Biochemical and molecular basis of differentiation in plant tissue culture

Madhusudan Dey, Sanjeev Kalia, Sagar Ghosh and Sipra Guha-Mukherjee*
Centre for Plant Molecular Biology, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India

Basic understanding of plant cell proliferation and differentiation is imminent for applying modern techniques of genetic transformations. Although remarkable progress has been made in the area of gene transfer technology, little is known as to how plant cells differentiate in cultures. Plant tissue culture has therefore remained an empirical science. In this review, an attempt has been made to assimilate the current knowledge of the various biochemical and molecular parameters, which play an important role in differentiation.

Gene transfer technology in plants promises to have a significant impact on crop improvement. The major advance in this technology is the development of explant-based regeneration system. Despite the fact that plant cells display a remarkable potential for cellular totipotency, behaviour of plant cells or explants in tissue culture medium is unpredictable. It is assumed that differentiated plant cells retain their ability to revert to embryogenic condition and generate a complete new plant through somatic embryogenesis or organogenesis. This unique property offers an opportunity to investigate cellular, physiological, biochemical and molecular basis of differentiation. Very little is known about the molecular mechanism of in vitro differentiation. The lack of reliable molecular markers is a serious constraint for extensive use of genetic engineering in plants. This review attempts to bring together the current information on biochemical, cellular and molecular mechanism underlying differentiation in plant tissue culture.

Biochemical regulation of differentiation

Visible manifestation of cell differentiation includes greening of callus, variation in the cell wall thickness and biogenesis of certain cytoplasmic organelles, such as plastids. Some tissues are specifically adapted for specialized functions, such as, secretion, storage, mechanical support and protection. Differentiation in such tissues involves differences in the basic metabolic pathways. The precise requirement for metabolites to bring about altered development can be fulfilled within the cell itself or through transport. Thus, explants require critical supply of metabolites: vitamins, phytoregulators and nutrients when grown in aseptic condition. Similarly, callus cultures of certain plants require external supply of auxin and cytokinin to maintain cell division. These phenomena strongly support the tenet that cell differentiation involves the activation of certain genes and repression of others, which control different basic metabolic or anabolic pathways. Besides hormones, several low molecular weight compounds, namely amino acids, oligosaccharides and polyamines are also known to be involved in differentiation.

Amino acid and polyamines in differentiation

Many metabolic and anabolic pathways are operative in a plant cell, e.g. photosynthesis, respiration and biosynthetic pathways for amino acids, polyamines and ethylene. These pathways are well connected with different cellular processes. The small change in metabolites of these pathways could bring about a dramatic change in various physiological processes. Amino acids, for instance, have been shown to be specific stimulators of somatic embryogenesis and differentiation. The role of amino acids in growth and differentiation is known to a considerable extent. Amino acids may induce or inhibit cell proliferation or differentiation. In Brassica, leucine and isoleucine were reported to promote differentiation, whereas methionine and threonine activated proliferation. Figure 1 depicts how different amino acids, supplied exogenously, affect proliferation and differentiation in Brassica culture. A little change in amino acid content could bring about different morphogenetic responses. However, higher concentrations of amino acids have been shown to be general growth inhibitors in Nicotiana silvestris and Cicer arietinum. Thus, a balance in amino acid composition is very crucial for organized growth. Some regulatory enzymes like aspartate kinase, homoserine dehydrogenase, threonine dehydrogenase, maintain this balance.

Polyamines play an important role in cell division and differentiation in eukaryotes. Rapid accumulation of polyamines occurs concurrently with the initiation of cell division and the inhibition of polyamine biosynthesis induces differentiation. Polyamines are implicated in...
a variety of physiological processes like flower development, plant defence and somatic embryogenesis. Some authors have even postulated polyamines as a type of plant growth regulator or hormonal second messenger. These studies have been substantiated by the use of inhibitors of their biosynthetic enzymes. Cloning of genes of polyamine biosynthetic pathway has given a new impetus to polyamine research. The effects of cellular perturbation of polyamine levels on plant developmental processes can be studied using transgenic approach. However, it is not yet clear whether polyamines act as developmental switches, which are indeed causal rather than consequential in their effects. Figure 2 shows growth and differentiation of Brassica callus maintained on spermidine containing medium.

