Cytohistological Studies During Cotton Somatic Embryogenesis With Brassinosteroid Application

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Abstract

The histology and chromosome alterations of somatic embryo initiation and development in cotton (*Gossypium hirsutum* L. cv. Coker) was examined during *in vitro* exogenous brassinosteroid (BR) application. Calli developed from hypocotyl explants were subjected to 0.5 μM BR. After 10 weeks of calli cultures with somatic embryos at different developmental stages were fixed for histological examinations. The histological examination showed that two types of embryogenic cells induced; epidermal or subepidermal. The embryogenic mass and somatic embryos were mostly derived from several morphologically competent cells from subepidermal cases; however single progenitor cells for epidermal situations. Chromosome behaviour of the cells surrounding embryogenic cells showed abnormal divisions and higher level of polyploidy on BR treated calli when compared to control group.

Keywords: *Gossypium hirsutum* L. cv. Coker, *in vitro* culture, brassinolide, regeneration, polyploidy.
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Introduction

During somatic to embryogenic transition, cells have to dedifferentiate, activate their cell division cycle and reorganize their physiology, metabolism and gene expression patterns (Feher et al. 2003), for this reason somatic embryogenesis is remarkable illustration of embryonic plant totipotency. Cotton (*Gossypium hirsutum* L. cv. Coker) is model cultivar for cotton somatic embryogenesis since Davidonis and Hamilton (1983), because of high regenerative response *in vitro* culture conditions. Considerable interest has been towards the genetic improving by using *in vitro* techniques in cotton for application of transgenic technologies (Leelavathi et al. 2004). Recently brassinolide which is the most biologically active of brassinosteroids was proved as a factor for the stimulation of somatic embryogenesis not only in cotton (Aydın et al. 2006) but also in different plant species such as *Cocos nucifera* (Azpetia et al. 2003), conifers and rice (Pulmann et al. 2003) and *Brassica* (Ferrie et al. 2005). Exogenous application of BR also effective for stimulation of cell divisions (Oh and Clouse 1998; Hu et al. 2000) however, little is known about the effect of BR on chromosome alterations. Analysis origin of embryogenic cells and cytohistological development of somatic embryos from these cells will give better understanding and utilization of cotton somatic embryogenesis towards to plant genetic improvement. The aim of the present study was to carry out histological/cytogenetical analysis of the

embryogenic process *in vitro* grown cotton calli during BR application, to find out the effects of BR on chromosome alterations and also investigate histological development of somatic embryos.

Materials and Methods

Cotton Somatic Embryogenesis

Cotton hypocotyls explants culturing, callus induction and somatic embryogenesis process was achieved as described before (Aydın et al. 2004, 2006). Seeds of cotton (*Gossypium hirsutum* L. cv. Coker) were delinted with sulphuric acid and washed with water for 3 h. For surface sterilization, seeds were initially deeped in 70 % ethanol for 2 min., rinsed with sterile distilled water for 3 min. and kept in 20 % commercial bleach for 20 min. After three rinse in sterile distilled water, seed coats were removed and cultured on (MS) medium (Murashige and Skoog, 1962) supplemented with 30 g l^{-1} sucrose and 2.2 g l^{-1} phytagel for germination. Hypocotyls were dissected from 10-day-old *in vitro* grown seedling and cultured for callus induction on MS basal medium + B_5 vitamins + 30 g l^{-1} sucrose+ 2.2 g l^{-1} phytagel+ 1 g l^{-1} PVP (Polyvinylpyrrolidone) + 1 mg l^{-1} BAP + 2 mg l^{-1} kinetin. 50 hypocotyl explants were used as starting material, one month - old calli pieces (0.4 g) from responding explants were transferred on same MS media in small petri dishes on a filter paper supplemented with 0.5 µM BR with their controls and incubated in the growth chamber under dark. Calli developed cotyledonary somatic embryos were taken periodically on hormone free MS media starting the 10th weeks till 29th weeks of cultures for maturation and the matured embryos were transferred to gibberellic acid (GA_3) containing MS media for plantlet formation.

Cytohistological Analyses

Samples were taken after 10th week of culture for histological analysis of callus and somatic embryogenesis which was previously shown as the most responsive date for somatic embryogenesis (Aydın et al. 2006). The specimens were fixed in acetic -ethanol (1:3 v/v) for 24 hours, rinsed 96% and stored in 70% ethanol respectively. Dehydrated in a set of increasing ethanol solutions (70, 95, 100 % v/v) for 30 min in each step and two times in 100%. Then the ethanol replaced by alcohol: xylene (2:1 v/v), alcohol: xylene (1:1 v/v), alcohol:xylene (1:2 v/v) and two times in xylene for 30 min in each step. The specimens were kept over night in xylene:paraffin (1:1 v/v) at 56 ^{o}C, transferred into a vessel that contained the paraffin wax and kept in 56 ^{o}C for at least 24 hours generally with two changes 48 hours. Finally the specimens were embedded in paraffine wax, sections (10µm) were cut using a rotary microtome and mounted on glass slides. The sections were de-waxed in xylene for 5-10 min., and then transferred reverse solutions of alcohol:xylene series (1:2 v/v, 1:1 v/v, 2:1 v/v) as performed previously for 10 min. in each step. 10 µm sections stained with haematoxlin and the covered by coverslips with a drop of entellan before examination under an Olympus BH-2 microscope. Nuclear volume was calculated by using the formula $4/3\ r_1r_2r_3\pi$ (Tschermak-Woess and Hasitschka 1953 ; Unal and Vardar 2006) at least 500 cells both in control and BR treated material. In the large, high poliploid nuclei the r_3 dimension represents the total sum of values from successive microtome sections and only nuclei of a regular shape were calculated. The preparations were analyzed with the Image pro-express software, assisted by an Evolution LC color camera and an Olympus BH-2 microscope.

