Biochemistry and molecular biology of competence of plant cell differentiation and regeneration *in vitro*—a review

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In spite of accumulation of voluminous literature on induction of cell division and differentiation in tissue culture, little information is available about the biochemical and molecular events that control it. In *Brassica oleracea*, exogenous supply of hormones, polyamines and certain amino acids lead to vigorous proliferation, whereas addition of inhibitors to inhibit polyamine or ethylene biosynthesis, transcription and translation, or abscisic acid, results in differentiation. Red/far-red light also induces proliferation or differentiation. There are also evidences to suggest that the phosphoinositide intracellular signalling system to generate second messengers, well established in the animal kingdom, also exists in plants. Some constituents of this cycle have been identified. It appears that through control of the cell cycle by arresting cells at G_1 or G_2 phases, it is possible to ensure differentiation in plant tissue culture.

In the last decade, major research efforts on the genetic engineering of plants and transformation methodologies have enabled the introduction of foreign genes into living cells. Most of the current strategies for the application of biotechnology to crop improvement envisage the regeneration of whole plants from single, genetically altered cells. Tissue and cell recalcitrance has been one of the major problems in obtaining regeneration or differentiation from transformed cells of various crop plants.

Differentiation, i.e. the development of single cells into complex multicellular organs and tissues, results through selective gene expression. Knowledge on the control of differentiation has hardly grown since the demonstration of differentiation of root and shoot in tissue culture by relative concentrations of auxin and cytokinin in tobacco[1] and control of somatic embryogenesis by maintaining the hormone levels[2]. However, in several systems, these hormones cause neoplastic growth by supporting rapid cell division, which fail to differentiate[3].

The elucidation of the biochemical and molecular changes which accompany differentiation, therefore, will be helpful in identifying the underlying mechanisms involved. In spite of voluminous literature on the factors controlling cell division and differentiation in tissue culture[4], very little is known regarding the biochemical and molecular events that control it.

The cell cycle—control point for cell differentiation

The key to understand cell differentiation is to know the regulatory mechanisms, which drive the cell cycle. Cell division and differentiation are controlled by several factors operating in G_1 and G_2 phases of the cell cycle[5,6]. Cells in the process of division move continuously from G_1-S-G_2 phases of the cell cycle and culminate in mitosis. Work with various animal and plant systems has shown that both entry into prolonged G_1 (G_0) phase and reinitiation are controlled by specific hormonal and nutrient factors[7,8]. However, if cells are arrested in G_1 or G_2 phase, they cease dividing and start differentiating. To ensure differentiation, the cell cycle is slowed down or partially arrested at G_1 or G_2 phase, when factors that operated during G_1 and G_2 become limiting[9]. Studies of transition from vegetative to floral meristem suggested the accumulation of cells in G_2 phase of the cell cycle[10]. Critical events for the induction of tracheary element differentiation in *Zinnia elegans* parenchyma, occurred in the early G_1 phase[11].

The changes in the pattern of secondary metabolites with respect to lag phase, cell division phase and stationary phase during the growth cycle show the accumulation of more secondary products in stationary phase resulting in differentiation. The dramatic change in metabolism during the stationary phase appears to be associated, in part at least, with the onset of structural organization. Results using metabolic inhibitors have supported the view that there is an inverse relationship between protein synthesis and the synthesis of certain secondary products. The restriction of growth by limiting the levels of essential nutrients also has similar effects (*see* Vasil)[12].

In our investigations, organized growth in *Brassica* cultures could be initiated by various classes of inhibitors, e.g. actinomycin-D (act-D), cordycepin and abscisic acid (ABA)[13] (Figure 1). Supplementation of trigonelline, *N*-methylnicotinic acid, which causes cellular arrest in G_1 or G_2 (ref. 14, 15) and theophylline,