Hormonal control of differentiation

In tissue culture, proper combinations of growth regulators elicit a wide range of responses. The switch from the undifferentiated cells (callus) to differentiated one in plant requires an early commitment to a specific cell fate. Plant growth regulators at low concentrations are
known to influence cell commitment and cell determination\textsuperscript{21}. Several auxin-regulated genes have been characterized and their possible roles in different cellular processes have been determined\textsuperscript{22}. Still, it is not clear whether hormones primarily influence differentiation by activating early response genes or are involved at relatively late stages such as during cell expansion or morphogenesis.

Of the various phytohormones known, ethylene is gaseous and is produced in trace amounts. As little as 10 ng/l of ethylene can induce fruit ripening. Besides promoting leaf/flower senescence, abscission, loss of geotropic sensitivity and sex determination in monoecious species, it controls many physiological processes in plants\textsuperscript{23,24}. Importance of ethylene in \textit{in vitro} cultures has been widely reported \textit{vis-à-vis} growth and differentiation\textsuperscript{25,26}. Ethylene influences growth and differentiation of \textit{in vitro} plant cell culture and high levels of ethylene inhibit shoot regeneration. Application of aminoethoxyvinylglycine and silver nitrate, the inhibitors of ethylene biosynthetic pathway cause high frequency regeneration from cultured explants of \textit{Zea mays}\textsuperscript{27}, \textit{Brassica campestris}\textsuperscript{28} and \textit{Brassica juncea}\textsuperscript{29}. Figure 3 shows the effect of AgNO\textsubscript{3} on \textit{in vitro} regeneration of \textit{Brassica juncea}\textsuperscript{6}.

\textbf{Cell division marker enzymes}

Enzyme glyoxalase I which catalyses the transformation of methylglyoxal and glutathione to S-lactoylglutathione is converted to D-lactic acid by glyoxalase II\textsuperscript{30}. Since glyoxal system has been found in cells of all organisms it is assumed that it must be having an important role in the developmental programme. However, the role of this enzyme is not clear, it probably is concerned with the detoxification of methylglyoxal, a potent cytotoxic metabolite\textsuperscript{31}. The glyoxalase I has been correlated to cell division in \textit{Datura}\textsuperscript{32}, \textit{coconut}\textsuperscript{33}, \textit{soybean}\textsuperscript{34} and \textit{Brassica}\textsuperscript{35}. Glyoxalase I level was reported to be high in proliferating cells of \textit{Brassica} and declined during differentiation induced by inhibitors of polyamine and ethylene biosynthesis\textsuperscript{36}.

\textbf{Molecular regulation of differentiation}

In recent years, a concerted effort is being made to understand the molecular control of cell differentiation. A set of genes orchestrates cell division, differentiation and somatic embryo development. The expressions of these genes are cell type, region and organ-specific\textsuperscript{36}. Moreover, the best approach for understanding the molecular mechanism is to identify molecular marker, which could be used to identify the early events of somatic embryogenesis and differentiation. The different approaches to explore the early events of differentiation are mutant analysis, differential screening of transcript and ectopic expression of regeneration-specific genes.

\textbf{Marker proteins for regeneration}

Gene products differentially expressed during somatic embryogenesis have been used as probe for differentiation. Two embryo-specific proteins (70 kDa and 43 kDa) were found in carrot embryogenic callus\textsuperscript{37}. Likewise, Stin and Jacobsen\textsuperscript{38} showed two embryogenic specific proteins (70 kDa and 45 kDa) in pea suspension culture. A polypeptide of 46 kDa was found exclusively in embryogenic barley cell culture\textsuperscript{39}.

\textbf{Embryogenic-specific genes}

A comparison of gene expression during embryogenesis has been utilized to identify developmental markers. During early stages of carrot embryogenesis EMB-1 mRNA starts accumulating. The accumulation of EMB-1 mRNA progressively increases as the embryo matures.

\textbf{Figure 2.} Growth and differentiation of \textit{Brassica} culture on spermidine (Spd) containing medium. \textit{a}, Basal medium with hormones (NAA, 5.37 \textmu M; BA, 1.4 \textmu M) and Spd, 1 \textmu M; \textit{b}, Basal medium with Spd (1 \textmu M).