Results and Discussion

One month old callus tissues developed on hypocotyl explants when transferred to the MS basal media with or without 0.5 µM BR, and observable somatic embryo development was started at the 10th weeks of the culture period. We were able to select the different developmental stages of bipolar structures of cotton somatic embryos morphologically (Fig. 1 A). During histological analyses it was shown

that the subepidermal cells of the compact callus converted to competent cells and started to divide which resulted in meristemoids (cluster of organogenic cells with cytoplasmic density, minimal vacuols and relatively large nuclei (Fig. 1 B). The meristemoids cells continued to divide to develop into primordia (Fig. 1 C). Subepidermal cells could also form the globular meristematic masses separated from the main calli cells. In additon to the subepidermal embryogenic cell masses we also observed epidermal cells with asymmetric divisions in a spesific pattern formations, suspensor like structures with round dividing embryogenic heads (Fig 1 D). Our histological observations reveal that the embryogenic cell mass and somatic embryos develop in most instances from several morphologically compotent adjacent cells in subepidermal cases as described by Lee et al. (1997). These cells generally produce small, dense cytoplasmic cells in the middle of the enlarged highly vacuoleted and polyploid cell niches (Fig. 1 E). However, in epidermal cases single unicellular origins with narrow suspensor like organs were prominent which was reported (Williams and Mahesweren 1986) previously. In these case it was possible to observe two adjacent epidermal cells producing two different extrusions (Fig. 1 D). Similar to our study In *Pistacia vera* L. somatic embryogenesis Onay et al. (2000) also pointed out the both single epidermal and subepidermal origin.

In BR treated calli, it was found that the nuclei in the surrounding cells of subepidermal embryogenic cells have larger in size with higher polyploid nuclei and not only various in sizes but also in number (Table 1). In control material while nuclei sizes changes (n-32n) in BR treated material they were in between (n-512n). In contrast to nuclei of uniform size in meristemoids therefore polyploidization has been suggested could be result of endoreduplication or cell fusions. Recently Ferrie et al. (2005) investigated the effects of BR on *Brassica* microspore embryogenesis and also pointed out the increase in somatic embryogenesis and chromosome doubling. Hase et al. (2005) pointed out the endoreduplications in petal tips in altered in fry1 sterol mutants because of altered sterol compositions. We can conclude the relation between sterol content and polyploidization of cells in culture. This phenomena could be the result of divisions without cell wall formation. There are contrasting reports on the role of different BR types in the regulation of root growth in different plant varieties under different experimental conditions. There are various reports on inhibitory effects (Guan and Roddick 1988; Ozdemir et al. 2004) and investigations on stimulating effects (Romani et al. 1983; Mussig et al. 2003; Kartal et al. 2009) also observed root growth stimulation at low concentrations whereas at high concentrations increased gravitropic curvature was detected as it inhibited primary root growth in culture grown *Arabidopsis* seeds. Howell et al. (2007) found out the changes in growth response of *Allium cepa* roots. At low doses (0.005 ppm) growth of roots were doubled in root lenghts. However, highest doses of EBR (0.5 ppm) decreased mean root lengths and number of mitoses. However, highest doses of EBR (0.5 ppm) decreased mean root lengths and number of mitoses. Kartal et al. (2009) showed that HBR application increased root length with increasing HBR concentrations. They also pointed out roots treated with HBR showed more mitotic activity, mitotic abnormalities and enlargements at the root tips.

Table 1: Volumes of the embryogenic calli nuclei and their degree of ploidy in control (A) and BR treated calli (B) of *Gossypium hirsutum* L.cv.Coker.

