuclei (Fig. 2). These results indicate that the G_1 peak of dedifferentiated cells represents chromatin relaxation rather than increased DNA content. Thus, the increased fluorescence intensity of propidium iodide–stained nuclei prior to entry of cells into the S phase resulted from chromatin decondensation, which appears to be an essential event for DNA replication initiation, determination of embryogenic cell fate, and acquisition of embryogenic competence.

During redifferentiation, we compared the FCM histograms of nuclei isolated from freshly prepared PEM and NEC. Similar to the dedifferentiation process, nuclei prepared from PEM cells (Fig. 2E, *G_1 nuclei) consistently showed an upward shift in fluorescence intensity compared with NEC nuclei, reflecting the condensation state of chromatin. Rather than cycling cotton cells entering G_1 (Fig. 2C), this upward shift shows the differentiated cells re-entering embryogenic developmental pathways and the developmental switch for SE induction. In addition, compared with primary redifferentiated embryogenic cells, G_1 nuclei of PEM had a nuclei fluorescence intensity coinciding with the position of the primary redifferentiated embryogenic cell nuclei. Therefore, the G_1 peak of dedifferentiated cells represents chromatin relaxation rather than increased DNA content. Thus, it appears that the upward shift in the G_1 peak represents a developmental switch at which point cells are competent to establish the apical-basal polarity and pattern formation of shoot and root meristems.

Chromatin decondensation is a common mechanism underlying cellular dedifferentiation and redif-
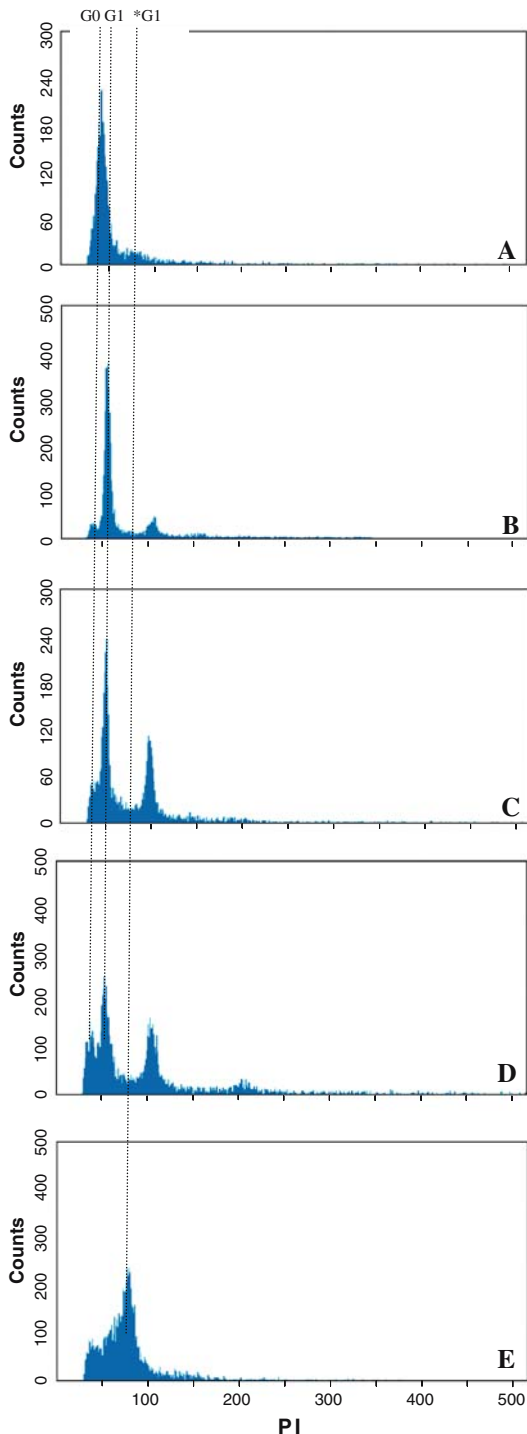
Fig. 2 Two phases of chromatin decondensation during dedifferentiation and redifferentiation. Nuclei were prepared from (**A**) cotton hypocotyls as explants, (**B**) freshly prepared primary dedifferentiated cells (t-48 h callus), and (**C**) cycling cells, stained, and subjected to flow cytometry analysis. Note the higher fluorescence intensity in dedifferentiated cells nuclei (G_1) compared with hypocotyl nuclei (G_0). An upward shift in the G_1 peak of dedifferentiated cells converges with the G_1 nuclei of cycling cells. (**D**) FCM histogram of a mixture of both types of nuclei in (**A**) and (**B**) (random unequal amounts in **A**, **B** and **D**). Calli were induced to redifferentiate by plant growth regulators and initiated the embryogenic developmental pathway switch, then displayed a second phase of chromatin decondensation. Note the upward shift in the G_1 peak of PEM cells (*G_1) in (**E**). This shift is characteristic of cells initiating genetic reprogramming and switching to a new embryogenic developmental pathway for establishment of the apical-basal polarity and embryogenesis. This transitory phase is necessary for activation of genes whose products are required for the establishment of polarity and pattern formation of shoot and root meristems, which results in totipotency and SE initiation