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\textbf{Figure 3.} Effect of AgNO\textsubscript{3} on shoot differentiation. The hypocotyl explant was placed on MS medium with or without AgNO\textsubscript{3} (50 \textmu M).
The expression of EMB-1 gene is detectable in zygote and somatic embryo as well. In fact, the spatial and temporal EMB-1 gene expression appears to be similar in both zygotic and somatic embryos. This suggests that normal embryogenesis process is independent of surrounding maternal tissue. Yoshida et al. cloned five regeneration-specific genes in rice by differential screening. One of them PCR-2 accumulates transiently in calli after the induction of embryogenesis. Besides, PCR-2 transcript specifically accumulates in both somatic and zygotic embryo. The expression of dormancy-related transcript specifically accumulates in both somatic and regenerative maternal tissue. In fact, the spatial and temporal EMB-1 gene expression appears to be similar and somatic embryo as well. In higher plants, there is a positive correlation between cdc2 level and meristematic activity, though cdc2 mRNA is also found in non-dividing tissue. Arabidopsis cdc48 was found to be highly expressed in meristematic and expanding cells, but not in morphologically differentiating cells. Besides, cdc48 is also involved in the cell growth process.

In dividing tobacco protoplasts, the proliferation marker enzyme-glyoxalase I was induced in a phase-dependent manner prior to the G2/M phases of the cell cycle. The expression of cell wall protein indicates its potential role during developmental processes. For instance, dicot HRGPs are usually expressed in dividing tissue which suggests that HRGPs play an important role in primary cell wall development and subsequent cell division. The expression of GRPs is closely associated with cells in the process of lignification. Therefore, these are most likely structural proteins associated with vascular system. The expression of a rice glycine rich cell wall protein gene, Osgrp-1 has been reported to be closely associated with cell elongation and expansion during post-mitotic cell differentiation.
nucleotide sequence analysis of glyoxalase I shows significant homology with auxin-inducible genes and a limited but strong similarity with the cdc25 binding domain of plant mitotic cyclins. Therefore, it suggests a possible role of glyoxalase I in auxin-induced cell division.

Role of homeotic genes in differentiation

Homeotic genes play crucial role in an orchestrated manner for cellular and regional differentiation of Drosophila. Several homeotic genes have also been isolated from maize, rice, Arabidopsis and soybean. The ectopic expression of the homeobox genes caused abnormal leaf development in transgenic plants. The plant homeotic genes are also directly involved in embryo development. A rice homebox gene, OSH1, is highly expressed before organ differentiation in a specific region during early embryogenesis. OSH1 is not directly associated with shoot development. The gene may function to specify cell identity and provide regional information of shoot and its adjacent tissue. Five hot (homeobox) genes from tobacco genetic tumours have been isolated by differential PCR. The profound expression of hot1 gene in tumour tissue indicates its positive regulation of cell growth and differentiation during early tumourogenesis. Maize homebox gene knotted-1 (kn-1) is a useful marker of meristem activity. The expression of kn-1 is reported on the dome of all meristeme. The first detectable expression of kn-1 occurs during embryogenesis before shoot meristem organization.

Future prospect and conclusion

One of the most important questions in developmental biology concerns the mechanism by which a single cell or a few cells coordinate division and differentiation to yield complex structure and organs found in multi-cellular organism. Callus culture of plants provides a unique tool to study differentiation. Upon induction with proper signals, callus cells coordinate themselves for division and differentiation to yield fully mature somatic embryo or shoot buds. Although there are reports on various embryogenic-specific genes, many interesting questions remain to be answered. Almost nothing is known regarding the signal transduction pathway operating during early events of embryogenesis and shoot bud formation and even during microscopic visibility. A schematic diagram (Figure 4) is given to show how cell differentiation is controlled according to present knowledge.


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Establishment of synchronization in carrot cell suspension culture and studies on stage specific activation of glyoxalase I

Sagar Ghosh1, Jayanti Sen2, Sanjeev Kalia2 & Sipra Guha-Mukherjee1
1Centre for Plant Molecular Biology, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India; 2National Centre for Plant Genome Research, New Delhi 110067, India

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Abstract. The present report summarizes and compares the effects of three cell cycle inhibitors, viz. aphidicolin, hydroxyurea and mimosine, in inducing synchronization of a rapidly proliferating suspension culture of carrot. These treatments efficiently synchronized the cell cycle as the doubling time of the cell population was roughly equal to the total length of one cell cycle. Protoplasts derived from mimosine treated cell suspension culture were resolved via flow cytometry to get an idea of the temporal organization of the cell cycle events. The biochemical analysis showed a rise in stage specific activity of glyoxalase I, an auxin inducible marker enzyme, activated at G2-M. This activity peak could be shifted to an early phase of interphase in response to auxin treatment.