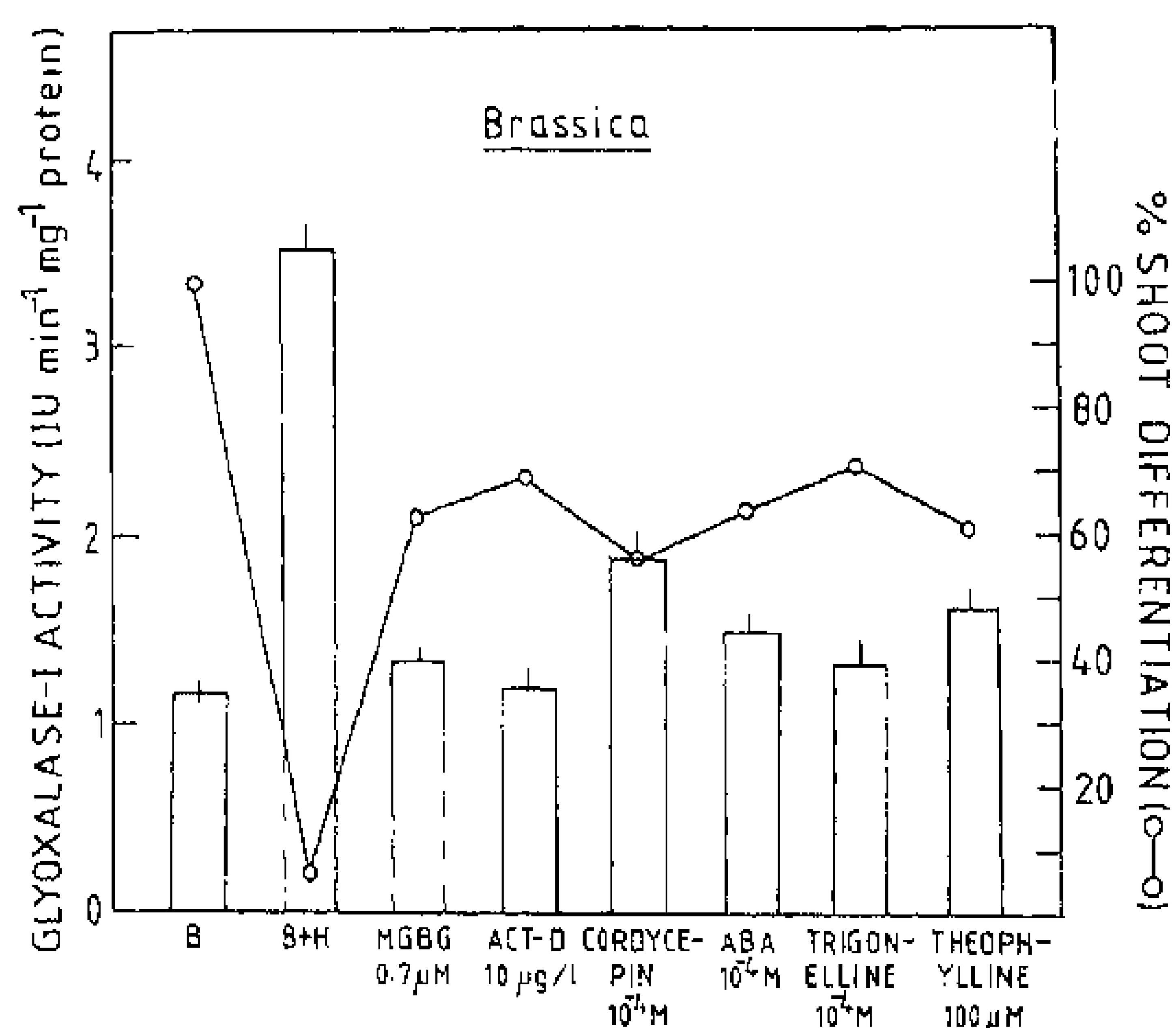

Figure 1. Histograms showing glyoxalase-I activity. Open circles show shoot differentiation in *Brassica oleracea* on the inhibitor-containing media.

an inhibitor of cyclic nucleotide phosphodiesterase, which is reported to induce a block of the cell cycle in G_1 and a temporary arrest in G_2 in roots of *Haplopappus*[16], also induced differentiation in *Brassica* cultures[13]. This work, when further extended to the model systems, *Datura* and *Nicotiana*, gave similar results[13]. The application of inhibitors slows down cell division thus changing the commitment of cells from division to differentiation. There are also a few reports on the effects of some inhibitors like act-D in cereal regeneration[17] and ABA in *Pennisetum*[18]. A set of proteins has been identified which accumulate gradually during normal embryogenesis of *Zea mays* and these polypeptides could be induced prematurely by ABA[19].