A) Degree of ploidy	Extreme values (μm^3)	Average (μm^3)	q	Number of nuclei
n	0,00014843-0,00029753	0,00022379	-	30
2n	0,00033113-0,00059560	0,00044827	2,0031	79
4n	0,00060257-0,00116756	0,00089556	1,9978	125
8n	0,00129140-0,00239911	0,00179052	1,9993	156
16n	0,00273659-0,00478616	0,00358363	2,0014	95
32n	0,00622680-0,00841003	0,00705384	1,9684	16

Total number of nuclei → 501 (CONTROL)

B) Degree of ploidy	Extreme values (μm^3)	Average (μm^3)	q	Number of nuclei
n	0,000019549-0,000043174	0,000028754	-	23
2n	0,000052106-0,000064724	0,000057459	1,9983	9
4n	0,000069602-0,000162328	0,000114195	1,9874	35
8n	0,000192097-0,000264222	0,000228935	2,0048	33
16n	0,000338511-0,000603572	0,000456427	1,9937	79
32n	0,000683721-0,001225434	0,000913364	2,0011	104
64n	0,001361370-0,002499863	0,001872924	2,0506	117
128n	0,002739178-0,004979512	0,003654399	1,9512	106
256n	0,006042256-0,009890119	0,007148695	1,9562	20
512n	0,011331830-0,016251707	0,013319008	1,8631	5

Total number of nuclei → 531 (BR TREATED)

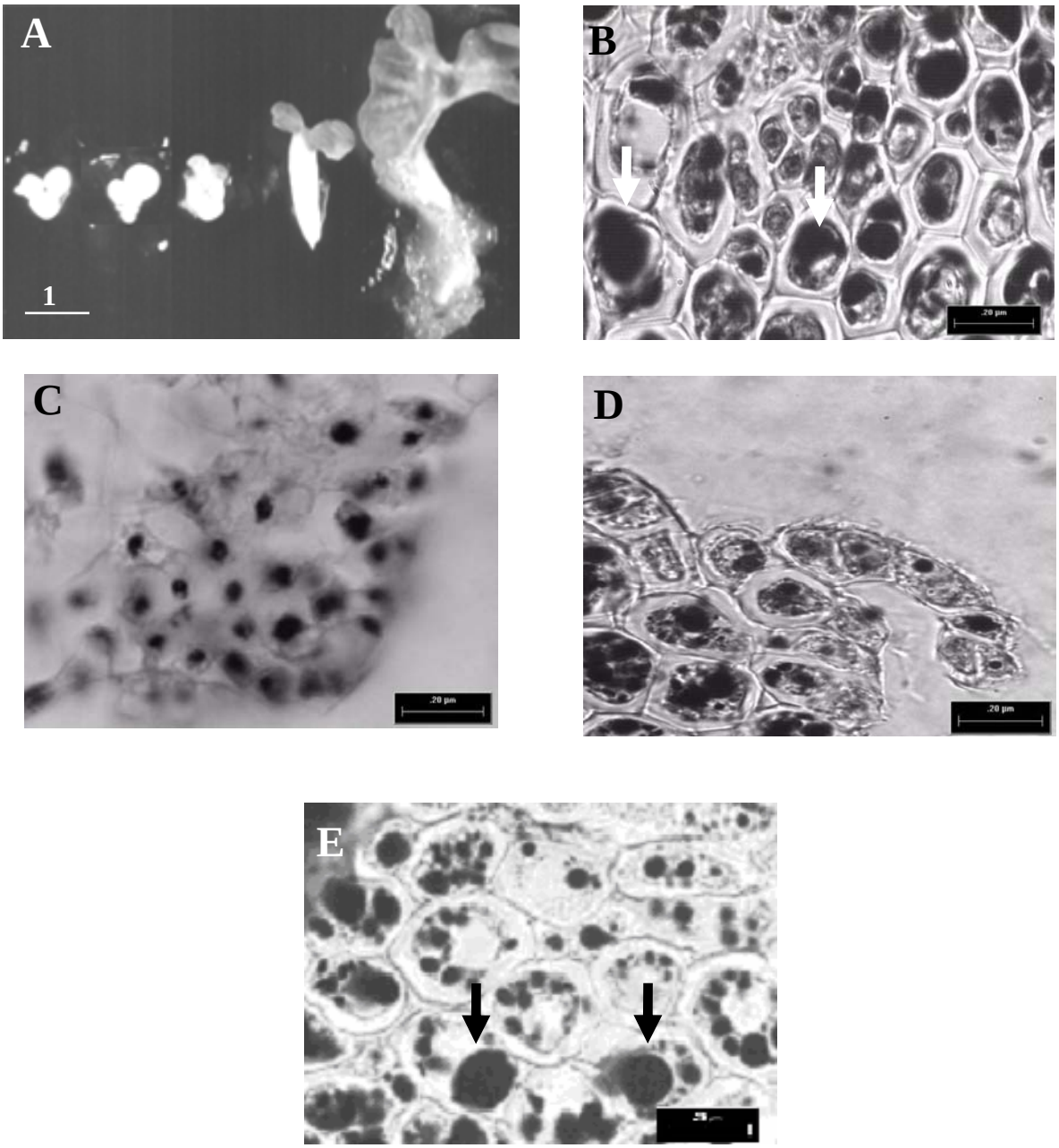

Figure 1. Somatic embryogenesis in control callus of cotton at different developmental stages A, subepidermal embryoid formation on 0.5 µM BR treated calli B, embryogenic cell mass C, embryogenic epidermal extrusions on 0.5 µM BR treated calli D, cells with polyploid nuclei and unequel divisions on 0.5 µM BR treated calli E .

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