Key words: Carrot cell suspension, Cell cycle study, Glyoxalase I activity, Inhibitors, Synchronization

1. Introduction

Basic understanding of the mechanism of cell division requires a complete analysis of the progression of the cell cycle. A synchronously dividing cell population is important for biochemical and molecular analysis of the cell cycle events. Several methods have been reported for synchronization of plant cells with varying degrees of success [1, 3, 6, 13, 15, 18–21, 30]. In most of these cases, however, mitotic index was only around 10–20% and chromosome aberration was often observed.

Recently a highly synchronizable tobacco BY2 (TBY2) cell suspension has been described [17]. In this cell line, synchrony was established using aphidicolin which showed high homogeneity and mitotic index of 70–80%. This line has been extensively used for studying cell cycle events [12, 25].

Aphidicolin, a specific inhibitor of eukaryotic DNA polymerase and the plant α-like DNA polymerase has been found to be very effective, non-toxic and versatile to induce synchronous growth in plant cell population [9, 11, 26, 27]. In Haplopappus aphidicolin treated cells showed mitotic index of 40%. In XD6S strain of Nicotiana tabacum cv. xanthi, mitotic index reached its maximum 52% and 40% following treatments with aphidicolin and hydroxyurea, respectively. During the treatments, the cells were arrested in G1/S phase of the cell cycle. In the aphidicolin induced system, almost 95% of the cell population resumed nuclear DNA synthesis after removal of the inhibitor. How long the synchrony was maintained in the cell cycle was not studied [26].

Besides aphidicolin and hydroxyurea, a plant amino acid, mimosine, was employed to induce synchronization [28]. Mimosine blocks cell cycle progression at or near G1/S boundary by inhibiting DNA replication. Mimosine has been reported to be more effective than aphidicolin in preventing entry into the S-phase in mammalian cells [16]. Mimosine is one compound or a class of inhibitors that apparently blocks cell cycle traverse by suppressing the formation of the rare amino acid hypusine, in the eukaryotic translation factor 4D [10, 29]. In Petunia, it has been shown that mimosine delivered to cells derived from protoplasts after 18 h, when early S-phase cells were appearing, can block these cells in between G1 and S phases. This does not have a toxic effect as 24 h after mimosine depletion 60% of the cells reached G2 [23].

The synchronized carrot cells were employed to examine the cell cycle phase dependent activation of the enzyme glyoxalase I, which was shown earlier to be auxin inducible and as a cell division marker enzyme activated during G2-M phase [14, 22].

In this paper, we report synchronization of cell suspension cultures of carrot using three inhibitors with a comparative account of their relative efficiency for inducing cell synchrony. The cell cycle phase dependent activation of glyoxalase I in synchronized cell cultures and the effect of auxin on this enzyme has also been studied.
2. Materials
A. Equipment
- Rotary shaker.
- Microscope.
- Cytofluorometer.
- Centrifuge.
- Homogenizer.
- Liquid scintillation counter.
- Spectrophotometer.
B. Glassware
- Culture tube.
- Conical flask.
C. Chemicals
- Aphidicolin.
- Hydroxyurea.
- Mimosine.
- RNase.
- Triton X-100.
- Sorbitol.
- Propidium iodide.
- Perchloric acid.
- Cellulase.
- Macerozyme.
- H\(^3\)-thymidine.
- Acetic acid.
- Orcein powder.
- Ethyl alcohol.