Involvement of polyamines, ethylene in proliferation/differentiation

The polyamines, spermidine, spermine and their diamine precursor putrescine have been implicated in an overwhelming array of plant growth and developmental processes[20,21]. Our studies revealed the role of hormones and polyamines in cell division and differentiation in *Brassica* cultures, which differentiated without any hormone. In the presence of hormones, only cell proliferation was achieved, which could be further enhanced by the addition of polyamines such as spermidine and spermine. Most biochemical and physiological studies of polyamines in plants and animals have depended on the availability of inhibitors. Methylglyoxal *bis*-(guanylhydrazone) (MGBG), an inhibitor of spermidine biosynthesis, had retarding effect on proliferation but was able to induce differentiation in *Brassica*[22]. Recent reports have shown that polyamine inhibitors difluoromethylornithine (DFMO) and difluoromethylarginine (DFMA) reduced polyamine content but did not affect embryogenesis in *Nicotiana*[23] and *Medicago*[24]. Similarly, DFMO promoted growth and organization with concomitant increase in bound putrescine and spermidine (ref. 25).

Since polyamine and ethylene biosynthesis share the propylamine group of *S*-adenosylmethionine (SAM)[26] suggesting that there may be regulatory interconnection between them, studies were also performed to determine the levels of ethylene with relation to proliferation and differentiation. *Brassica* cultures in which proliferation could be induced by polyamines, L-methionine, L-threonine, SAM and 1-aminocyclopropane-1-carboxylic acid (ACC) showed a concomitant increase in ethylene level compared to differentiating cultures raised on MGBG. Ethylene antagonists, aminoethoxyvinylglycine (AVG), silver nitrate and cobalt chloride, induced higher percentage shoot differentiation with declined ethylene level compared to the hormone control[27]. These studies corroborate earlier findings that ethylene inhibitors can stimulate the regenerating capacity of the cultures[28-30].

Signal transduction pathway for differentiation

As mentioned earlier, differentiation requires a harmonized activity of several genes and subsets of genes, which would require involvement of enzymes, membranes and finally the genome. Transition of cells from stationary phase to division and differentiation are accompanied by various biochemical changes inducing activation/inactivation of some crucial enzymes.

An interesting observation was made during investigations on the role of amino acid uptake modulating growth and differentiation, that the uptake of only one of the amino acids, L-leucine, was found to be phytochrome-mediated which was however, Ca^{2+}-dependent but calmodulin-independent[31]. These results led to the further work on the direct role of L-leucine and L-isoleucine on differentiation, if any. It was found that these amino acids inhibited the activity of threonine deaminase (TD), which converts L-threonine to α-ketobutyrate in the isoleucine biosynthetic pathway. Interestingly, a strong correlation was observed between cell division, differentiation and activity of TD. This is in contrary to the general dictum that L-leucine activates TD[32]. L-Leucine and L-isoleucine, which reduced TD level, induced differentiation. L-Methionine and L-threonine, which increased TD activity, enhanced proliferation. L-Cysteine and L-valine had no effect on the TD activity or proliferation/differentiation[33].

Besides TD, another enzyme which is not directly involved in the metabolism of the cell, glyoxalase-I, has also been employed as a marker enzyme to determine

the extent of proliferation and differentiation and the transition of one phase to another[22]. This enzyme that catalyses the conversion of ketoaldehyde into its respective thioester, was found to be associated with cell proliferation in cultures of *Datura innoxia*[34], *Amaranthus*[35] and *Brassica*[22]. One possibility as to how this enzyme initiates proliferation maybe by removal of its substrate, methylglyoxal, a cell division inhibitor, from the system.

The polyphosphoinositide intracellular signalling system is now well investigated in the animal kingdom, and it is generally agreed that its principal purpose is to generate the second messengers, diacylglycerol (DG) and inositol triphosphate (IP_3) (ref. 36). A number of pieces of experimental evidence suggest that the same signalling system may exist in plants, for example: several constituents of the cycle including inositol phospholipids (PI, PIP and PIP_2)[37], inositol phosphates (IP, IP_2 and IP_3)[38]. DG[39], kinase that catalyse the phosphorylation of PI to PIP and PIP to PIP_2 (ref. 40), phospholipase[41,42] and a kinase that resembles PKC[43] have been identified in plants.