found in cycling dedifferentiated cells or subcultured embryogenic cells. The stepwise manner by which chromatin reorganization occurs points to various levels of heterochromatin compaction; that is, heterochromatin may be a heterogeneous structure displaying different degrees of chromatin condensation. This assumption is supported by recent findings demonstrating variations in chromatin (de)condensation within heterochromatin segments in meiotic chromosomes of *Arabidopsis* (Fransz et al. 2000). We propose that the second phase of chromatin reorganization is transitory during embryogenic development (Fig. 2E). When PEM cells, however, were incubated until they became redifferentiated embryogenic calli, the majority of nuclei shifted to the original G_1 position. This pattern suggests that the upward shift in the G_1 peak of PEM cells represents the stage at which nuclei are competent to re-enter the embryogenic developmental phase.

Our findings suggest that the two phases of chromatin decondensation during dedifferentiation and redifferentiation are related but functionally distinct and divergent processes. On one hand, the events of the cell cycle are common to both dedifferentiation and redifferentiation; that is, the two phases of chromatin decondensation represent a common mitotic cycle. On the other hand, G_1 nuclei of PEM cells had a higher fluorescence intensity compared with nuclei of primary dedifferentiated cells (including all other stages during SE); that is,

ferentiation in both plants and animals. The transitory upward shift in the G_1 peak of PEM cells appears to be a unique feature of dedifferentiated cells re-entering the redifferentiation process, one that is not

PEM cells show the highest degree of chromatin reorganization, causing the most relaxed chromatin structure for its special function.

Zhao et al. (2001) suggested that border chromatin might have biological significance in cellular plasticity, namely the interplay between differentiation, proliferation, and cell death. Our study of cotton cells revealed one phase of chromatin reorganization during primary dedifferentiation, whereas Zhao et al. (2001) reported two phases of chromatin decondensation during dedifferentiation of tobacco cells. Thus, the different materials used in these experiments (i.e., intact cotton somatic cells vs. tobacco protoplasts devoid of cell wall due to treatment with cell wall-degrading enzymes) could be under different genetic and physiological processes. Conversely, the different results could reflect differences in SE versus cell plasticity.

Chromatin reorganization and remodeling likely play key roles in embryogenic developmental pathways, and thus can be considered as main regulators of the developmental switch and a change in cell fate during somatic embryo induction (for review, see Grossniklaus et al. 2001). It appears that SE proceeds by two functionally distinct but related phases of chromatin decondensation. During dedifferentiation, chromatin reorganization confers on the somatic cells the ability to re-enter the cell cycle, determine embryogenic cell fate, and acquire embryogenic competence, a process whereby existing transcriptional and translational profiles are erased or altered to allow cells to set a new developmental program. During the initiation of redifferentiation, chromatin reorganization plays a vital role in the establishment of the apical-basal polarity and pattern formation of shoot and root meristems, as well as activation of vigorous cell division. In fact, cell division and differentiation are related to development.

An essential switch in developmental pathways may be facilitated in dividing cells by a more open chromatin structure, altered metabolism, and physiological homeostasis, compared to differentiated cells. Therefore, chromatin reorganization and remodeling appears to be intrinsically involved in the regulation of nuclear processes, especially transcription. Chromatin structure changes in a dynamic way and is continuously remodeled during development. Thus, chromatin remodeling is necessarily linked with the cellular events and switching of cell fate during cellular dedifferentiation and redifferentiation. Chromatin remodeling seems to play two major roles during the early stages of SE. Dedifferentiation requires unfolding of the supercoiled chromatin structure to allow the expression of genes inactivated by heterochromatinization during differentiation, and subsequent chromatin remodeling can result in the specific activation of a set of genes required for embryogenic development during redifferentiation and somatic embryo development. These findings may have implications for a wide range of SE cellular processes in higher eukaryotes.

Distinct endogenous auxin/cytokinin biochemical marker remodeling cellular programming and the developmental switch

Among factors that affect plant tissue culture, including growth media (sterility, minerals, growth factors, carbon source, hormones) and environmental factors (light, temperature, photoperiod), endogenous hormones are the most likely regulators of developmental switches. In plants, auxins and cytokinins are the main regulators of cell division and differentiation. The influences of exogenous auxin/cytokinin on the induction of SE, particularly 2,4-D, are well known. Although little is known about endogenous hormones during the SE process, especially during primary dedifferentiation and redifferentiation during the initiation and progression of SE, endogenous auxin/cytokinin are likely more important than exogenous factors because they directly determine the progression of SE (Fehér A et al. 2002).