3. Procedure
A. Plant material and cell suspension culture
*Daucus carota* L. cv. Pusa Keshar callus cultures are initiated from the hypocotyl explants of 14 day old seedlings on B\(_2\) medium [8] with 1 mg/L of NAA and 0.5 mg/L BAP, pH 5.8, solidified with 0.8% agar as reported earlier [18] with slight modification. Proliferative friable green calli are subcultured in 50 ml liquid B\(_2\) medium with 1 mg/L of NAA and 0.5 mg/L BAP. Subcultures are maintained by transferring one-third culture every 50 ml liquid B\(_2\) medium every 10 days. The suspension cultures are maintained under standard conditions on a rotary shaker at 120 strokes/min, 2.5 eccentricity, 26 °C and under dimlight. Log phase culture is taken for the experimental purposes.
B. Synchronization of cell suspension cultures
Early exponential phase carrot cell cultures are transferred to fresh medium (basal B\(_2\) medium) so as to reduce the external hormone level in the suspension cultures, allowed to grow for 4–5 days to achieve a good single cell suspension. The cultures are grown for 12 hours in standard conditions in dark after the addition of three inhibitors, aphidicolin, mimosine and hydroxyurea added separately twice at 12 h intervals in varying concentrations (5, 15, 30 and 60 μg/ml). After 24 hours, inhibitors are removed by washing the cells 2–3 times in basal B\(_2\) medium and grown in the same medium under standard growth conditions (in dimlight). To study the activity of glyoxalase I enzyme, cells are grown in the presence of NAA (2 mg/L) after removal of the inhibitor. Samples are retrieved regularly at 4 h intervals for 48 h for studying mitotic index. Each experiment is repeated at least twice with two replicates in each case.

C. Cell number and mitotic index (MI) determination
A 0.5 ml aliquot of cell suspension is collected at regular intervals as indicated in the figures. The cell number is determined by counting the nuclei under a microscope. The MI is determined by counting the cells in late prophase to telophase after fixation with acetic acid: ethyl alcohol (1:3 v/v) overnight and stained with 1% aceto-orcein:1N HCl (9:1) relative to the total cell number.

D. Flowcytometric analysis of synchronized cell cycle
For flowcytometric analysis, 1 ml enzyme solution (1% cellulase, 0.5% macerozyme dissolved in B\(_2\) medium with 1M sorbitol) is directly added to 0.5 ml cell culture and incubated for 30 min at 4 °C with continuous shaking in order to partially remove the cell wall. After washing out the enzyme solution with excess B\(_2\) medium, nuclei are liberated by vigorously pipetting in Galbraith’s buffer [7] supplemented to 0.6% Triton X-100, followed by RNase treatment (5 μg/ml) and staining with propidium iodide [4]. In this process pelleted samples are resuspended in 500 μl autoclaved double distilled water and treated with tween 20 (0.5%) for two times and then propidium iodide (200 μl from a stock solution of 50 μg/ml) is added along with RNase followed by a 15 min incubation. Different DNA contents are identified by EPICS V cytofluorometer. The resulting histograms are analysed by ‘Multicycle’ software (Phoenix flow system, USA) univariate deconvolution program to obtain the distribution of cells in various cell cycle stages.

E. Incorporation of \(^3\)H-thymidine into the DNA fraction
Synchrony of DNA synthesis is examined by monitoring the incorporation of \(^3\)H-thymidine into the DNA fraction at regular time intervals. Three replicates of 1 ml culture are incubated with 3 μCi of \(^3\)H-thymidine (25 Ci/mM, Amersham) at 26 °C for 40 min on a shaker at designated time intervals. Incubation is stopped by the addition of 4 ml of ice-cold ethanol. The suspension is then homogenized with a teflon homogenizer and centrifuged at 2,000 g for 10 min. The insoluble fraction is washed twice with 80% ethanol and 0.2 M cold PCA. Nucleic acid is extracted twice with 0.5 M PCA at 80 °C for 15 min. The extract is centrifuged at 2,000 g for 10 min and an aliquot
of supernatant is transferred to a scintillation vial. The radioactivity is measured in a Beckman liquid scintillation counter.

F. Extraction and assay of glyoxalase I

The extraction and assay of glyoxalase I is done essentially according to the procedure of Ramaswamy et al. [24]. Cells are extracted in 0.1 M sodium phosphate buffer, pH 7.0 at 4 °C in a pre-chilled mortar. The homogenate is centrifuged at 15,000 rpm for 20 min in a Sorvall RC5B centrifuge at 4 °C. The supernatant is used for the activity assay.