Earlier studies of comparison of the membrane lipid composition of *Datura* cultures at different stages of differentiation revealed significant differences in the level of PI[44,45]. Recently, it was found that the levels of glycerophosphoinositol increased in homogenates of *Brassica* cultures during proliferation, induced by polyamines or SAM or amino acids like L-methionine or L-threonine, whereas there was a decrease in the level of glycerophosphoinositol (GPI) and increase in the level of glycerophosphoinositol phosphate (GPIP) and glycerophosphoinositol biphosphate ($GPIP_2$) during differentiation induced by MGBG or by the amino acids L-leucine or L-isoleucine[46]. The increased level of GPIP and $GPIP_2$ along with decreased level of GPI in differentiating cultures indicates the higher turnover of PI compared to the proliferating cultures. Such turnover can lead to enhanced levels of secondary messengers being released in the systems as a result of hydrolysis of PIP_2 into DG and IP_3 and each of these metabolites possesses the potential to initiate a cascade of biochemical processes. In animal systems, polyamines have also been found to stimulate the phosphorylation of PI, by activating PIP kinase, leading to the formation of PIP_2 (ref. 47, 48).

Manoharan *et al.*[48] demonstrated light-induced changes in the membrane lipid composition and among the plasma membrane lipids, PI showed an increase. A strong correlation between phytochrome-regulated induction in proliferation, differentiation and PI turnover has also been demonstrated[50]. Red-light-induced proliferation and far red-induced differentiation are likely to be the consequences of alteration in plasma membrane polyphosphoinositides. Therefore, as in animal systems, the agonist-stimulated inositol phospholipid turnover maybe a crucial step in the signal transduction for altering the pattern of plant growth and development.

Molecular controls of differentiation

There are some evidences to show that special types of proteins are formed in a callus committed to produce somatic embryoids. The polypeptide pattern alteration during somatic embryogenesis in callus cultures of two genotypes of *Pisum sativum* was studied. Two protein bands (70 kDa and 45 kDa) were found to be present in a nodular yellowish type of callus which formed somatic embryoids[51]. Choi *et al.*[52] could probe differentiation at the molecular level. They isolated three distinct cDNA clones through differential immuno-adsorption techniques which may be playing key roles in differentiation of embryoids in somatic cells cultures of carrot. The progression of the cell cycle is primarily controlled by sequential expression of various genes. Kodama *et al.*[53] present a comprehensive study of the changes in gene expression during the cell cycle of a higher plant by providing a catalogue of constituent polypeptides, of their relative rates of synthesis and of the population of translatable mRNAs.

In studies from this laboratory, satellite DNA from hypocotyl tissue were found to be highly methylated (CmCgg) whereas satellite DNA of cultured cells was undermethylated or demethylated. Demethylation of some of the satellite DNA sequences appears to be a necessary condition before regulatory steps leading to proliferation and differentiation of explants can occur *in vitro* conditions[54].

Conclusions

Figure 2 summarizes our observations in induction of proliferation and differentiation in *B. oleracea*. Methionine, SAM, ACC and polyamines, when supplied