We surveyed the endogenous content of auxin/cytokinin in different representative staged cultured tissue in vitro during dedifferentiation and redifferentiation during the initiation and progression of SE (Table 1). A noteworthy feature of the endogenous auxin/cytokinin content was that IAA, iPAs, and their ratio surged at SE initiation (primary embryogenesis callus), which is very interesting during this process. Previous studies have also shown that sharp changes in endogenous auxin levels may be one of the first signals leading to SE (Zhang et al. 1992; Thomas et al. 2002). Redifferentiation was clearly correlated with a sharp increase in auxin responses in cotton cells, and we found direct evidence for the significance of an endogenous auxin pulse in the expression of cellular totipotency.

Table 1 Endogenous content of growth regulatory substances during dedifferentiation and redifferentiation during somatic embryogenesis (SE) initiation and progression

Sample	IAA (pmol g FW^{-1})	IPAs (pmol g FW^{-1})	IAA/iPAs
Hypocotyls (explant)	1797.06 ± 8.01	58.60 ± 0.62	30.67 ± 0.51
Primary dedifferentiated callus	1088.41 ± 1.54	26.03 ± 0.54	41.81 ± 1.08
Late nonembryogenic callus	952.41 ± 7.35	46.39 ± 0.44	20.53 ± 0.04
Primary redifferentiated embryogenic callus	98,976.09 ± 15.90	62.32 ± 0.32	1588.19 ± 7.09
Long-term Subcultured embryogenic callus	3191.91 ± 11.59	11.36 ± 0.11	280.98 ± 5.43

During the dedifferentiation process and subsequent division of callus cells, the IAA content decreased continuously until redifferentiation (Table 1). However, at another representative phase during transitory primary dedifferentiation (48 h after induction), iPAs content was at its lowest level, whereas the IAA/iPAs ratio showed its highest value before redifferentiation (Table 1). In long-term subcultured embryogenic calli, although the IAA/iPAs ratio and IAA content were higher than in tissues at all other stages before primary redifferentiation, these values showed sharp decreases. Especially, the iPAs content was the lowest during the entire SE process. Thus, it appears that this pattern of hormone change is a distinct feature of long-term subculture.

Auxin and cytokinin are required to induce cell division in plant tissue cultures and are indispensable for the progression of dedifferentiation and redifferentiation, which cause embryogenic competence (totipotency) of somatic plant cells and SE during cellular adaptation; determination of embryogenic cell fate; establishment of the apical-basal polarity; and pattern formation of shoot and root meristems. The cotton cell culture system provides an outstanding experimental tool for studying the biochemical and molecular bases for cellular SE. Hormones are the most likely regulators of developmental switches. Auxins and cytokinins, which are involved in the regulation of cell division and differentiation, are the main growth regulators in plants. The influences of exogenous growth regulators, particularly 2,4-D, on the induction of SE are well documented. Endogenous auxin/cytokinin are more important, however, because they directly determine cell division and differentiation after induction by exogenous growth regulators.

The present study showed that a sharp increase in endogenous auxin level promoted redifferentiation. Increase in the ratio of IAA, iPAs at the stage of cell redifferentiation and redifferentiation process serve as distinct biochemical maker of dedifferentiation and SE initiation with the unique feature. During the early phase of SE, endogenous auxin/cytokinin levels are the main factors that determine the specificity of cellular responses to exogenous growth regulators, rather than general exoteric stimuli.

Cross talk among genetic, cytological, and biochemical dynamic activity

A noteworthy finding of this study is that during the dedifferentiation and redifferentiation process during SE, there is distinct cross talk among cytological and biochemical dynamic activity in cotton. The two phases of chromatin decondensation during dedifferentiation and redifferentiation cause genetic, metabolic, and physiological reprogramming, which results in the embryogenic competence (totipotency) of somatic plant cells. The chromatin decondensation corresponds respectively with the increase in the ratio of IAA, iPAs (result from distinct iPAs decrease and an auxin surge) during these two crucial phases. This discovery indicates that developmental changes in cells, induced by environmental or developmental signals, are dependent on the original DNA content, chromatin reorganization, and the differentiation state of cells. These two major aspects of cytological and biochemical dynamic activity are strongly related and cooperate with each other during the entire SE process in cotton. Our results suggest the two phases of chromatin decondensation may be associated with endogenous auxin/cytokinin dynamic activity of cultured cell, which may underlie dedifferentiation

and redifferentiation during the entire somatic embryogenesis process in cotton.

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