The reaction cocktail is set in a final volume of 1 ml containing 0.5 ml sodium phosphate buffer (0.1 M) (pH 7.5), 0.2 ml methylglyoxal (17.6 mM), 0.1 ml GSH (17 mM), 0.1 ml MgSO₄·7H₂O (160 mM) and 0.08 ml water making the final concentration of reaction mixture as 100 mM sodium phosphate buffer, 3.5 mM methylglyoxal, 1.7 mM GSH and 16.0 mM MgSO₄·7H₂O. The mixture is incubated for 7 min before the addition of the enzyme (0.02 ml). The formation of thioester, which shows maximum absorption at 240 nm, is measured in a spectrophotometer. The enzyme unit (IU) is defined as the amount of enzyme catalysing the formation of 1 μmol of S-lactoylglutathione from methylglyoxal and reduced glutathione per min at 25 °C. Specific activity of the enzyme is determined by calculating the enzyme per mg protein.

The total protein concentration is quantified by the method of Bradford [2] with bovine serum albumin (BSA) as standard. For extraction, sodium phosphate buffer pH 7.0 is employed. Cells are crushed in cold with the help of mortar and pestle in extraction buffer. The extract is centrifuged at 15,000 rpm for 20 min at 4 °C. Supernatant is used for the assay.

4. Results and discussion

4.1. Cell cycle synchronization

Hydroxyurea (HU) inhibits DNA synthesis and collects cells at the G1/S boundary. Addition of HU at 15 μg/ml concentration induced partial synchrony of cell cycle during the course of the 36 h study (Figure 1). The highest MI was approximately 58% achieved at about 12 h after removal of HU from the culture medium. The incorporation of isotopic thymidine in the DNA fraction attained a peak at 6 h preceding the MI peak (Figure 2). HU used at a lower or higher concentrations (30 and 60 μg/ml) resulted in lower MI (44% and 33% respectively).

Aphidicolin, another G1/S phase inhibitor, was found to be more effective as compared to HU yielding a MI peak of 60% at a dose rate of 5 μg/ml. At higher doses, lesser cells were found to be in the mitotic phase during the first cycle of synchronized division. Cell count reached its peak in 18-20 h after removal of aphidicolin (15 μg/ml) (Figure 3). The ³H-thymidine incorporation peak was obtained at the entry of S phase after the withdrawal of aphidicolin.

Maximum value of MI (72%) was attained after removal of mimosine at concentration of 15 μg/ml.

Figure 1. Growth curve and mitotic index (MI) of carrot suspension cells at different time intervals treated with 15 μg/ml of hydroxyurea (twice). Growth was monitored by counting cell numbers. Data represent the mean value of three experiments. S.E. < ± 0.5%.
Figure 2. Thymidine incorporation into carrot suspension cells after release from hydroxyurea. Data represent the mean value of three experiments. S.E. < ± 0.5%.

Figure 3. Growth curves and mitotic indices (MI) of carrot suspension cells at different time intervals treated with 5 μg/ml of aphidicolin. Growth was monitored by counting cell numbers. Data represent the mean value of three experiments. S.E. < ± 0.5%.

Higher and lower concentrations showed a decrease in MI. The maximum cell number was observed at 18–20 h following removal of mimosine (15 μg/ml) (Figure 4). Radioactive thymidine incorporation into DNA fraction resumed immediately after removal of the inhibitor (6 h) and the peak dropped to almost zero before the mitotic phase started (Figure 5).

During the course of above study, true synchrony was differentiated from periodic kinetic increases in the mitotic behaviour on the basis of well-defined and
Figure 4. Growth curves and mitotic indices (MI) of carrot suspension cells at different time intervals treated with 15 μg/ml of mimosine. Growth was monitored by counting cell numbers. Data represent the mean value of three experiments. S.E. < ± 0.5%.