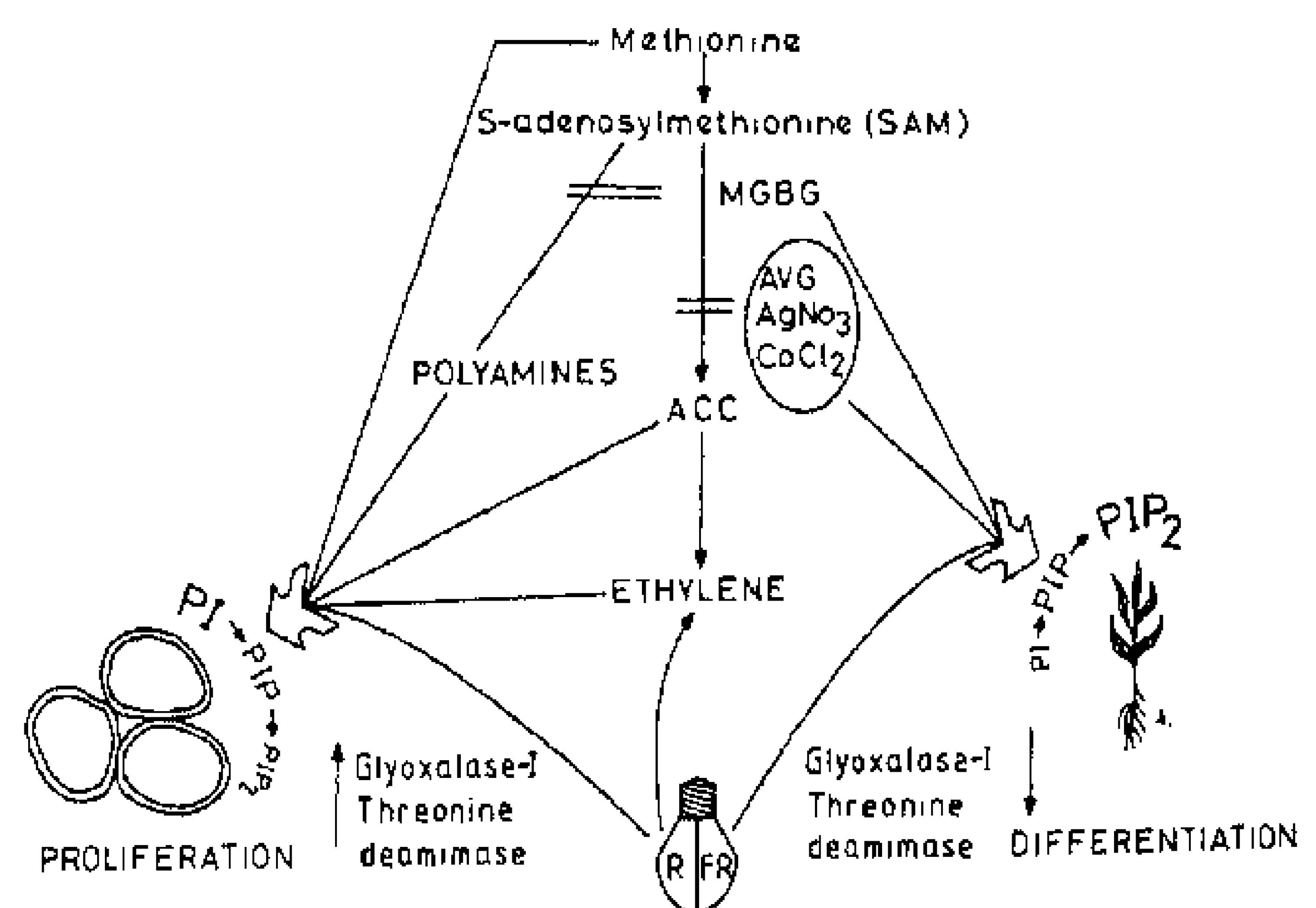

Figure 2. Summarizing figure showing the role of factors regulating cell division and differentiation in *Brasica oleracea.*

exogenously in the presence of hormones, induce rapid cell division, which unless checked by growth inhibitors, such as ethylene antagonists—AVG, $AgNO_3$ and $CoCl_2$ and polyamine inhibitor, MGBG, differentiation cannot occur. Red light also induced proliferation which could be reversed by FR light. The signal transduction leading to differentiation involves PI turnover and a decrease in some marker enzymes like glyoxalase-I and TD. We have also recognized amino acids as important growth-regulators which can perhaps initiate the chain of transformation in cell commitment.

In tissue culture, addition of growth hormones may result in prolific cell division, and often due to positive feedback by cytokinins, more cytokinins are produced[55] and tissue remain in the potential phase of growth. Such tissues often become neoplastic and fail to differentiate. However, on depletion of nutrients or with exogenous supply of inhibitors, cells may enter stationary phase, where cell organization may occur resulting in differentiation. To understand the metabolic switches controlling cell cycle and differentiation, it is essential that a concerted effort is made not only by tissue culturists but also by biochemical and molecular biologists.

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