Figure 5. Thymidine incorporation into carrot suspension cells after withdrawal from mimosine. Data represent the mean value of three experiments. S.E. < ± 0.5%.

tall peaks of MI, coupled with a true ladder or step ups in the relative cell counts. Since mimosine was found to show the highest MI at a dose of 15 μg/ml in synchronized carrot suspension culture, further studies on cell cycle were performed only with the same dose of mimosine.
4.2. Flow cytometric analysis of synchronized cell cycle

Cell cycle analysis was conducted on freshly isolated protoplasts from carrot cell suspension cultures with and without mimosine treatment (15 μg/ml) and analysed for separation into various cell stages in order to understand the stage specific activation of glyoxalase I. Propidium iodide stained nuclei were isolated on EPICS V cytofluorometer (Coulter, USA) and the resulting histograms were analyzed by 'Multicycle' (Phoenix Flow System) univariate deconvolution program. The various subpopulations of cells were identified as G_1, S, G_2-M stage cells on the basis of differences in DNA content. The asynchronously dividing cell culture (normal cell suspension culture growing on B_5 medium without any inhibitor supplement) resolved the G_1, S and G_2-M populations as 34.91%, 39.59% and 25.51%, respectively. Mimosine treatment arrested approximately 81% cells at the boundary of G_1/S phase (Table 1). The resolution of various cell stage subpopulations after removal of mimosine gave an insight into the temporal kinetics of the cell division cycle in carrot, showing the duration of G_1 phase as about 6 h followed by an S phase of 6-12 h duration and G_2-M phase reaching its peak at 18 h.

4.3. Induction of glyoxalase I activity

Induction of glyoxalase I activity was first examined in cells grown on basal B_5 medium containing no hormones after removal of mimosine (considered 0 h). The activity of glyoxalase I showed an increase after 12 h of growth and reached its peak within 18-24 h, which was immediately followed by a peak

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<td>6</td>
<td>30</td>
<td>32.03 ± 0.29</td>
<td>32.30 ± 0.98</td>
<td>29.67 ± 0.09</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>45.53 ± 0.66</td>
<td>54.26 ± 1.01</td>
<td>14.57 ± 0.76</td>
</tr>
</tbody>
</table>

Data represent the mean value of three independent experiments ± S.E.

Figure 6. Growth curve and enzyme activities of glyoxalase I in carrot suspension cells grown on basal B_5 medium at different time intervals after release from 15 μg/ml of mimosine. Growth was monitored by counting cell numbers. Filled boxes represent G_2/M phase of cell cycle. Data represent the mean value of three experiments. S.E. < ± 0.5%.
increase in cell number (Figure 6). However, when 2 mg/L NAA was added to the basal medium cell division cycle was reduced and a rise in glyoxalase activity occurred in 12 h (Figure 7). Glyoxalase I, a cell division marker enzyme [14], was thus activated during G2-M phase and its activity could be triggered by application of auxin. Triggering of cell division and induction of glyoxalase I activity, as observed here by treatment with mimosine, has also been reported in Nicotiana suspension culture [5].

The experimental data show that two repeated treatments with appropriate dose (15 μg/ml) of mimosine cause the accumulation of cultured carrot suspension cells at the G2/S boundary of cell cycle. The blockage is complete and reversible resulting in immediate and synchronous resumption of nuclear DNA synthesis following its removal. A synchronous mitotic division of 72% cells is also observed after 18 h of removal of mimosine. Thus, mimosine, a reversible cell cycle inhibitor as reported earlier in other plants, could be used successfully to synchronize carrot cell suspension in the present experiment.

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Notes on suppliers

1. Rotary Shaker, Hoeffer, San Francisco, USA
2. Nikon Alphaphot 2, YS2 microscope, M/S. Nikon Corporation, Fuji Bldg., 2-3 Marunouchi, 3-0 Chome, Chiyoda-Ku, Tokyo-100, Japan
3. Cytoscourimeter, Coulter, USA
4. Sorvall RCS Centrifuge, Du Pont Instruments, USA
5. Homogenizer Yorco (High speed homogenizer), Yorco Scientific Industries, Delhi 110 006, India
6. Beckman liquid scintillation counter, Model Wallac LSC 1409 (Wallac Oy, 20101, Turkey, Finland)
7. Hitachi U2000 spectrophotometer, Japan
8. Vensil Glass works (P) Ltd., Dooravaninagar, Bangalore-16, India
9. Sigma Chemical Company, St Louis, USA
11. Amersham International PLC, Amersham, UK
12. Central Drug House (P) Ltd., Bombay, India

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**Author for correspondence:** Sipra Guha-Mukherjee, Centre for Plant Molecular Biology, School of Life Sciences, Jawaharlal Nehru University, New Delhi 110 067, India
Phone: +91 11 6187175/6170016; Fax: +91 11 6165886
E-mail: sipra@jnuniv.ernet.in